



UNIVERSITÀ
DI PAVIA

UNIVERSITY OF PAVIA
FACULTY OF ENGINEERING

DEPARTMENT OF CIVIL ENGINEERING AND ARCHITECTURE

PhD program in Design, Modeling, and Simulation in Engineering

XXXVII Cycle

Advanced optical methods in tissue engineering

PhD Dissertation of:
Melissa Pezzotti, 509876

Advisor: Prof. Francesco Pasqualini
Co-Advisor: Prof. Moises Di Sante

A.Y 2025/2026

Contents

List of figures	v
List of abbreviations and acronyms	xi
List of symbols	xv
Abstract	xix
Thesis motivation and structure	xxi

1 Engineering toolkits for high-throughput and high-content phenotyping

1.1 Introduction	1
1.2 HT/HC phenotyping framework in cell biology	4
1.2.1 The biological readout space: cell-cycle, structure, function	7
1.2.2 Labeling, reporters, and model systems as determinants of phenotypic readouts	10
1.2.3 Transforming multiplexed fluorescence data into quantitative HT/HC phenotypes	13
1.3 Live-cell imaging strategies for high-throughput and high-content phenotyping	16
1.3.1 Live-cell imaging readouts: proliferation, migration, and morphogenesis	19
1.3.2 Core imaging architectures for HT/HC live-cell phenotyping	22
1.3.3 Adaptive acquisition and computational reconstruction in HT/HC live-cell imaging	24
1.4 Engineered microenvironments and external modulation	28
1.4.1 EM's readout space: cell-cycle gating, guided migration, and morphogenetic control	31
1.4.2 Fabrication toolkits and light-enabled EM's control	33
1.4.3 Dynamic microenvironment actuation and perturbation for causal phenotyping	35
1.5 Discussion and outlook	37
1.6 How to read this thesis	41
1.7 Bibliography	42

2 FUCCIsmart: Event-driven imaging routine for 3D spindle acquisitions

2.1 Introduction	57
2.2 Cell biology: reporter engineering, cell culture, and traditional imaging	62
2.3 FUCCIsmart design and implementation	65
2.3.1 Algorithm: decision-level performance with live imaging	68
2.4 FUCCIsmart mitosis coverage vs. phototoxicity	72
2.5 Discussion	74

2.6 Materials and Methods	77
2.7 Bibliography	82

3 A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement

3.1 Introduction	86
3.2 Microscopy-based automatic selection for engineered reporter line	88
3.3 Baseline simultaneous assessment of cell-cycle (CC) progression and migration	92
3.4 Implementation of Fab2Mic, a correlative pipeline for photofabrication and imaging	97
3.5 Quantifying confinement effects on CC progression and proliferation	101
3.6 Discussion	106
3.7 Materials and Methods	110
3.8 Bibliography	128

4 TE-DaXi: design a custom light-sheet microscope for tissue engineering applications

4.1 Introduction	133
4.2 TE-DaXi microscope design	137
4.3 Construction and alignment	141
4.4 Preliminary tests and validation	144
4.5 Discussion	151
4.6 Materials and Methods	152
4.7 Bibliography	159

5 Developing engineering tools for 3D HT/HC functional and structural phenotyping

5.1 Introduction	162
5.2 Experimental toolkits for volumetric HT/HC phenotyping workflow: from spheroids to cardioids	165
5.2.1 Optimizing sample preparation and volumetric acquisition	166
5.2.2 High-throughput screening JOB for rapid morphological QC	168
5.2.3 Static and dynamic volumetric imaging of cardioids	170
5.3 Image analysis toolkits for 3D structural and functional phenotyping	173
5.3.1 Unified 3D analysis pipeline for coupled functional-structural phenotyping	173
5.3.2 Functional 3D readout: cardioids' proliferative state at different stages	178
5.3.3 Structural 3D readout: cardioids' volumetric growth and multicavity morphologies across differentiation	180

5.4 Multicellular Phase-field Model of lumen morphogenesis	183
5.5 Discussion	189
5.6 Materials and Methods	193
5.7 Bibliography	206
 6 Conclusions	 208
 Annotated bibliography	 218
 Appendix A Additional figures	 290
 A1 Chapter 2 - FUCCIsmart performance evaluation	291
A2 Chapter 3 - FUCCIplex cells, Fab2Mic, and 2D CC-aware motility and morphology	292
A3 Chapter 5 - MCPFM simulations and phase-field regimes	311
 Appendix B Additional tables/methods	 314
 B1 Chapter 2 - FUCCIsmart extended data	315
B2 Chapter 4 - Oblique-Plane Microscope alignment protocol	319
B3 Chapter 5 - Multicellular Phase-Field Model for lumen morphogenesis	326
B4 Chapter 2/3 - Live cell microscopy protocol	331
 Appendix C Codes	 342
 C1 Chapter 2 - FUCCIsmart JOBs pseudocode	343
C2 Chapter 3 - Fab2Mic routine	351
C3 Chapter 3 - FUCCIphase_morphology	362
C4 Chapter 3 - Tubulin spread analysis macro	369
C5 Chapter 2/3 - Image processing macro for 2D visualization	372
C6 Chapter 4 - TE-DaXi light-sheet tilt analysis	380
C7 Chapter 5 - Cavity analysis	388
 Appendix D Academic Curriculum	 393

List of figures

- **Figure a Advanced optical methods in tissue engineering.** Graphical abstract with the main thesis contribution summarized in four interconnected modules.

Chapter 1

- **Figure 1.1 High-throughput and high-content phenotyping framework in cell biology.** Conceptual map linking cell-cycle reporters, structural imaging, functional organoid readouts, multiplex labeling, genome editing, and plate-scale screening. Shows how segmentation/feature extraction turns images into quantitative phenotypes.
- **Figure 1.2 Live-cell imaging strategies for high-throughput and high-content phenotyping.** Survey of optical modalities and imaging constraints (speed, phototoxicity, resolution), linked to biological readouts and throughput scaling.
- **Figure 1.3 Engineered microenvironments and external modulation of cell behavior.** Overview of biomaterial and microenvironment engineering strategies (geometry, stiffness, biochemical cues) and perturbation layers used to modulate and probe phenotypes.
- **Figure 1.4 Dataset passport table for HT/HC phenotyping workflows.** Compact dataset reporting structure for HT/HC phenotyping in tissue engineering.

Chapter 2

- **Figure 2.1 FUCCIsmart: CC-aware event-triggered imaging routine for 3D spindle acquisition.** Concept of event-driven volumetric imaging that switches from low-dose screening to high-resolution acquisition when a mitotic event is detected.

- **Figure 2.2 HaCaT FUCCIplex cell line: reporter engineering, cell culture, traditional imaging.** Reporter integration and validation workflow, culture conditions, and traditional imaging outputs.
- **Figure 2.3 FUCCIsmart algorithm flowchart.** Decision pipeline defining nuclear masking, intensity gates, timing rules, and trigger conditions.
- **Figure 2.4 FUCCIsmart decision outcomes: performance and representative cases.** Examples of successful event capture and failure modes, linking trigger logic to imaging outcomes.
- **Figure 2.5 FUCCIsmart benchmark across four 24-h acquisition schedules.** Quantitative comparison of acquisition schedules showing performance trade-offs between capture probability and dose/file size.

Appendix A1

- **Figure A1 FUCCIsmart performance evaluation tables.** Summary tables of schedules and metrics used for performance evaluation and comparison.

Chapter 3

- **Figure 3.1 Engineering and rescue of a four-reporter CALIPERS-enabled HT1080 line.** CRISPR/editing workflow and final reporter configuration enabling multiplex CC-aware phenotyping.
- **Figure 3.2 Confinement-guided CC and motility phenotyping in free space vs patterns.** Representative trajectories and cell-cycle distributions comparing unconstrained migration versus engineered confinement.
- **Figure 3.3 Fab2Mic workflow: fabrication-to-microscopy coordinate correlation.** Coordinate-guided mapping between patterned substrates and microscope acquisition, enabling automated targeting and reproducible imaging.
- **Figure 3.4 Time-resolved motility and morphology under confinement.** Quantitative time-course analysis of migration and morphology metrics across confinement geometries.

- **Figure 3.5 Confinement-induced CC bias and proliferation changes.** Comparison of CC phase distributions and division dynamics linked to confinement geometry and spatial context.

Appendix A2

- **Figure A2.1 HT1080 CALIPERS four fluorophores generation.** Reporter integration and validation workflow in a cancer cell line
- **Figure A2.2 Validation of FUCCIplex phase assignment and timing consistency.** Controls validating FUCCI-based phase calls and timing behavior. Supports reliability of phase-resolved phenotyping.
- **Figure A2.3 Free space 48h long-term population validation.** Longer-duration baseline dataset showing population-level stability over extended imaging. Supports that observed effects are not short-window artifacts.
- **Figure A2.4 Synchronization time-dependent analysis.** Time-aligned analysis for synchronized populations. Supports interpretation of phase-dependent dynamics beyond asynchronous mixtures.
- **Figure A2.5 Population-average profiles in normal vs abnormal synchronized cells.** Comparative time profiles distinguishing normal and abnormal cycle trajectories. Strengthens links between dynamics and fate outcomes.
- **Figure A2.6 Fab2Mic validation and single-cell occupancy in square islands.** Confirms pattern fidelity and occupancy statistics. Supports QC of confinement assignments.
- **Figure A2.7 Additional nuclear/cytoskeletal morphology features across confinements.** Expanded feature set beyond primary metrics. Supports robustness of confinement-associated phenotypes.
- **Figure A2.8 Normal cell-cycle dynamics under ECM confinement.** Phase timing and progression metrics under confinement for “normal” trajectories. Anchors what “normal” looks like per geometry.
- **Figure A2.9 Extended phenotyping of normal vs abnormal events under confinement.** Representative trajectories plus linked dynamics (e.g., velocity/area) across sizes. Connects fate class to measurable time-resolved phenotypes.

- **Figure A2.10 Nuclear dynamics across ECM sizes (normal vs abnormal).** Time-resolved nuclear area/perimeter/aspect ratio across conditions. Supports nuclear remodeling as a sensitive readout of abnormality.
- **Figure A2.11 Cell morphological dynamics across ECM sizes (normal vs abnormal).** Time-resolved cell perimeter/circularity/aspect ratio across conditions. Supports geometry-dependent morphological instability signatures.
- **Figure A2.12 Physiologically inspired ECM islands and related abnormalities.** Validation of physiologically shaped islands and example phenotypes observed on them. Extends confinement beyond squares toward morphology-realistic constraints.

Chapter 4

- **Figure 4.1 Tissue-engineering platforms suitable for the DaXi microscope.** Positioning of TE-DaXi in the tissue-engineering imaging landscape and target use-cases.
- **Figure 4.2 TE-DaXi optical layout.** System schematic detailing excitation/detection paths and key optical planes.
- **Figure 4.3 TE-DaXi virtual assembly model.** Mechanical/optical integration overview supporting build validation and alignment planning.
- **Figure 4.4 TE-DaXi construction and alignment.** Building steps and alignment checkpoints are used to reach stable imaging operation.
- **Figure 4.5 Test for detection-path alignment and imaging.** Alignment verification and representative imaging outputs validating the detection arm.
- **Figure 4.6 Illumination tilt from axial/lateral beam displacement.** Illustration of beam misalignment modes producing illumination tilt and associated corrective strategy.
- **Figure 4.7 TE-DaXi performance-reporting framework for quantitative 3D tissue-engineering imaging.** Main performance metrics and validation tests

required to move from prototype-level image formation to quantitative 3D phenotyping.

Chapter 5

- **Figure 5.1 Low-melting agarose embedding stabilizes spheroids for static + long-term LSFM.** Embedding workflow and stability outcomes, enabling volumetric acquisition with reduced motion artifacts.
- **Figure 5.2 Daily HT screening JOB for cardioid differentiation for early rapid QC.** Plate-scale imaging strategy for longitudinal monitoring and early QC gating before volumetric LSFM.
- **Figure 5.3 Static LSFM volumes of FUCCIplex cardioids across differentiation.** Representative 3D datasets showing structural organization and fluorophore readouts at matched stages.
- **Figure 5.4 Long-term LSFM time-lapse of a FUCCIplex cardioid (24h).** Example of low-dose volumetric time-lapse capturing CC dynamics and structural remodeling.
- **Figure 5.5 Unified 3D analysis pipeline for coupled functional-structural phenotyping.** End-to-end pipeline from preprocessing to segmentation and extraction of linked CC and cavity morphology descriptors.
- **Figure 5.6 ARIVIS Cloud deep-learning model for cavity segmentation.** Deep learning workflow and performance evaluation for cavity segmentation in volumetric datasets.
- **Figure 5.7 FUCCIplex cell-cycle proliferation state mapping in 3D across differentiation.** 3D CC state mapping and quantification across stages, enabling functional stratification of morphologies.
- **Figure 5.8 3D reconstruction of cardioid morphology and cavity architecture.** Surface reconstruction and cavity topology analysis capturing transitions from compact to fragmented/complex cavities.

- **Figure 5.9 Quantitative evolution of cardioid volume and cavity architecture.** Time-course morphometrics describing growth and cavity organization (counts, occupancy, size distributions).
- **Figure 5.10 Representative MCPFM morphologies and trajectories (spherical vs complex/branched).** Simulated outcomes capturing two qualitative regimes: compact single-cavity versus branched/multicavity structure.
- **Figure 5.11 Phase-field regime mapping of cavity morphogenesis in (τ_v, ξ) space.** Regime map summarizing cavity occupancy outcomes across the control-parameter sweep.
- **Figure 5.12 Experiments to MCPFM simulations: qualitative regime comparison (branched/multicavity).** Interpretation layer connecting experimentally extracted descriptors to representative model regimes.

Appendix A3

- **Figure A3.1 MCPFM spherical regime trajectories in compact/spherical regime.** Additional trajectories and stability examples in the compact/spherical regime.
- **Figure A3.2 MCPFM branched regime trajectories in complex/branched regime.** Additional trajectories and stability examples in the complex/branched regime.
- **Figure A3.3 Phase-field regime mapping of cavity morphogenesis in (τ_v, ξ) space.** Additional visualization of the regime map and extracted occupancy metric.

Chapter 6

- **Figure 6.1. Key thesis contributions.** Summary of the main HT/HC bottlenecks, the corresponding engineering contribution, where each module is validated, and the primary metric or capability improved

List of abbreviations and acronyms

2D	Two-dimensional.
3D	Three-dimensional.
4D	Three-dimensional plus time (3D over time).
AI	Artificial intelligence.
APS	Anterior primitive streak (cardioid differentiation stage).
BSA	Bovine serum albumin.
CALIPERS	Cell-cycle-aware live imaging for phenotyping experiments and regeneration studies.
CARE	Content-aware image restoration (deep-learning denoising).
CC	Cell-cycle.
CFP	Cyan fluorescent protein.
DaXi	Dual-axial light-sheet microscope architecture.
DDM	Data-driven microscopy.
diSPIM	Dual-view inverted selective plane illumination microscope.
DMD	Digital micromirror device.
DMEM	Dulbecco's modified Eagle medium.
DPBS	Dulbecco's phosphate-buffered saline.
ECM	Extracellular matrix.
EGFP	Enhanced green fluorescent protein.
EM	Engineered microenvironment.
ERC	European Research Council.
Fab2Mic	Fabrication-to-microscopy correlation pipeline.
FBS	Fetal bovine serum.
FN	False negative.
FOV	Field of view.
FP	False positive.
FUCCI	Fluorescent ubiquitination-based cell-cycle indicator.
FUCCIplex	Multiplexable FUCCI reporter with CFP and iRFP nuclear fluorescence

probes. **FUCCIsmart** Cell-cycle-aware, event-driven imaging routine.

G1	G1 cell-cycle phase.
GFP	Green fluorescent protein.
GFR	Growth factor reduced (e.g., GFR Matrigel).
HaCaT	Human keratinocyte cell line.
hiPSC	Human induced pluripotent stem cell (plural: hiPSCs).
HT/HC	High-throughput / high-content.
HT1080	Human fibrosarcoma cell line.
iRFP	Infrared fluorescent protein.
ISM	Image-scanning microscopy.
LIMAP	Light-induced molecular adsorption of proteins.
LMA	Low-melting agarose.
LSFM	Light-sheet fluorescence microscopy.
MCPFM	Multi-cellular phase-field model (cavity morphogenesis model used in this thesis).
NA	Numerical aperture.
ND	Neutral density (optical filter).
OPM	Oblique plane microscopy.
OPSIM	Oblique-plane structured illumination microscopy.
P/S	Penicillin/streptomycin.
PBS	Phosphate-buffered saline.
PFS	Perfect Focus System.
PI	Propidium iodide.
POPSIM	Projective oblique-plane structured illumination microscopy.
PSF	Point spread function.
QC	Quality control.
RFP	Red fluorescent protein.
RNP	Ribonucleoprotein complex (e.g., Cas9-sgRNA).
ROI	Region of interest (plural: ROIs).
S/G2/M	Combined S, G2, and M phases (FUCCIplex - derived class).
SCAPE	Swept confocally-aligned planar excitation microscopy.
sCMOS	Scientific complementary metal-oxide-semiconductor (sCMOS) camera.

SDCM	Spinning-disk confocal microscopy.
sgRNA	Single-guide RNA.
smartLLSM	Smart lattice light-sheet microscopy.
TE	Tissue engineering.
TE-DaXi	Tissue-engineering implementation of the DaXi light-sheet platform.
UCSFi001-A-1	hiPSC line identifier used in the thesis.
ULA	Ultra-low attachment (cultureware).
VsLFM	Virtual-scanning light-field microscopy.
WF	Widefield microscopy.
WT	Wild type.
WTC-11	hiPSC line name used in the thesis.

List of symbols

Global

μm	Micrometer.
μM	Micromolar.
μL	Microliter.
μg	Microgram.
Δ	Change / difference.
λ	Wavelength.
σ	Sigma (e.g., standard deviation or interfacial/surface tension).
τ	Time constant.

Chapter 2

TP	True positive.
TN	True negative.
FP	False positive.
FN	False negative.
Z-step	Axial sampling step size (μm).
t	Time variable.

Chapter 3

FOV	Field of view.
px	Pixel (image coordinate units).
$\Delta\text{x}, \Delta\text{y}$	Displacement along x/y.

Chapter 4

NA	Numerical aperture.
WD	Working distance.
4f	Relay optical system (two lenses separated by the sum of focal lengths).

Chapter 5

τ_v	Model control parameter used in Chapter 5 MCPFM regime mapping.
ξ	Model control parameter used in Chapter 5 MCPFM regime mapping.
ΔP	Pressure difference (context-dependent; permeability/pressure driver proxy).
V_t	Cavity area/volume proxy extracted from the phase-field.
V_m	Area/volume of cell m in the phase-field representation.
$\sum_m V_m$	Total cell area/volume summed over all cells.
$\phi(\mathbf{r})$	Summed cell-indicator/occupancy field as a function of spatial coordinate $\mathbf{r}$.
$R(t)$	Cavity radius computed from cavity area/volume over time.
λ_w	Effective water permeability parameter.

Abstract

Tissue engineering increasingly depends on live-cell fluorescence imaging to connect cellular behavior with engineered microenvironments and to measure how state, structure, and function change over time. However, scaling phenotyping toward high-throughput and high-content (HT/HC) experiments remains limited by three ongoing challenges: phototoxicity and accumulated photodamage during information-rich acquisition; weak compatibility between microenvironment fabrication and microscopy workflows; and the difficulty of obtaining quantitative, cell-state-aware readouts in complex 3D models under practical constraints of cost, standardization, and imaging stability. This thesis tackles these challenges from an engineering perspective by developing modular, compatible, and dose-controlled tools to improve phenotype interpretability and measurement comparability as experimental platforms, acquisition modes, and analysis pipelines evolve.

First, we present a cell biology-guided adaptive imaging strategy to reduce illumination load during highly phototoxic mitotic imaging. By using the multiplexed FUCCIplex cell-cycle (CC) reporter to identify informative time windows, confocal acquisition is focused both in time and space, allowing high-resolution observation of mitotic processes while minimizing unnecessary exposure outside the relevant phase.

Second, the thesis advances substrate engineering as a controlled external modulation and develops a vertically integrated workflow for CC-aware phenotyping under planar confinement. Using LMAP-patterned confinement and multiplexed genome-edited readouts, the developed Fab2Mic fabrication-to-microscopy correlation pipeline translates fabrication coordinates into imaging coordinates, enabling long-term, automated phenotyping that links microenvironment geometry to structural and cell-cycle behaviors within defined planar confinement assays.

Third, we introduce TE-DaXi, a custom oblique-plane light-sheet microscope developed as a design and prototyping effort to support volumetric phenotyping in tissue-engineering specimens. TE-DaXi is conceived to combine fast, high-resolution

volumetric bursts with long-term, low-dose volumetric imaging for morphology and cell-cycle tracking, providing a platform onto which experimental and analytical workflows can be transferred after further calibration and validation.

Lastly, the thesis focuses on a 3D phenotyping workflow using a volumetric imaging system, prioritizing robustness and interpretability to establish an end-to-end pipeline for self-organizing hiPSC-derived cardiac organoids (cardioids). The workflow integrates sample handling, gentle volumetric imaging, and quantitative analysis to link cavity architecture to proliferative state via coupled structural-functional readouts. A two-phase model is adopted as an interpretive layer for observed trends, providing a qualitative framework for comparing cavity morphogenesis regimes across conditions. Together, these contributions connect genetic engineering, microenvironment design, instrument development, and quantitative analysis toward more controlled, auditable, and context-aware phenotyping workflows in live-cell tissue engineering. This thesis provides practical modules and design principles that can support future scaling and cross-platform comparability when validated across additional models, perturbations, and imaging systems.

Advanced optical methods in tissue engineering applications

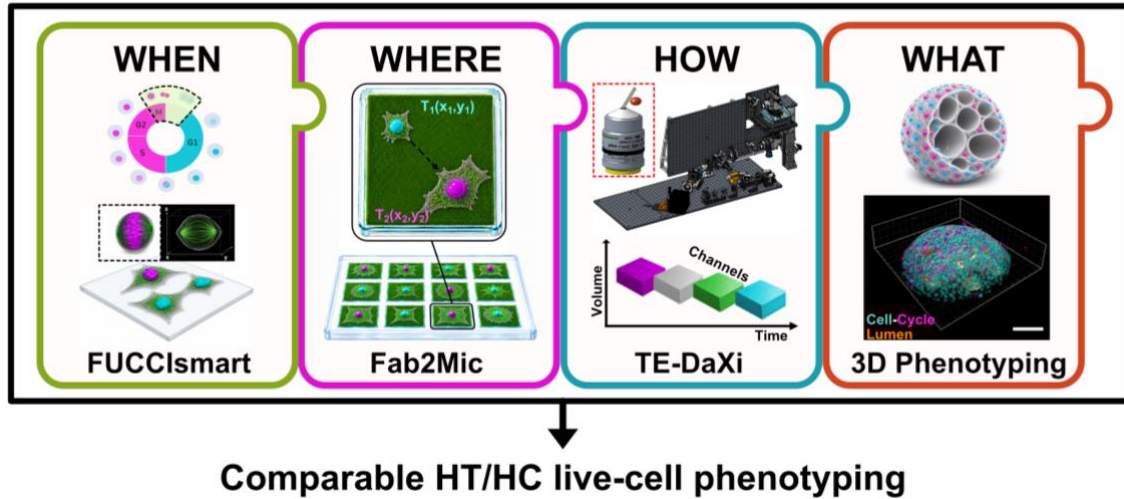


Figure a. Advanced optical methods in tissue engineering applications. The thesis contributions are organized into four connected modules: when to image through FUCCIsmart state-aware acquisition, where to image through Fab2Mic spatial targeting, how to image in 3D through TE-DaXi volumetric microscopy, and what to extract and interpret through coupled structural-functional 3D phenotyping.

Thesis motivation and structure

High-throughput and high-content (HT/HC) phenotyping is increasingly central to modern tissue engineering. The field asks not only whether an engineered system works but also how and why it behaves under different conditions, time scales, and perturbations. The promise is straightforward: to capture multiparametric cellular behaviors at scale while preserving single-cell context, thus narrowing the gap between elegant in vitro demonstrations and results that are valid in real-world applications. In practice, however, the work is more challenging than the promise suggests. As workflows scale, variation from fabrication, illumination strategies, and computational processing can become comparable to or greater than the biological effects being measured. This often results in a familiar failure mode: large, expensive datasets that are difficult to compare across platforms and challenging to interpret mechanistically.

Over the past decade, the component technologies needed for HT/HC phenotyping have matured rapidly. Morphological profiling frameworks can compress rich image content into measurable feature sets; live-cell and volumetric imaging techniques can observe dynamics rather than static endpoints; and modern analysis pipelines can automate segmentation, tracking, and representation learning. These improvements enable the measurement of trajectories, such as cell-cycle transitions, structural changes, migration, and morphogenesis, under controlled conditions. However, feasible is not synonymous with reliable. Many pipelines still rely on fixed-cell endpoints, limited multiplexing, and incomplete reporting of acquisition parameters, including photon dose. These gaps weaken reproducibility and complicate biological interpretation. More fundamentally, fluorescent reporters, imaging techniques, engineered microenvironments, and analysis software often develop as parallel tracks rather than interoperable modules. This fragmentation slows progress, introduces confounders that are not always explicit, and limits the ability to turn phenotypic measurements into evidence that is usable for robust comparisons. This thesis takes an engineering view of HT/HC phenotyping as an end-to-

end system composed of linked modules: biological readouts, imaging strategies, and engineered perturbations that must be designed to function together.

Chapter 1 reviews the state of the art through this lens and motivates a practical requirement: if a phenotype is to be comparable across time, sites, and platforms, then the workflow must make its sources of variance explicit. Fabrication tolerances, illumination dose, and analysis provenance must be measured and reported, and quality control and metadata must be treated as first-class outputs alongside biological readouts.

Three persistent limitations recur throughout the literature and motivate the tools developed in this thesis. First, cell populations are heterogeneous in cell-cycle state, and cell-cycle mixing can mask or misattribute morphological and functional phenotypes unless the proliferative state is measured and used as an explicit coordinate. Second, long-term live-cell imaging accumulates phototoxicity and photodamage; as observation windows extend and model complexity increases, illumination dose becomes an experimental variable that can perturb physiology and bias sensitive phenotypes. Third, limited interoperability between photofabricated substrates and microscopy workflows wastes acquisition time and reduces comparability, particularly when large patterned areas must be reproducibly mapped to imaging coordinates. The topics discussed in this chapter were adapted from a literature review currently under revision. The highlighted constraints are exacerbated in three-dimensional constructs, where volumetric imaging is required, motion artifacts become more prominent, and quantitative, cell-cycle-aware structural and functional phenotyping remains underdeveloped. The work in this thesis targets this interface directly. Rather than treating biology, fabrication, imaging, and analysis as separate pipelines, it develops integrable tools that coordinate *when*, *where*, and *how* measurements are collected and interpreted. The guiding premise is pragmatic: if the microenvironment is precisely defined, if the biological state can inform acquisition timing, and if volumetric imaging is both gentle and reproducible, then live-cell HT/HC measurements are more likely to remain interpretable and comparable within defined operating ranges.

Accordingly, the thesis advances four mutually reinforcing contributions (Fig. a): adaptive acquisition strategies; a vertically integrated fabrication-to-microscopy pipeline

for engineered microenvironments; a single-objective light-sheet microscope developed for tissue-engineering applications; and 3D live-cell phenotyping workflows integrating structural and functional readouts. The chapters are organized as follows: **Chapter 2** introduces FUCCIsmart, an event-driven, cell-cycle-aware acquisition framework that uses fluorescent cell-cycle reporters to trigger high-resolution three-dimensional imaging when mitosis is imminent. This strategy concentrates photon dose where information density is highest while suppressing unnecessary illumination elsewhere. The work is associated with a bioRxiv preprint posted in December 2024, and the manuscript is currently under revision.

Chapter 3 presents Fab2Mic, a correlative light-fabrication-to-microscopy pipeline for confined phenotyping. By combining multiplexed reporter engineering, fabrication-imaging correlation, and longitudinal single-cell tracking, this chapter quantifies how geometric confinement influences motility, morphology, and cell-cycle dynamics during long-term fluorescence imaging. This work was adapted from the published work in *APL Bioengineering* in March 2026.

Chapter 4 describes TE-DaXi, a modular, single-objective oblique-plane light-sheet microscope inspired by axial swept light-sheet designs and tailored for tissue-engineering use cases. TE-DaXi is developed as a design and prototyping effort to support dose-governed volumetric imaging, enabling fast volumetric acquisition over short windows as well as long-term, low-dose imaging for morphology and cell-cycle readouts through explicit mode and channel governance. This chapter is adapted from a contribution included in the *Ninth National Congress of Bioengineering* proceedings (GNB 2025).

Chapter 5 develops engineering tools for three-dimensional HT/HC structural and functional phenotyping. It introduces an HT/HC volumetric imaging workflow from sample preparation to controlled long-term light-sheet imaging, along with quantitative pipelines that connect structural cavity morphometrics to cell-cycle state distributions in 3D hiPSC-derived cardioids. In addition, a two-phase model describing proliferation-driven growth and interface transport mechanisms in cavity formation is adopted as an interpretative reference layer for interpreting 3D readouts and supporting qualitative model-data comparison.

Finally, **Chapter 6** synthesizes the contributions and outlines future research directions. Collectively, this thesis proposes an integrated framework that connects genetic reporters, substrate engineering, two- and three-dimensional live-cell imaging, and quantitative analysis to improve information use, reduce avoidable measurement variability, and support more controlled live-cell HT/HC phenotyping workflows in tissue engineering. Broader scalability, causal interpretation, and cross-platform comparability are presented as future directions that require further validation across additional biological models, perturbations, and imaging systems.

Note on prior publications and reuse of material

Portions of this thesis are based on work that has been previously published, deposited as a preprint, submitted, or is under review for publication. Where text, figures, tables, or other material have appeared elsewhere, the original source is explicitly cited.

1

Engineering toolkits for high-throughput and high-content phenotyping

1.1 Introduction

High-throughput and high-content phenotyping (HT/HC) has established the value of image-based, multiparametric measurement for analyzing cellular behavior at scale while preserving single-cell context^{1–5}. In standard 2D systems, these approaches have demonstrated the potential of quantitative imaging to support systematic phenotypic discovery and screening⁶. However, simplified 2D cultures on glass do not always capture the geometry, mechanics, multicellular organization, and dynamic behavior that shape phenotype in tissue-engineering platforms and organoid systems^{3,7}. As a result, more physiologically relevant models are increasingly needed when the goal is to study structure-function relationships, microenvironment-dependent behavior, or morphogenetic processes.

That transition, however, makes HT/HC phenotyping more difficult. Live-cell imaging, multiplexed fluorescent readouts, engineered substrates, and 3D culture models increase the biological richness of the assay, but they also introduce multiple sources of technical variation. Fabrication variability can affect experimental consistency, photon dose can alter live-cell measurements, and computational choices can influence how phenotypes are interpreted^{3,7–10}. Under these conditions, the challenge is to design workflows in which image-derived measurements remain biologically meaningful despite increased experimental complexity.

Over the past decade, HT/HC phenotyping has advanced substantially through robust profiling approaches such as Cell Painting¹¹, dose-efficient live and volumetric imaging modalities including light-sheet microscopy¹², and ML/AI-enabled pipelines for segmentation, tracking, restoration, and representation learning¹³. These developments have expanded the range of measurable phenotypes, including cell-cycle transitions, structural remodeling, migration, contractility, and morphogenesis, particularly when reporter choice, acquisition design, and assay duration are aligned. A recurring limitation is continued reliance on endpoint or snapshot-style readouts in many screening workflows. In parallel, live-cell implementations often remain constrained by modest multiplexing and incomplete reporting of illumination dose, limiting reproducibility and weakening biological interpretation^{10,14,15}. Open and scalable tools such as CellProfiler/Analyst, ilastik, napari, and DeepCell, together with FAIR data practices and cloud-native bioimaging formats, provide important enabling infrastructure for large-scale phenotyping^{16–21}. Even so, adoption remains uneven across the bioimaging community^{22–24}. As a result, a persistent challenge is fragmentation. In many workflows, reporter design, acquisition strategy, substrate engineering, and data analysis still operate as adjacent technical layers rather than as a controlled measurement system. This layered setup can influence the measured phenotype to a degree that weakens biological interpretation^{8,9,25,26}.

Scope and roadmap of the state of the art review

In this chapter, we discuss HT/HC live-cell phenotyping as a workflow problem linking biological readouts (Fig. 1.1), imaging strategies (Fig. 1.2), and engineered microenvironments (Fig. 1.3), because these components respectively define *what must be measured*, *how it can be observed*, and *which perturbations can be imposed*. Recent reviews have already covered individual domains in depth, including large-scale phenotyping pipelines, single-cell profiling, and hiPSC-based assay models^{27,28}. Here, we examine how these domains integrate into live-cell HT/HC workflows to support reproducibility, comparability, and biological interpretation across 2D, 2.5D, and 3D models, while recognizing that cross-platform transfer requires assay-specific validation. The following sections, therefore, aim to connect biological questions to measurable axes, dose-aware imaging strategies to reliable signal acquisition, and mechanical, optical, or

biochemical perturbations to experiments that can strengthen mechanistic interpretation when linked to live readouts. The overall goal is not to define a universal live-cell HT/HC framework, but to outline an engineering-oriented set of design considerations for assembling phenotyping workflows that are more auditable, context-aware, and transferable under defined experimental conditions.

1.2 HT/HC phenotyping framework in cell biology

HT/HC phenotyping begins by defining which biological variables need to be measured, because that choice determines the requirements for labeling, imaging, perturbation, and analysis. In this review, we treat cell-cycle state, structure, and function as the core readout space. Cell-cycle state provides temporal context, helping distinguish regulated transitions from asynchronous variation. Structural readouts describe how phenotype is organized across scales, from nuclear architecture and cytoskeletal arrangement to cell shape and tissue geometry. Functional outputs, including motility, contractility, calcium dynamics, and voltage dynamics, capture what cells and tissues do over time within those structural and proliferative constraints (Fig. 1.1A-C).

These biological axes must then be converted into measurable signals. The fluorescence layer (Fig. 1.1D-F) does so through dyes, reporters, genome engineering, and model choice, which together determine signal specificity, multiplexing depth, and compatibility with live imaging. The analysis layer (Fig. 1.1G-I) converts images into quantitative phenotypes through 2D pipelines and scalable 3D-4D workflows that extract morphometric, spatial, and dynamic features across large cell and organoid datasets. Together, these components define the experimental conditions under which biological state can be observed, quantified, and compared, provided that reporter performance, acquisition settings, and analysis assumptions are validated within the relevant assay context.

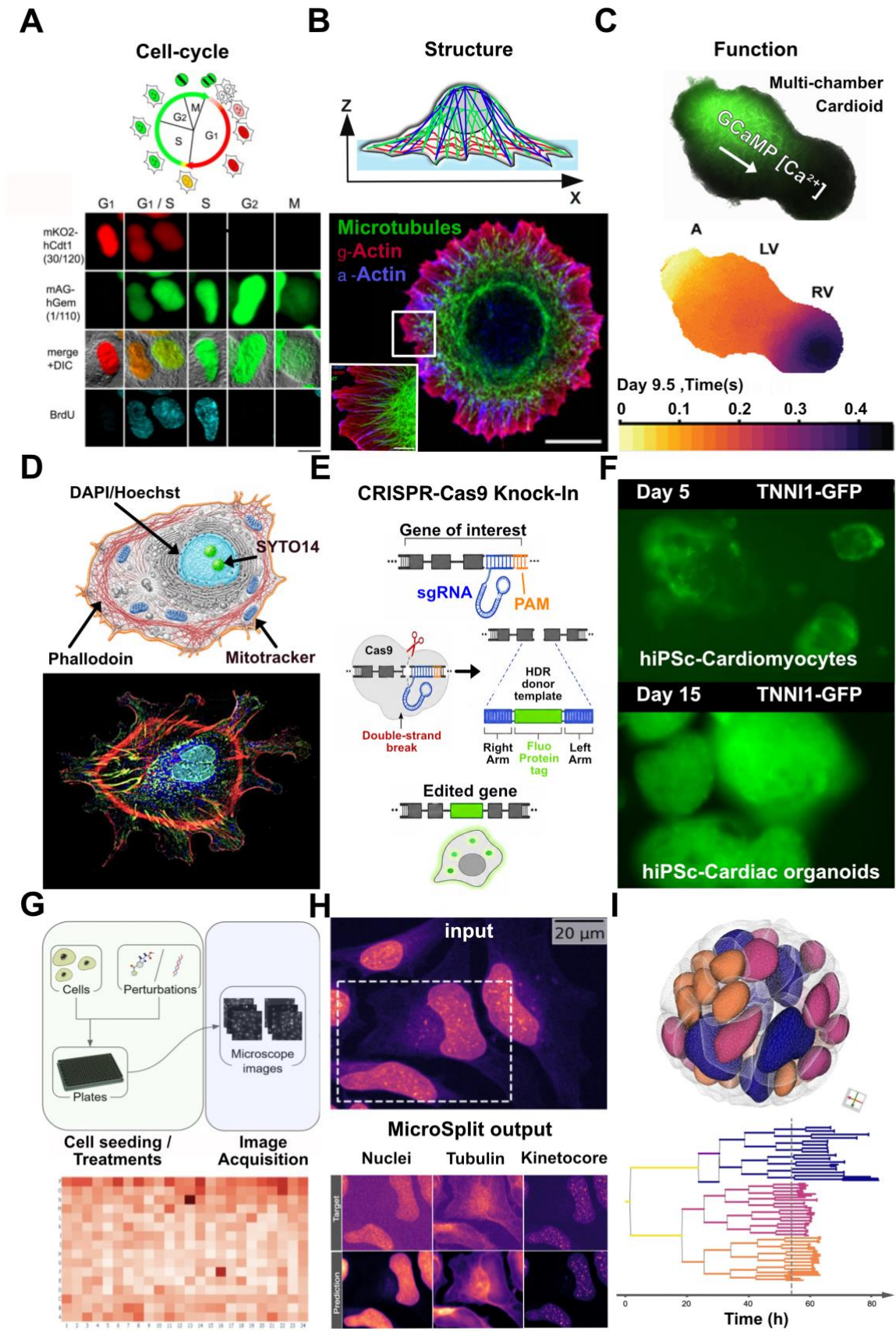


Figure 1.1. High-throughput and high-content phenotyping framework in cell biology

(A) Cell-cycle dynamics resolved by FUCCI reporters and BrdU incorporation. (B) Structural phenotyping of cytoskeletal organization with multicolor fluorescence imaging of microtubules and actin. (C) Functional readouts in 3D cardioids using GCaMP calcium reporters to track chamber-specific activity. (D) Multiplex labeling with classical dyes highlights nuclei, nucleoli, cytoskeleton, and mitochondria. (E) CRISPR-based genome editing enables endogenous tagging of proteins to systematically generate reporter cell lines. (F) Differentiation of hiPSC-derived cardiomyocytes and cardiac organoids visualized with TNNT1-GFP reporters. (G) High-throughput, high-content image-based screening workflow using ultra-high-throughput plate format. (H) Automated image segmentation (MicroSplit) extracts features of nuclei, tubulin, and kinetochores. (I) Multiscale light-sheet organoid imaging framework with segmented nuclei and cell meshes simultaneously visualized with lineage trees. Panels A-I were adapted or created from the indicated sources. Panel A adapted from Sakaue-Sawano, A. et al., “Visualizing spatiotemporal dynamics of multicellular cell-cycle progression,” *Cell* 132, 487-498 (2008), Copyright 2008, with permission from Elsevier. Panel B adapted from Dugina, V. et al., “Interaction of microtubules with the actin cytoskeleton via crosstalk of EB1-containing +TIPs and γ -actin in epithelial cells,” *Oncotarget* 7, 72699-72715 (2016); licensed under a Creative Commons Attribution license. Panel C adapted from Schmidt, C. et al., “Multi-chamber cardioids unravel human heart development and cardiac defects,” *Cell*, 186, 5587–5605.e27 (2023); licensed under a Creative Commons Attribution license. Panel D adapted from Chandrasekaran, S. N. et al., “Three million images and morphological profiles of cells treated with matched chemical and genetic perturbations,” *Nature Methods* 21, 1114-1121 (2024); licensed under a Creative Commons Attribution 4.0 license. Panel E was created by the authors. Panel F adapted from Prondzynski, M. et al., “Efficient and reproducible generation of human iPSC-derived cardiomyocytes and cardiac organoids in stirred suspension systems,” *Nature Communications* 15, 5929 (2024); licensed under a Creative Commons Attribution 4.0 license. Panel G adapted from Corbe, M. et al., “Transfer learning for versatile and training-free high content screening analyses,” *Scientific Reports* 13, 22599 (2023); licensed under a Creative Commons Attribution license. Panel H adapted from Ashesh, A. et al., “MicroSplit: Semantic Unmixing of Fluorescent Microscopy Data,” *Nature Methods* 23, 1047-1057 (2026); licensed under a Creative Commons Attribution 4.0 license. Panel I adapted from De Medeiros, G. et al., “Multiscale light-sheet organoid imaging framework,” *Nature Communications* 13, 4864 (2022); licensed under a Creative Commons Attribution 4.0 license.

1.2.1 The biological readout space: cell-cycle, structure, function

HT/HC imaging can generate thousands of image-derived features per cell. Across platforms, these measurements are most interpretable when organized around cell-cycle state, structure, and function (Fig. 1.1A-C).

Cell-cycle progression is a major source of latent heterogeneity in phenotypic assays because it reshapes nuclear architecture, chromatin state, and transcriptional programs. As a result, differences in morphology, motility, or signaling may reflect asynchronous progression rather than a true perturbation. High-content workflows, therefore, often incorporate cell-cycle analysis through two complementary strategies: endpoint markers and live reporters. Endpoint readouts infer phase from fixed-cell measurements, most commonly DNA content combined with replication or proliferation markers. EdU incorporation and markers such as Ki-67 remain widely used because they are scalable, relatively easy to standardize, and broadly applicable across cell types and assay formats, including settings where genetic engineering is impractical^{29,30}. Their limitation is that they provide only single-time-point phase assignment and do not resolve transition kinetics or phase durations within the same cell trajectory.

Live reporters instead convert the cell-cycle into a time-resolved coordinate that can be aligned with morphology and behavior^{31,32}. The canonical FUCCI system introduced by Sakaue-Sawano et al. uses mKO2-hCdt1(30/120) and mAG-hGeminin(1/110) to distinguish G1 from S/G2/M (Fig. 1.1A), with validation against BrdU incorporation³³. Later versions, including FUCCI4, PIP-FUCCI, and mVenus-p27K, expanded phase coverage and improved temporal precision from quiescence through mitosis^{34–36}. These reporters are especially useful for trajectory-level phenotyping and time-alignment of transient events, as shown in large-scale live-imaging efforts such as Mitocheck, although their performance remains constrained by reporter validation, tracking robustness, and photon dose^{10,31,37}. They also introduce practical costs, including genetic engineering, expression-dependent variability, additional spectral burden, and greater risk of phototoxicity under repeated acquisition^{38–40}. Together, endpoint and live strategies define a practical design space: endpoint assays maximize throughput and portability,

whereas live reporters preserve temporal continuity and support more mechanistic interpretation^{27,41}.

When nuclear cell-cycle reporters are used, they define cell-cycle timing, while cytoskeletal structural reporters report the spatial organization associated with the mechanical context in which that timing is phenotypically expressed (Fig. 1.1B)^{42,43}. This matters because many functional outcomes, including migration mode, division geometry, and mechanosensitive signaling, depend on the organization of the cytoskeleton, adhesions, and intracellular architecture^{44,45}. Among structural targets, F-actin and microtubules are especially informative because they report complementary aspects of cell architecture: actin regulates cortex tension, protrusions, and adhesion coupling, whereas microtubules contribute to polarity, intracellular trafficking, and organelle positioning^{42,46,47}. As with cell-cycle readouts, structural phenotyping can be performed in endpoint or live formats. Fixed-cell labeling provides robust, standardized maps suitable for large screens, whereas live structural labels track cytoskeletal remodeling over time. In both cases, the aim is to extract interpretable descriptors of shape, polarity, texture, and subcellular organization that can be compared across perturbations and aggregated from single cells to populations^{48,49}. For example, Dugina et al. showed that epithelial spreading involves complementary organization of β - and γ -actin, with microtubules aligning preferentially to γ -actin-enriched domains associated with stabilized spreading⁵⁰ (Fig. 1.1B). At finer scales, live-cell super-resolution with probes such as SiR-actin and SiR-tubulin can resolve filament bundling, alignment, and remodeling dynamics that are blurred in diffraction-limited imaging⁵¹. At screening scale, Cell Painting shows that multi-channel structural fingerprints can group compounds by mechanism, including perturbations targeting microtubule regulation^{11,52}. Structural phenotyping therefore links spatial organization to assay features with mechanistic interpretability.

Functional readouts become increasingly important as model complexity rises and gross morphology becomes less predictive. In 3D tissues, calcium transients, voltage propagation, and contractile force report how geometry, lineage patterning, and mechanics are integrated into physiological behavior^{53,54}. Cardioids provide a clear example: hiPSC-derived tissues self-organize chamber-like architectures, and exhibit

synchronized Ca^{2+} transients, where transient periodicity, rise and decay kinetics, and inter-region synchrony serve as quantitative proxies for maturation and coupling⁵³ (Fig. 1.1C). In this setting, morphology alone does not guarantee coordinated excitation-contraction, whereas calcium dynamics directly report whether the tissue behaves as a coupled electromechanical system. These phenotypes can be measured with genetically encoded indicators, which support longitudinal tracking, or with small-molecule dyes, which are easier to deploy in screening workflows. GCaMP and red-shifted variants such as jRGECO and jRCaMP support repeated calcium measurements across differentiation and maturation, whereas dyes such as Fluo-4 and Cal-520 provide strong signal-to-noise when genetic engineering is impractical^{55–57}. Voltage indicators such as Archon extend this logic to action-potential waveforms and propagation patterns, enabling quantification of activation timing, conduction heterogeneity, and spatiotemporal stability across tissues^{58–60}. Because these signals are fast and photophysics-limited, modality choice becomes part of the phenotype design itself. Consequently, fast volumetric imaging regimes, including light-sheet implementations, are increasingly used to preserve temporal bandwidth while limiting phototoxicity^{61,62}.

At a larger scale, platforms such as Heart Dyno demonstrate how functional phenotyping can support reproducible assay-level comparison by integrating optical dynamics with mechanical outputs across large organoid cohorts⁶³. Rather than reporting isolated traces, these systems assemble multi-metric readouts such as rhythm regularity, synchrony, beating robustness, and force-related proxies to map performance landscapes under perturbation or maturation cues. In engineered 3D tissues, morphology alone is often insufficient; therefore, functional imaging becomes necessary when the goal is to measure physiological competence or emerging behavior.

1.2.2 Labeling, reporters, and model systems as determinants of phenotypic readouts

Fluorescence phenotyping in HT/HC workflows cannot be determined by imaging alone. It is set upstream by how signals are encoded and by the biological context in which they are measured. Labeling and model choice sit upstream of the measurement itself: they determine whether the signal is trustworthy, how much the assay perturbs the sample, and how long the phenotype can be followed (Fig. 1.1D-F). These are not interchangeable implementation choices. They strongly condition which phenotypes can be measured with confidence.

At the simplest end of this design space, dyes and probes remain attractive because they are fast to deploy, broadly compatible with standard imaging workflows, and readily multiplexed^{4,11,64}. Their strength lies not only in convenience but also in portability. Cell Painting is the clearest example: a small set of inexpensive dyes labels nuclei/DNA, nucleoli/RNA, ER/Golgi, mitochondria, and actin/membrane compartments across five to six channels, generating dense morphological fingerprints that scale well across large screens^{1,4,11,64} (Fig. 1.1D). These profiles support more than organelle-level description⁶⁵. They can capture higher-order phenotypic structure, including proxies for cell-cycle progression, chromatin organization, and cytoskeletal remodeling, enabling perturbations to be grouped by mechanism rather than by single-marker response. This makes dye-based schemes well-suited to standardized endpoint profiling and rapid comparative screening. That same portability defines their limits. In live and repeated imaging, dye-based readouts can become less trustworthy because the label itself begins to shape the measured state. DNA-binding probes can increase phototoxic burden under repeated excitation, some live nuclear stains have been reported to interfere with normal cell-cycle progression and mitosis, and mitochondrial dyes can produce misleading intensity changes because their accumulation depends on uptake and membrane potential^{66,67}. Practically, dyes are effective when speed, standardization, and cross-assay transfer matter most, but they are less reliable when the phenotype depends on sustained observation with minimal disturbance.

When the assay requires longer temporal access or more specific molecular interpretation, genetically encoded and hybrid reporters become more compelling. Their main advantage is the stability of the assignment over time, in addition to specificity. Self-labeling systems such as Halo and SNAP, especially when paired with Janelia Fluor dyes, provide bright and photostable labeling of defined proteins while preserving more control over signal origin than many diffuse stains^{68–72}. Fluorescent-protein fusions similarly support persistent tracking of structures and processes that are difficult to capture with conventional probes, including spindle dynamics, mitochondrial remodeling, and ER organization. Functional reporters extend this logic further by converting activity into time-resolved optical signals: GCaMPs and jRGECO/jRCaMPs report calcium dynamics, ASAP- and Archon1-type sensors report membrane voltage, and FRET- or FLIM-based kinase reporters such as AKAR and EKAR convert signaling activity into measurable trajectories^{55,57,60}. These strategies offer greater temporal depth and mechanistic specificity, but only at the cost of more demanding construct design, validation, and optical compatibility.

Genome engineering addresses a related problem: not only how to label, but how faithfully the label reflects the native system. CRISPR/Cas knock-in strategies improve positional fidelity by inserting tags at endogenous loci, preserving native regulation, stoichiometry, and subcellular context more effectively than overexpression-based approaches^{73,74} (Fig. 1.1E). This matters when quantitative phenotyping is sensitive to dosage, localization, or protein complex balance. The OpenCell atlas illustrates the broader value of this approach by systematically tagging hundreds of human proteins with split mNeonGreen, it transformed endogenous labeling from a single-gene method into a scalable high-content microscopy resource, with utility for localization priors, segmentation benchmarking, and cross-protein comparison under perturbation⁷⁵. Newer frameworks such as CRISPaint and ORANGE further reduce the barrier to endogenous tagging by simplifying insertion design or extending accessibility to settings poorly served by homology-directed repair^{76,77}. Even so, endogenous tagging is not intrinsically neutral. Inserted tags can still disrupt folding, interactions, or function, so validation remains important to ensure that the resulting phenotypes are biologically trustworthy⁷⁸.

Lentiviral delivery occupies a different part of the same design space. Its appeal lies in speed, scalability, and combinatorial flexibility. Reporter populations can be generated rapidly across multiple cell types and experimental conditions, making lentiviral systems particularly useful when assay deployment matters more than exact locus control^{78–81}. The trade-off is that expression becomes harder to model cleanly. Insertion site, copy number, and cell-to-cell heterogeneity can all distort quantitative comparisons and complicate downstream interpretation⁸². Safe-harbor targeting and insulator strategies can reduce some of these positional effects, but they do not erase the underlying compromise⁸³. In practical terms, CRISPR knock-ins and lentiviral systems should not be treated as competing technologies so much as different design choices: one prioritizes positional fidelity and mechanistic control, the other rapid deployment and screening scalability. Hybrid strategies increasingly combine both, reflecting the need for reporter platforms that are mechanistically informative without becoming operationally prohibitive².

The same design logic applies at the level of the biological model (Fig. 1.1F). Phenotype is always measured in context, and model choice sets that context before any image is acquired. Immortalized cell lines, primary cells, and stem-cell-derived systems differ not only in biological realism, but in baseline phenotype, experimental tractability, accessible timescales, and analytical difficulty. Immortalized lines such as HaCaT and HT1080 remain indispensable for method development because they are robust, scalable, and genetically tractable, allowing standardized perturbations and large-n imaging^{84,85}. Their weakness is biased representativeness: fixed genetic lesions, such as TP53 H179Y/R282 in HaCaT or NRAS Q61K in HT1080, can reshape proliferation, stress response, and signaling, so observed phenotypes may reflect lineage-specific wiring rather than broader tissue principles^{86,87}. Primary cells often offer better preservation of lineage identity and native differentiation state, improving physiological interpretability, but this gain comes with reduced assay stability. Donor variability, limited expansion, and more difficult genetic manipulation all constrain throughput and reproducibility^{84,88}. hiPSC-based systems partly rebalance that trade-off by combining patient-linked genetics with expandable experimental platforms in 2D and 3D settings, making them attractive for disease modeling and systematic perturbation^{84,89,90}. Yet these systems introduce their own uncertainty, including immature functional states, batch variability during

differentiation, and, in larger 3D formats, diffusion-related transport constraints^{91–93}. Engineered culture formats and suspension-based systems can improve uniformity and support more reproducible tissue assembly, but greater physiological complexity often comes with reduced standardization, lower screening throughput, and more demanding imaging requirements^{94–97}.

Probes, reporters, engineering strategy, and model system determine what signal exists, how stable it is, how strongly it perturbs the specimen, and how far its interpretation can be generalized. The remaining challenge is computational: converting these assay-dependent signals into quantitative phenotypes that remain comparable across experiments.

1.2.3 Transforming multiplexed fluorescence data into quantitative HT/HC phenotypes

Multiplexed fluorescence supports high-content phenotyping only when the images can still be interpreted across conditions. The computational problem is measurement fidelity: segmentation, tracking, feature extraction, and representation must remain robust to heterogeneity, photophysics, and model complexity without severing the link to biology. In practice, this is what helps determine whether multiplexed imaging yields comparable phenotypes or merely more data (Fig. 1.1G-I). In 2D assays (Fig. 1.1G), analysis pipelines generally follow an established sequence: illumination correction and segmentation to identify biological objects, followed by feature extraction, normalization, and representation learning for comparison across perturbations. CellProfiler set a community standard by enabling handcrafted, interpretable morphology features through modular pipelines for segmentation and feature calculation that could be shared and reused across laboratories¹⁷. Latest works formalized an equally important preprocessing step: batch-aware normalization and correction, showing that without explicit management of plate and batch effects, phenotype patterns can be masked by acquisition artifacts rather than biological differences⁹⁸. Building on this operational framework, Pycytominer automated critical steps needed for scalable profiling and aggregation, enabling consistent generation of both single-cell distributions and well-level summaries for downstream statistical analysis and machine learning⁹⁹. Profiling also extends beyond average shifts:

distribution-aware metrics, such as Wasserstein distances, were introduced to preserve heterogeneity and improve phenotype detection when perturbations change variance, multimodality, or rare subpopulations, rather than only shifting the central tendency¹⁰⁰. More recently, representation learning has expanded this field: transfer-learned CNN embeddings can capture detailed phenotypic signatures from large cell datasets and have been reported to outperform handcrafted features in specific benchmarks and tasks such as mechanism-of-action prediction, while also reducing the need to predefine features¹⁰¹. As a comprehensive resource, the Cell Painting playbook combines standardized acquisition, preprocessing, and machine-learning workflows, addressing reproducibility by improving profile comparability across studies and locations^{1,11,102}.

As the biological question expands from one structure to many structures and functions, multiplex depth can become a major bottleneck (Fig. 1.1H). Physical and optical methods increase the number of distinguishable signals by using additional contrast dimensions beyond color alone. Techniques such as multi-excitation spectral imaging, optimized detector setups, linear unmixing, and fluorescence lifetime separation have pushed live-cell multiplexing toward the 8-10 channel range in selected implementations, by increasing the amount of distinguishable information obtainable within a given photon budget^{103,104}. Alongside hardware improvements, computational methods increasingly treat multiplexing as an inference problem: model-based unmixing algorithms separate overlapping spectra using calibrated system responses and priors on fluorophore behavior, enhancing accuracy when channels are not perfectly orthogonal¹⁰⁴. More radically, deep semantic unmixing reframes multiplexing as a prediction task, reconstructing multiple structures from limited measurements by learning statistical relationships between compartments, thereby increasing the effective channel capacity without additional optics. However, this approach requires careful validation to avoid introducing biological assumptions unsupported by the measurement¹⁰³. These methods show that multiplexing depends on optical design, inference strategy, and validation, rather than solely on the hardware itself.

For 3D organoids, the analysis challenge changes considerably: pipelines often need to scale not only in sample number but also in geometry, topology, and time (Fig. 1.1I). Modern frameworks therefore combine organoid-level segmentation with nuclear and

cellular segmentation and topology-aware feature extraction, enabling screening of thousands of 3D cultures while maintaining clear links between tissue architecture and cellular composition¹⁰⁵. Representative toolchains illustrate the increase in throughput: StarDist-3D workflows support high-volume analysis of around 10^5 organoids and over 10^6 nuclei, making volumetric phenotyping feasible at screening scales that were previously more common in 2D assays¹⁰⁶. Spatial “cartography” adds a layer that accounts for neighborhood effects by mapping epithelial morphology and polarity relative to local microenvironments, enabling spatially resolved phenotypes rather than global averages¹⁰⁷. In large-scale industrial applications, automated imaging pipelines combine standardized culture handling with analytics to reduce process variability and support systematic perturbation studies in complex 3D systems¹⁰⁸. Finally, multiscale descriptors can connect organoid architecture to function, such as linking structural organization to downstream physiological or activity readouts in brain organoid models, broadening phenotyping from morphology to a more comprehensive view of tissue state¹⁰⁹.

In these 3D-4D regimes, segmentation and tracking remain key rate-limiting steps: without consistent object definitions across space and time, feature extraction becomes unstable, and comparisons degrade. Generalist segmentation tools like StarDist-3D^{110,111}, Cellpose¹¹², and nnU-Net¹¹³ are increasingly used as shared infrastructure because they can adapt across datasets and imaging conditions when properly trained or tuned. For time-resolved systems, lineage-aware tracking platforms, such as TrackMate⁷¹¹⁴, ulTrack¹¹⁵, and ELEPHANT¹¹⁶, support tracking of hundreds of thousands of cells in 4D, enabling analysis of trajectory-level phenotypes and lineage-based statistics in organoids and tissues. The resulting outputs, typically organoid volume, cavity size, polarity indices, sphericity, and topology graphs, make morphogenesis and tissue assembly quantifiable and support comparative studies that relate perturbations to changes in both cellular states and overall architecture.

Multiplexed fluorescence supports high-content phenotyping only when biological intent, signal design, and computational extraction remain aligned. The value of these pipelines lies less in the number of measured variables than in whether they preserve interpretable links among state, structure, and function across scales and perturbations.

1.3 Live-cell imaging strategies for high-throughput and high-content phenotyping

Live-cell fluorescence imaging determines which phenotypes can be measured, at what resolution, for how long, and at what biological cost. Foundational work in optical microscopy established the coupled trade-offs among spatial resolution, temporal resolution, field of view, depth penetration, and photostability, showing that no single modality can optimize all of these simultaneously^{117–119}. In practice, improving one measurement dimension often compromises another, so imaging strategy must be matched to the biological process, sample geometry, and experimental timescale. In HT/HC settings, this problem becomes more demanding because the same workflow may need to accommodate monolayers, crowded 2.5D interfaces, and thick 3D organoids while maintaining sufficient signal, limiting dose, and preserving comparability across scales^{120,121}. Imaging regimes are best distinguished by the readouts they must preserve. Different phenotypes impose different observational burdens (Fig. 1.2A-C). Cell-cycle progression requires temporal continuity to resolve state transitions^{33,122}. Migration places greater emphasis on lateral coverage, population statistics, and sustained live compatibility¹²³. Morphogenesis in organoids and embryos typically requires volumetric imaging, where depth penetration and photon economy directly condition biological validity^{89,124}. Modality choice, therefore, reflects a trade-off among photon dose, temporal sampling, spatial resolution, depth access, and dependence on downstream computation (Fig. 1.2D-F). Within this space, widefield and spinning-disk confocal are often favored when speed and field size dominate, whereas light-sheet approaches become more suitable when long-term 3D viability and dose efficiency are limiting requirements^{125–127}. By the same logic, phototoxicity should be treated as an experimental variable, acquisition should be adapted to sample geometry and developmental stage, and computational restoration should be evaluated as part of the measurement system when information is recovered from low-photon data^{10,118,119,128,129} (Fig. 1.2G-I). Accordingly, the reviewed literature helps examine which imaging trade-off best preserves the phenotype, given the experimental readout to be uncovered.

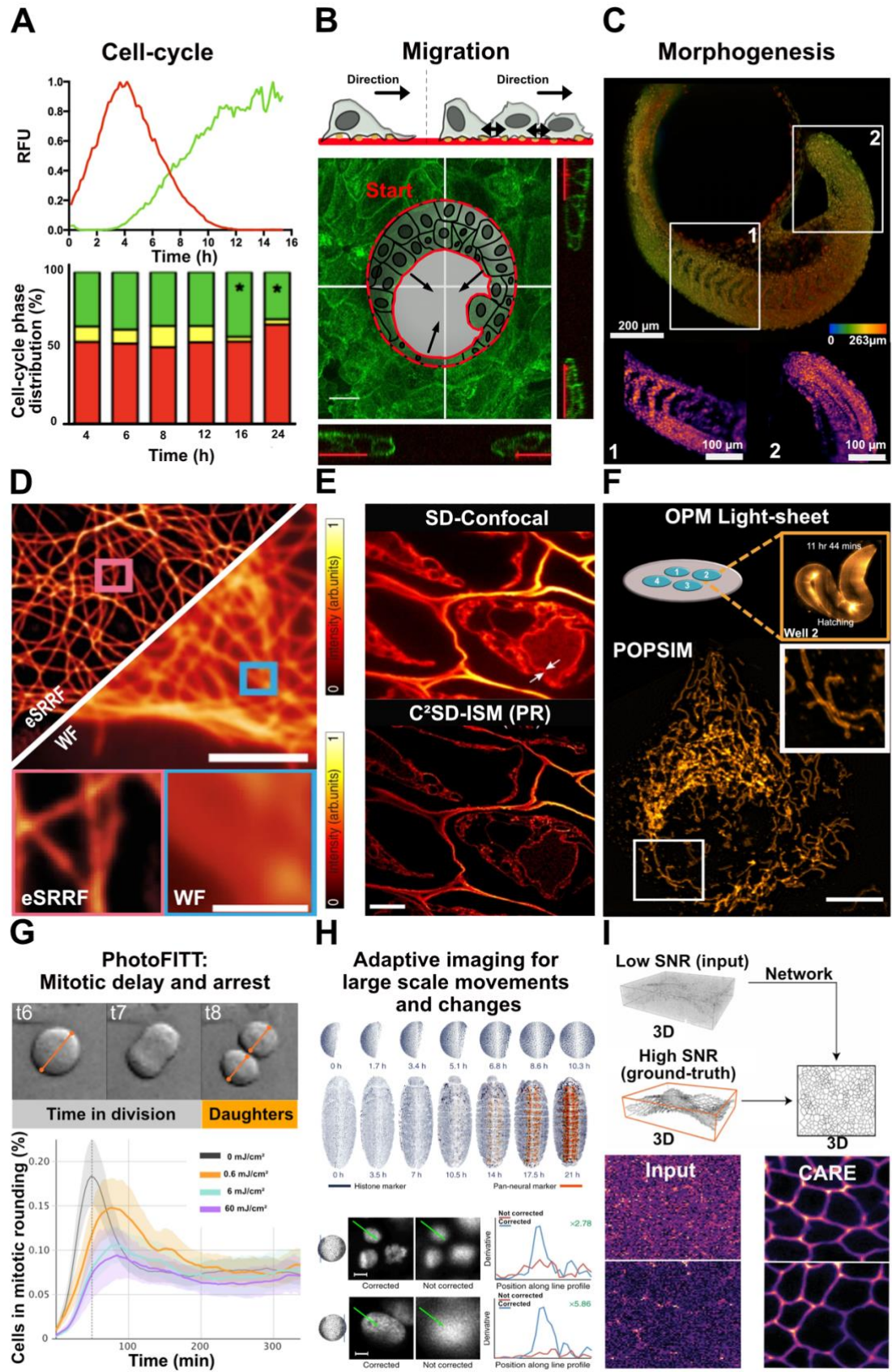


Figure 1.2. Live-cell imaging strategies for high-throughput and high-content phenotyping (A) Time-resolved FUCCI reporters quantify cell-cycle dynamics at single-cell and population levels. (B) Collective migration is visualized by tracking cells moving inward from a circular edge. (C) Morphogenetic remodeling captured in 3D organoid-like structures with high-resolution fluorescence imaging. (D) Subcellular architecture resolved with eSRRF processing compared to widefield. (E) Resolution enhancement of vascular-like networks with C²SD-ISM relative to spinning-disk confocal. (F) OPM light-sheet imaging adapted for multiwell acquisition enables long-term volumetric imaging, exemplified by embryo hatching, with POPSIM processing for enhanced detail. (G) The PhotoFiTT assay reveals mitotic delay and arrest with increasing light exposure, quantifying phototoxicity. (H) Adaptive light-sheet microscopy for long-term high-resolution imaging, enabling long-term developmental tracking with reduced perturbation. (I) Deep-learning-based denoising (CARE) restores high-resolution features from low-SNR input, expanding measurable information in live 3D imaging. Panel A adapted from Marcus, J. M. et al., “Longitudinal tracking of single live cancer cells to understand cell cycle effects of the nuclear export inhibitor, selinexor,” *Scientific Reports* 5, 14391 (2015); licensed under a Creative Commons Attribution 4.0 license. Panel B adapted from Vedula, S. R. K. et al., “Mechanics of epithelial closure over non-adherent environments,” *Nature Communications* 6, 6111 (2015); licensed under a Creative Commons Attribution 4.0 license. Panel C adapted from Yang, B. et al., “DaXi—high-resolution, large imaging volume and multi-view single-objective light-sheet microscopy,” *Nature Methods* 19, 461-469 (2022); licensed under a Creative Commons Attribution 4.0 license. Panel D adapted from Laine, R. F. et al., “High-fidelity 3D live-cell nanoscopy through data-driven enhanced super-resolution radial fluctuation,” *Nature Methods* 20, 1949-1956 (2023); licensed under a Creative Commons Attribution 4.0 license. Panel E adapted from Liang, Q. et al., “High-fidelity tissue super-resolution imaging achieved with confocal² spinning-disk image scanning microscopy,” *Light: Science & Applications* 14, 260 (2025); licensed under a Creative Commons Attribution 4.0 license. Panel F adapted from Chang, B.-J. et al., “Projective oblique plane structured illumination microscopy,” *npj Imaging* 1, 2 (2023); licensed under a Creative Commons Attribution 4.0 International License. Panel G adapted from Del Rosario, M. et al., “PhotoFiTT: a quantitative framework for assessing phototoxicity in live-cell microscopy experiments,” *Nature Communications* 16, 11401 (2025); licensed under a Creative Commons Attribution 4.0 license. Panel H adapted from Royer, L. A. et al., “Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms,” *Nature Biotechnology* 34, 1267-1278 (2016), reproduced with permission from Springer Nature Customer Service Centre GmbH. Panel I adapted from Weigert, M. et al., “Content-aware image restoration: pushing the limits of fluorescence microscopy,” *Nature Methods* 15, 1090–1097 (2018), reproduced with permission from Springer Nature Customer Service Centre GmbH.

1.3.1 Live-cell imaging readouts: proliferation, migration, and morphogenesis

Across HT/HC fluorescence assays, proliferation, migration, and morphogenesis impose distinct observational demands. Each readout class imposes a distinct imaging requirement, and its interpretation depends on preserving the corresponding biological features during measurement. Otherwise, phase-dependent behavior collapses into mixed-population averages, transient motility is reduced to net displacement, and developing structure is sampled as incomplete morphology. Accordingly, imaging regime selection is part of the biological problem because different regimes vary in how well they preserve the processes being measured.

Cell-cycle progression is a major source of latent heterogeneity in fluorescence phenotyping because it continuously reshapes transcriptional state, chromatin organization, cytoskeletal architecture, and mechanics. Without explicit phase assignment, structural and functional readouts are readily averaged across biologically distinct states. For this phenotype to remain interpretable, imaging must preserve temporal phase identity with sufficient resolution and acceptable spectral and phototoxic cost. FUCCI provided a practical solution by separating G1 from S/G2/M in living cells through anticorrelated reporter trajectories compatible with long-term imaging^{33,130,131} (Fig. 1.2A). FUCCI4 increased phase specificity by resolving all four phases, improving identification of G2-to-M progression and mitosis, but with greater spectral burden and stricter exposure constraints³⁴. PIP-FUCCI addressed the same interpretive need from another direction by sharpening S-phase boundary detection through replication-coupled degrons rather than additional color channels³⁵. These differences matter because therapeutic and environmental perturbations often produce phase-biased outcomes that disappear in population means. Gross et al. showed that targeted therapies generate distinct cell-cycle-resolved responses¹³², while Caicedo et al. showed that some cells arrest in G2 whereas others proceed through one final division or undergo apoptosis¹. Hunter et al. further showed that geometric confinement can alter cell-cycle progression without genetic change, and that detecting these shifts required long-term, low-dose 3D imaging rather than more perturbative acquisition strategies¹³³. In this setting, the

microscope must preserve phase-resolved temporal continuity well enough for downstream phenotypes to be assigned to the correct biological state.

Migration imposes a different burden. Here, the problem is the preservation of transient spatial dynamics across scales, from protrusion cycles in single cells to coordinated motion in collectives. Motility becomes mechanistically interpretable only if imaging resolves the events that generate displacement rather than displacement alone. At the single-cell level, this requires sufficient temporal sampling to retain protrusion-retraction dynamics, adhesion turnover, and shifting polarity. Barry et al. formalized this requirement with ADAPT, which extracts protrusion velocities, adhesion lifetimes, and shape dynamics from actin and focal adhesion reporters such as LifeAct, SiR-actin, paxillin-GFP, and vinculin-mApple¹³⁴. Bolado-Carrancio et al. showed that Rho-GTPase activity propagates as periodic waves organizing front-rear polarity, illustrating how easily oscillatory structure is lost under sparse sampling¹³⁵. In collectives, the requirement shifts toward low-dose imaging sustained over many hours so that coordination, force transmission, and signaling propagation can be followed without distorting the behavior being measured. Trepap et al. showed that traction forces arise several rows behind the leading edge, demonstrating that epithelial sheets migrate as mechanically coupled systems rather than as independent border cells¹²³. Hino et al. then visualized ERK waves propagating across monolayers over hundreds of microns, linking coordinated motion to long-range signaling dynamics¹³⁶. Migration assays also often couple fast cytoskeletal responses to slower behavioral consequences. Pittman et al. showed that lamellipodial ruffling acts as a mechanosensor of extracellular fluid viscosity, with only ruffling-competent cells increasing adhesion turnover and traction¹³⁷. In practical terms, interpretable migration imaging generally needs to preserve both rapid local dynamics and longer-timescale behavioral integration, ideally under assay geometries that reduce uncontrolled starting-state variability, as in photosensitized wound substrates with defined initial boundaries¹³⁸ (Fig. 1.2B).

Morphogenesis places the heaviest burden because it combines proliferation, migration, and signaling into a developing three-dimensional structure over days. Here, interpretability depends on preserving both cellular dynamics and tissue-scale geometry over prolonged observation, typically under stringent phototoxicity limits and with

sufficient volumetric fidelity for longitudinal reconstruction (Fig. 1.2C). Gastruloids, cardioids, and engineered epithelial organoids illustrate distinct developmental programs, but they converge technically in demanding structurally faithful imaging across multicellular length scales. Hamazaki et al. showed that RA-induced human gastruloids recapitulate posterior embryonic morphogenesis, including neural-tube-like structures and segmented somites under WNT/BMP modulation¹³⁹. Hofbauer et al. showed that cardioids self-organize chambers, lumenized cavities, and early cardiac patterning through mechano-chemical feedback⁵³. Mitrofanova et al. described a mini-colon platform in which chip-imposed biochemical gradients drive compartmentalized epithelial differentiation¹⁴⁰. What differs across these systems is not the need for longitudinal structural preservation, but the scale and mode at which it must be achieved. Gastruloids and cardioids undergo large geometric transformations, including axis elongation and chamber formation, and therefore require wide fields of view, gentle illumination, and uninterrupted imaging over 48-96 hours. More spatially organized epithelial systems may be more geometrically stable, but still require high-resolution 3D imaging and, often, tiling across patterned compartments. For high-throughput translation, platforms such as JeWells help standardize sample placement and parallelize light-sheet imaging across many organoids under comparable optical conditions^{141,142}. Light-sheet microscopy is well matched to these demands because selective plane illumination reduces bleaching and phototoxicity while preserving volumetric continuity over time, as established by Huisken and Stainier¹⁴³. Yang et al. extended this principle with DaXi, a single-objective light-sheet configuration that combines submicron lateral resolution with millimeter-scale coverage at low phototoxicity¹⁴⁴. Yet image acquisition alone does not secure interpretability in morphogenesis. That depends equally on computational continuity across denoising, segmentation, lineage mapping, surface reconstruction, and longitudinal atlas building, as shown in multiscale light-sheet workflows for developmental systems^{145,146}. In morphogenesis, the microscope often needs to preserve viability and image quality while maintaining geometric continuity over time.

These phenotype classes impose different observational burdens: cell-cycle readouts require temporal-state fidelity, migration requires preservation of transient dynamics, and morphogenesis requires sustained volumetric continuity under low perturbation.

1.3.2 Core imaging architectures for HT/HC live-cell phenotyping

For HT/HC live-cell phenotyping, widefield, spinning-disk confocal, and light-sheet microscopy occupy distinct operating regimes within a trade space defined by depth, speed, optical sectioning, field coverage, and photon burden (Fig. 1.2D-F). Their relevance depends on which combination of these factors best preserves the phenotype of interest with acceptable perturbation.

Widefield remains the baseline low-perturbation regime. In many long-term experiments, failure is driven less by instantaneous power than by cumulative dose effects, including bleaching, phototoxicity, and physiological drift. Laissue and colleagues argued that live imaging conditions should therefore be judged by explicit dose reporting and by biology-based sentinels such as mitotic fidelity, morphology stability, and function¹⁰. Within this framework, widefield fluorescence microscopy remains valuable because it provides a gentle route to sustained observation across large populations. Recent developments extend that role. ASTER improves quantitative consistency across larger fields by generating more uniform flat-top illumination and reducing hotspots¹⁴⁷. Widefield FLIM expands the same architecture toward functional readouts, including FRET, metabolism, and molecular crowding, by exploiting lifetime contrast rather than absolute intensity¹⁴⁸. At the high-information edge of this regime, computational super-resolution approaches such as enhanced super-resolution radial fluctuation microscopy (eSRRF; Fig. 1.2D) show that widefield-derived live-cell datasets can be pushed beyond the diffraction limit by extracting fluctuation-based information from image time series. This makes eSRRF a relevant example of how photon-efficient acquisition and data-driven reconstruction can extend live super-resolution while keeping the assay compatible with dynamic biological samples¹⁴⁹. Widefield detection is therefore most useful when prolonged observation, field size, and photon economy dominate the design problem.

Spinning-disk confocal occupies an intermediate regime in which out-of-focus background and axial discrimination matter, but the specimen still requires live-compatible speed and moderate dose. By parallelizing excitation and detection through pinholes and a camera, SDCM preserves optical sectioning without the full temporal and phototoxic penalties of point-scanning confocal microscopy. This makes it well suited to

dynamic 2D and 2.5D systems, crowded monolayers, patterned interfaces, and relatively thin multicellular constructs. Several developments extend its range. Increased inter-pinhole spacing reduces crosstalk, and two-photon implementations improve background rejection in scattering samples^{150,151}. Image-scanning extensions further increase structural detail while preserving live compatibility. Camera-based CSD-ISM modules and related protocols provide near-confocal sectioning with improved lateral resolution and signal-to-noise at rates still compatible with dynamic imaging^{152,153}. More recent variants, including confocal² and s²ISM (Fig. 1.2E), move this regime closer to fast super-resolved 3D imaging, in some cases with depth penetration and throughput relevant to organoids and multicellular tissues^{154,155}. In practice, SDCM is most effective when widefield no longer provides sufficient sectioning, but a fully volumetric light-sheet workflow is unnecessary or impractical.

Light-sheet microscopy defines the volumetric low-perturbation regime. When the biological problem requires repeated 3D acquisition over hours or days, and sample viability depends on minimizing out-of-plane excitation, light-sheet microscopy becomes especially effective. Its core advantage is dose geometry. By restricting excitation largely to the detection plane, it reduces the penalty associated with stack depth and field size and supports long-term imaging of fragile 3D systems such as organoids, cardioids, gastruloids, and embryos. SCAPE established this principle in a single-objective format by enabling rapid volumetric imaging without translating the sample mechanically, addressing a major limitation of classical SPIM geometries for moving specimens¹⁵⁶. As engineered tissues and organoid platforms became more important, sample geometry and mounting compatibility emerged as central constraints. OPM variants addressed this by bringing light-sheet-like sectioning onto conventional inverted platforms and into standard chamber formats while retaining high-NA detection and compatibility with microdevices and organoid workflows¹⁵⁷. Throughput then became the next bottleneck. JeWells-based and related pipelines showed that dense organoid cultures can be paired with automated 3D imaging to generate larger and more standardized live datasets¹⁰⁵. Resolution-enhanced variants such as OPSIM and POPSIM (Fig. 1.2F) extend the same regime when finer structural detail must be recovered without giving up the low out-of-focus excitation that supports long-term live imaging^{158,159}. Accessibility remains a practical limitation, which is why simplified and retrofit-friendly systems such as LITE

remain important despite lower ultimate performance¹⁶⁰. In this context, light-sheet microscopy appears especially useful when volumetric continuity and long-term 3D viability are experimental priorities.

These architectures differ mainly in the measurement constraints they handle best. Widefield is most effective for prolonged, low-perturbation observation across many cells. Spinning-disk confocal becomes advantageous when optical sectioning and moderate depth access are required without losing live compatibility. Light-sheet is best suited to phenotypes that are inherently volumetric and must remain measurable under dose budgets compatible with development, remodeling, or prolonged 3D viability. Across all three regimes, the performance is set by how much acquisition and computation are balanced against biological tolerance.

1.3.3 Adaptive acquisition and computational reconstruction in HT/HC live-cell imaging

Adaptive control and computational reconstruction become important once the biological space and imaging regime have been defined (Fig. 1.2A-F). In long-term live-cell imaging, the central problem is to preserve biological validity while extracting usable information from a limited photon budget. Three issues follow directly: phototoxicity should be measured rather than assumed, acquisition should be adapted to biological context, and reconstruction methods should be evaluated as part of the measurement system (Fig. 1.2G-I).

Long-term imaging is reliable only when illumination remains within the biological tolerance of the sample. Photobleaching reports fluorophore fatigue and phototoxicity reflects the more consequential failure mode in which excitation perturbs motility, cytoskeletal dynamics, and division before overt damage appears. Because bleaching and toxicity do not necessarily covary, and because toxicity can depend on irradiance as well as total dose, direct assessment is required. Laissue et al. argued that live imaging conditions should therefore be evaluated by explicit reporting of intensity, exposure, and biological sentinels such as mitotic fidelity, morphology stability, and function¹⁰. Subsequent studies support the same conclusion, showing that increased excitation can

suppress motility and induce oxidative stress responses even when endpoint failure is not yet evident. In HT/HC phenotyping, phototoxicity is therefore an experimental variable.

PhotoFiTT provides a practical implementation of this logic. Del Rosario et al. linked a dose-gradient design to automated extraction of toxicity-associated markers, including motility, persistence, division hazard, EB3 comet density, and morphology variance, directly from the fluorescence channels already used in the assay¹⁶¹ (Fig. 1.2G). By comparing these signatures across irradiance levels, PhotoFiTT estimates operational windows in which predefined biological behaviors remain stable within tolerance under the specific assay conditions tested. Its relevance for HT/HC workflows lies in its plate compatibility and intended use in organoid and gastruloid screening, where dose assessment should be integrated into acquisition design rather than treated as a one-off calibration step. Exposure is therefore not simply chosen and assumed to be safe; here, it is tested against measurable biological stability criteria.

Sampling imposes the next constraint. Imaging too slowly misses rare or transient events; imaging too quickly increases phototoxicity. Adaptive imaging addresses this by making acquisition conditional. A low-dose survey is acquired first, analyzed in real-time, and intensified locally only when informative events occur. This can involve higher frame rates, finer resolution, or a shift in modality. Early smart-microscope concepts established this logic by coupling analysis to acquisition control¹⁶². A key example is the AutoPilot framework introduced by Royer et al., which continuously optimized light-sheet and detection-plane alignment during long-term *in vivo* imaging. By adapting the microscope to large-scale morphogenetic changes in the developing embryo, AutoPilot improved resolution and signal strength while reducing unnecessary exposure and preserving volumetric fidelity (Fig. 1.2H)¹¹⁹. Subsequent developments extended this principle. Daetwyler and Fiolka showed that adaptive light-sheet imaging can correct sheet alignment, focus, and cadence during embryo growth, reducing dose while preserving volumetric fidelity¹⁶³. André et al. extended the same principle through data-driven microscopy, in which survey images are analyzed on the fly to identify informative cells or regions for higher-resolution capture¹⁶⁴ (Fig. 1.2H).

Event-driven implementations show how this strategy expands measurable phenotypes. Mahečić et al. coupled neural-network detection to instant SIM, allowing low-dose

monitoring until mitochondrial or bacterial division triggered fast SIM bursts¹⁶⁵. Alvelid et al. used the same logic in STED, restricting high-intensity illumination to brief Ca^{2+} flashes or protein-recruitment events¹⁶⁶. Ibrahim et al. extended escalation across modalities by predicting aggregation onset from fluorescence cues and triggering Brillouin imaging when mechanical transformation began¹⁶⁷. In smart lattice light-sheet microscopy, Shi et al. combined rapid 2D survey imaging with event-triggered 3D lattice light-sheet bursts to capture rare dynamics such as spindle assembly and immune-synapse formation across large populations without compromising long-term viability¹⁶⁸. Together, these studies show that adaptive acquisition can reduce redundant exposure while improving access to rare and transient biological events.

Adaptive acquisition redistributes photon use but does not eliminate the underlying limit. Computational reconstruction addresses the same constraint from the analysis side by recovering usable information from low-dose or low-information measurements. Its value lies in extending the usable imaging regime when direct acquisition is limited by dose, speed, or scanning burden. CARE and VsLFM are representative examples, but their value depends on whether the recovered information remains biologically trustworthy. In dose-reduction workflows, CARE restores subcellular detail from low-dose input data and can extend experiment duration when illumination must remain gentle (Fig. 1.2I). Trained on paired examples, CARE suppresses noise and can recover near-isotropic 3D structure from under-sampled stacks. Across widefield, confocal, and light-sheet datasets, Weigert et al. reported dose reductions of 10–60 $\times$ in specific benchmarks while maintaining downstream segmentation and tracking performance¹²⁹. Snapshot volumetric methods push the same logic further by recovering 3D information without z-scanning. VsLFM combines light-field optics, a dedicated deep-learning engine, and digital adaptive optics to reconstruct high-resolution volumes from a single snapshot. Lu et al. reported diffraction-limited reconstructions at hundreds of volumes per second in systems such as beating zebrafish hearts, *Drosophila* brains, and migrating immune cells, where mechanical scanning would either bleach the sample or miss transient events¹⁶⁹.

These approaches are defensible when validation remains explicit. Belthangady and Royer outlined the main rules for deep-learning-based reconstruction, including documentation of training data, testing under unseen conditions, reporting of failure modes, and, where possible, estimation of uncertainty¹⁷⁰. Without those controls,

reconstruction can generate false structural confidence and weaken interpretation. The critical distinction is therefore not raw versus enhanced data, but validated versus unvalidated inference.

Adaptive acquisition and computational reconstruction both extend what can be measured under limited photon budgets. The first concentrates exposure on informative events, while the second recovers signal from sparse or low-dose data. In both cases, the gain is defensible only when it remains tied to explicit dose conditions, biological sentinels, and validated operating limits. Live-cell phenotyping therefore depends on treating dose, sampling, and inference as coupled parts of experimental design.

1.4 Engineered microenvironments and external modulation

Engineered microenvironments (EM) define the external boundary conditions under which phenotype is expressed and measured in HT/HC workflows. In practice, geometry, mechanics, and external modulation act as coupled control variables that shape adhesion, force transmission, signaling, and state transitions^{171–173}. Through biomaterials, microfabrication, and microfluidics, these approaches can control extracellular matrix (ECM) identity, stiffness, viscoelasticity, topography, soluble-factor gradients, and ECM-to-cell or cell-cell interactions. By specifying these external variables, they delimit the accessible space of cell behavior, state, and fate, and support reproducible, high-throughput phenotyping through scalable fabrication and photo-functionalization strategies^{174–176}.

Platforms differ primarily in how much control they provide over phenotype evolution. Some systems standardize spatial and mechanical starting conditions, some make them digitally specifiable and easier to remodel, and some enable direct perturbation during ongoing biological change. This progression is relevant across cell-cycle control, guided migration, and morphogenesis in 2D and 3D systems (Fig. 1.3A-C). It also motivates the fabrication and control strategies used to build these platforms, from 2D patterning methods such as soft lithography to nonlinear 3D fabrication approaches that support volumetric structuring and modulation within living matrices (Fig. 1.3D-F)^{177–179}. In addition, microfluidic implementations extend this control to delivery, residence time, and treatment exposure in screening-oriented workflows¹⁸⁰.

As with imaging regime selection, engineered control is most informative when its effects remain quantitatively interpretable. Perturbation design should therefore remain linked to readout and analysis through imaging, segmentation, tracking, feature extraction, and batch-aware pipelines that preserve comparability across experiments^{1,181–183} (Fig. 1.3G-I).

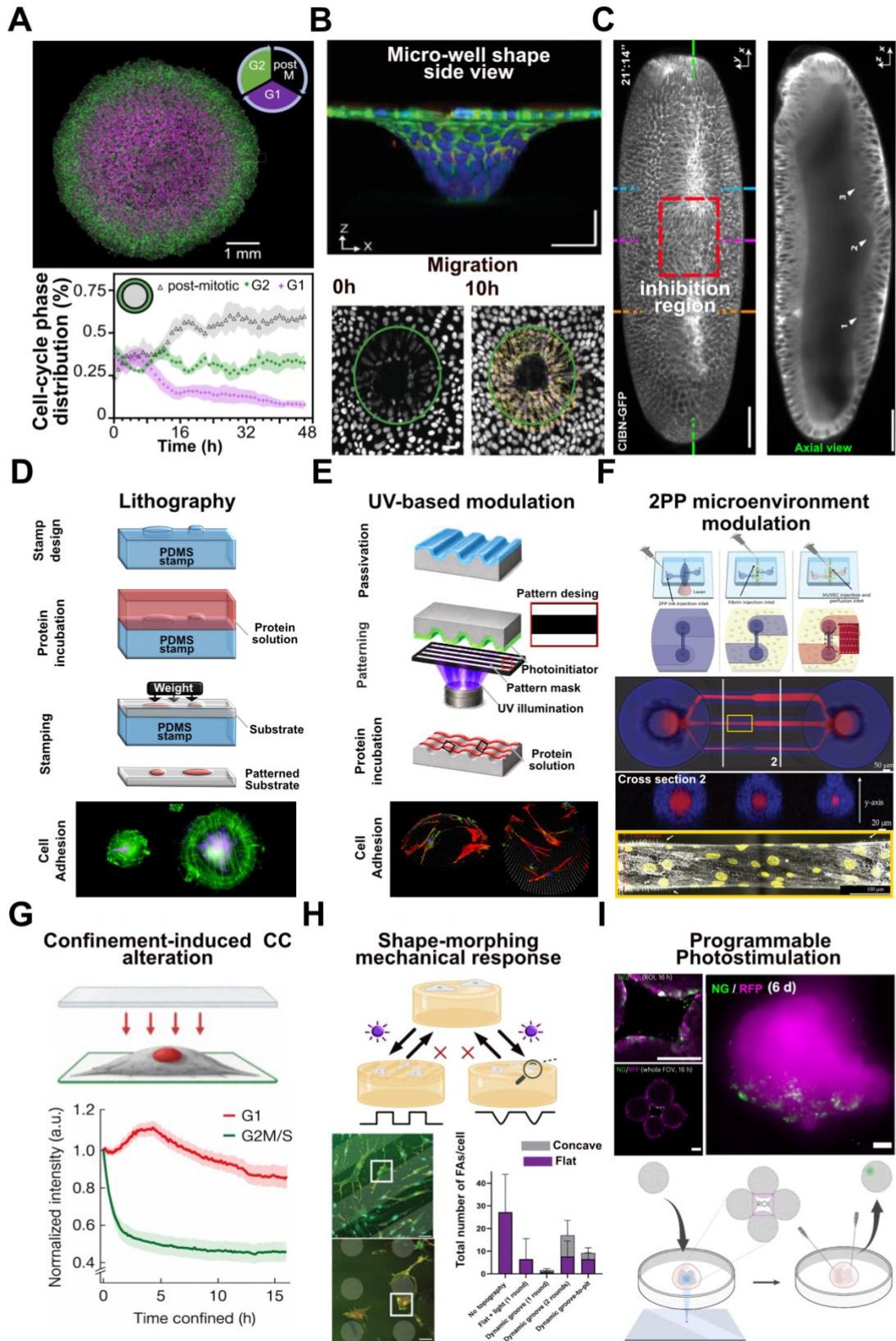


Figure 1.3. Engineered microenvironments and external modulation of cell behavior

(A) Cell-cycle dynamics in confined spheroids tracked with FUCCI reporters. (B) Microwell geometry influences migration, with cells adapting to curvature and moving inward over time. (C) Localized optogenetic inhibition in embryos indicates spatial control of morphogenesis. (D) Lithography-based protein stamping of ECM patterns for controlled cell adhesion. (E) UV-based photopatterning enables the creation of user-defined adhesion sites with high spatial precision. (F) Two-photon modulation sculpts collagen hydrogel microvasculature model by combining the precision of 2PP and the cell compatibility of hydrogel casting (G). By linking mechanics to a cell-cycle biological readout, researchers showed that confinement alters cell-cycle (CC) progression. (H) Shape-morphing substrates reveal mechanical feedback on focal adhesion formation. (I) Spatially targeted photostimulation at the organoid scale. Panels A-I were adapted from the indicated sources. Panel A adapted from Heinrich, M. A. et al., "Size-dependent patterns of cell proliferation and migration in freely-expanding epithelia," *eLife* 9, e58945 (2020); licensed under a Creative Commons Attribution 4.0 license. Panel B adapted from Luciano, M. et al., "Mechanoresponse of Curved Epithelial Monolayers Lining Bowl-Shaped 3D Microwells," *Advanced Healthcare Materials* 13, 2203377 (2024), with permission from John Wiley and Sons. Panel C adapted from De Medeiros, G. et al., "Cell and tissue manipulation with ultrashort infrared laser pulses in light-sheet microscopy," *Scientific Reports* 10, 1942 (2020); licensed under a Creative Commons Attribution 4.0 license. Panel D adapted from Théry, M., "Micropatterning as a tool to decipher cell morphogenesis and functions," *Journal of Cell Science* 123, 4201-4213 (2010), adapted with permission from The Company of Biologists. Panel E adapted from Werner, M. et al., "Surface curvature differentially regulates stem cell migration and differentiation via altered attachment morphology and nuclear deformation," *Advanced Science* 4, 1600347 (2017); licensed under a Creative Commons Attribution 4.0 license. Panel F adapted from Cantoni, F. et al., "A perfusable multi-hydrogel vasculature on-chip engineered by 2-photon 3D printing and scaffold molding to improve microfabrication fidelity in hydrogels," *Advanced Materials Technologies* 9, 2300718 (2024); licensed under a Creative Commons Attribution 4.0 license. Panel G adapted from Hunter, M. V. et al., "Mechanical confinement governs phenotypic plasticity in melanoma," *Nature* 647, 517-527 (2025); licensed under a Creative Commons Attribution 4.0 license. Panel H adapted from Bril, M. et al., "Shape-Morphing Photoresponsive Hydrogels Reveal Dynamic Topographical Conditioning of Fibroblasts," *Advanced Science* 10, 2303136 (2023); licensed under a Creative Commons Attribution license. Panel I adapted from Legnini, I. et al., "Spatiotemporal, optogenetic control of gene expression in organoids," *Nature Methods* 20, 1544–1552 (2023); licensed under a Creative Commons Attribution 4.0 license.

1.4.1 EM's readout space: cell-cycle gating, guided migration, and morphogenetic control

Engineered microenvironments become more mechanistically informative when they impose defined geometric, adhesive, and mechanical starting conditions on cell behavior. In this context, three readout axes are especially useful: cell-cycle gating, guided migration, and morphogenetic organization. Together, they capture how external constraints shape proliferative decisions, movement, and multicellular architecture^{184–187}.

In 2D, geometry and adhesion directly influence proliferation. Chen et al. showed that cell spreading regulates survival and proliferation, identifying adhesion as a mechanical input into growth control rather than passive anchoring¹⁸⁵. Zhu et al. further linked adhesion to G1 progression by showing that it is required for Cyclin D1/E induction and Rb phosphorylation, thereby connecting adhesive state to canonical G1-S commitment¹⁸⁸. Geometry also affects how division is deployed in space. Théry and colleagues showed that extracellular matrix layout guides spindle orientation, so that division axes align with adhesive cues and influence daughter-cell positioning and tissue organization^{179,189}. In practice, micropatterns can therefore be used to probe both proliferation and division orientation, especially when paired with live cell-cycle reporters such as FUCCI that resolve G1 versus S/G2/M occupancy over time. This logic extends to collective growth under confinement. Heinrich et al. showed that expanding epithelia develop size-dependent internal patterns in which edge and interior dynamics decouple reproducibly¹⁹⁰ (Fig. 1.3A), and Devany et al. later showed that confinement can inhibit growth and induce compensatory behaviors, including volume-reducing divisions¹⁹¹.

In 2.5D systems, the dominant readout shifts from proliferation to migration mode. Microtopographies, curvature fields, stiffness gradients, partial confinement, and microwells reorganize polarity and cytoskeletal architecture, remaining experimentally reproducible. Isenberg and colleagues showed that the strength of durotaxis depends on gradient steepness rather than absolute stiffness alone, making stiffness a tunable parameter for migratory selection¹⁹². Hartman et al. added that this response depends on matrix composition, with robust migration bias on fibronectin but not necessarily on laminin¹⁹³. Curvature acts as a second guidance variable. Vassaux and colleagues

described curvotaxis with a physical model in which cells bias migration across curvature landscapes, often toward concave features¹⁹⁴. In bowl-shaped hydrogel microwells, Luciano et al. showed that edge curvature increases centripetal alignment, straightness, and speed, whereas Gaussian curvature promotes supracellular actin organization, vertical nuclear reorientation, and chromatin compaction¹⁹⁵ (Fig. 1.3B). Topography can further scale local guidance into collective order: Leclech's work demonstrated that grooves induce long-range alignment and laning-like collective migration¹⁹⁶. More recently, Kalukula et al. showed that repeated confinement can stabilize a persistent mechanical state, with the actin cortex storing a form of morphological memory that affects future migration behavior¹⁹⁷.

In 3D systems, the same design logic extends from guided behavior to morphogenesis. Here, boundary conditions influence proliferative expansion, stress accumulation, curvature, and tissue rearrangement simultaneously. Di Meglio et al. showed that epithelia grown under confinement accumulate pressure that inhibits proliferation above a threshold, directly linking mechanical state to cell-cycle regulation in 3D¹⁹⁸. Karzbrun et al. further showed that imposed geometry and domain coupling determine folding fidelity and morphogenetic outcome in neural tube-like systems, demonstrating that organ-on-chip constraints can prescribe rather than merely bias tissue organization¹⁹⁹. Optical perturbation studies support the same conclusion at tissue scale: optogenetic control of contractility or Rho signaling enables spatially restricted manipulation of morphogenetic movements, whereas laser ablation reveals pre-existing tension through recoil and remodeling analysis^{200–203} (Fig.1.3C). In this setting, engineered constraints define the conditions under which tissue organization can be stabilized, redirected, and tested.

Across these examples, EM act as boundary-condition systems. In 2D, patterned adhesion and geometry regulate cell-cycle progression and division orientation. In 2.5D, stiffness, curvature, and topography affect migration modes and, in some cases, stabilize persistent mechanical states. In 3D, imposed constraints control how proliferation, stress, and geometry interact during morphogenesis. These boundary conditions are shown to be biologically important: an open challenge is to determine how precisely they can be specified, reproduced, and remodeled during experiments.

1.4.2 Fabrication toolkits and light-enabled EM's control

Fabrication strategy determines how precisely boundary conditions can be imposed, reproduced, and modified in EM^{204,205}. Different fabrication approaches provide different levels of control over the initial state and its later evolution²⁰⁶. Microcontact printing stabilizes initial adhesion geometry and standardizes the conditions from which spreading, polarity, traction, and fate decisions emerge (Fig. 1.3D)^{179,204,207}. Light-based patterning extends this control by allowing boundary conditions to be specified digitally, revised rapidly, and, in some cases, distributed in three dimensions (Fig. 1.3E)^{208,209}. More advanced optical implementations go further by reshaping matrix architecture, altering local mechanics, or modulating cell-state programs after the experiment has begun (Fig. 1.3F)^{209–211}.

Microcontact printing (μ CP) remains the practical baseline for controlling 2D starting conditions in mechanobiology. By transferring ECM proteins onto defined regions while passivating the surrounding surface, it produces reproducible adhesion geometries that constrain cell spreading and organization^{179,204,212}. Protocols such as those described by Théry and Piel helped establish μ CP as a robust and scalable platform for standardizing experimental input¹⁷⁹. Its main strength is reproducibility: islands, lanes, rings, and junctions can be generated at scale and used to isolate geometric effects from other variables. McBeath et al. showed that restricting spreading and contractility influences RhoA/FAK-associated signaling and mesenchymal stem cell lineage decisions, while Fu and coworkers combined micropatterning with micropost arrays to separate adhesion geometry from force generation and thereby map traction under controlled boundary conditions^{207,213,214}. Its main limitation is that the pattern is fixed once fabricated, making μ CP effective for standardizing the initial state but not for modifying it during an experiment^{179,209,210}.

Light-based patterning replaces static replication with digital specification. In LIMAP, projected UV illumination locally removes passivation and defines where proteins are immobilized, making adhesive layouts software-defined rather than stamp-defined²¹⁵ (Fig. 1.3E). DMD-based implementations such as PRIMO extend this logic by allowing patterns to be drawn, revised, and iterated rapidly, with greater flexibility for gradients,

multi-protein arrangements, and complex geometries than conventional stamping. This makes boundary conditions easier to tune, align, and adapt to imaging or screening workflows. Van der Putten and colleagues extended this approach to structured 2.5D surfaces, showing that biochemical patterning can be combined with topographic constraint in the same system²¹⁶. In 3D, the same framework can be used to localize bioactive cues within living matrices. Batalov et al. demonstrated covalent immobilization of proteins within cell-laden hydrogels, showing that light-based patterning can specify cues throughout matrix volume rather than only at exposed interfaces²¹⁷. These methods expand experimental flexibility, but they also introduce photochemistry, alignment accuracy, exposure dose, and batch variability as variables that must be controlled explicitly^{218–219}.

Optical methods are also used to intervene after self-organization has begun. Enrico et al. used femtosecond-laser cavitation in collagen to generate stable channels and cavities within living matrices, showing that ECM architecture can be remodeled locally without external molds²²⁰. A parallel two-photon polymerization approach is also presented by Martinelli et al. to write defined 3D microarchitectures inside a perfused, imaging-accessible microphysiological device, providing a complementary route based on direct fabrication rather than local remodeling²²¹ (Fig. 1.3F). At the biological level, optogenetic systems extend this control from material architecture to cell-state regulation by allowing illumination to trigger signaling or mechanically relevant pathways in defined regions²²². Laser ablation offers a different kind of intervention by disrupting junctions or tissue regions and measuring recoil, thereby converting light into a direct probe of pre-existing force transmission²²³. These approaches differ from μ CP and digital patterning in that perturbation is introduced during phenotype evolution rather than at the start of the experiment.

These fabrication strategies differ primarily in when and how boundary conditions can be specified or revised. μ CP standardizes the starting state, light-based patterning makes it digitally specifiable and partly reconfigurable, and optical actuation enables localized intervention during ongoing phenotype organization.

1.4.3 Dynamic microenvironment actuation and perturbation for causal phenotyping

EMs become especially informative when boundary conditions can be perturbed during phenotype evolution rather than fixed at the start. In this setting, the key advantage is causal testing: perturbation-response experiments in which modeling, fabrication, imaging, and, in some cases, photomodulation are combined within the same workflow²²⁴. This experimental design is particularly useful when variables such as shear stress, stiffness distributions, or transport-driven gradients are difficult to measure directly, because simulation-informed design can convert them into quantitative and testable boundary conditions^{225–228}. These systems are most valuable when they link ECM mechanics, cytoskeletal force generation, and downstream nuclear or transcriptional states within the same experimental framework^{133,208,229}.

On the ECM-to-cell side, several studies illustrate this causal logic. Théry et al. showed that spindle orientation can be quantitatively explained by cortical force generators that respond to geometric and adhesive constraints, making the microenvironment an instruction layer for division orientation rather than a passive setting¹⁸⁹. Li et al. then linked engineered 3D geometry to nuclear deformation and transcriptional regulation by showing that cytoskeletal reorganization and local compressive forces can stretch nuclear pores and enhance YAP nuclear import in prism- and cuboid-shaped micropatterns²³⁰. Wang et al. extended this mechanotransduction axis by showing that stiff ECM drives nuclear flattening and promotes a mechanically grounded mode of nuclear regulation²³¹. Together, these studies demonstrate how geometry, adhesion, and mechanics are translated into intracellular decision-making. More directly, Hunter et al. showed that mechanical confinement can govern phenotypic plasticity in melanoma, highlighting how physical boundary conditions can regulate cell state and phenotype under constrained microenvironments¹³³ (Fig. 1.3G).

Since these readouts should be measured within the same sample, the bottleneck often shifts from biology to workflow integration. Dynamic microenvironments are more difficult to reproduce consistently when fabrication, positioning, stimulation, and imaging are handled through separate workflows. A more scalable solution is to incorporate fabrication into the imaging pipeline, ensuring that geometry, perturbation, and

measurement are part of a single experiment^{224,232}. Toro-Nahuelpan et al. demonstrated this by extending photo-micropatterning to EM grids, which improved control over cell attachment points and allowed for more systematic targeting in downstream cryo-EM and cryo-ET workflows²³². At the material level, Bril et al. showed that shape-shifting photoresponsive hydrogels can be reconfigured on demand using localized blue light, enabling repeated and timed changes in topography during live imaging²³³ (Fig. 1.3H). These examples are valuable because they maintain spatial control and experimental traceability of perturbations while the phenotype continues to evolve.

A further step is to make stimulation computationally controllable. In that regime, perturbation is no longer fully pre-scripted: it can be updated according to the evolving state of the sample under defined measurement constraints. The limiting factor then becomes the control infrastructure, including segmentation, tracking, device orchestration, and feedback logic²²⁴. MicroMator provides a representative example. Fox et al. developed it as an open platform for reactive microscopy, with modular event definitions, trigger-effect logic, and real-time feedback for illumination control²²⁴. Legnini et al. extended this direction to organoids by demonstrating programmable spatiotemporal optogenetic stimulation through live “printing” of illumination patterns, enabling targeted perturbation as tissues move and reorganize²³⁴ (Fig. 1.3I). These systems point toward closed-loop stimulation workflows in which targets are updated dynamically rather than predefined once.

What distinguishes these platforms is the shift from static culture control to programmable, time-dependent causal testing. Some systems primarily standardize boundary conditions, while others introduce dynamic environments and track perturbation-response relationships as cell and tissue states change. The most advanced platforms allow intervention during this process, enabling direct provocation and measurement of state transitions. For HT/HC phenotyping, the goal remains the same: readout, imaging, perturbation, and analysis must be closely integrated so that causal conclusions are both interpretable and scalable.

1.5 Discussion and outlook

In this chapter, we reviewed the recent evolution of HT/HC live-cell phenotyping across assay design, imaging, computation, and EM. Three points follow from this review. First, major advances in individual components have not, on their own, resolved many workflow-level challenges in HT/HC phenotyping. In many practical settings, the main limitation is no longer the absence of assays, imaging modalities, or analysis tools, but their integration into coherent workflows. Phenotyping quality depends less on the strength of any single component than on how readouts, acquisition, perturbation, and downstream analysis are assembled into a controlled and documented measurement workflow. The literature reviewed here suggests that reproducibility depends on metadata, QC, and analysis provenance as well as on optical performance and image quality: standards such as REMBI, OME/Bio-Formats, and QUAREP-LiMi are valuable because they help make workflows more auditable, transferable, and interpretable across instruments, sites, and biological models^{10,20,25,118,235–238}.

Second, in live-cell phenotyping, measurement is often itself an intervention. Photobleaching and phototoxicity are not background nuisances, but experimental constraints that can alter mitosis, migration, cytoskeletal organization, and transcriptional state before overt cell death is observed^{10,118}. Acquisition settings therefore cannot be interpreted independently of phenotype, and imaging conditions are most defensible when chosen against measurable biological tolerances rather than assumed safety margins. In practice, photon budget links multiple workflow layers at once: it constrains modality choice, sampling strategy, QC, and downstream interpretation. The literature reviewed in this article suggests that live-cell imaging is more reliable when dose, sampling strategy, and biological validation are designed together.

Third, scaling computation does not eliminate the need for experimental control: it shifts more of that burden to validation, versioning, and monitoring. Event-based and conditional microscopy can allocate photons more efficiently by increasing sampling during informative events, while restoration and reconstruction methods can expand what is measurable at low-dose^{129,165,169,239,240}. Generalist deep-learning models have also lowered barriers to segmentation and tracking, but they make workflow reliability more

dependent on verification under changing imaging conditions^{241,242}. From a workflow perspective, algorithms should therefore be treated as calibrated components whose versions, scope, and failure modes remain visible if phenotypic outputs are to retain biological meaning.

Despite the substantial progress reviewed here, unresolved constraints in dose comparability, interoperability, AI generalizability, operational accessibility, and transferability across model complexity continue to limit comparability across HT/HC studies. These limitations help explain why closed-loop phenotyping remains promising but is still in an emerging phase. Conditional microscopy and state-dependent control strategies show that perturbations and acquisition can, in principle, be coupled to measured biological state. However, this remains an emerging operational mode, and its broader value will depend on whether state estimation, QC gating, and perturbation provenance become robust enough for routine use.

Based on these studies, we propose five practical design rules for HT/HC live-cell phenotyping workflows, which informed the best practices adopted throughout the experimental work in this thesis.

- **Define phenotype coordinates before choosing tools.** Phenotype axes should be declared before reporters, modalities, perturbations, or computational outputs are selected.
- **Treat photons as a budget, not a setting.** Illumination should be handled as a constrained resource tied to biological tolerances.
- **Use multiplexing only when it increases inference.** Additional channels, restoration steps, and deep-learning layers should be included only when they materially improve interpretability or decision-making.
- **Map perturbations in space, time, and dose if causal claims are intended.** Perturbations strengthen causal interpretation when their delivery, timing, and intensity can be explicitly linked to the measured phenotypic trajectory.
- **Adopt minimum standards from the start.** Acquisition metadata, calibration and QC outputs, dose descriptors, and analysis provenance should accompany the dataset from the beginning rather than being reconstructed retrospectively.

In addition, the literature reviewed here highlights recurring gaps in metadata, dose reporting, QC, and analysis provenance that limit interpretability beyond the local conditions in which datasets are generated. In response to those recurring limitations, we propose a minimum operational standard for HT/HC live-cell phenotyping datasets. In our view, four types of information should be routinely recorded and retained: acquisition and sample metadata, photon-dose descriptors, QC gates, and analysis provenance. At the reporting level, these elements could be bundled into a single dataset-linked record, referred to here as a ***dataset passport***, and used as a practical scaffold for reproducible acquisition and downstream analysis

Section	Subsection	What to record
A. Acquisition & sample metadata	Sample identity	Cell line/model; passage/age; matrix/ECM; geometry/microenvironment; perturbation (where, when, how much)
A. Acquisition & sample metadata	Imaging context	Modality; objective NA; pixel size/voxel size; temperature/CO ₂ control; time interval; z-step/volume rate; channels/filters
A. Acquisition & sample metadata	Calibration	Illumination uniformity / flat-field method; stage drift characterization; depth-dependent attenuation note (for 3D)
B. Photon dose accounting	Per-channel dose proxy	Irradiance or power at sample; exposure time; frame count; sampling frequency
B. Photon dose accounting	Adaptation rule	When and why sampling escalates (event-driven); hard stop conditions
C. QC gates	Imaging QC	Focus stability; bleaching trend; drift threshold; saturation rate; SNR floor per channel
C. QC gates	Biological sentinels	Division timing/fidelity; morphology stability; motility suppression; stress markers if available; explicit declaration of which sentinel was used
C. QC gates	Exclusion rules	Pre-registered thresholds for dropping wells, FOVs, tracks, or samples
D. Analysis provenance (versioned, auditable)	Pipeline	Code repository / notebook version; parameter file(s)
D. Analysis provenance (versioned, auditable)	Model provenance (DL)	Model name; version/hash; training domain summary; inference settings; proxy metric such as confidence, residuals, or feature drift
D. Analysis provenance (versioned, auditable)	Outputs	Feature schema and units; aggregation rule (cell, well, organoid); batch-correction method if any
E. Reporting bundle	Export	Single file bundling metadata, dose descriptors, QC report, and analysis provenance, linked to raw and processed data

Figure 1.4. Dataset passport table for HT/HC phenotyping workflows. The figure defines a compact, table-format reporting structure that integrates acquisition/sample metadata, photon-dose descriptors, QC gates, analysis provenance, and exportable reporting bundles to improve reproducibility, comparability, and downstream interpretability across imaging datasets.

This proposed **dataset passport** (Fig. 1.4) provides a compact reporting structure for preserving interpretability, reuse, and supporting cross-platform comparability. Making such minimum standards routine will require reducing the implementation burden through templates, acquisition software support, automated QC capture, version-aware analysis logging, and interoperable reporting structures. This is also the level at which community standards such as REMBI, OME/Bio-Formats, and QUAREP-LiMi are most likely to have practical effects^{25,235,237,238}. Beyond proposing these design rules and reporting elements at the conceptual level, this thesis also adopted them in practice through reproducibility-oriented live-cell imaging workflows implemented in the lab. In particular, standardized imaging protocols, routine laser power measurements and calibration checks, and structured recording and storage of key experimental parameters were incorporated to reduce hidden variability across sessions and instruments (Appendix B4). These practices do not eliminate all sources of technical variation, but they help ensure that image-derived phenotypes are more interpretable, comparable, and auditable within and across experiments.

Looking ahead, near-term progress will likely come from improved interoperability across workflows rather than from any single new modality, reporter, or algorithm. Longer term, state-aware and closed-loop phenotyping may improve photon efficiency and support stronger perturbation-response interpretation, especially in heterogeneous live systems. But that future should extend minimum standards for metadata, dose descriptors, QC gates, and provenance. Otherwise, closed-loop control may become more technically sophisticated without becoming more comparable or more interpretable. Within this scope, this chapter defines a set of operational design considerations for live-cell HT/HC phenotyping workflows in tissue-engineering contexts, while recognizing that broader causal interpretation and cross-platform transfer require further validation across biological models, perturbations, instruments, and analysis pipelines.

1.6 How to read this thesis

Here, high-throughput/high-content (HT/HC) phenotyping is treated as an end-to-end workflow in which biological readouts, engineered perturbations, imaging strategies, and analysis choices jointly influence how image-derived phenotypes are measured and

interpreted. Each chapter contributes a module focused on reducing specific sources of variance discussed in **Chapter 1**: variation in cell-cycle state; photon dose-dependent perturbation; limited interoperability between fabricated substrates and microscopy coordinates; and the practical difficulty of extracting coupled structural-functional readouts in three-dimensional models.

The chapters progress from *when* measurements should be made, to *where* they can be made reproducibly, to *how* these measurements can be supported by instrument architectures tailored to tissue-engineering constraints, and finally to *how* to interpret and quantify volumetric phenotypes. **Chapter 2** introduces FUCCIsmart, an event-driven acquisition framework that uses fluorescent cell-cycle reporters to trigger high-resolution 3D imaging, aiming to concentrate photon dose into short, information-dense windows. **Chapter 3** presents Fab2Mic, a fabrication-to-microscopy correlation pipeline that connects patterned microenvironments to imaging coordinates and supports longitudinal single-cell tracking to quantify motility, morphology, and cell-cycle dynamics. **Chapter 4** describes *TE-DaXi*, a modular single-objective oblique-plane light-sheet microscope developed as a design and prototyping effort for tissue-engineering use cases, intended to support both fast volumetric acquisition over short windows and long-term low-dose imaging through explicit mode and channel governance. **Chapter 5** develops a 3D HT/HC imaging workflow that links structural descriptors (e.g., cavity morphometrics) with cell-cycle state distributions in volumetric models; a minimal two-phase model is then adopted as an interpretive reference layer to support qualitative comparison between experimental phenotypes and simulated morphogenesis regimes. **Chapter 6** synthesizes these contributions, discusses their limitations, and outlines future directions for extending these modules toward more controlled, context-aware, and auditable phenotyping workflows.

1.7 Bibliography

1. Caicedo, J. C. et al. Data-analysis strategies for image-based cell profiling. *Nat. Methods* 14, 849-863 (2017).
2. Chandrasekaran, S. N. et al. Three million images and morphological profiles of cells treated with matched chemical and genetic perturbations. *Nat. Methods* 21, 1114-1121 (2024).

3. Hofer, M. & Lutolf, M. P. Engineering organoids. *Nat. Rev. Mater.* 6, 402-420 (2021).
4. Seal, S. et al. Integrating image-based profiling with cell health data as a foundation for interpretable prediction of biological phenotypes. *Mol. Biol. Cell* 35, ar15 (2024).
5. Way, G. P. et al. Evolution and impact of high content imaging. *SLAS Discov.* 28, 292-305 (2023).
6. Stossi, F. et al. High throughput microscopy and single cell phenotypic image-based analysis in toxicology and drug discovery. *Biochem. Pharmacol.* 216, 115770 (2023).
7. Lukonin, I., Zinner, M. & Liberali, P. Organoids in image-based phenotypic chemical screens. *Exp. Mol. Med.* 53, 1495-1502 (2021).
8. Arevalo, J. et al. Evaluating batch correction methods for image-based cell profiling. *Nat. Commun.* 15, 6516 (2024).
9. Eliceiri, K. W. et al. Biological imaging software tools. *Nat. Methods* 9, 697-710 (2012).
10. Laissue, P., Alghamdi, R., Tomancak, P., Reynaud, E. & Shroff, H. Assessing phototoxicity in live fluorescence imaging. *Nat. Methods* 14, 657-661 (2017).
11. Bray, M.-A. et al. Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. *Nat. Protoc.* 11, 1757-1774 (2016).
12. Wan, Y., McDole, K. & Keller, P. J. Light-sheet microscopy and its potential for understanding developmental processes. *Annu. Rev. Cell Dev. Biol.* 35, 655-681 (2019).
13. Durkee, M. S. et al. Artificial intelligence and cellular segmentation in tissue microscopy images. *Am. J. Pathol.* 191, 1693-1701 (2021).
14. Kasprzycka, W., Magidson, V. & Khodjakov, A. New approaches for low phototoxicity imaging of living cells and tissues. *BioEssays* 46, e2300122 (2024).
15. Lampart, F. L., Iber, D. & Doumpas, N. Organoids in high-throughput and high-content screenings. *Front. Chem. Eng.* 5, 1120348 (2023).
16. Berg, S. et al. ilastik: interactive machine learning for (bio)image analysis. *Nat. Methods* 16, 1226-1232 (2019).
17. Carpenter, A. E. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100 (2006).
18. Ellenberg, J. et al. A call for public archives for biological image data. *Nat. Methods* 15, 849-854 (2018).
19. Greenwald, N. F. et al. Whole-cell segmentation of tissue images with human-level performance using large-scale data annotation and deep learning. *Nat. Biotechnol.* 40, 555-565 (2022).
20. Moore, J. et al. OME-NGFF: a next-generation file format for expanding bioimaging data-access strategies. *Nat. Methods* 18, 1496-1498 (2021).
21. Sofroniew, N. et al. napari: a multi-dimensional image viewer for Python (2025).
22. Kemmer, I. et al. Building a FAIR image data ecosystem for microscopy communities. *Nat. Methods* 20, 1712-1718 (2023).
23. Schmidt, C. et al. Research data management for bioimaging: the 2021 NFDI4BIOIMAGE community survey. *F1000Research* 11, 638 (2022).

24. Sivagurunathan, S. et al. Bridging imaging users to imaging analysis - a community survey. *J. Microsc.* 1-15 (2023).
25. Swedlow, J. R. et al. A global view of standards for open image data formats and repositories. *Nat. Methods* 18, 1440-1446 (2021).
26. Williams, E. et al. Image Data Resource: a bioimage data integration and publication platform. *Nat. Methods* 14, 775-781 (2017).
27. Mattiazzi Usaj, M., Yeung, C. H. L., Friesen, H., Boone, C. & Andrews, B. J. Single-cell image analysis to explore cell-to-cell heterogeneity in isogenic populations. *Cell Syst.* 12, 608-621 (2021).
28. Menduti, G. & Boido, M. Recent advances in high-content imaging and analysis in iPSC-based modelling of neurodegenerative diseases. *Int. J. Mol. Sci.* 24, 14689 (2023).
29. Salic, A. & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 105, 2415-2420 (2008).
30. Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J. Cell. Physiol.* 182, 311-322 (2000).
31. Held, M. W. et al. CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging. *Nat. Methods* 7, 747-754 (2010).
32. Wang, J. et al. A concise guide to fluorescent cell cycle reporters for live-cell imaging. *Front. Cell Dev. Biol.* 13, 1702230 (2025).
33. Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487-498 (2008).
34. Bajar, B. T. et al. Fluorescent indicators for simultaneous reporting of all four cell cycle phases. *Nat. Methods* 13, 993-996 (2016).
35. Grant, G. D. et al. Accurate delineation of cell cycle phase transitions in living cells with PIP-FUCCI. *Cell Cycle* 17, 2496-2516 (2018).
36. Oki, T. et al. A novel cell-cycle-indicator, mVenus-p27K-, identifies quiescent cells and visualizes G0-G1 transition. *Sci. Rep.* 4, 4012 (2014).
37. Neumann, B. et al. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature* 464, 721-727 (2010).
38. Pargett, M. & Albeck, J. G. Live-cell imaging and analysis with multiple genetically encoded reporters. *Curr. Protoc. Cell Biol.* 78, 4.36.1-4.36.19 (2018).
39. Specht, E. A., Braselmann, E. & Palmer, A. E. A critical and comparative review of fluorescent tools for live-cell imaging. *Annu. Rev. Physiol.* 79, 93-117 (2017).
40. Tosheva, K. L. et al. Between life and death: strategies to reduce phototoxicity in super-resolution microscopy. *J. Phys. D Appl. Phys.* 53, 163001 (2020).
41. Yang, M. & Du, Z. To see and to know: the power of live imaging in illuminating and decoding biological complexity. *J. Genet. Genomics* (2025).
42. Gardel, M. L., Schneider, I. C., Aratyn-Schaus, Y. & Waterman, C. M. Mechanical integration of actin and adhesion dynamics in cell migration. *Annu. Rev. Cell Dev. Biol.* 26, 315-333 (2010).

43. Jones, M. C., Zha, J. & Humphries, M. J. Connections between the cell cycle, cell adhesion and the cytoskeleton. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20180227 (2019).
44. Conboy, J. P. How cytoskeletal crosstalk makes cells move. *Biophys. Rev.* 5, 021307 (2024).
45. Godard, B. G. et al. Combined effect of cell geometry and polarity domains determines the orientation of unequal division. *eLife* 10, e75639 (2021).
46. Etienne-Manneville, S. Microtubules in cell migration. *Annu. Rev. Cell Dev. Biol.* 29, 471-499 (2013).
47. Sallee, M. D., Pickett, M. A. & Feldman, J. L. Apical PAR complex proteins protect against programmed epithelial assaults to create a continuous and functional intestinal lumen. *eLife* 10, e64437 (2021).
48. Boutros, M., Heigwer, F. & Laufer, C. Microscopy-based high-content screening. *Cell* 163, 1314-1325 (2015).
49. Scheeder, C., Heigwer, F. & Boutros, M. Machine learning and image-based profiling in drug discovery. *Curr. Opin. Syst. Biol.* 10, 43-52 (2018).
50. Dugina, V., Zwaenepoel, I., Gabbiani, G., Clement, S. & Chaponnier, C. Interaction of microtubules with F-actin in cell protrusions depends on F-actin bundling by fascin. *J. Cell Sci.* 122, 2791-2801 (2009).
51. Lukinavičius, G. et al. Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat. Methods* 11, 731-733 (2014).
52. Akbarzadeh, M. et al. Morphological profiling by means of the Cell Painting assay enables identification of tubulin-targeting compounds. *Cell Chem. Biol.* 29, 1053-1064.e3 (2022).
53. Hofbauer, P. et al. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021).
54. Lewis-Israeli, Y. R. et al. Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021).
55. Dalangin, R. et al. Far-red fluorescent genetically encoded calcium ion indicators. *Nat. Commun.* 16, 3318 (2025).
56. Mills, R. J. et al. Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. *Proc. Natl. Acad. Sci. U. S. A.* 114, E8372-E8381 (2017).
57. Zhang, Y. et al. Fast and sensitive GCaMP calcium indicators for imaging neural populations. *Nature* 615, 884-891 (2023).
58. Dvinskikh, L. et al. Remote-refocusing light-sheet fluorescence microscopy enables 3D imaging of electromechanical coupling of hiPSC-derived and adult cardiomyocytes in co-culture. *Sci. Rep.* 13, 3342 (2023).
59. Hochbaum, D. R. et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat. Methods* 11, 825-833 (2014).
60. Milosevic, M. M., Jang, J., McKimm, E. J., Zhu, M. H. & Antic, S. D. In vitro testing of voltage indicators: Archon1, ArcLightD, ASAP1, ASAP2s, ASAP3b, Bongwoori-

- Pos6, BeRST1, FlicR1, and Chi-VSFP-Butterfly. *eNeuro* 7, ENEURO.0060-20.2020 (2020).
61. Boutin, M. E. et al. A multiparametric calcium signal screening platform using iPSC-derived cortical neural spheroids. *SLAS Discov.* 27, 209-218 (2022).
 62. Skardal, A. et al. Drug compound screening in single and integrated multi-organoid body-on-a-chip systems. *Biofabrication* 12, 025017 (2020).
 63. Pocock, M. W. et al. Maturation of human cardiac organoids enables complex disease modeling and drug discovery. *Nat. Cardiovasc. Res.* 4, 821-840 (2025).
 64. Tang, Q. et al. Morphological profiling for drug discovery in the era of deep learning. *Brief. Bioinform.* 25, bbae284 (2024).
 65. Way, G. P. et al. Morphology and gene expression profiling provide complementary information for mapping cell state. *Cell Syst.* 13, 911-923.e9 (2022).
 66. Sen, O., Saurin, A. T. & Higgins, J. M. G. The live cell DNA stain SiR-Hoechst induces DNA damage responses and impairs cell cycle progression. *Sci. Rep.* 8, 7898 (2018).
 67. Tovar-Ferrero, O. et al. Measuring mitochondrial membrane potential. *EMBO J.* 44, 7334-7345 (2025).
 68. Albert, L. et al. Nanobodies equipped with HaloTag variants enable rapid and straightforward one-step immunofluorescence lifetime multiplexing. *bioRxiv* [Preprint] (2026).
 69. Grimm, J. B. et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat. Methods* 14, 987-994 (2017).
 70. Los, G. V. et al. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3, 373-382 (2008).
 71. Keppler, A., Pick, H., Arrivoli, C., Vogel, H. & Johnsson, K. Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9955-9959 (2004).
 72. Kühn, S. et al. SNAP-tag2 for faster and brighter protein labeling. *Nat. Chem. Biol.* 21, 1754-1761 (2025).
 73. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819-823 (2013).
 74. Nguyen, D. N. et al. Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. *Nat. Biotechnol.* 38, 44-49 (2020).
 75. Cho, N. H. et al. OpenCell: endogenous tagging for the cartography of human cellular organization. *Science* 375, eabi6983 (2022).
 76. Schmid-Burgk, J. L., Höning, K., Ebert, T. S. & Hornung, V. CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat. Commun.* 7, 12338 (2016).
 77. Willems, J. et al. ORANGE: a CRISPR/Cas9-based genome editing toolbox for epitope tagging of endogenous proteins in neurons. *PLOS Biol.* 18, e3000665 (2020).
 78. Shalem, O. et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343, 84-87 (2014).

79. Cockrell, A. S. & Kafri, T. Gene delivery by lentivirus vectors. *Mol. Biotechnol.* 36, 184-204 (2007).
80. De Wannemaeker, L. et al. Standardization of fluorescent reporter assays in synthetic biology across the visible light spectrum. *ACS Synth. Biol.* 12, 3591-3607 (2023).
81. Naldini, L. et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272, 263-267 (1996).
82. Pannell, D. & Ellis, J. Silencing of gene expression: implications for design of retrovirus vectors. *Rev. Med. Virol.* 11, 205-217 (2001).
83. Sadelain, M., Papapetrou, E. P. & Bushman, F. D. Safe harbours for the integration of new DNA in the human genome. *Nat. Rev. Cancer* 12, 51-58 (2012).
84. Awada, B. et al. Phenotypic screening with primary and human iPSC-derived neurons. In *Assay Guidance Manual* (eds Markossian, S., Grossman, A., Baskir, H. et al.) (Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, 2025).
85. Zingales, V. et al. The growing importance of three-dimensional models and microphysiological systems in the assessment of mycotoxin toxicity. *Toxins* 15, 422 (2023).
86. Boukamp, P. et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106, 761-771 (1988).
87. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. & Gardner, M. B. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* 33, 1027-1033 (1974).
88. Harper, J. M. Primary cell culture as a model system for evolutionary molecular physiology. *Int. J. Mol. Sci.* 25, 7905 (2024).
89. Lancaster, M. A. & Knoblich, J. A. Generation of cerebral organoids from human pluripotent stem cells. *Nat. Protoc.* 9, 2329-2340 (2014).
90. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872 (2007).
91. Gintant, G. et al. Use of human induced pluripotent stem cell-derived cardiomyocytes in preclinical cancer drug cardiotoxicity testing: a scientific statement from the American Heart Association. *Circ. Res.* 125, e75-e92 (2019).
92. Karakikes, I., Ameen, M., Termglinchan, V. & Wu, J. C. Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. *Circ. Res.* 117, 80-88 (2015).
93. Zhao, Z. et al. Organoids. *Nat. Rev. Methods Primers* 2, 94 (2022).
94. Cho, J., Lee, H., Rah, W., Chang, H. J. & Yoon, Y.-S. From engineered heart tissue to cardiac organoid. *Theranostics* 12, 2758-2772 (2022).
95. Gjorevski, N., Ranga, A. & Lutolf, M. P. Bioengineering approaches to guide stem cell-based organogenesis. *Development* 141, 1794-1804 (2014).
96. Garreta, E. et al. Rethinking organoid technology through bioengineering. *Nat. Mater.* 20, 145-155 (2021).

97. Joddar, B. et al. Engineering approaches for cardiac organoid formation and their characterization. *Transl. Res.* 250, 46-67 (2022).
98. Stirling, D. R., Carpenter, A. E. & Cimini, B. A. CellProfiler Analyst 3.0: accessible data exploration and machine learning for image analysis. *Bioinformatics* 37, 3992-3994 (2021).
99. Serrano, E. et al. Reproducible image-based profiling with Pycytominer. *Nat. Methods* 22, 677-680 (2025).
100. Pearson, Y. E. et al. A statistical framework for high-content phenotypic profiling using cellular feature distributions. *Commun. Biol.* 5, 1409 (2022).
101. Corbe, M., Boncompain, G., Perez, F., Del Nery, E. & Genovesio, A. Transfer learning for versatile and training free high content screening analyses. *Sci. Rep.* 13, 22599 (2023).
102. Seal, S. et al. Cell Painting: a decade of discovery and innovation in cellular imaging. *Nat. Methods* 22, 254-268 (2025).
103. Ashesh, A. et al. MicroSplit: semantic unmixing of fluorescent microscopy data. *Nat. Methods* (2026)
104. Huang, X., Gao, X. & Fu, L. BINGO: a blind unmixing algorithm for ultra-multiplexing fluorescence images. *Bioinformatics* 40, btae052 (2024).
105. Ong, H. T. et al. Digitalized organoids: integrated pipeline for high-speed 3D analysis of organoid structures using multilevel segmentation and cellular topology. *Nat. Methods* 22, 1343-1354 (2025).
106. Mukashyaka, P. et al. High-throughput deconvolution of 3D organoid dynamics at cellular resolution for cancer pharmacology with Cellos. *Nat. Commun.* 14, 8406 (2023).
107. Andrés-San Román, J. A. et al. CartoCell, a high-content pipeline for 3D image analysis, unveils cell morphology patterns in epithelia. *Cell Rep. Methods* 3, 100597 (2023).
108. Bozal, S. B. et al. Development of an automated 3D high content cell screening platform for organoid phenotyping. *SLAS Discov.* 29, 100182 (2024).
109. Albanese, A. et al. Multiscale 3D phenotyping of human cerebral organoids. *Sci. Rep.* 10, 21487 (2020).
110. Schmidt, U., Weigert, M., Broaddus, C. & Myers, G. Cell detection with star-convex polygons. In *Medical Image Computing and Computer Assisted Intervention - MICCAI 2018* (eds Frangi, A. F., Schnabel, J. A., Davatzikos, C., Alberola-López, C. & Fichtinger, G.) 265-273 (Springer International Publishing, Cham, 2018).
111. Weigert, M., Schmidt, U., Haase, R., Sugawara, K. & Myers, G. Star-convex polyhedra for 3D object detection and segmentation in microscopy. In *2020 IEEE Winter Conference on Applications of Computer Vision (WACV)* 3655-3662 (IEEE, 2020).
112. Stringer, C. & Pachitariu, M. Cellpose3: one-click image restoration for improved cellular segmentation. *Nat. Methods* 22, 592-599 (2025).

113. Isensee, F., Jaeger, P. F., Kohl, S. A. A., Petersen, J. & Maier-Hein, K. H. nnU-Net: a self-configuring method for deep learning-based biomedical image segmentation. *Nat. Methods* 18, 203-211 (2021).
114. Ershov, D. et al. TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nat. Methods* 19, 829-832 (2022).
115. Bragantini, J. et al. Ultrack: pushing the limits of cell tracking across biological scales. *Nat. Methods* 22, 2423-2436 (2025).
116. Sugawara, K., Çevrim, Ç. & Averof, M. Tracking cell lineages in 3D by incremental deep learning. *eLife* 11, e69380 (2022).
117. Booth, M. J. Adaptive optical microscopy: the ongoing quest for a perfect image. *Light Sci. Appl.* 3, e165 (2014).
118. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017).
119. Royer, L. A. et al. Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016).
120. Power, R. M. & Huisken, J. A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nat. Methods* 14, 360-373 (2017).
121. Reynaud, E. G., Peychl, J., Huisken, J. & Tomancak, P. Guide to light-sheet microscopy for adventurous biologists. *Nat. Methods* 12, 30-34 (2015).
122. Sakaue-Sawano, A. et al. Genetically encoded tools for optical dissection of the mammalian cell cycle. *Mol. Cell* 68, 626-640.e5 (2017).
123. Treppe, X. et al. Physical forces during collective cell migration. *Nat. Phys.* 5, 426-430 (2009).
124. Lancaster, M. A. et al. Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373-379 (2013).
125. Galland, R., Greci, G., Aravind, A., Viasnoff, V., Studer, V. & Sibarita, J.-B. 3D high- and super-resolution imaging using single-objective SPIM. *Nat. Methods* 12, 641-644 (2015).
126. Gustafsson, M. G. L. et al. Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. *Biophys. J.* 94, 4957-4970 (2008).
127. York, A. G. et al. Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat. Methods* 10, 1122-1126 (2013).
128. Cabillic, M. et al. In-depth single molecule localization microscopy using adaptive optics and single objective light-sheet microscopy. *Nat. Commun.* 16, 8362 (2025).
129. Weigert, M. et al. Content-aware image restoration: pushing the limits of fluorescence microscopy. *Nat. Methods* 15, 1090-1097 (2018).
130. Koh, S.-B. et al. A quantitative FastFUCCI assay defines cell cycle dynamics at a single-cell level. *J. Cell Sci.* 130, 512-520 (2017).
131. Zielke, N. & Edgar, B. A. FUCCI sensors: powerful new tools for analysis of cell proliferation. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 469-487 (2015).

132. Gross, S. M. et al. Analysis and modeling of cancer drug responses using cell cycle phase-specific rate effects. *Nat. Commun.* 14, 3450 (2023).
133. Hunter, M. V. et al. Mechanical confinement governs phenotypic plasticity in melanoma. *Nature* 1-11 (2025).
134. Barry, D. J., Durkin, C. H., Abella, J. V. & Way, M. Open source software for quantification of cell migration, protrusions, and fluorescence intensities. *J. Cell Biol.* 209, 163-180 (2015).
135. Bolado-Carrancio, A. et al. Periodic propagating waves coordinate RhoGTPase network dynamics at the leading and trailing edges during cell migration. *eLife* 9, e58165 (2020).
136. Hino, N. et al. ERK-mediated mechanochemical waves direct collective cell polarization. *Dev. Cell* 53, 646-660.e8 (2020).
137. Pittman, M. et al. Membrane ruffling is a mechanosensor of extracellular fluid viscosity. *Nat. Phys.* 18, 1112-1121 (2022).
138. Azzam, S. et al. A high-precision wound healing assay based on photosensitized culture substrates. *Sci. Rep.* 14, 9103 (2024).
139. Hamazaki, N. et al. Retinoic acid induces human gastruloids with posterior embryo-like structures. *Nat. Cell Biol.* 26, 1790-1803 (2024).
140. Mitrofanova, O. et al. Bioengineered human colon organoids with in vivo-like cellular complexity and function. *Cell Stem Cell* 31, 1175-1186.e7 (2024).
141. Beghin, A. et al. Automated high-speed 3D imaging of organoid cultures with multi-scale phenotypic quantification. *Nat. Methods* 19, 881-892 (2022).
142. Moshksayan, K. et al. A microfluidic platform for culturing and high-content imaging of adult stem cell-derived organoids. *Sci. Rep.* 15, 40162 (2025).
143. Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J. & Stelzer, E. H. K. Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305, 1007-1009 (2004).
144. Yang, B. et al. DaXi-high-resolution, large imaging volume and multi-view single-objective light-sheet microscopy. *Nat. Methods* 19, 461-469 (2022).
145. De Medeiros, G. et al. Multiscale light-sheet organoid imaging framework. *Nat. Commun.* 13, 4864 (2022).
146. Malin-Mayor, C. et al. Automated reconstruction of whole-embryo cell lineages by learning from sparse annotations. *Nat. Biotechnol.* 41, 44-49 (2023).
147. Mau, A., Friedl, K., Leterrier, C., Bourg, N. & L  v  que-Fort, S. Fast widefield scan provides tunable and uniform illumination optimizing super-resolution microscopy on large fields. *Nat. Commun.* 12, 3077 (2021).
148. Ronceray, N. et al. Wide-field fluorescence lifetime imaging of single molecules with a gated single-photon camera. *Light Sci. Appl.* 14, 258 (2025).
149. Laine, R. F. et al. High-fidelity 3D live-cell nanoscopy through data-driven enhanced super-resolution radial fluctuation. *Nature Methods* 20, 1949–1956 (2023).
150. Elliott, A. D. Confocal microscopy: principles and modern practices. *Curr. Protoc. Cytom.* 92, e68 (2020).

151. Shimozawa, T. et al. Improving spinning disk confocal microscopy by preventing pinhole cross-talk for intravital imaging. *Proc. Natl. Acad. Sci. U. S. A.* 110, 3399-3404 (2013).
152. Goswami, M., Lachmann, R., Kretschmer, R. & Heintzmann, R. simpleISM - a straight forward guide to upgrade from confocal to ISM. *PLOS ONE* 17, e0279378 (2022).
153. Schulz, O. et al. Resolution doubling in fluorescence microscopy with confocal spinning-disk image scanning microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 110, 21000-21005 (2013).
154. Liang, Q. et al. High-fidelity tissue super-resolution imaging achieved with confocal2 spinning-disk image scanning microscopy. *Light Sci. Appl.* 14, 260 (2025).
155. Zunino, A. et al. Structured detection for simultaneous super-resolution and optical sectioning in laser scanning microscopy. *Nat. Photonics* 19, 888-897 (2025).
156. Bouchard, M. B. et al. Swept confocally-aligned planar excitation (SCAPE) microscopy for high-speed volumetric imaging of behaving organisms. *Nat. Photonics* 9, 113-119 (2015).
157. Sapoznik, E. et al. A versatile oblique plane microscope for large-scale and high-resolution imaging of subcellular dynamics. *eLife* 9, e57681 (2020).
158. Chang, B.-J. et al. Real-time multi-angle projection imaging of biological dynamics. *Nat. Methods* 18, 829-834 (2021).
159. Chen, B. et al. Resolution doubling in light-sheet microscopy via oblique plane structured illumination. *Nat. Methods* 19, 1419-1426 (2022).
160. Fadero, T. C. et al. LITE microscopy: tilted light-sheet excitation of model organisms offers high resolution and low photobleaching. *J. Cell Biol.* 217, 1869-1882 (2018).
161. Del Rosario, M. et al. PhotoFiTT: a quantitative framework for assessing phototoxicity in live-cell microscopy experiments. *Nat. Commun.* 16, 11401 (2025).
162. Madhusoodanan, J. Smart microscopes spot fleeting biology. *Nature* 614, 378-380 (2023).
163. Daetwyler, S. & Fiolka, R. P. Light-sheets and smart microscopy, an exciting future is dawning. *Commun. Biol.* 6, 1-11 (2023).
164. André, O., Ahnlide, J. K., Norlin, N., Swaminathan, V. & Nordenfelt, P. Data-driven microscopy allows for automated context-specific acquisition of high-fidelity image data. *Cell Rep. Methods* 3, 100419 (2023).
165. Mahečić, D. et al. Event-driven acquisition for content-enriched microscopy. *Nat. Methods* 19, 1262-1267 (2022).
166. Alvelid, J., Damenti, M., Sgattoni, C. & Testa, I. Event-triggered STED imaging. *Nat. Methods* 19, 1268-1275 (2022).
167. Ibrahim, K. A. et al. Self-driving microscopy detects the onset of protein aggregation and enables intelligent Brillouin imaging. *Nat. Commun.* 16, 6699 (2025).

168. Shi, Y. et al. Smart lattice light-sheet microscopy for imaging rare and complex cellular events. *Nat. Methods* 21, 301-310 (2024).
169. Lu, Z. et al. Virtual-scanning light-field microscopy for robust snapshot high-resolution volumetric imaging. *Nat. Methods* 20, 735-746 (2023).
170. Belthangady, C. & Royer, L. A. Applications, promises, and pitfalls of deep learning for fluorescence image reconstruction. *Nat. Methods* 16, 1215-1225 (2019).
171. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* 126, 677-689 (2006).
172. Hannezo, E. & Heisenberg, C.-P. Mechanochemical feedback loops in development and disease. *Cell* 178, 12-25 (2019).
173. Sonn, A., Tomba, C., Selhuber-Unkel, C. & Schamberger, B. 3D fabrication of artificial cell microenvironments for mechanobiology. *Curr. Opin. Biomed. Eng.* 33, 100574 (2025).
174. Barthes, J. et al. Cell microenvironment engineering and monitoring for tissue engineering and regenerative medicine: the recent advances. *Biomed Res. Int.* 2014, 921905 (2014).
175. Torchia, E. et al. Fabrication of cell culture hydrogels by robotic liquid handling automation for high-throughput drug testing. *Commun. Eng.* 4, 222 (2025).
176. Enrico, A. et al. LigHTS: massively parallel biomimetic photo-functionalization for imaging-based ultra-high-throughput screening. *bioRxiv [Preprint]* (2025).
177. Ho, C. M. B., Mishra, A., Hu, K., An, J., Kim, Y.-J. & Yoon, Y.-J. Femtosecond-laser-based 3D printing for tissue engineering and cell biology applications. *ACS Biomater. Sci. Eng.* 3, 2198-2214 (2017).
178. Qin, D., Xia, Y. & Whitesides, G. M. Soft lithography for micro- and nanoscale patterning. *Nat. Protoc.* 5, 491-502 (2010).
179. Théry, M. Micropatterning as a tool to decipher cell morphogenesis and functions. *J. Cell Sci.* 123, 4201-4213 (2010).
180. Piunti, C. et al. Microfluidic loading of verteporfin into extracellular vesicles for neuroblastoma therapy. *Lab. Chip* 25, 1718-1727 (2025).
181. Bugaj, L. J. & Lim, W. A. High-throughput multicolor optogenetics in microwell plates. *Nat. Protoc.* 14, 2205-2228 (2019).
182. Supatto, W., Truong, T. V., Débarre, D. & Beaurepaire, E. Advances in multiphoton microscopy for imaging embryos. *Curr. Opin. Genet. Dev.* 21, 538-548 (2011).
183. Treppe, X. & Sahai, E. Mesoscale physical principles of collective cell organization. *Nat. Phys.* 14, 671-682 (2018).
184. Bera, K. et al. Extracellular fluid viscosity enhances cell migration and cancer dissemination. *Nature* 611, 365-373 (2022).
185. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, D. E. Geometric control of cell life and death. *Science* 276, 1425-1428 (1997).

186. Dike, L. E. et al. Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro Cell. Dev. Biol. Anim.* 35, 441-448 (1999).
187. Mooney, D. J., Langer, R. D. & Ingber, D. E. Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix. *J. Cell Sci.* 108, 2311-2320 (1995).
188. Zhu, X., Ohtsubo, M., Böhmer, R. M., Roberts, J. M. & Assoian, R. K. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J. Cell Biol.* 133, 391-403 (1996).
189. Théry, M. et al. The extracellular matrix guides the orientation of the cell division axis. *Nat. Cell Biol.* 7, 947-953 (2005).
190. Heinrich, M. A. et al. Size-dependent patterns of cell proliferation and migration in freely-expanding epithelia. *eLife* 9, e58945 (2020).
191. Devany, J., Falk, M. J., Holt, L. J., Murugan, A. & Gardel, M. L. Epithelial tissue confinement inhibits cell growth and leads to volume-reducing divisions. *Dev. Cell* 58, 1462-1476.e8 (2023).
192. Isenberg, B. C., Dimilla, P. A., Walker, M., Kim, S. & Wong, J. Y. Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. *Biophys. J.* 97, 1313-1322 (2009).
193. Hartman, C. D., Isenberg, B. C., Chua, S. G. & Wong, J. Y. Vascular smooth muscle cell durotaxis depends on extracellular matrix composition. *Proc. Natl. Acad. Sci. U. S. A.* 113, 11190-11195 (2016).
194. Vassaux, M., Pieuchot, L., Anselme, K., Bigerelle, M. & Milan, J.-L. A biophysical model for curvature-guided cell migration. *Biophys. J.* 117, 1136-1144 (2019).
195. Luciano, M. et al. Cell monolayers sense curvature by exploiting active mechanics and nuclear mechanoadaptation. *Nat. Phys.* 17, 1382-1390 (2021).
196. Leclech, C. et al. Topography-induced large-scale antiparallel collective migration in vascular endothelium. *Nat. Commun.* 13, 2797 (2022).
197. Kalukula, Y. et al. The actin cortex acts as a mechanical memory of morphology in confined migrating cells. *Nat. Phys.* 21, 1451-1461 (2025).
198. Di Meglio, I. et al. Pressure and curvature control of the cell cycle in epithelia growing under spherical confinement. *Cell Rep.* 40, 111227 (2022).
199. Karzbrun, E. et al. Human neural tube morphogenesis in vitro by geometric constraints. *Nature* 599, 268-272 (2021).
200. De Medeiros, G. et al. Cell and tissue manipulation with ultrashort infrared laser pulses in light-sheet microscopy. *Sci. Rep.* 10, 1942 (2020).
201. Guglielmi, G., Barry, J. D., Huber, W. & De Renzis, S. An optogenetic method to modulate cell contractility during tissue morphogenesis. *Dev. Cell* 35, 646-660 (2015).

202. Izquierdo, E., Quinkler, T. & De Renzis, S. Guided morphogenesis through optogenetic activation of Rho signalling during early *Drosophila* embryogenesis. *Nat. Commun.* 9, 2366 (2018).
203. Marshall, A. R. et al. Two-photon cell and tissue level laser ablation methods to study morphogenetic biomechanics. *Methods Mol. Biol.* 2438, 217-230 (2022).
204. Hu, X. & Bao, M. Advances in micropatterning technology for mechanotransduction research. *Mechanobiol. Med.* 2, 100066 (2024).
205. Miklosic, G., Ferguson, S. J. & D'Este, M. Engineering complex tissue-like microenvironments with biomaterials and biofabrication. *Trends Biotechnol.* 42, 1241-1257 (2024).
206. Truong, H. D., Ge, Z., Chng, E., Tran, Y.-V., Zhang, Y. & Lim, C. T. Strategies to control cellular spatial organization in microphysiological systems. *Microsyst. Nanoeng.* 12, 85 (2026).
207. McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K. & Chen, C. S. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* 6, 483-495 (2004).
208. Li, Y., Wong, I. Y. & Guo, M. Reciprocity of cell mechanics with extracellular stimuli: emerging opportunities for translational medicine. *Small* 18, e2107305 (2022).
209. Wang, H., Wang, W. & Xie, Z. Patterning meets gels: advances in engineering functional gels at micro/nanoscales for soft devices. *J. Polym. Sci.* 60, 2679-2700 (2022).
210. Bril, M., Fredrich, S. & Kurniawan, N. A. Stimuli-responsive materials: a smart way to study dynamic cell responses. *Smart Mater. Med.* 3, 257-273 (2022).
211. Davletshin, E. et al. Optogenetic approaches for neural tissue regeneration: a review of basic optogenetic principles and target cells for therapy. *Neural Regen. Res.* 21, 521-533 (2026).
212. Obenaus, A. M., Mollica, M. Y. & Sniadecki, N. J. (De)form and function: measuring cellular forces with deformable materials and deformable structures. *Adv. Healthc. Mater.* 9, 1901454 (2020).
213. Fu, J. et al. Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nat. Methods* 7, 733-736 (2010).
214. Oakes, P. W., Banerjee, S., Marchetti, M. C. & Gardel, M. L. Geometry regulates traction stresses in adherent cells. *Biophys. J.* 107, 825-833 (2014).
215. Melero, C. et al. Light-induced molecular adsorption of proteins using the PRIMO system for micro-patterning to study cell responses to extracellular matrix proteins. *J. Vis. Exp.* 60092 (2019).
216. Van der Putten, C. et al. Protein micropatterning in 2.5D: an approach to investigate cellular responses in multi-cue environments. *ACS Appl. Mater. Interfaces* 13, 25589-25598 (2021).

217. Batalov, I., Stevens, K. R. & DeForest, C. A. Photopatterned biomolecule immobilization to guide three-dimensional cell fate in natural protein-based hydrogels. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2014194118 (2021).
218. Bretherton, R. C. & DeForest, C. A. The art of engineering biomimetic cellular microenvironments. *ACS Biomater. Sci. Eng.* 7, 3997-4008 (2021).
219. Tian, C., Zhang, J., Gu, J., Li, W. & Cao, Y. Light controlled biomaterials for regulating cell migration and differentiation. *Smart Mater. Med.* 3, 209-216 (2022).
220. Enrico, A. et al. 3D microvascularized tissue models by laser-based cavitation molding of collagen. *Adv. Mater.* 34, 2109823 (2022).
221. Martinelli, C. et al. A 3D millifluidic model of a dermal perivascular microenvironment on a chip. *Lab. Chip* 25, 423-439 (2025).
222. Kumar, S., Beyer, H. M., Chen, M., Zurbriggen, M. D. & Khammash, M. Image-guided optogenetic spatiotemporal tissue patterning using μ PatternScope. *Nat. Commun.* 15, 10469 (2024).
223. Pinheiro, D. & Bellaïche, Y. Mechanical force-driven adherens junction remodeling and epithelial dynamics. *Dev. Cell* 47, 3-19 (2018).
224. Fox, Z. R. et al. Enabling reactive microscopy with MicroMator. *Nat. Commun.* 13, 2199 (2022).
225. Crossley, R. M. et al. Modeling the extracellular matrix in cell migration and morphogenesis: a guide for the curious biologist. *Front. Cell Dev. Biol.* 12, 1354132 (2024).
226. Di Gravina, G. M., Loi, G., Auricchio, F. & Conti, M. Computer-aided engineering and additive manufacturing for bioreactors in tissue engineering: state of the art and perspectives. *Biophys. Rev.* 4, 031303 (2023).
227. Keshavanarayana, P., Aparicio-Yuste, R., Spill, F., Gomez-Benito, M. J. & Bastounis, E. E. Leveraging computational modeling to explore epithelial and endothelial cell monolayer mechanobiology. *Trends Cell Biol.* 35, 799-813 (2025).
228. Su, W., Zhao, Y., Yeboah, S., Li, X. & Wang, L. Numerical models for organ-on-a-chip: a systematic review and analyses. *Biomicrofluidics* 19, 041501 (2025).
229. Doyle, A. D., Nazari, S. S. & Yamada, K. M. Cell-extracellular matrix dynamics. *Phys. Biol.* 19, 021002 (2022).
230. Li, Y. et al. 3D micropattern force triggers YAP nuclear entry by transport across nuclear pores and modulates stem cells paracrine. *Natl. Sci. Rev.* 10, nwad165 (2023).
231. Wang, T.-C. et al. Matrix stiffness drives drop like nuclear deformation and lamin A/C tension-dependent YAP nuclear localization. *Nat. Commun.* 15, 10151 (2024).
232. Toro-Nahuelpan, M. et al. Tailoring cryo-electron microscopy grids by photo-micropatterning for in-cell structural studies. *Nat. Methods* 17, 50-54 (2020).
233. Bril, M. et al. Shape-morphing photoresponsive hydrogels reveal dynamic topographical conditioning of fibroblasts. *Adv. Sci.* 10, 2303136 (2023).
234. Legnini, I. et al. Spatiotemporal, optogenetic control of gene expression in organoids. *Nat. Methods* 20, 1544-1552 (2023).

- 235. Boehm, U. et al. QUAREP-LiMi: a community endeavor to advance quality assessment and reproducibility in light microscopy. *Nat. Methods* 18, 1423-1426 (2021).
- 236. Kiepas, A., Voorand, E., Mubaid, F., Siegel, P. M. & Brown, C. M. Optimizing live-cell fluorescence imaging conditions to minimize phototoxicity. *J. Cell Sci.* 133, jcs242834 (2020).
- 237. Linkert, M. et al. Metadata matters: access to image data in the real world. *J. Cell Biol.* 189, 777-782 (2010).
- 238. Sarkans, U. et al. REMBI: recommended metadata for biological images - enabling reuse of microscopy data in biology. *Nat. Methods* 18, 1418-1422 (2021).
- 239. Chiron, L. et al. CyberSco.Py an open-source software for event-based, conditional microscopy. *Sci. Rep.* 12, 11579 (2022).
- 240. Di Sante, M. et al. CALIPERS: cell-cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* [Preprint] (2025).
- 241. Stringer, C., Wang, T., Michaelos, M. & Pachitariu, M. Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* 18, 100-106 (2021).
- 242. Zimmermann, J. et al. Bioimage analysis for multiplexed FUCCI acquisitions powered by deep learning. *npj Imaging* 4, 27 (2026).

2

FUCCIsmart: Event-driven imaging routine for 3D spindle acquisitions

This chapter is based on part of the work disseminated as a bioRxiv preprint: *Di Sante et al., CALIPERS: Cell cycle-aware live imaging for phenotyping experiments and regeneration studies, bioRxiv (2024), doi: 10.1101/2024.12.19.629259*, currently submitted and under review. Parts of the text, figures, or tables may overlap with the corresponding manuscript. Such overlap is presented here transparently for academic completeness

As outlined in Chapter 1, the first practical limitation concerns the timing of acquisition in live-cell experiments. When the biological events of interest are transient and cell populations are asynchronous, fixed-cadence imaging distributes photon dose inefficiently and risks perturbing the system under observation. This chapter tackles this challenge by moving from descriptive imaging to state-aware control, where the cell-cycle serves as a real-time context signal that governs when high-dose 3D acquisition is allowed. Accordingly, FUCCIsmart is introduced as an event-driven routine that concentrates volumetric confocal sampling around the mitotic window while keeping the remainder of the experiment in low-dose surveillance, maximizing information-per-photon under viability constraints.

2.1 Introduction

In tissue engineering and regenerative medicine, understanding how cells proliferate and behave over time is important for designing effective therapies and models. Advanced live-cell imaging platforms (ranging from 2.5D microfluidic chips to 3D organoids) now enable observation of cell dynamics in their natural environment¹. However, the cell-

cycle (CC) state of each cell is often not explicitly accounted for in these studies. Cell-cycle progression affects gene expression and morphology and can influence cell fate decisions. For instance, the orientation and timing of cell division can influence tissue architecture and developmental outcomes²⁻⁶. A cell in G1 may respond differently to stimuli than one in G2 or M phase (mitosis), so ignoring CC context can distort phenotypic evaluations. In *in vitro* experiments and high-content imaging screens, this means that without CC-aware methods, we risk misinterpreting data because phenotypes can be altered or confounded, as a mix of cells in various phases may show diverse responses.

Traditional fluorescence tools, such as Fluorescence Ubiquitin Cell-cycle Indicators (FUCCI)⁷, have advanced cell-cycle research by color-coding cells by phase^{8,9}. FUCCI typically uses two fluorescent proteins: a green fluorescent protein (GFP) and a red fluorescent protein (RFP), fused to cell-cycle regulators that are degraded in G1 or S/G2/M, respectively. This results in green nuclei during G1 and red nuclei during S/G2/M, enabling real-time visualization of cell-cycle progression. A faster FUCCI assay developed by Koh *et al.* refined this method for single-cell dynamics^{10,11}. Despite their advantages, current FUCCI systems face a major limitation: they occupy the standard green and red fluorescence channels. To address this, alternative techniques such as temporally multiplexed imaging (TMI) use specialized fluorescent proteins with unique kinetics (e.g., photoswitchable proteins) to encode multiple signals within a single channel^{12,13}, as reported in the introductory literature review in Chapter 1. Although powerful, TMI requires complex hardware control and computation, making it difficult to implement on standard microscopes. Therefore, there is an unmet need for a cell-cycle indicator that is easier to combine with common structural and functional reporters, without monopolizing the critical green and red channels, and that can be integrated into conventional imaging setups. The recently developed CALIPERS¹⁴ framework by the Synthetic Physiology Lab was developed to address this challenge in live-cell imaging, particularly the spectral overlap in the green/red bands used by most structural and functional reporters. Within CALIPERS, we introduced FUCCIplex, a spectrally re-engineered two-color FUCCI sensor that shifts the cell-cycle readout away from green and red channels, keeping these key channels available for phenotype reporters.

Specifically, the FUCCIplex cassette includes CFP-Cdt1 (G1 marker) with cyan nuclear signal, and iRFP-Geminin (S/G2/M marker), with far-red nuclear signal.

By using CFP/iRFP rather than GFP/RFP, FUCCIplex preserves the green and red channels for simultaneous structural readouts (e.g., EGFP-tagged tubulin and RFP LifeAct for Actin). This spectral economy reduces the conflicts that limit classic FUCCI implementations and is central to the multiplexing aim of this chapter

Another technological challenge in live-cell imaging and CC-aware phenotyping is capturing three-dimensional (3D) events, such as mitosis, over long periods. In adherent cells, interphase microtubules form a largely planar network. Still, as a cell enters mitosis, the microtubule cytoskeleton reorganizes into the mitotic spindle, a 3D structure necessary for chromosome segregation^{4,5,15}. Visualizing the full 3D architecture of the mitotic spindle is crucial for understanding cell division mechanics and has implications for tissue engineering (e.g., proper spindle positioning influences symmetric vs. asymmetric division and thus tissue structure). Spinning-disk confocal microscopy and other volumetric imaging methods can reveal these 3D structures at high resolution^{16,17}. However, continuous volumetric imaging is phototoxic. Frequent z-stack acquisitions expose cells to much more light than 2D time-lapse imaging, leading to increased photobleaching and stress, or even cell death, during the cell-cycle. In long-term experiments (24-72 hours or more), a straightforward strategy of acquiring 3D stacks at short intervals (e.g., every few minutes or even every hour) can disturb cells and may lead to cell-cycle arrest or abnormal behavior due to light-induced damage¹⁸. Conversely, capturing stacks very infrequently (e.g., every 6-8 hours) misses many short-lived events like mitosis. Therefore, researchers face a trade-off between the temporal coverage of events and the risk of phototoxicity.

In this chapter, we propose FUCCIsmart, a CC-aware, event-driven imaging routine that leverages FUCCIplex sensors to address that trade-off. The workflow operates in two tiers: (i) low-dose widefield (WF) snapshots provide nuclear masks and per-cell FUCCIplex intensities; (ii) when these signals indicate a window likely to include mitosis, the system switches hardware to short time-window confocal (SD) Z-stack bursts on the structural channels (e.g., EGFP-Tubulin, RFP-Actin) to capture the spindle in 3D.

Thanks to the CALIPERS framework and FUCCIphase package, we related CFP/iRFP nuclear intensities to a quantitative cell-cycle percentage using reference trajectories of the HaCaT FUCCIplex prototype cell line. These calibration-derived thresholds allow us to express measurements in absolute units or relative to cycle means, and to apply consistent decision rules across plates and days. Conceptually, FUCCIsmart concentrates photons where information density is highest (around mitosis) and avoids expensive 3D sampling elsewhere, with the aim of increasing spindle captures per photon while preserving cell viability.

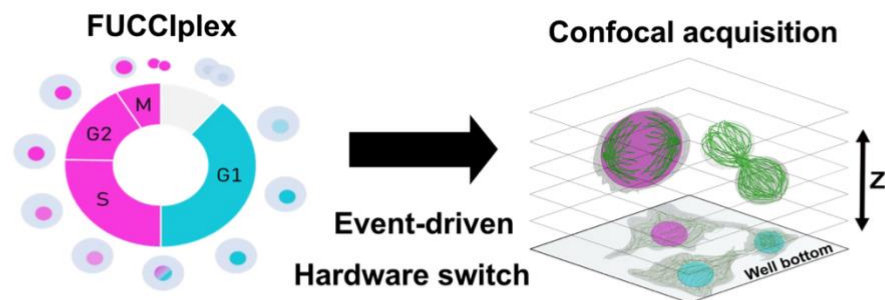
Given that FUCCIsmart was developed as part of the CALIPERS project, this work first focuses on wet-lab and imaging skills using the HaCaT FUCCIplex prototype line (CFP-Cdt1 for G1; iRFP-Geminin for S/G2/M) and was then extended to a four-color model with EGFP- β -tubulin and RFP-LifeAct. First, I established a personal wet-lab foundation in cell culture, reporter engineering, sample preparation, and standard fluorescence imaging techniques for both static and long-term experiments. Iterative tuning of seeding density, medium handling, illumination dose, autofocus policy, and acquisition cadence was used to maintain viability while retaining the temporal resolution required for cell-cycle dynamics.

Building on this expertise, we then designed and developed FUCCIsmart, an event-triggered imaging workflow implemented in commercial microscope software. Using FUCCIphase, we converted FUCCIplex nuclear intensities into cell-cycle percentage and set up phase-aware gates to trigger a switch from low-dose widefield surveillance to short spinning-disk Z-stacks on the preserved green/red structural channels. The routine integrates detection logic and device control for channel configuration, exposure/laser power, autofocus, and Z-stack management, as illustrated in Fig. 2.1A. Together, the implementation provides a reproducible acquisition pipeline suitable for high-throughput and long-term experiments.

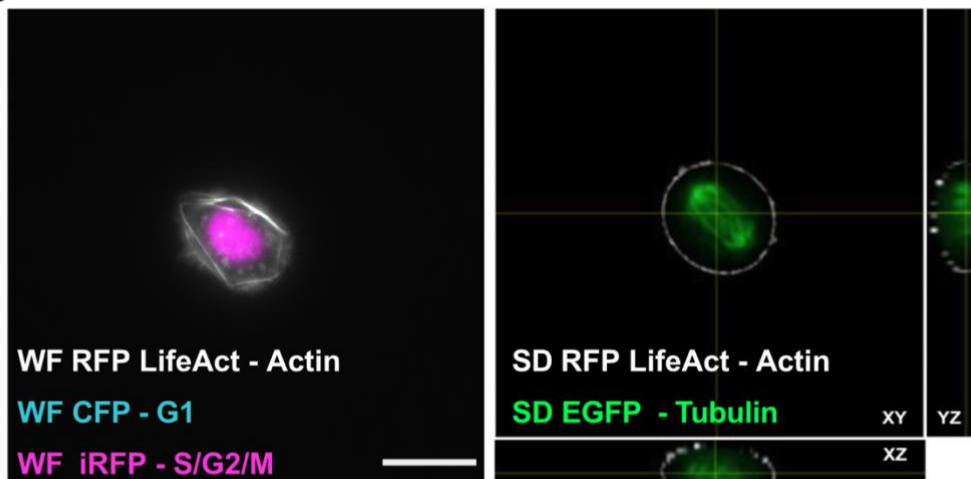
Finally, we evaluated FUCCIsmart on two levels. At the decision level, we defined mitosis labels and calculated TP/FP/FN/TN to measure detection accuracy during long-term acquisitions. At the systems level, we benchmarked against periodic 3D schedules (e.g., stacks every 1 h or 6 h), comparing (i) mitotic coverage, as the fraction of true

divisions captured with analyzable 3D spindle geometry, and (ii) phototoxicity endpoints, defined here as cell death and cell-cycle timing disruptions measured within the same experiment. Together, these tests evaluate whether a CC-aware adaptive imaging routine increases information per photon relative to conventional automation.

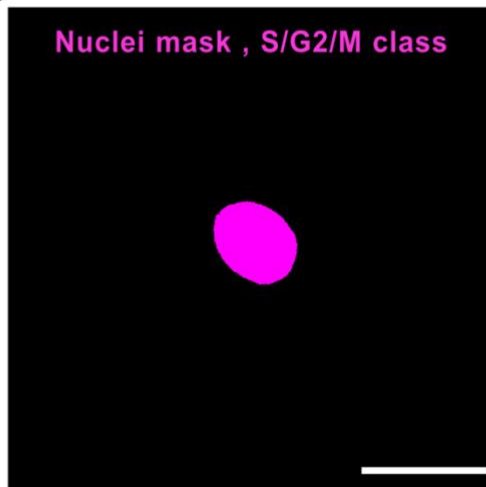
A



B



C



D

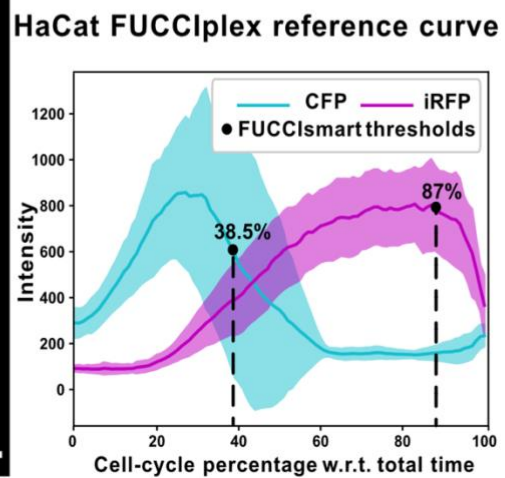


Figure 2.1. FUCCIsmart: CC-aware event-triggered imaging routine for 3D spindle acquisition. (A) Schematic of FUCCIsmart event-driven hardware switching. Nuclear CFP (G1) and iRFP (S/G2/M) provide CC context; when mitosis is likely, the system switches from low-dose widefield (WF) surveillance to spinning disk (SD) 3D confocal acquisition (Z-stack). (B) Left, Example of WF RFP LifeAct (actin) with nuclear CFP (G1) and iRFP (S/G2/M). Right, triggered SD Z-stack showing EGFP (Tubulin) and RFP (Actin) with orthogonal views (XY/YZ/XZ) (C) Nuclear intensity-based segmentation and class assignment (S/G2/M) used by FUCCIsmart. (D) FUCCIphase reference curve for HaCaT FUCCIplex cell line: CFP and iRFP intensity vs normalized cell-cycle time. Black markers indicate FUCCIsmart gating landmarks (e.g., 38.5% and 87%) used to calibrate WF screening and trigger SD acquisition.

2.2 Cell biology: reporter engineering, cell culture, and traditional imaging

FUCCIplex sensor was introduced as a spectrally re-engineered FUCCI that preserves green/red for structural probes: CFP-Cdt1 marks G1, and iRFP-Geminin marks S/G2/M. The HaCaT line used here was generated in the Synthetic Physiology Lab by lentiviral delivery and antibiotic selection and was extended to a four-color model with an EGFP tag for β -tubulin (via CRISPR/Cas9 knock-in), and RFP-LifeAct for actin. We validated the edited cell line using fluorescence microscopy, as illustrated in Fig. 2.2A. Following the reporter-engineering phase (cloning, transduction/selection, clonal screening), we optimized handling and preparation of the HaCaT model to support a robust live-imaging system.

We established the wet-lab and microscopy foundations needed for multi-day imaging of an adherent line. This included medium preparation, passaging, and counting; standardizing seeding density on fibronectin-coated #1.5H glass; and optimizing imaging medium to suppress background while retaining components essential for viability during long acquisitions.

Beyond culture and sample preparation, we defined imaging parameters to balance readout quality with minimal light burden, explicitly accounting for the signal-toxicity trade-off. Widefield (WF) nuclear screening was configured in CFP (440-450/470-500 nm) and iRFP (630-650/660-690 nm) bands, reserving EGFP (488/520 nm) and RFP (561/590 nm) for structural readouts and confocal Z-stacks. Channels were executed from

longer to shorter wavelengths to reduce cumulative exposure to higher-energy. Illumination ceilings and exposure times were adjusted to reduce saturation and limit dose, while mitigating minor CFP-EGFP bleed-through in G1. For long-term WF runs (24-48 h), we aimed to minimize dose while maintaining acceptable SNR, then optimized the cadence (15 min) and duty cycle to keep survival high and phototoxic levels low while still resolving cell-cycle transitions. Environmental control was refined by equipping the microscope with a 37 °C stage/top incubation and 5% CO₂ gas mixing to match incubator conditions and maintain physiology throughout imaging.

With sample preparation, imaging, and environmental controls in place, we conducted both static (WF/SD snapshots and Z-stacks) and dynamic acquisitions. Across asynchronous populations imaged by WF, phase occupancy was typically near 40% G1 and 60% S/G2/M, consistent with snapshot FUCCIplex and DNA-content controls. In 40-hour dynamic datasets, individual cells exhibited clear CFP-to-iRFP transitions, accompanied by remodeling of RFP-actin. Additionally, at the highest resolution, spinning-disk (SD) Z-stacks resolved 3D microtubule architecture, from planar arrays in interphase to bright EGFP spindles at mitotic entry, as shown in Fig. 2.2C.

The result is a viable, low-toxicity four-color imaging protocol for the HaCaT FUCCIplex model, suitable for both short and long-term experiments, together with a calibrated, quantitative CC timeline (via FUCCIphase) that provides the operational foundation for CC-aware phenotyping and subsequent FUCCIsmart development.

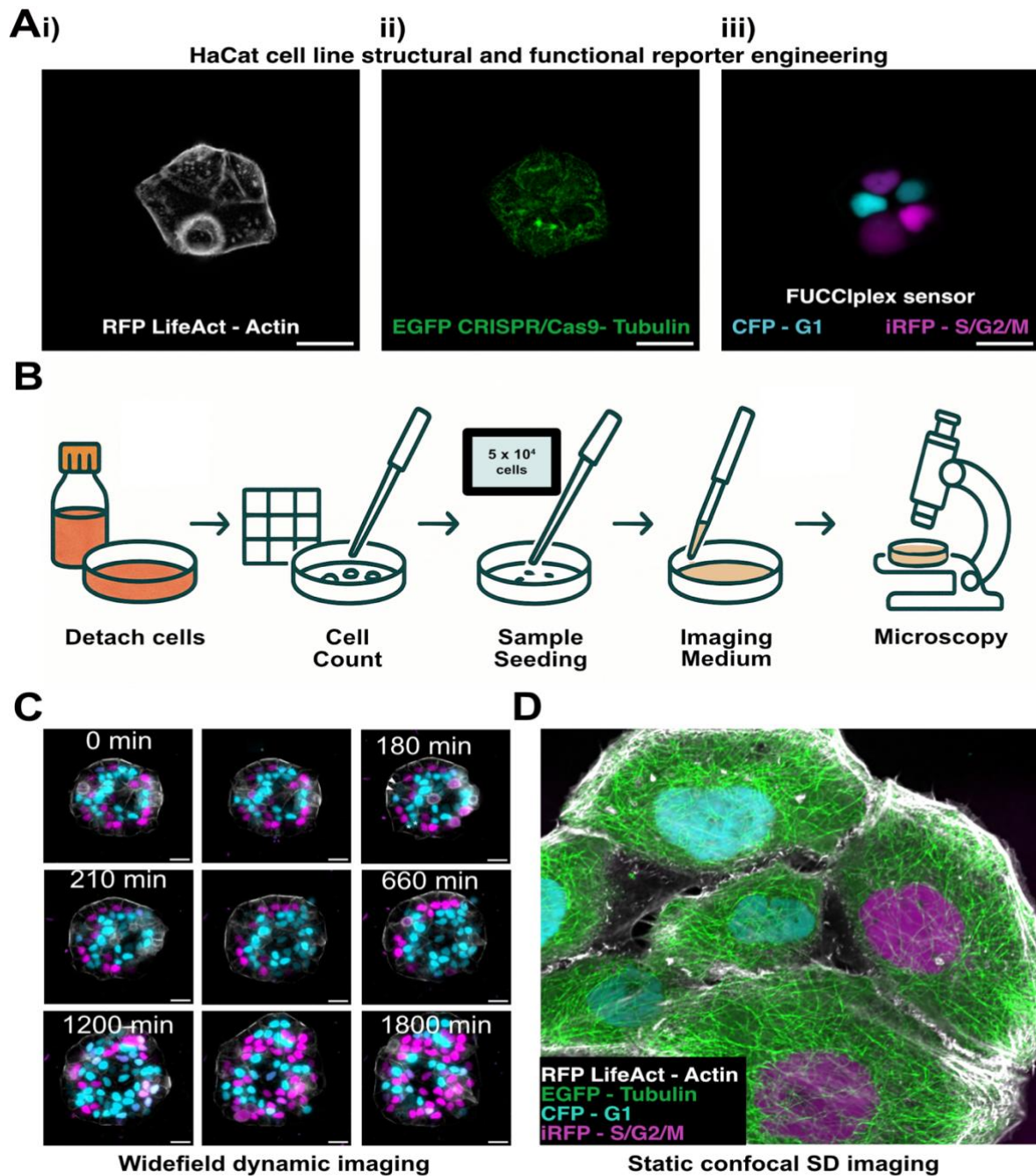


Figure 2.2. HaCaT FUCCIplex cell line: reporter engineering, cell culture, traditional imaging (A) reporter engineering steps: i) RFP LifeAct (actin), ii) EGFP β -tubulin (CRISPR/Cas9 knock-in), iii) FUCCIplex nuclear sensor (CFP = G1, iRFP = S/G2/M). Scale bars, 25 μ m. (B) Sample preparation pipeline for live imaging: enzymatic detach, cell count, seeding at defined density (e.g., 5×10^4 cells/dish) on fibronectin-coated #1.5H glass, exchange to imaging medium, and microscopy. (C) Traditional widefield imaging (WF) 30h dynamic imaging sequence showing population-level CC progression over time. (D) spinning-disk confocal (SD) static image resolving cytoskeleton and nuclear CC state simultaneously (RFP-LifeAct, EGFP-tubulin, FUCCIplex CFP/iRFP). Scale bars, 25 μ m.

2.3 FUCCIsmart design and implementation

Long-term 3D imaging is often needed to resolve the dynamic configuration of the mitotic spindle, but brute-force confocal stacks either bleach and stress cells or miss events entirely. We hypothesized that, since FUCCIplex provides a real-time readout of cell-cycle progression, we could use it to identify when a cell is about to divide and trigger 3D acquisition only then. To address this limitation, we used FUCCIplex not only as a label but also as a control signal, allowing the biological state to select the acquisition modality. This way, instead of blindly capturing volumes on a fixed schedule, the microscope could remain in a low-impact monitoring mode (e.g., low-dose 2D imaging or idle) and switch to a high-resolution volumetric mode at the key moment. In practice, the microscope stays in low-dose widefield (WF) while cells progress through interphase and switches into short spinning-disk (SD) Z-stacks only as nuclei reach late G2/early M. Because FUCCIplex shifts the cycle readout off green/red, EGFP-tubulin imaging at mitosis remains clean, avoiding CFP bleed-through at the trigger time.

We developed the FUCCIsmart algorithm for the NIS-Elements JOBS Module as a tiered scheduler with explicit gates. Fig. 2.3 shows the algorithm flowchart: a coarse loop (default 6 h) sweeps pre-selected fields with two fast WF shots with iRFP and CFP under PFS autofocus. On each pass, the routine segments nuclei, measures mean intensities, and assigns cell-cycle phases. If no nucleus appears close to division, the system stands down until the next coarse tick, keeping the dose low. As soon as a nucleus crosses the S/G2 gate (iRFP high, CFP low, calibrated with FUCCIphase), FUCCIsmart narrows focus to those fields and enters a fine loop (default 1 h). When a nucleus clears the M-probable gate ($\approx 95\%$ FUCCIphase, corroborated by nuclear area growth), the program promotes that target and switches modes: it pauses WF at that FOV, engages the Crest Optics V3 Spinning disk SD, and acquires a compact Z-stack (typical 20 μm range, 2 μm step) in EGFP Tubulin (477 nm) and RFP-Actin (546 nm) using the same 100 $\times$ silicon oil objective. After recording the event, the routine resumes widefield monitoring without requiring user intervention.

The system runs in multi-position, enabling the algorithm to be extended to high-throughput pipelines. As proof of concept during development, we scheduled on average 10 fields of view (FOV) per 24-hour session. All the initial settings, threshold gates, timeloops duration, Z-stack range, exposure/laser ceilings, and file template sit in a single initialization block, so the user can retune quickly (e.g., 8 h/30 min loops for slower/faster cycles). In this configuration, the spectral economy of FUCCIplex was beneficial: once cells advanced into S/G2/M, EGFP spindles were cleanly rendered at the trigger moment, without cyan signal.

We ran three experiments to test the algorithm and assess the match between design and performance. FUCCIsmart detected impending mitoses and captured 3D spindles with seamless WF-to-SD transitions that were stable in our runs. From a hardware standpoint, it's important to note that our microscope was equipped with an automated stage and focus maintenance, which are prerequisites for multi-site, long-term adaptive imaging. The objective's high numerical aperture (1.35) provided the needed resolution for spindle imaging, and the PFS kept the focal plane steady despite thermal or mechanical drifts. We also used Nikon's Denoise module during image acquisition for the 2D images to improve nuclear signal-to-noise while limiting exposure. Notably, FUCCIsmart was developed and calibrated using the HaCaT FUCCIplex prototype dataset, where CFP/iRFP nuclear intensities were mapped to cell-cycle percentage and used to derive phase-aware trigger gates. These thresholds are therefore not assumed to be universal across reporter constructs, cell lines with atypical cycle dynamics, or different acquisition settings. In a new framework, the practical transfer step is to acquire a short calibration time-lapse to re-estimate the CFP/iRFP reference trajectories and re-set the intensity/area gates, then re-check decision performance against ground-truth mitoses before running long experiments. We provide implementation-oriented threshold definitions and JOB logic in Appendix C1, and extended evaluation tables are in Appendix B1.

In conclusion, we designed and implemented FUCCIsmart, an event-driven imaging algorithm that uses the FUCCIplex cell-cycle signal to guide microscope behavior in real time. The system was designed to keep the microscope in a low-light widefield mode for most of the experiment and switch to confocal 3D mode primarily around the moments of interest (mitosis).

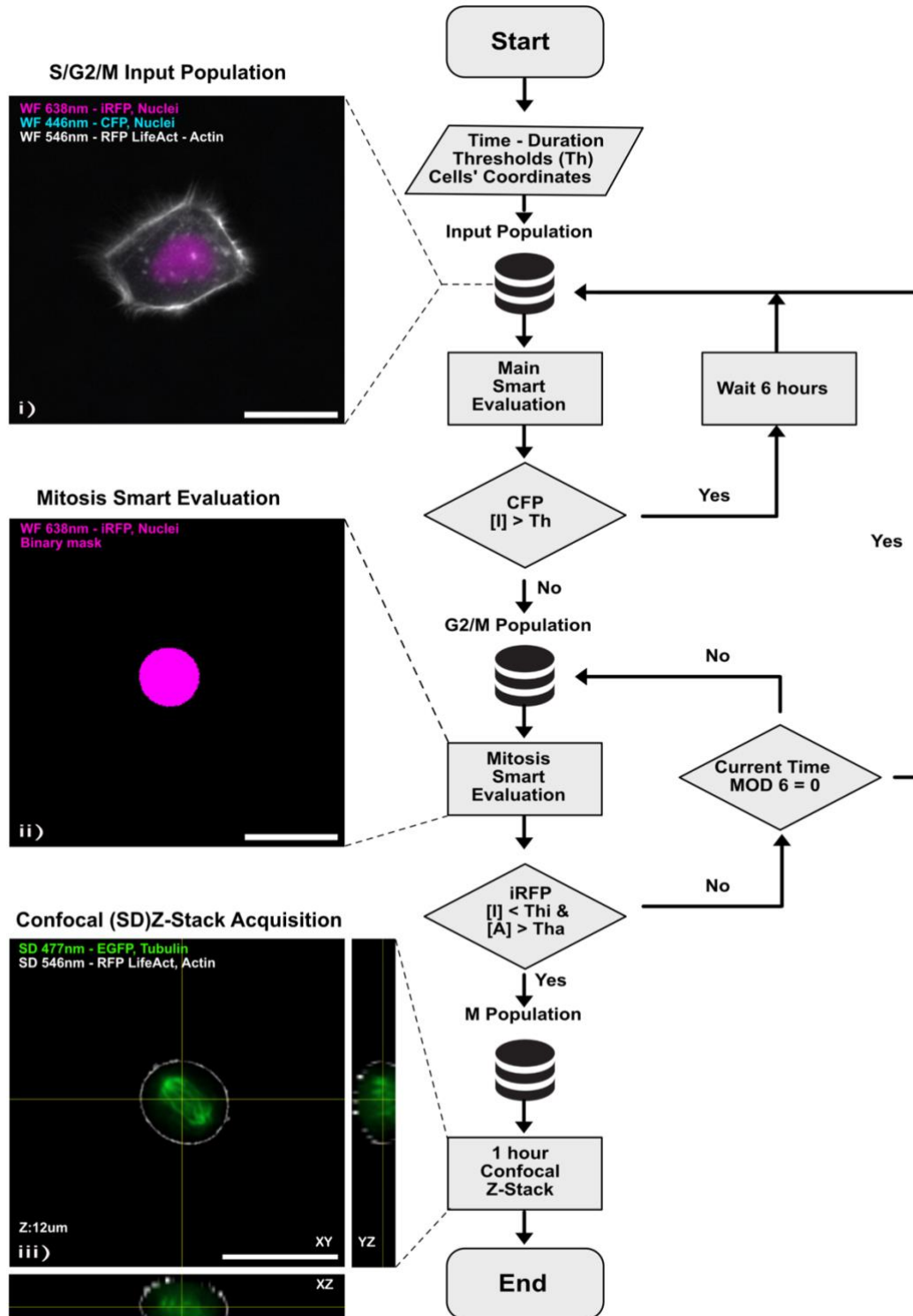


Figure 2.3. FUCCIsmart flowchart i) *Widefield screening (input population).* Low-dose WF images acquired per FOV in iRFP 638 nm (nuclei), CFP 446 nm (nuclei), and RFP 546 nm (LifeAct-actin) establish nuclear masks and intensities. Main Smart Evaluation: nuclei with CFP intensity > CFP threshold gate (Th) are classified as

S/G2/M candidates (CFP low). If no candidates are found, the routine idles and rechecks every 6 h (Current Time MOD 6 = 0). ii) Mitosis Smart Evaluation, on 6-hour ticks, S/G2/M candidates are tested for mitosis using a dual gate: iRFP intensity < iRFP threshold gate (late G2/entry to M) and nuclear area $[A] > 2 \times \text{Mean nuclei area}$. Passing nuclei are labeled as the M population and queued for high-resolution spinning-disk capture. Binary mask shown (magenta). iii) Triggered 3D capture. Upon M detection, FUCCIsmart switches hardware to SD confocal and acquires a compact Z-stack time series (~1 h; example Z-range 20 μm) in EGFP-tubulin (SD 477 nm) and RFP-LifeAct (SD 546 nm). Orthogonal views (XY/YZ/XZ) resolve spindle architecture while limiting light dose by restricting volumetric imaging to the mitotic window. Scale bars, 25 μm .

2.3.1 Algorithm: decision-level performance with live imaging

Before comparing 3D capture yield or phototoxicity, we examined whether FUCCIsmart makes an appropriate decision at an appropriate time. Decision-level evaluation isolates the control loop from imaging quality: if the trigger is incorrect, downstream metrics are affected. We thus evaluated the algorithm against ground-truth mitoses under two operational modes. Multi-stack sessions test scalability by rotating across an average of 10 fields, while single-stack sessions simulate an expert operator focusing on one region, revealing the effects of gates and cadence without the overhead of multi-site management within the same mitotic window.

For each nucleus, we analyzed the first algorithm decision within a ± 60 -minute window around the true mitosis and assigned standard labels: true positive (TP) when a trigger happened in the window; false positive (FP) when a trigger occurred without a mitosis in that window; false negative (FN) when a mitosis happened without a trigger; and true negative (TN) when neither event occurred. We also marked a subset of FPs as early triggers if the cell divided within the next hour.

In this decision-layer framing, TP rate reflects sensitivity (recall) for detecting mitoses within the target window, whereas FP rate reflects the cost of reduced specificity (unnecessary triggers). False negatives are the most consequential error mode for phenotype interpretation because missed mitoses reduce the effective sampling of division-associated morphology and dynamics and can bias conclusions if misses are state or cadence-dependent.

Across Multi-FOV runs, FUCCIsmart triggered correctly for a substantial portion of events, with performance mainly influenced by fine-loop cadence. In Experiment 1 (Exp1) shown in Fig. 2.4A, decisions were distributed as TP 56%, TN 11%, FP 22%, FN 11%. Exp 2 shows TP 35%, TN 0%, FP 26%, FN 3, while Exp 3 reported: TP 37%, TN 19%, FP 31%, FN 13%. Many divisions completed between 1-hour checks, resulting in higher FN rates. Accordingly, we interpret false negatives, FN, primarily as a cadence and workflow-limited sampling error, rather than as evidence of systematic classifier failure, although the variability across runs indicates that performance is also influenced by field load and experiment-specific timing. This motivates cadence tightening and more explicit prioritization logic when less biased mitosis sampling is required. Apparently, FPs generally reflected early firing on bright S/G2 nuclei; most of those cells entered mitosis within about 1 hour, so the additional stack often served as a pre-mitosis reference rather than an unnecessary dose.

Focusing algorithm attention to Single-FOV improved performance. Experiment 1 achieved TP 78%, TN 0%, FP 22%, FN 0%. The second run achieved TP 67%, TN 33%, FP 0%, FN 0%, while run 3 showed TP 55%, TN 18%, FP 9%, FN 18%, again reflecting cadence limits rather than gate failure. In all cases, triggers clustered before or near the actual mitosis timepoint, producing analyzable spindle stacks while the system stayed dark during non-mitotic periods.

These results identify two tractable behaviors. First, early triggers are the costs of precision in prioritizing recall near the mitotic window; they can be intentionally retained when pre-mitotic organization is valuable or suppressed by raising the M-gate threshold. Second, cadence-limited misses occur when mitoses complete between fine-loop checks. Tightening the fine loop from 60 to 30 minutes addresses this issue with minimal light overhead, as monitoring remains low-dose and widefield. In sum, the classifier generally performs as intended: it fires when information density peaks and stands down otherwise, with residual errors that are explainable and correctable by simple parameter tuning (gates and cadence). This decision-layer validation motivates the next analysis stages: system-level benchmarking of 3D mitotic capture and phototoxic load against fixed-interval confocal schedules.

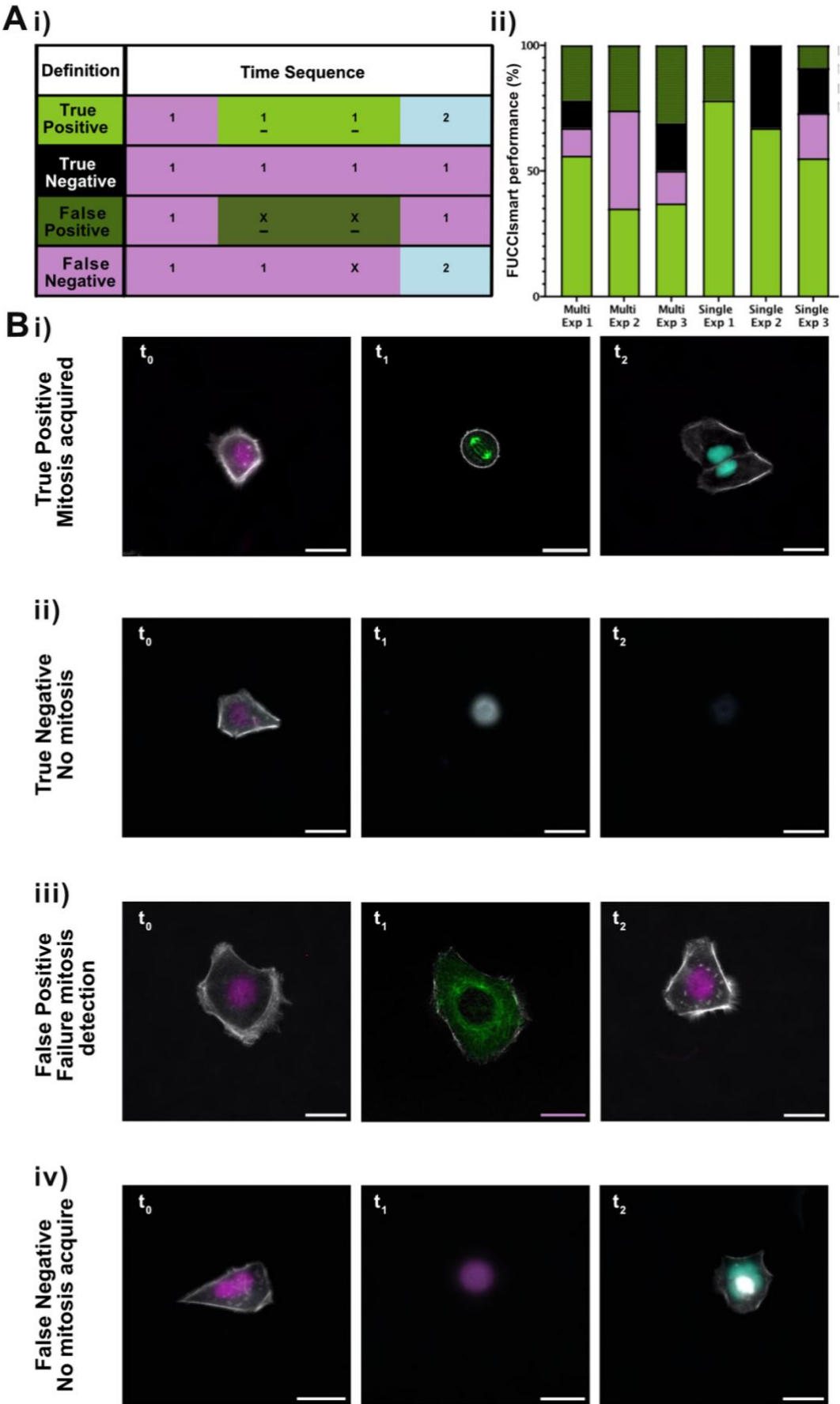


Figure 2.4. FUCCIsmart routine decision outcomes: performances and representative cases. (A) i) definition matrix and time-sequence schematic for true positive (TP), true negative (TN), false positive (FP), and false negative (FN) decisions. TP = trigger within ± 60 min of a ground-truth mitosis. FP = trigger without a mitosis in that window; FN = mitosis without a trigger; TN = neither trigger nor mitosis. ii) stacked bars summarizing decision fractions for six 24-h sessions (Multi stack Exp1-3; Single stack Exp1-3). (B) Representative fields for each outcome in time sequence: i) TP, mitosis acquired; ii) TN, no mitosis; iii) FP, trigger without mitosis in the window; iv) FN, mitosis not acquired. Fluorophores: iRFP nuclei (magenta, S/G2/M), CFP nuclei (cyan, G1), EGFP tubulin (green), RFP LifeAct (white, actin). Scale bars, 25 μ m

2.4 FUCCIsmart mitosis coverage vs. phototoxicity

We set out to test whether a cell-cycle-aware trigger can outperform fixed schedules in two key aspects of long-term mitosis imaging: capturing the event and maintaining cell viability. Conceptually, the benchmarks frame a common trade-off. A cautious 6-hour confocal stack minimally perturbs the sample but misses divisions that occur in between; an hourly stack reduces misses but increases light burden. FUCCIsmart is designed to avoid both extremes: it screens in widefield and allocates photons only when FUCCIplex indicates late G2/early M. To probe this, we ran three replicates of two fixed schedules in 24-hour experiments with identical optics and powers that differed only in timing, and we quantified the fraction of divisions recorded in 3D and the fraction of cells showing phototoxicity, which here is operationally defined as death or cycle prolongation beyond FUCCIplex baselines.

We also tested two FUCCIsmart modes: a single-stack capture at detection and a short, selective time series of 5 stacks across the mitotic window.

The data indicate that the trigger improves the yield-toxicity balance under these conditions. FUCCIsmart-single captured $66.67 \pm 11.50\%$ of mitoses while limiting phototoxic events to $7.33 \pm 8.74\%$. Hourly automation achieved a similar capture rate ($69.67 \pm 8.33\%$) but with a $60.33 \pm 6.43\%$ phototoxicity burden. In this dataset, FUCCIsmart-single delivered a similar yield with substantially lower phototoxicity. FUCCIsmart-multi, which acquires five stacks across M-phase to build a brief 3D time-series, captured $43.00 \pm 11.36\%$ of mitoses, approximately $3.3\times$ the yield of the sparse 6-hour schedule ($13.00 \pm 2.65\%$), while keeping phototoxicity at $15.67 \pm 4.16\%$, close to the 6-hour baseline ($13.33 \pm 3.79\%$). Thus, even the richer multi-stack mode remains far gentler than hourly automation while substantially improving event coverage over sparse imaging.

These outcomes are consistent with tying acquisition to biology: FUCCIsmart treats mitosis as a time-limited target and allocates photons primarily around the predicted window. The single-stack occupies a high-yield/low-toxicity regime relative to fixed

schedules, approaching the hourly strategy for event capture without its collateral damage, whereas the multi-stack mode offers time-resolved 3D spindle dynamics at an exposure cost comparable to cautious imaging. Because all groups used the same hardware and per-frame settings, the observed differences reflect the timing policy rather than changes in optics or exposure parameters.

In sum, the quantitative comparison supports that state-aware control mitigates the fixed-schedule trade-off in this setting. FUCCIsmart captures a larger fraction of divisions with lower phototoxicity in these experiments, and scales from single snapshots at mitotic entry to compact mitosis movies without a proportional increase in phototoxicity. Overall, these results link the control strategy to measurable gains and motivate the use of CC-aware acquisition for long-term phenotyping.

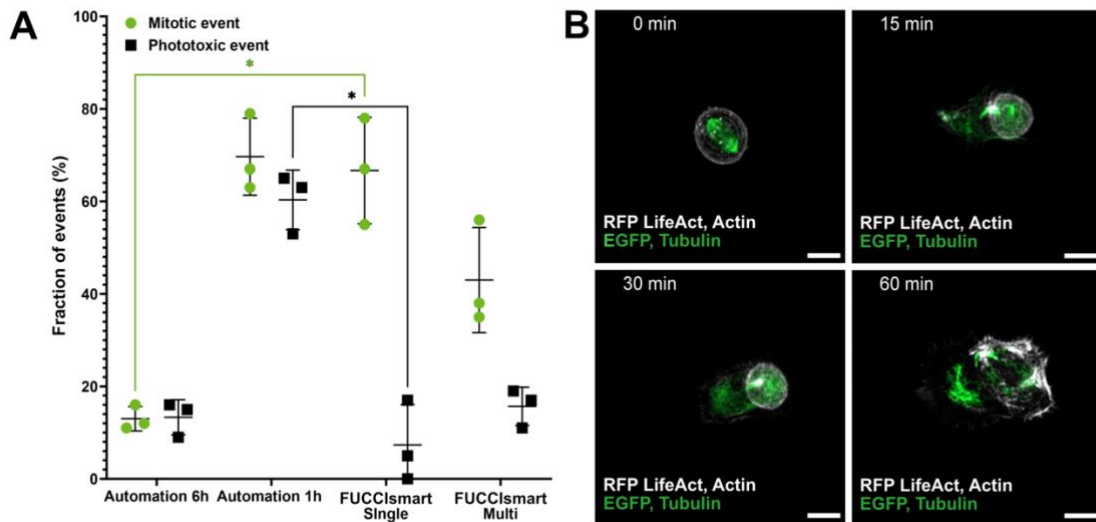


Figure 2.5. FUCCIsmart quantitative benchmark across four 24-h acquisition schedules.

(A) Green circles: fraction of mitotic events captured in 3D; black squares: phototoxic events (cell death or cycle-length prolongation). Conditions: Automation 6 h (sparse fixed schedule), Automation 1 h (frequent fixed schedule), FUCCIsmart Single (one Z-stack at detection), and FUCCIsmart Multi (five short Z-stacks over ~1 h around mitosis). FUCCIsmart-Single achieves hourly-level capture with markedly lower phototoxicity; FUCCIsmart-Multi performs better than the 6 h schedule in capture while maintaining near-baseline toxicity. Points show independent experiments; bars indicate mean $\pm$ SD. (B) Representative FUCCIsmart-Multi sequence (0, 15, 30, 60 min) showing time-resolved spindle and actin remodeling: EGFP tubulin (green) and RFP LifeAct Actin

(white) acquired by triggered spinning-disk Z-stacks during the mitotic window. Scale bar: 25 μm .

2.5 Discussion

The FUCCIplex sensor, recently developed in the lab, places the cell-cycle at the center of live phenotyping, where phase serves as the context that shapes morphology, signaling, and cellular decision-making. This context becomes particularly important when the event of interest is intrinsically three-dimensional, like the mitotic spindle. Continuous 3D confocal can reveal spindle architecture but imposes a high light cost; sparse schedules reduce dose but miss many divisions. In this chapter, we introduce FUCCIsmart, an event-driven algorithm that addresses this trade-off by allowing the cell's state to guide acquisition: low-dose widefield imaging while cells traverse interphase, and short spinning-disk Z-stacks only when FUCCIplex indicates late G2/early M.

Our validation supports FUCCIsmart as a proof-of-concept for state-aware, event-driven mitosis imaging rather than as a fully stabilized acquisition framework. Under the conditions tested, FUCCIsmart improved the trade-off between mitotic-event capture and phototoxicity relative to fixed schedules. In particular, FUCCIsmart-single captured $66.67 \pm 11.50\%$ of mitoses while keeping phototoxic events at $7.33 \pm 8.74\%$, whereas hourly automation achieved a similar capture rate at a markedly higher phototoxicity burden. FUCCIsmart-multi, which acquired five stacks across M phase, also improved event recovery relative to the sparse 6 h schedule while maintaining phototoxicity close to that lower-dose baseline. These results support the central premise of the method: concentrating 3D acquisition around biologically informative time windows can increase information yield per photon.

At the same time, the decision-level results indicate that performance remains cadence-dependent and sensitive to the operational context of the experiment. In particular, false negatives should not be interpreted solely as a classifier failure, but neither should they be attributed uncritically to cadence alone. The variability observed across runs, especially in Multi-FOV mode, suggests that missed events arise from a combination of factors, including temporal undersampling, differences in cell-cycle timing across fields,

and the practical overhead of managing multiple positions within the same mitotic window. Accordingly, the present implementation should be viewed as an initial operational version whose performance depends on the selected fine-loop interval, field load, and gate calibration.

This method shows practical advantages, including higher event yield at lower dose, clean spindle contrast enabled by FUCCIplex's spectral efficiency, and easy deployment on commercial systems, but it also has limitations. First, the threshold gates are intensity-based (calibrated for the HaCaT FUCCIplex line, around $> 37\%$ S/G2 and 95% M) and will require recalibration for different lines or cell-cycle sensors. Second, the fine-loop cadence is limited and may need to be increased for ultra-fast mitoses, and third, consistent FUCCIplex expression is essential.

FUCCIsmart demonstrates the value of coupling a live cell-cycle readout to adaptive microscope control, but the current benchmarks establish a feasible regime and identify the main tuning parameters for future optimization. In practice, tighter fine-loop cadence, adaptive prioritization across competing FOVs, and more robust trigger logic should reduce run-to-run variability and improve capture consistency, particularly in higher-load experiments. Looking ahead, three extensions follow directly from this framework. First, static thresholds could be replaced with machine learning classifiers on widefield nuclei to anticipate prophase earlier and reduce both false triggers and misses. Second, the freed green/red channels could be used to add functional reporters (tension, kinase activity, Ca^{2+}) and make the trigger multi-factor, for example coupled information from mitosis AND signaling pulse. Third, the approach can be scaled in time and space toward high-throughput and high-content screening. In this direction, it should be possible to run 48-72 hour sessions with dozens of FOVs and simple schedulers to manage multiple events simultaneously, leveraging the low monitoring dose. In summary, FUCCIplex provides the cell-cycle readout, and FUCCIsmart uses it to steer acquisition. The result is long-term, high-content imaging intended to be informative while limiting dose: mitotic coverage comparable to hourly sampling without the phototoxicity burden of frequent volumetric imaging, and compact mitosis movies at exposure costs comparable to those of careful imaging. By combining biosensor readouts with real-time control, this chapter

frames a shift from passive observation to context-aware microscopy, providing a practical basis for studying dynamic, rare events in tissue engineering.

The FUCCI smart approach addresses the temporal dimension of acquisition, but it does not resolve the spatial constraint that arises in engineered assays. Indeed, in patterned microenvironments, throughput and reproducibility depend on the ability to map fabrication layouts onto microscopy coordinates. This limitation is addressed in Chapter 3.

2.6 Materials and Methods

HaCaT cell line and media

HaCaT cells were kindly provided by Professor Hugo de Jonge (Department of Molecular Medicine, University of Pavia) and cultured in DMEM/F12 no phenol red (catalog# 21041-025, Gibco) with 10% Fetal Bovine Serum (FBS) (catalog # 10270-106, Gibco) and 1% Penicillin/Streptomycin (P/S) (catalog# A001, HiMedia). Cells were never allowed to reach full confluency and were split at 70% of confluence.

HaCaT FUCCIplex-prototype generation:

The generation of the HaCaT FUCCIplex-prototype clonal cell line consisted of 3 distinct, sequential steps. First, HaCaT cells were infected with the FUCCIplex lentivirus and positively selected with antibiotics. A second lentiviral infection with a genetically encoded LifeAct-ACTB-RFP probe created a double reporter cell line that has the (F-)actin filaments tagged with a red fluorescent reporter in addition to the cell-cycle indicator FUCCIplex. After antibiotic selection, cells were genome-edited to tag alpha-tubulin with an EGFP fluorophore at the N-terminus; this new 3-reporters cell line was then clonally expanded and selected. More in detail, HaCaT cells were plated at the density of 6×10^4 cells per well in a 12-well plate in complete DMEM/F12. The following day, the medium from the tissue culture plate was removed by aspiration and replaced with a fresh complete medium containing 8 $\mu\text{g/ml}$ polybrene (VectorBuilder). Next, 10 μL of concentrated FUCCIplex lentivirus particles ($>10^8$ TU/ml, VectorBuilder) were resuspended in 500 μL of complete medium and added directly into the well. Cells were incubated overnight at 37 °C and 5% CO₂, and the next morning the transduction medium was aspirated and replaced with a fresh complete medium. After 4 days, positively transduced cells were selected with 20 $\mu\text{L/ml}$ of Hygromycin B (50 mg/ml in PBS) (catalog # 10687010, Invitrogen). HaCaT FUCCIplex cells were then clonally seeded and expanded. Positive cells were evaluated by PCR and confocal imaging to assess the correct expression of the different reporters. For the LifeAct-ACTB-RFP lentivirus infection (rLV Ubi-LifeAct-TagRFP, 1×10^7 TU/ml, 10 μL), cells underwent the same

procedure with the only difference that 0.62 µl/ml of Puromycin (1 µg/µl) was used to select positive cells. Finally, HaCaT cells were genome-edited using the ThermoFisher Scientific TrueTag system. The donor sequence and primers with specific homology arms for GFP-tagging the N terminus of alpha-tubulin (TUBA1B, NM006082.3) were designed online with the assistance of the ThermoFisher Scientific TrueDesign Genome Editor tool. Briefly, the linear TrueTag dsDNA donor oligo with the TUBA1A homology arms was amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix, TrueTag Donor DNA Kit, GFP (catalog# A42992, Thermo). The TrueTag homology arm primer forward sequence is:

TCCTGTCGCCTTCGCCTCCTAATCCCTAGCCACTATGGGAGGTAAGCCCTTG
CATTCG;

The reverse is:

CCTGAAAGCAGCCGGGAGCCGCACGGCTTACTCACACCGCTTCCACTACCT
GAACC). Next, the TrueGuide Synthetic gRNA (sgRNA) (ThermoFisher Scientific, sequence: GCACGGCTTACTCACCATAG) and True cut CAS9 Protein V2 (catalog# A36496, ThermoFisher Scientific) were combined to form the ribonucleoprotein (RNP) complex (CAS9+gRNA). True cut CAS9 Protein V2 (2 µg, 12 pmol), sgRNA (400 ng, 12 pmol), TrueTag donor (500 ng) were resuspended in 10 µL of Resuspension Buffer R (catalog# MPK1025, ThermoFisher Scientific) and then combined with 8 x 10⁴ cells resuspended in 8 µl of Resuspension Buffer R for a total final volume of 18 µL. 10 µL of this mix was used to electroporate the cells with the Neon transfection system (catalog# MPK5000, ThermoFisher Scientific). The conditions for electroporation were the following: 1200 V / 20 ms / 4 pulses. Electroporated cells were seeded in a 24-well plate filled with 0.5 ml of DMEM/F12 medium containing 10% FBS without antibiotics. The plate was incubated at 37 °C in a humidified CO₂ incubator. Positive cells were then clonally plated and expanded.

Widefield and confocal imaging

Imaging was performed using a commercial fully motorized Nikon Ti-2 widefield inverted microscope stand with the Perfect Focus System integrated with a Crest V3 X-Light spinning disk confocal module. The microscope is equipped with a Nikon linear-

encoder motorized stage with a Mad City Labs 100 μm range Z piezo insert (catalog #NI-2-C312, Mad City Labs MCL). Fluorescence was achieved using a Lumencore Celesta Light Engine (405 nm, 477 nm, 520 nm, 546 nm, 577 nm, 638 nm, 740 nm, catalog# TSX5030FV), with 7 solid-state laser lines that deliver up to 800 mW of power at the fiber tip. This work used solid-state directly modulated 638, 546, 477 and 446 nm laser lines. Excitation light passes through the dichroic mirror wheel, hitting either a hard-coated Full Multi Band Penta CELESTA -DA/FI/TR/Cy5/Cy7-A (catalog #MXR00543, Nikon) or a Full Multiband dual CELESTA -CFP/YFP-A (catalog #MXR00544, Nikon). Depending on the specific laser line, the light was subsequently filtered through either a Multiband Full Penta FF01-391/477/549/639/741 (catalog #FL-416877, Semrock) or a Full Multiband dual FF01- 449/520 (catalog #FL-411981, Semrock). The sample emitted fluorescence is then collected by a Teledyne Photometrics Kinetix CMOS camera through various objective lenses depending on the experimental requirements, including Nikon CFI Plan Apo 20X objective (air-immersion, N.A. 0.75, WD 1 mm, catalog# MRD00205, Nikon), Nikon CFI Plan Apo Lambda S 25XC Sil objective (oil-immersion, N.A. 1.05, WD 0.55 mm, catalog# MRD73250, Nikon) Nikon CFI Plan Apo Lambda S 40XC Sil objective (oil-immersion, N.A. 1.25, WD 0.3 mm, catalog# MRD73400, Nikon) Nikon CFI SR HP Plan Apo Lambda S 100XC Sil objective (oil-immersion, N.A. 1.35, WD 0.31 - 0.28 mm, catalog# MRD73950, Nikon) within Nikon silicon immersion oil 30cc (catalog #MXA22179, Nikon).

The detector collected proper filtered light using one of the matching emission filters placed in the wheel: Single band FF01-484/561 (catalog #FL-412124, Semrock) for mTurquoise2 emission collection, Single band FF01-685/40-25 nm (catalog #FL-011482, Semrock) for the Geminin signal, Single band FF01-595/31 (catalog #FL-004391, Semrock) for RFP, or FF01-511/20-25 (catalog # FL-004306, Semrock) for EGFP fluorescence. The signals from each channel were acquired sequentially, following a far-red (638nm) to cyan (446nm) laser line order. The effective number of pixels (3200x3200) was cropped to 2700x2700 (6.5 μm pixel size) and used with a 16-bit digitizer and no binning. Image acquisition was managed via Nikon Elements AR NIS software (version 5.42.03). Data were saved as ND2 files.

According to the sample preparation, all experiments were performed under live imaging conditions using a phenol red-free imaging medium. Samples were prepared on #1.5H glass coverslips coated with 0.2% porcine gelatine (catalog # G2625-500G, Sigma-Aldrich) in a round dish, 8-well, 96-well formats (catalog #81158, #80807, #89626, Ibidi), and 96-well nanosurface plate format (catalog# ANFS-0096, CuriBio). The microscope was housed in an Okolab enclosure to maintain physiological conditions during imaging. The enclosure maintained a temperature of 37°C with 5% CO₂ and passive humidification. Confocal and widefield imaging were both used for different tasks. Typical experiments were set up with sequential multichannel acquisitions, multiple fields of view (FOV), and time-lapse imaging. The shutter remained closed during stage movements or when idle, and the Perfect Focus system was chosen as the focus maintenance device. For Z-stack imaging, Z-stacks were acquired using the MCL Piezo Z-device by capturing each channel across the entire Z-stack or each focal plane across all channels.

FUCCIphase calibration

For HaCaT FUCCIplex image analysis, FUCCIplex signals were calibrated using an in-house software plugin called FUCCIphase, a Python package developed in our lab in the CALIPERS framework¹⁴ to facilitate cell-cycle analysis of single-cell FUCCI data. FUCCIphase converts the two fluorescence intensities (CFP and iRFP nuclear signals) into a quantitative cell-cycle percentage (0% at the start of G1 and 100% at the end of M) by referencing an average intensity vs. time curve. We established the reference by tracking several cells through full cycles and by cross-validating against fixed-cell-cycle stage assays (e.g. DNA content)

FUCCIsmart sample preparation culture

All cell culture and imaging was done in standard conditions: cells were grown on #1.5H high-precision glass coverslips coated with 20 µg/mL fibronectin (catalog# F0895, Sigma-Aldrich) in dishes, seeded at low density ($\sim 1.5 \times 10^4$ cells, ~ 15 h before imaging to allow attachment and cell-cycle re-entry after plating). DMEM F-12 without phenol red was chosen as Imaging medium for fluorescence microscopy (catalog #21041025, Gibco)

FUCCIsmart statistical analysis

The percentages of mitosis acquired and the phototoxicity levels generated in the Single FUCCIsmart routine and Multi FUCCIsmart routine are compared against Z-stack automation every 1h and every 6 hours as positive controls. The percentage of mitosis acquired is calculated over the number of cells present at the beginning of the experiment (N). In contrast, the events of phototoxicity are calculated over the expected number of cells at the end of experiment 2N.

Each experiment class was repeated 3 times, with 13 Fields of view (FOVs) excluded from the analysis due to imaging issues or cells escaping from the imaging FOV. Through the work, our results are presented as mean $\pm$ standard deviation (SD). The following tables present summary and descriptive statistics for the dataset. Graph represented in Fig. 2.5A, and statistical analyses were performed using GraphPad Prism (version 10.3.01)¹⁹. After checking for normality and variance differences, a two-way ANOVA was used to compare the source of variation between the group and the readouts of Mitosis acquired and the Phototoxicity yield. Statistical power was defined at an alpha level of 0.05, and statistical significance was defined as $p < 0.05$. To confirm the assumption of a normal distribution, the acquired datasets were checked for normality using the Shapiro-Wilk test.

Shapiro-Wilk Test on Mitosis Acquired dataset	FUCCIsmart Single	FUCCIsmart Multi	Automation 1h	Automation 6h
W	0.9994	0.8547	0.9231	0.8929
P value	0.9521	0.2530	0.4633	0.3631
Passed normality test alpha =0.05	Yes	Yes	Yes	Yes

Shapiro-Wilk Test on Phototoxicity dataset	FUCCIsmart Single	FUCCIsmart Multi	Automation 1h	Automation 6h
W	0.9465	0.9231	0.8710	0.8547
P value	0.5542	0.4633	0.2983	0.2530
Passed normality test alpha =0.05	Yes	Yes	Yes	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3100	3	1033	F (3, 16) = 16.98	P<0.0001
Row Factor	3432	1	3432	F (1, 16) = 56.38	P<0.0001
Column Factor	8446	3	2815	F (3, 16) = 46.25	P<0.0001

Multiple comparisons were made using Bonferroni's correction and are shown in the related appendix. Statistical differences were shown in the tested groups:

- Mitosis Acquired (%): FUCCIsmart Single vs. Automation 6h with a $p < 0.0001$, represented in Fig. 2.5A.
- Mitosis Acquired (%): FUCCIsmart Multi vs. Automation 1h with a $p=0.0042$
- Mitosis Acquired (%): Automation 6h vs. Automation 1h with a $p < 0.0001$
- Phototoxic events (%): FUCCIsmart Single vs. Automation 1h with a $p<0.001$, represented in Fig. 2.5A).
- Phototoxic events (%): FUCCIsmart Multi vs. Automation 1h with a $p < 0.0001$
- Phototoxic events (%): Automation 6h vs. Automation 1h with a $p < 0.0001$

2.7 Bibliography

1. Théry, M. *et al.* The extracellular matrix guides the orientation of the cell division axis. *Nat. Cell Biol.* 7, 947-953 (2005).

2. Lechler, T. & Mapelli, M. Spindle positioning and its impact on vertebrate tissue architecture and cell fate. *Nat. Rev. Mol. Cell Biol.* 22, 691-708 (2021).
3. Morin, X. & Bellaïche, Y. Mitotic Spindle Orientation in Asymmetric and Symmetric Cell Divisions during Animal Development. *Dev. Cell* 21, 102-119 (2011).
4. Noatynska, A., Gotta, M. & Meraldi, P. Mitotic spindle (DIS)orientation and DISease: Cause or consequence? *J. Cell Biol.* 199, 1025-1035 (2012).
5. Kline-Smith, S. L. & Walczak, C. E. Mitotic Spindle Assembly and Chromosome Segregation: Refocusing on Microtubule Dynamics. *Mol. Cell* 15, 317-327 (2004).
6. Siller, K. H. & Doe, C. Q. Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* 11, 365-374 (2009).
7. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell.* (2008).
8. Zielke, N. & Edgar, B. A. FUCCI sensors: powerful new tools for analysis of cell proliferation. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 469-487 (2015).
9. Koh, S.-B. *et al.* Quantitative FastFUCCI assay defines cell cycle dynamics at single-cell level. *J. Cell Sci.* jcs.195164 (2016) doi:10.1242/jcs.195164.
10. Sakaue-Sawano, A. *et al.* Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. *Cell* 132, 487-498 (2008).
11. Marcus, J. M., Burke, R. T., DeSisto, J. A., Landesman, Y. & Orth, J. D. Longitudinal tracking of single live cancer cells to understand cell cycle effects of the nuclear export inhibitor, selinexor. *Sci. Rep.* 5, 14391 (2015).
12. Chai, F., Cheng, D., Nasu, Y., Terai, T. & Campbell, R. E. Maximizing the performance of protein-based fluorescent biosensors. *Biochem. Soc. Trans.* 51, 1585-1595 (2023).
13. Qian, Y., Celiker, O. T., Wang, Z., Guner-Ataman, B. & Boyden, E. S. Temporally multiplexed imaging of dynamic signaling networks in living cells. *Cell* 186, 5656-5672.e21 (2023).
14. Di Sante, M. *et al.* CALIPERS: Cell cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* 2024.12.19.629259 (2024)
15. Petry, S. Mechanisms of Mitotic Spindle Assembly. *Annu. Rev. Biochem.* 85, 659-683 (2016).
16. Spinning-disk confocal microscopy: present technology and future trends. in *Methods in Cell Biology* vol. 123 153-175 (Academic Press, 2014).
17. Chen, B. *et al.* Resolution doubling in light-sheet microscopy via oblique plane structured illumination. *Nat. Methods* 19, 1419-1426 (2022).
18. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017).
19. Home - GraphPad. <https://www.graphpad.com/>.

3

A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement

This chapter is adapted from: Pezzotti M., et al. *A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement*. *APL Bioengineering* 10, 016114 (2026). doi: 10.1063/5.0306480. The published article is distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) license. This chapter has been rephrased for consistency with the thesis narrative, notation, and cross-references, while preserving the underlying scientific content. As introduced in Chapter 1, engineered microenvironments expand the measurable “readout space” by shaping cell state and behavior, but they also amplify the need for standardized, comparable measurements, particularly when cell-cycle (CC) gating is itself modulated by geometry and mechanics. Therefore, HT/HC phenotyping under an engineered microenvironment, such as patterned confinement, fails not only because imaging is limited by dose, but also because fabrication parameters, coordinate registration, and downstream analysis are rarely executed as a reproducible end-to-end pipeline. Building on Chapter 2, where the cell-cycle was used as a real-time gating signal to determine *when* and *what to image* under phototoxicity constraints, this chapter addresses the next scalability requirement: deciding *where to image* and ensuring that “where” can be traced from pattern design to field selection. Accordingly, we present a vertically integrated system that combines multiplexed biological reporters with substrate engineering and a correlative fabrication-to-microscopy pipeline suitable for high-content imaging/analysis to quantify CC-migration and morphology coupling under defined planar confinement.

3.1 Introduction

Recent estimates still report cancer as a leading cause of mortality, with 20 million new cases and 9.7 million deaths in 2022, the majority after metastatic spread¹. Beyond genetic alterations, tumor progression is critically shaped by the tumor microenvironment, where the extracellular matrix (ECM), stromal barriers, and tissue architecture impose physical constraints on cell behaviors. In vivo, these microenvironmental features govern how cells migrate, invade, and proliferate during dissemination². Among them, geometric confinement challenges cell migration³, affects nuclear integrity^{4,5}, and can alter cell-cycle (CC) progression^{6,7}. However, in vitro assays generally measure either migration or proliferation: standard 2D assays, such as scratch and transwell assays⁸⁻¹⁰, report collective migration or transmigration but do not detail cell-cycle phase; more advanced 3D in vitro models (e.g., spheroids, collagen invasion assays) better mimic tissue architecture but likewise seldom provide real-time information on cell-cycle stage¹¹. Conversely, endpoint readouts such as EdU and Ki-67 provide snapshots of the proliferative state of the cells but cannot track CC dynamics over time^{12,13}. Furthermore, ECM cues, such as substrate stiffness¹³⁻¹⁵, chemical composition with force transmission¹⁶, and cell shape and geometry¹⁷, have been shown to regulate adhesion, cytoskeletal tension¹⁸⁻²⁰, and proliferation programs²¹⁻²⁴. Each of these has been linked to either proliferative or migratory phenotypes, underscoring the importance of contextual mechanical inputs in determining cell fate.

To study how confinement reshapes CC-migration coupling^{7,25-27}, it is crucial to integrate confined migration assays with live CC readouts. However, open technical challenges are preventing this integration. First, most fluorescent CC reporters rely on GFP and RFP²⁸, the same spectral channels used by common structural and functional sensors. This competition limits multiplexing, making it difficult to track the CC and migration-driving cytoskeletal dynamics simultaneously. Second, photopatterning approaches such as LIMAP and PRIMO now allow fabrication of engineered ECM islands with precise geometry²⁹. However, aligning these patterns with microscope fields of view still requires manual trial-and-error, reducing throughput and reproducibility³⁰. Finally, it has been

difficult to obtain accurate long-term quantification when cells can move out of the field of view over hours of imaging³¹⁻³³. These obstacles span distinct competencies: reporter engineering, photopatterned microfabrication, and high-content imaging and analysis, which require different skill sets and infrastructure. Bringing them together within one workflow imposes a substantial coordination and tooling burden, which has constrained routine, large-throughput execution of end-to-end CC-aware confinement assays.

To address this gap, we introduce a vertically integrated platform that simultaneously tracks migration and assesses the cell-cycle, unifying multiplexed reporters, photopatterned microfabrication, and high-content live-cell imaging. This system builds directly on our CALIPERS³⁴ framework. CALIPERS denotes the multiplexed, cell-cycle-aware live-imaging phenotyping framework built around the FUCCIplex reporter (CFP/iRFP) and additional structural readouts, which leverages FUCCIplex, a cell-cycle sensor spectrally multiplexable with diverse structural and functional reporters. Here, we extend this concept to a migratory HT1080 fibrosarcoma model and integrate it with Fab2Mic. This fabrication-to-microscopy correlative pipeline engineers ECM islands and registers them to acquisition coordinates, enabling automated imaging of single-cell migration and proliferation on each island. Custom image analysis scripts segment and track individual cells and nuclei over time, allowing quantification of how 2D geometric confinement modulates migration-CC coupling.

In a broad tissue engineering context, microenvironments are design variables used to bias cell state, organization, and function. A practical bottleneck is converting microenvironment features (geometry, ligand presentation, boundary conditions) into reproducible, comparable phenotypes at the screening scale. The Fab2Mic + CALIPERS workflow addresses this by providing coordinate-traceable microenvironment provenance and longitudinal, cell-cycle-resolved phenotyping that can be reused as a decision layer for selecting microenvironment designs before moving to more expensive 2.5D/3D constructs.

3.2 Microscopy-based automatic selection for engineered reporter line

We set out to engineer HT1080 fibrosarcoma cells into a four-reporter reference line that simultaneously encodes CC state and a migration-supporting cytoskeletal structure (Fig. 3.1A). In this work, we used HT1080 cells carrying EGFP LifeAct construct, edited the endogenous TUBA1B locus with a red fluorescent protein (RFP) tag via CRISPR/Cas9 editing, and introduced the FUCCIplex cassette via lentiviral infection under the EF1 α promoter (Methods). The FUCCIplex design replaces the RFP and GFP markers for the G1 and S/G2/M phases in the traditional FUCCI sensor²⁸ with cyan and far-red variants (CFP and iRFP), enabling simultaneous readout of CC state alongside actin and tubulin reporters.

Following the selection of engineered cells using hygromycin, we confirmed that actin filaments, microtubules, and nuclei, marked in G1 (CFP) or S/G2/M (iRFP), were fluorescently tagged using high-resolution microscopy and PCR (Fig. 3.1A, Fig. A2.1A,C). In terms of nuclear markers, the mixed population featured CFP localized robustly to nuclei. In contrast, iRFP frequently exhibited cytoplasmic retention (Fig. 3.1B). In cancer cells, such as HT1080, nuclear localization failures can arise from multiple causes, ranging from altered nuclear import machinery^{4,5,35} to deregulated protein degradation³⁶.

Experimentally, across 898 annotated cells (26 fields of view), $55\% \pm 5\%$ expressed CFP and $45\% \pm 5\%$ expressed iRFP (Fig. 3.1C); however, only $15\% \pm 5\%$ of iRFP⁺ cells showed predominantly nuclear signal (Fig. 3.1D), indicating that correct FUCCI nuclear localization represented a limiting quality attribute of the engineered population.

Because downstream phenotyping assays rely on four-channel live-cell imaging, we used our vertically integrated microscopy workflow to qualify and select a working HT1080 CALIPERS clone under the same operational regime used for prolonged live-cell phenotyping. A microscopy-guided enrichment increased the frequency of cells with nuclear iRFP by ~ 3 -fold (Fig. 3.1E-i). We then performed automated high-throughput clonal screening and applied an image-based quality filter that retained only clones with

concordant tubulin-based cell counts and FUCCIplex-derived nuclear masks (Fig. 3.1E; full screening logic and thresholds in Methods). More than half of the screened clones passed this automated qualification, and manual inspection of brightfield/fluorescence overlays confirmed the algorithm's calls. We banked five validated four-color lines and selected clone E4 as the working HT1080 CALIPERS line for all subsequent experiments (Fig. A2.1E).

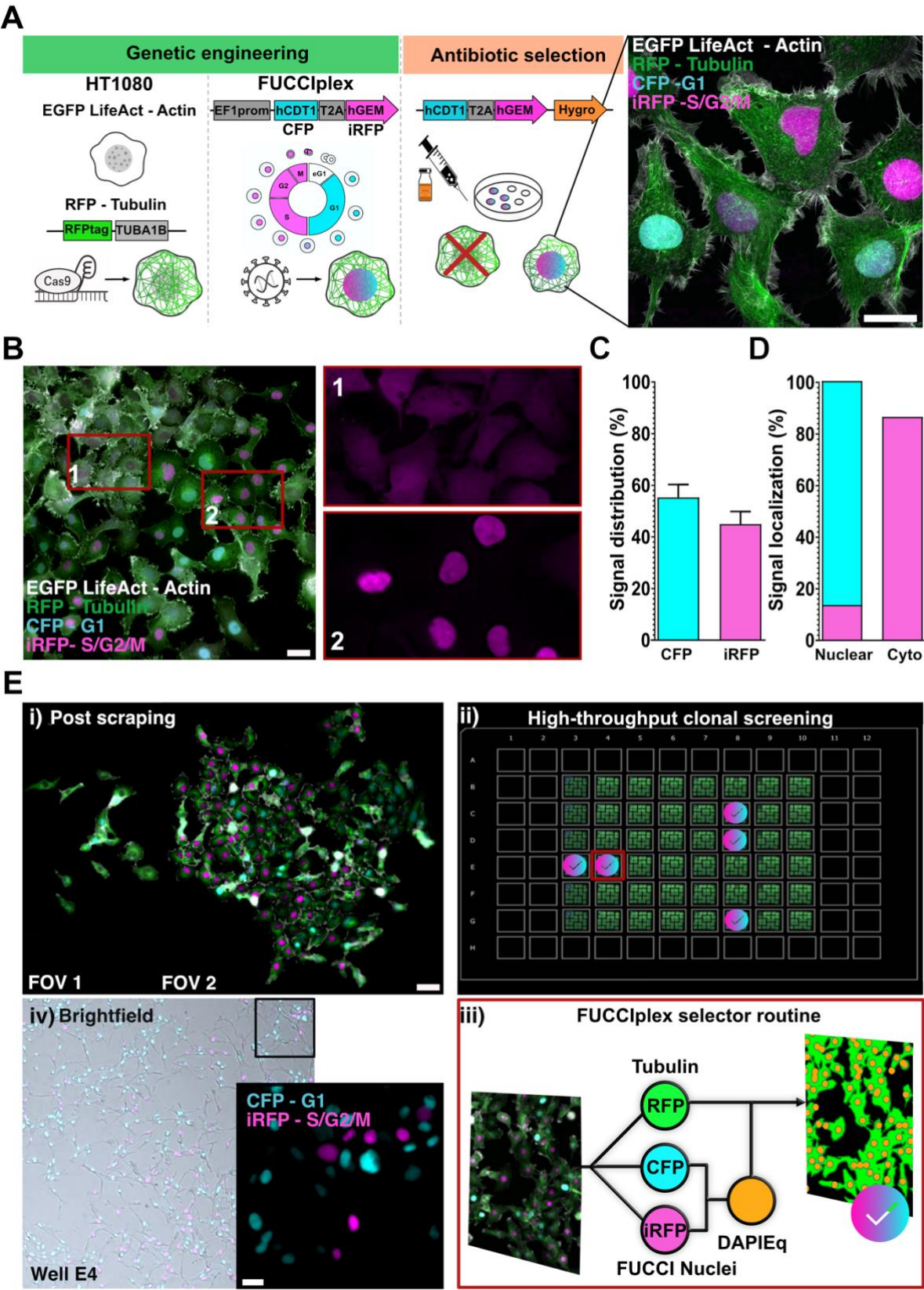


Figure 3.1. Engineering and rescue of a four-reporter CALIPERS-enabled HT1080 reference line. (A) Schematic of the genetic engineering pipeline. HT1080 fibrosarcoma cells pre-engineered with EGFP LifeAct were genome-edited at the endogenous TUBA1B locus to introduce RFP-tubulin and transduced with the FUCCIplex cassette (CFP-G1 and iRFP-S/G2/M). Lentiviral infection and hygromycin selection yielded a mixed population expressing four channels (actin, tubulin, G1, S/G2/M). High-resolution confocal imaging confirmed robust actin filaments, tubulin networks, and phase-marked nuclei. (B) Widefield imaging revealed anomalous cytoplasmic iRFP signal in many cells (example in inset 1) compared to correctly localized nuclear iRFP (inset 2). (C) Quantification of signal distribution in the mixed population ($n = 898$ cells, 26 FOVs) showed $55\% \pm 5\%$ of total cells CFP+ and $45\% \pm 5\%$ of total cells iRFP+. (D) Localization analysis confirmed 100% nuclear localization of CFP, whereas only 15% of the iRFP signal was nuclear, with the majority retained in the cytoplasm. (E) Rescue pipeline. (i) Post-scraping enrichment of cells with nuclear iRFP yielded approximately $3\times$ higher nuclear signal. (ii) Clonal seeding of the scraped population in 96-well format enabled systematic high-throughput screening. (iii) An automated selector routine segmented tubulin as ground truth and generated a DAPI-equivalent mask from CFP and iRFP nuclear signals, calling a clone only when nuclear counts matched $> 80\%$ of the tubulin-defined cell count. (iv) Manual inspection of brightfield and fluorescence confirmed nuclear expression of both CFP and iRFP in selected clones. Five reference lines were banked, with clone E4 chosen as the working HT1080 CALIPERS line.

3.3 Baseline simultaneous assessment of cell-cycle (CC) progression and migration

To establish a reference baseline for CC-aware migratory phenotyping in free space, we first imaged HT1080 CALIPERS cells seeded on fibronectin-coated glass substrates.

Qualitatively, our analysis highlights how cellular heterogeneity emerges from the combined influence of cell-cycle state and morphology. Cell 1, shown in Fig. 3.2A and captured in G1, displays a relatively smaller projected area. In contrast, Cell 2 in Fig. 3.2B, which is in S/G2/M, exhibits a markedly larger projected area. Conversely, Cell 3 in Fig. 3.2C shares the same S/G2/M phase as Cell 2 but shows a substantially smaller projected area, underscoring the variability that exists even within a single cell-cycle phase.

We then quantified CC distribution and morphology across three independent experiments at early (4 h) and late (24 h) time points to control for seeding bias. A total of 60 cells were analyzed in the early condition and 82 in the late condition. G1 and S/G2/M comprised, respectively, approximately 37-39% and 58-61% of the population at both time points (Fig. 3.2D), consistent with near-steady-state cycling, where the fraction of cells observed in each phase reflects the relative time that cells spend in that phase across the population, which indicates ergodicity. Normality ($p=0.822$) and variance ($p=0.418$) tests were passed; two-way ANOVA with Tukey correction confirmed statistical differences only between phases within each condition ($*p < 0.05$). The average cell area trended downward from early to late time points but without significance: early G1 $2039 \pm 228 \mu\text{m}^2$ vs. late G1 $1802 \pm 184 \mu\text{m}^2$; early S/G2/M $2257 \pm 178 \mu\text{m}^2$ vs. late S/G2/M $2025 \pm 139 \mu\text{m}^2$ (normality $p=0.492$, variance $p=0.907$; no factor-level effect, Fig. 3.2E). Tubulin spread was consistently higher in S/G2/M than in G1 but declined between early and late: early G1 $976 \pm 82 \mu\text{m}^2$ vs. late G1 $655 \pm 110 \mu\text{m}^2$; early S/G2/M $1216 \pm 117 \mu\text{m}^2$ vs. late S/G2/M $718 \pm 62 \mu\text{m}^2$ (Fig. 3.2F). These data suggest that tubulin remodeling is the most sensitive morphological feature to the passage of time in culture.

To capture how migration evolves through phases, we performed long-term CC-aware live imaging for 15 h at 30 min interval (three independent experiments, ≥ 3 FOVs each). Representative trajectories (Fig. 3.2G) show two cells tracked across CFP-iRFP transitions. Overall, across 54 free space tracks, cells spent on average 4.5 ± 2.0 h in G1 and 5.2 ± 2.0 h in S/G2/M, respectively. Velocity traces revealed an average velocity of 0.37 ± 0.20 $\mu\text{m}/\text{min}$ in G1 and 0.32 ± 0.20 $\mu\text{m}/\text{min}$ in S/G2/M, with a mean path length of 192 ± 22 μm . Instantaneous velocity peaked during G1, decreased at the G1-S/G2/M transition, and rose again before mitosis (Fig. 3.2H), indicating that migration speed is coupled to cell-cycle state, potentially reflecting higher motility during growth, reduced dynamics during DNA replication, and cytoskeletal reorganization preceding division. In parallel, projected cell area increased toward S/G2/M, consistent with the static imaging results (Fig. 3.2I). Importantly, these phase-dependent trends are not limited to the representative traces shown in Fig. 3.2H-J: a 48 h free space acquisition enabling quantification of 50 complete cell-cycles reproduced the same temporal modulation of velocity and area when computed as a population average with phase-aligned trajectories (Fig. A2.3D-E)

Tubulin spread fluctuated substantially, often changing by 1.5-fold within an hour, reflecting intracellular remodeling which could not be captured from static snapshots (Fig. 3.2J). A small G0/G1-arrested subpopulation was present but excluded from this analysis, as arrested cells remain outside the proliferative cycle and thus do not engage in the dynamic remodeling observed in cycling populations; these are addressed in Fig. 3.4.

Taken together, these results suggest that in free space, HT1080 CALIPERS cells maintained a near steady-state distribution of approximately 40% in G1 and 60% in S/G2/M, in line with previous reports^{27,37,38} and the detailed FUCCIplex cross-validation at the population level reported in Fig. A2.2-3. Dynamic assays complement the picture by directly linking motility to cell-cycle transitions. Existing in vivo and ex vivo imaging studies³⁹ demonstrate that CC-dependent regulation of invasion occurs. At the same time, in vitro longitudinal tracking approaches like ours^{34,37,40} are valuable as they can capture

instantaneous velocity, trajectory, and intracellular variability, inaccessible to static endpoints.

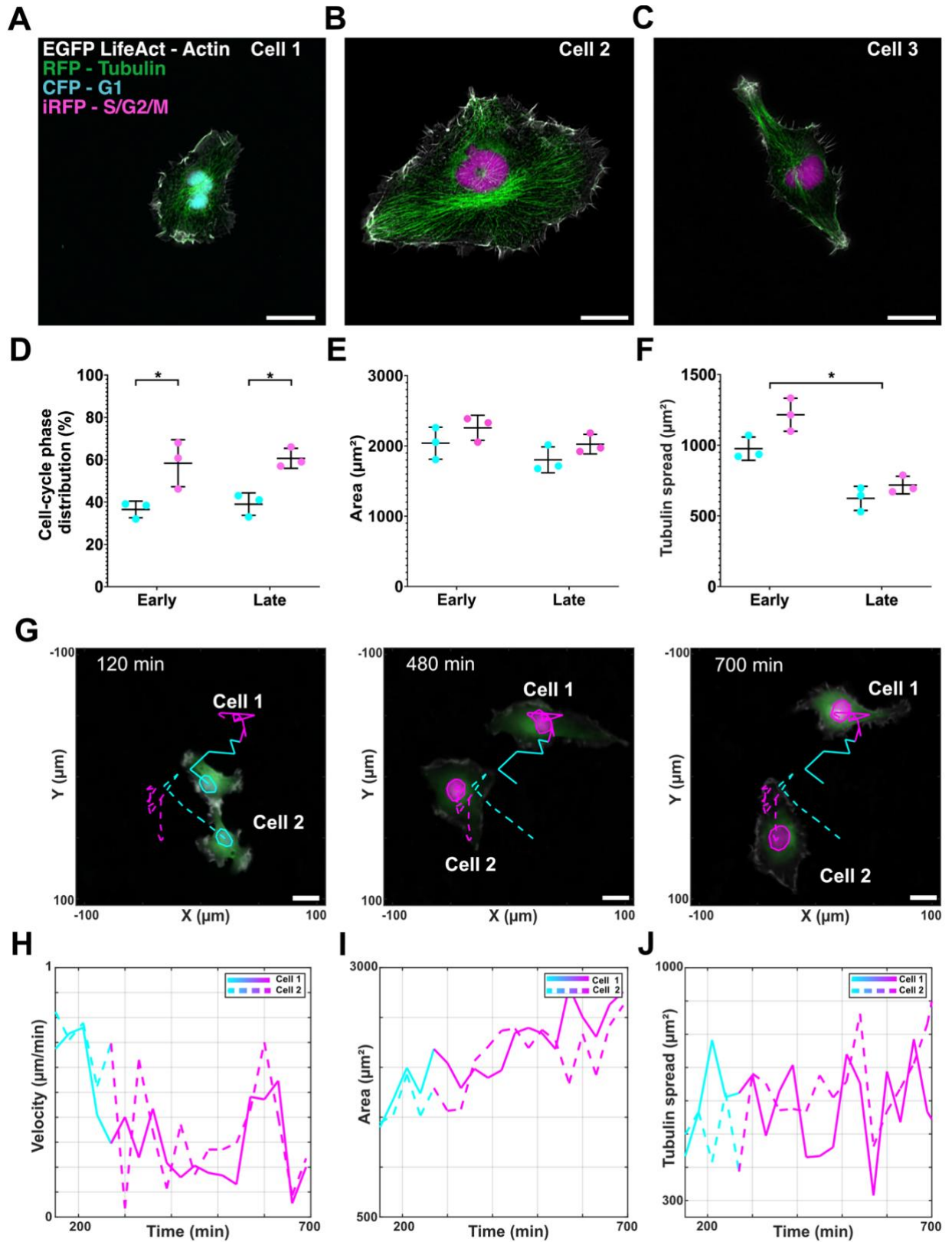


Figure 3.2. CC-aware phenotyping of HT1080 CALIPERS in free space. (A-C) Representative four-channel fluorescence images of HT1080 CALIPERS cells in G1 (CFP, Cell 1) and S/G2/M (iRFP, Cells 2-3) with actin (EGFP LifeAct) and tubulin (RFP). Scale bars, 25 μm . (D-E) Quantification of static phenotyping at early (4 h, $n = 60$ cells) and late (24 h, $n = 82$ cells) time points. (D) CC phase distribution: $37\% \pm 4\%$ G1 vs. $58\% \pm 11\%$ S/G2/M (early) and $39\% \pm 5\%$ G1 vs. $61\% \pm 5\%$ S/G2/M (late). (E) Cell area: early G1 $2039 \pm 228 \mu\text{m}^2$, S/G2/M $2257 \pm 178 \mu\text{m}^2$; late G1 $1802 \pm 184 \mu\text{m}^2$, S/G2/M $2025 \pm 139 \mu\text{m}^2$. (F) Tubulin spread early G1 $976 \pm 82 \mu\text{m}^2$, S/G2/M $1216 \pm 117 \mu\text{m}^2$; late G1 $655 \pm 110 \mu\text{m}^2$, S/G2/M $718 \pm 62 \mu\text{m}^2$. $*p < 0.05$ between phases within conditions (two-way ANOVA; time/area effect n.s.). (G) Dynamic phenotyping tracks of two cells across 700 min showing G1-S/G2/M transitions. Population level 48 h time-lapse in (H-J) Dynamic quantification of (H) velocity (average $0.35 \mu\text{m}/\text{min}$, mean path length $192 \pm 22 \mu\text{m}$, $n = 53$ tracks), (I) area (increase toward S/G2/M), and (J) tubulin spread (high variability, frequent 1.5-fold changes within 1 h).

3.4 Implementation of Fab2Mic, a correlative pipeline for photofabrication and imaging

Having characterized our HT1080 CALIPERS reporter line in free space, we set out to study how cell migration and morphology evolve under confinement, by engineering adhesive ECM patterns via photofabrication that constrain cell migration over time. We realized that manually performing photofabrication and imaging as separate steps was both error-prone and inefficient. Mapping fabricated arrays and imaging fields of view (FOVs) via manual trial-and-error could lead to inaccuracy, which wasted imaging or analysis time. Instead, we developed Fab2Mic, a correlative fabrication-to-microscopy pipeline that directly translates photofabrication layouts into imaging coordinates, ensuring efficient and reproducible alignment between fabrication and live imaging (Fig. 3.3A).

The algorithm takes the fabrication design (.tif) and metadata (.xml) as input, extracts feature centroids and array geometry, and exports an xml file that can be used immediately for multi-point acquisition in most microscopes via commercial software or Micro-Manager. Smaller features, less than 500 μm in size, are centered in the imaging FOV, whereas larger features are tiled seamlessly to minimize redundancy. We validated this method on a 50 μm square design (5×5 array, 15 mJ/mm^2 exposure) and confirmed that centroids (red) and imaging FOVs (green) matched the fabricated array on inspection (Fig. 3.3A). As a proof-of-concept, we also fabricated a 2D grayscale rendering of a picture of the Ponte Coperto in Pavia, coated it with FITC-fibronectin, and imaged a composite of 4×3 stitched FOVs (Fig. A2.6A-B). The Fab2Mic routine automatically generated the coordinate map and enabled acquisition without any manual adjustment.

We then applied this pipeline to fabricate engineered ECM islands of three sizes (10000, 2500, and 625 μm^2) arranged as 25×25 arrays (Fig. 3.3B-D). Widefield 477 nm imaging of FITC-fibronectin confirmed sharp island boundaries and reproducible array geometry. Importantly, the surrounding inter-island background remained passivated and non-

adhesive. Thus, adherent cells localized to ECM-positive islands rather than attaching in unpatterned regions, as shown in Fig. 3.3E-J. For all ECM-confinement analyses, we included only single-cell islands and excluded rare boundary-straddling or off-island adherent cells by manual curation of the dataset.

Because single-cell occupancy is essential for unbiased phenotyping, we adopted a probabilistic seeding strategy that deliberately favors single-cell islands. On average, more than 50% of the occupied islands contain a single cell, although this comes at the cost of an overall occupancy of about 20% of the available islands (Fig. A2.6D-E). This trade-off was strategic: by combining probabilistic seeding with the automated Fab2Mic pipeline, we ensured reproducible access to single-cell islands while substantially reducing manual settings and registration time. After seeding HT1080 CALIPERS cells at optimized densities ($4\times$ array size for 625 and 2500 μm^2 , $2\times$ for 10000 μm^2), cells adhered selectively to the islands. Finally, high-resolution imaging centered on individual islands revealed actin filaments aligned with fibronectin boundaries, microtubule networks spanning the cytoplasm, and CC phases marked by CFP (G1) or iRFP (S/G2/M).

Taken together, these results validate Fab2Mic as a correlative pipeline that reduces alignment errors in multi-point automated acquisitions, enables multiplexed imaging, and streamlines assay execution by saving time. By demonstrating robust single-cell occupancy across engineered ECM islands of decreasing size, we established the feasibility of combining photofabrication and CC-aware live imaging for quantitative studies of confined migration.

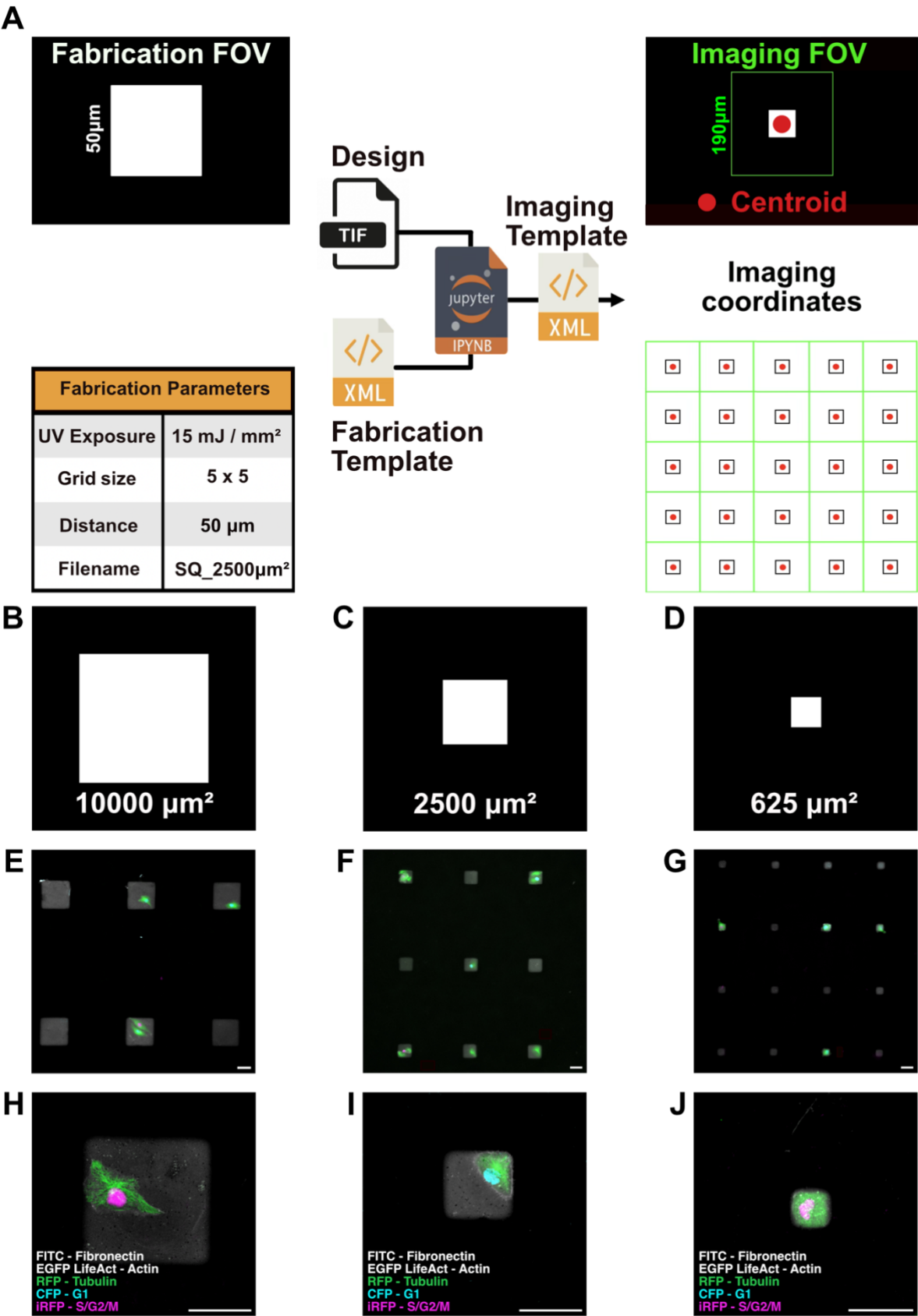


Figure 3.3. Fab2Mic pipeline for correlative fabrication-to-microscopy of engineered ECM islands. (A) Schematic of Fab2Mic workflow translating fabrication layouts (.tif + .xml) into imaging coordinates (.xml). Example shows a $50\ \mu\text{m}$ square mask ($SQ\_2500\ \mu\text{m}^2$, 5×5 array) with centroids (red) and imaging FOVs (green). (B-D) Representative photomasks to impose graded planar confinement: $10000\ \mu\text{m}^2$ ($100 \times 100\ \mu\text{m}$), $2500\ \mu\text{m}^2$ ($50 \times 50\ \mu\text{m}$), and $625\ \mu\text{m}^2$ ($25 \times 25\ \mu\text{m}$). (E-G) Low-resolution multichannel views showing single-cell occupancy on ECM island arrays for each confinement regime. The adhesive islands are visualized with FITC-labeled fibronectin, allowing direct identification of island boundaries and the surrounding non-adhesive background. (H-J) High-resolution acquisition illustrating cell spreading constrained by the island boundary and cellular readouts on individual islands.

3.5 Quantifying confinement effects on CC progression and proliferation

The combination of a validated reporter line, free space baselines, and the Fab2Mic pipeline for high-content correlation between fabrication and imaging enables the systematic study of the influence of planar confinement on both migration and proliferation. Using engineered ECM islands of 10000, 2500, and 625 μm^2 , we quantified CC distribution, morphology, and cytoskeletal dynamics under defined geometric constraints, comparing directly with free space conditions. Here, we set 2D planar confinement exclusively by restricting the adhesive area on a passivated substrate, such that cells are laterally constrained by the island boundary while remaining uncompressed in the vertical dimension. The three island sizes span distinct confinement regimes relative to the free space projected area: 625 μm^2 enforces strong footprint restriction, 2500 μm^2 approximates a near-physiological spread area but prevents long-range displacement, and 10000 μm^2 permits intra-island exploration while still bounding migration to a finite domain. From the outset, imaging was restricted to islands occupied by single cells, ensuring unbiased phenotyping at the individual cell level.

Cells adapted to the adhesive island boundaries based on their free space area: cells retained large spread morphologies on 10000 μm^2 islands (Fig. 3.4A, roughly five times their free space area), while at 625 μm^2 the cytoskeleton filled the available area (Fig. 3.4C). Interestingly, the CC distribution and cytoskeletal architecture show a scaling in confinement (Fig. 3.4D-F). This scaling suggests that confinement acts as a continuum rather than a binary on/off state, with gradual modulation of both morphology and CC fidelity. In free space, approximately 40% of cells were in G1 and about 60% in S/G2/M. On the engineered ECM islands, the G1 fraction decreased to $26\% \pm 17\%$ (10000 μm^2) and $23.5\% \pm 9\%$ (2500 μm^2), with partial recovery to $33\% \pm 25\%$ at 625 μm^2 , suggesting a relative accumulation in pre-mitotic phases under confinement. Notably, variability increased with confinement, particularly in G1, where standard deviation rose to $\pm 25\%$ at 625 μm^2 (Fig. 3.4D). Cell area followed a similar trend: from over 2000 μm^2 in free space

to $1663 \pm 639 \mu\text{m}^2$ (G1) and $1483 \pm 425 \mu\text{m}^2$ (S/G2/M) at $10000 \mu\text{m}^2$, $1803 \pm 867 \mu\text{m}^2$ and $1559 \pm 137 \mu\text{m}^2$ at $2500 \mu\text{m}^2$, and just $711 \pm 106 \mu\text{m}^2$ and $688 \pm 128 \mu\text{m}^2$ at $625 \mu\text{m}^2$ (Fig. 3.4E). Tubulin spread also decreased with confinement, roughly halving from $659 \pm 246 \mu\text{m}^2$ (G1, $10000 \mu\text{m}^2$) to $383 \pm 80 \mu\text{m}^2$ (G1, $625 \mu\text{m}^2$) (Fig. 3.4F). Dynamic phenotyping revealed how these morphological effects intersect with CC fidelity (Fig. 3.4G-I, Fig. A2.9).

To test whether the reduced G1 fraction observed in static imaging reflected an artifact, we performed dynamic imaging as described in Fig. 3.2 and evaluated cells with normal and abnormal CC events separately. Across 156 tracked cells (31 at $10000 \mu\text{m}^2$, 58 at $2500 \mu\text{m}^2$, 67 at $625 \mu\text{m}^2$), 42 cells exhibited normal CC progression (Fig. A2.8), defined by canonical FUCCI phase progression with a single monotonic G1-S/G2/M transition and no abnormal CC events, with average phase durations of $\sim 39\%$ G1 and $\sim 61\%$ S/G2/M, even under confinement. Notably, this phase partition is consistent with prior HT1080 FUCCI time-lapse reports, which describe G1 lasting $\sim 4\text{-}6$ h and S/G2 lasting $\sim 8\text{-}10$ h with ~ 1 h mitosis³⁷. However, abnormal CC events rose with increasing confinement, from a baseline of 17% in free space to 78% at $625 \mu\text{m}^2$ (Fig. 3.4I). Abnormal events were classified as Long G1, Long S/G2/M, Long Mitosis, or S/G2/M-G1 slippage (see classification of abnormal cell-cycle in Methods section). While Long G1 and S/G2/M-G1 slippage dominated under confinement, rare instances of Long S/G2/M and Long Mitosis were also observed, together accounting for approximately 5% of all abnormal events. Long G1 phases dominated confinement-induced anomalies, increasing from 37% of abnormal events in free space to 75% at $625 \mu\text{m}^2$. Conversely, S/G2/M-G1 mitotic slippage emerged^{41,42}, but its incidence decreased with tighter constraints (21% at $10000 \mu\text{m}^2$ to 4% at $625 \mu\text{m}^2$). In the context of this study, S/G2/M-G1 slippage was observed in this dataset under confined conditions and was therefore treated as a confinement-associated cell-cycle behavior. Representative trajectories at $2500 \mu\text{m}^2$ (Fig. 3.4G-H), similar to the projected area of cells in free space, highlight the divergence: a normal cell progressed from G1 to S/G2/M with G1 velocity averaging $0.44 \pm 0.22 \mu\text{m}/\text{min}$, while a Long G1 cell had a lower motility ($0.29 \pm 0.23 \mu\text{m}/\text{min}$) (Fig. 3.4J). Morphologically, abnormal G1 cells maintained $2\times$ larger areas ($912 \pm 127 \mu\text{m}^2$ vs. $465 \pm 98 \mu\text{m}^2$) (Fig. 3.4K) and broader tubulin spread ($489 \pm 122 \mu\text{m}^2$ vs. $309 \pm 56 \mu\text{m}^2$),

lacking the $2\times$ cytoskeletal expansion observed in normal S/G2/M transitions ($416 \pm 98 \mu\text{m}^2$) (Fig. 3.4L).

Taken together, these results indicate that confinement modulates both morphology and CC fidelity, consistent with prior reports^{7,19,43} showing that reduced adhesive area compresses cytoskeletal assembly. It increases the frequency of abnormal phenotypes, particularly prolonged G1 states (Long G1)⁴⁴. Notably, while static imaging suggested a reduced representation of normal G1 under confinement, dynamic imaging revealed two distinct populations: normal and abnormal cycling cells. This suggests that ergodicity assumptions should be applied with caution. Automated cell tracking and cell-cycle phase assignment, as used here, provide critical value in distinguishing these subpopulations.

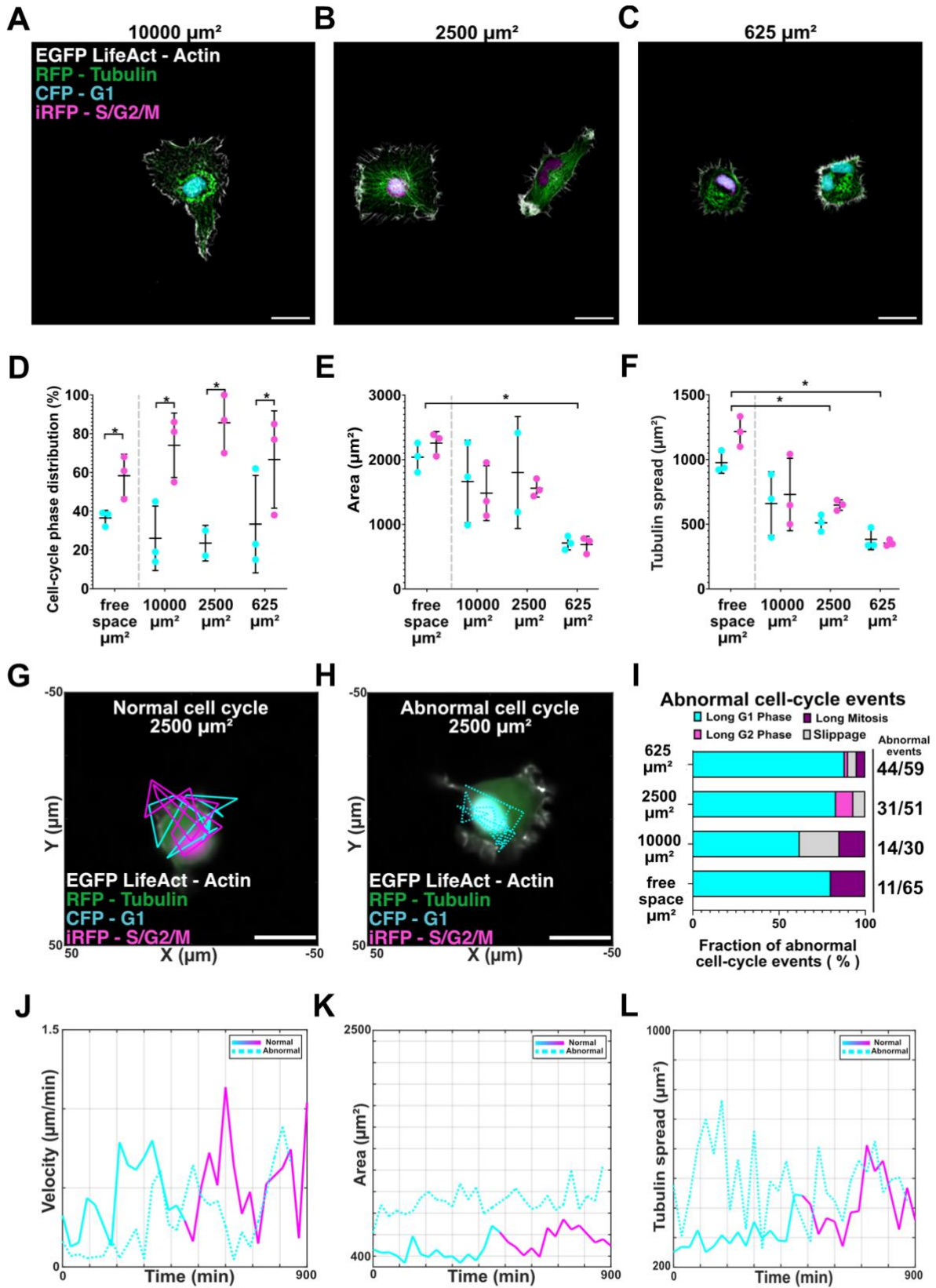


Figure 3.4. CC-aware phenotyping of HT1080 CALIPERS under confinement reveals scaling of morphology and cell-cycle fidelity. (A-C) Representative images of HT1080 CALIPERS cells seeded on engineered ECM islands of (A) 10000, (B) 2500, and (C) 625 μm^2 , showing actin (EGFP LifeAct), tubulin (RFP), and nuclei in G1 (CFP) or S/G2/M (iRFP). Scale bars, 25 μm . (D-F) Static phenotyping across free space and confinement. (D) CC phase distribution: free space (40% G1, 60% S/G2/M) shifts under confinement, with G1 reduced to $26\% \pm 17\%$ at 10000 μm^2 and $23.5\% \pm 9\%$ at 2500 μm^2 , with variability peaking at 625 μm^2 ($33\% \pm 25\%$). (E) Cell area decreased progressively with confinement, from $>2000 \mu\text{m}^2$ in free space to $711 \pm 106 \mu\text{m}^2$ at 625 μm^2 . (F) Tubulin spread followed the same trend, halving from $659 \pm 246 \mu\text{m}^2$ (G1, 10000 μm^2) to $383 \pm 80 \mu\text{m}^2$ (G1, 625 μm^2). * $p < 0.05$ between phases within conditions. (G-H) Representative dynamic trajectories at 2500 μm^2 show normal CC progression (G) and a prolonged G1 abnormality (Long G1) (H). (I) Frequency of abnormal CC events increased with confinement: 17% in free space, 48% at 10000 μm^2 , 66% at 2500 μm^2 , and 78% at 625 μm^2 . Long G1 phases dominated at small islands (75% of abnormal events at 625 μm^2), while S/G2/M-G1 slippage emerged but decreased with island size (21% at 10000 μm^2 , 5% at 2500 μm^2 , and 4% at 625 μm^2). Slippage was observed only under confined conditions and was interpreted as a confinement-associated vulnerability. Rare Long S/G2/M and Long Mitosis events were also observed, together accounting for about 5% of all abnormal events. Percentages were calculated over the total number of tracked cells pooled across three independent experiments. Cell death events were also recorded (5 in free space, 1 on 10000 μm^2 islands, 7 on 2500 μm^2 islands, and 9 on 625 μm^2 islands) but not included in the visualization for clarity of abnormal CC phenotypes. (J-L) Dynamic comparisons at 2500 μm^2 between a normal and abnormal (Long G1) cell. (J) Instantaneous velocity was higher in normal G1 ($0.44 \pm 0.22 \mu\text{m}/\text{min}$) than in Long G1 ($0.29 \pm 0.23 \mu\text{m}/\text{min}$). (K) Abnormal cells maintained $2\times$ larger G1 area ($912 \pm 127 \mu\text{m}^2$ vs. $465 \pm 98 \mu\text{m}^2$). (L) Tubulin spread expanded $2\times$ during normal S/G2/M progression (309 ± 56 to $416 \pm 98 \mu\text{m}^2$) but fluctuated broadly without net growth in Long G1 cells ($489 \pm 122 \mu\text{m}^2$).

3.6 Discussion

This study presents a vertically integrated platform that unifies multiplexed CC reporters, engineered ECM islands, and correlative high-content imaging to examine the coupling between migration and CC under geometric confinement. By combining spectral reallocation through FUCCIplex (freeing the green/red channels for structural phenotyping), bridging the registration-throughput gap with Fab2Mic, and enabling longitudinal single-cell tracking, we treat confinement into a tunable parameter within the tested design space. Across the conditions evaluated, as the adhesive area decreases, the G1 fraction declines, cytoskeletal organization becomes compressed, and variability increases. Embedding CC resolution directly into high-content morphological analysis provides a practical framework for systematic phenotyping of migration and proliferation under constraint, extending classic adhesion-^{22,23} and geometry-dependent control of proliferation¹⁷ including nuclear volume-G1 coupling on micropatterns^{25,26}. Our results also support the importance of including live-cell imaging experiments to provide dynamic phenotyping, which revealed single-cell confinement-associated behaviors invisible to static imaging: including Long G1 or Long S/G2/M phases and S/G2/M-G1 mitotic slippage. These phenotypes are consistent with reports that mechanical stress impairs mitotic progression and promotes slippage-like outcomes under constraint^{42,45,46}.

More broadly, our platform builds on the framework established by Ingber, who demonstrated how adhesion geometry and cytoskeletal tension regulate proliferation¹⁷. By integrating CC resolution through FUCCIplex and confinement control via Fab2Mic, we translate this principle into a high-content, imaging-based screening format. This provides a framework to interrogate how physical context may shape proliferative fate decisions. The approach is compatible with large-scale image-based screens, where CC-aware analysis can be applied to evaluate compound effects on CC-migration coupling, or to design microenvironments that guide morphogenesis in engineered tissues⁴⁷. Time-resolved high-content screening workflows have precedent (e.g., genome-scale live-cell imaging of mitosis), demonstrating the feasibility and value of temporal phenoprints⁴⁸⁻⁵⁰. However, scaling dynamic, multi-channel assays demands a strengthened quality-control

framework. Segmentation errors, tracking identity swaps, and channel registration drift remain partial bottlenecks, and manual curation was still necessary in this study for a subset of the data (~20%). Future integration of AI-based segmentation and standardized quality control (QC) benchmarks (e.g., error rates, reproducibility metrics) will be essential to translate CC-aware assays into robust screening pipelines, including multi-metric artifact detection and image-level QC (focus/illumination/saturation checks), which are being democratized via open guidance/toolkits for large-scale screens⁵¹. For example, cell-level QC workflows utilize clustering coupled with one-class support vector machines (SVMs) to flag segmentation artifacts and salvage valid cells from partially corrupted images⁵². Overall, these results connect classical mechanobiology with next-generation tissue engineering, providing both a conceptual extension and a practical toolkit for probing how cells “read” geometry and confinement to balance migration and growth.

To contextualize these findings, it is important to acknowledge key limitations of the present work and the most direct paths for improvement. First, our confinement model intentionally simplifies the *in vivo* mechanical landscape: cells are constrained on 2D ECM-patterned substrates with defined adhesive boundaries. This design isolates geometry-dependent effects but does not recapitulate the hallmark features of 3D tissues, including matrix porosity, viscoelasticity, degradability, and multicellular remodeling. Second, we primarily interrogate a single cell type and a limited set of ECM architectures; therefore, the quantitative phase-migration relationships reported here should be interpreted as platform-specific phenotypes within a controlled design space rather than universal rules across tissues or microenvironments. Third, dynamic multichannel imaging remains constrained by practical trade-offs among throughput, phototoxicity, segmentation/tracking fidelity (especially in dense fields), and reporter-specific limitations (spectral crosstalk, expression variability, and robust phase calling over long acquisitions). These constraints motivated partial manual curation in this study.

Accordingly, future extensions should prioritize translating confinement into more physiologically relevant settings (2.5D/3D matrices, microchannel-based confinement, and patterned ECM networks with tunable stiffness/viscoelasticity); integrating additional mechanical and biochemical cues (e.g., strain, shear, stiffness gradients, and

co-culture contexts); and strengthening the computational stack through automated QC, event-aware tracking, and uncertainty-aware reporting to enable unbiased, scalable, CC-aware phenotypic profiles.

Despite these limitations, the present work provides practical, extensible toolkits for CC-aware phenotyping within a defined design space. We introduce an integrated, open-format workflow that links engineered multi-reporter cells, ECM microenvironment fabrication-to-imaging validation, and phase-resolved quantitative readouts into a single experimental-computational pipeline. By keeping both the readouts modular and the analysis transferable across experiments, this platform can be extended to additional cell types, ECM architectures, and higher-dimensional microenvironments.

In the cancer context, our study contributes by introducing a four-color reporter HT1080 line that enables systematic exploration of CC-migration coupling. We deliberately selected conditions that emphasize long-term behaviors, whereas actual metastatic migration in vivo is typically a transient, short-lived event. The value of our approach lies less in reproducing metastatic dissemination per se and more in providing a controlled system to study how CC dynamics interact with migratory states. This perspective aligns with the “go-or-grow” framework^{22,40,53}, and with pathology observations that invasive fronts are often slow-cycling while cores are proliferative^{24,54,55}. In vivo, metastatic cells “go” to a distant site and then “grow,” whereas in our system, cells “go” but remain confined, leading to gradual accumulation of CC stress.

Although caution is warranted when extrapolating to clinical applications, the CC abnormalities revealed under prolonged confinement indicate an increased frequency of abnormal cell-cycle events, a state that, in principle, could prevent the development of a large metastatic mass. By contrast, recent findings show that confinement-induced states can also be more drug-tolerant, as HMGB2-high melanoma cells under mechanical confinement resist Taxol and BRAF/MEK inhibitors⁵⁴. Reconciling these observations highlights a critical dependency on therapeutic context: most cytotoxic drugs preferentially target rapidly proliferating cells, so confinement-driven cell-cycle exit may simultaneously suppress metastatic outgrowth (beneficial) but blunt the efficacy of conventional chemotherapy (detrimental). Mechanistically, Taxol triggers mitotic arrest;

therefore, non-dividing, confined cells are inherently less affected, and confinement-driven HMGB2 programs slow cycling and tolerance, with HMGB2 overexpression impairing BRAF/MEK response *in vivo*⁷. This tension highlights the importance of considering CC-migration coupling within the broader “go-or-grow” framework and evaluating confinement as both a source of mechanistic vulnerability and a driver of therapy resistance. Notably, some *in vitro* studies do not observe a strict trade-off: FUCCI-based analyses reported comparable motility in G1 vs S/G2/M and even under pharmacologic arrest⁴⁰, and cross-line surveys found migration can correlate with proliferation in certain cancers⁵³. Our results suggest that the appropriate geometric confinement can be leveraged to play along the go-or-grow continuum. This framing opens the door to thinking of designing confinement with a therapeutic goal. Analogous to immuno-engineered hydrogels designed to capture or prime immune cells⁵⁶⁻⁵⁸, one could envision biomaterials tailored as exploratory “capture sites” for metastatic cells.

The framework developed here enables reproducible, state-aware phenotyping in engineered 2D systems. The next limitation arises when the specimen becomes volumetric, because acquisition is constrained by the limited availability of architectures suited to controlled 3D imaging in tissue engineering platforms. Chapter 4 addresses this requirement.

3.7 Materials and Methods

Cell line generation

HT1080 human fibrosarcoma cells (Ibidi, #HT-1080-LifeAct-TagGFP2, catalog# 40101) were genome-engineered to generate a four-color CALIPERS reporter line.

CRISPR/Cas9 Tubulin tagging: Endogenous α -tubulin (TUBA1B, NM_006082.3) was tagged with tagRFP using the Thermo Fisher Scientific TrueTag system. The donor sequence and primers with locus-specific homology arms for RFP tagging of the N-terminus of α -tubulin (TUBA1B, NM_006082.3) were designed with the assistance of the TrueDesign Genome Editor tool (Thermo Fisher Scientific)⁵⁹. The linear TrueTag dsDNA donor was amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (TrueTag Donor DNA Kit, RFP, Thermo Fisher Scientific, catalog# A42993). The ribonucleoprotein (RNP) complex was assembled by combining TrueGuide synthetic sgRNA (Thermo Fisher Scientific; target sequence: GCACGGCTTACTCACCATAG) with TrueCut Cas9 Protein V2 (Thermo Fisher Scientific, catalog# A36496). TrueCut Cas9 Protein V2 (2 μ g, 12 pmol), sgRNA (400 ng, 12 pmol), and the TrueTag donor (500 ng) were resuspended in 10 μ L of Resuspension Buffer R (Thermo Fisher Scientific, catalog# MPK1025) and subsequently mixed with 8×10^4 HT1080 cells resuspended in 8 μ L of the same buffer, yielding a final reaction volume of 18 μ L. A 10 μ L aliquot of this mixture was electroporated using the Neon Transfection System (Thermo Fisher Scientific, catalog# NEON1S) with the following parameters: 1200 V, 20 ms, 4 pulses. Following electroporation, cells were seeded into 24-well plates containing 0.5 mL of Dulbecco's Modified Eagle Medium / Ham's F-12 Nutrient Mixture without phenol red (DMEM F-12, Gibco, catalog# 21041-025), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, catalog# 10270-106). Cultures were incubated at 37 °C in a humidified CO₂ atmosphere. RFP-positive cells were subsequently isolated and expanded in a clonal manner. Edited clones were validated via live-cell fluorescence microscopy and PCR (Fig. A2.1A,C)

FUCCIplex sensor integration: The FUCCIplex cassette was derived from pBOB-EF1-FastFUCCI-Puro (Addgene, plasmid #86849) by replacing mKO2 (Cdt1 degron) with mTurquoise2 and hmAzami Green (Geminin degron) with mRFP670. The cassette was cloned into a third-generation lentiviral backbone (pLV[Exp]-Hygro-EF1A, VectorBuilder, catalog# VB210825-1211nbd) with hygromycin resistance. Lentiviral particles were produced in HEK293T packaging cells (ATCC, catalog# CRL-1573), seeded at 2.5×10^6 cells per 10 cm dish pre-coated with 0.2% gelatin (catalog# G1393, Sigma-Aldrich). Transfection was performed with Lipofectamine 2000 (Thermo Fisher Scientific, catalog# 11668019). Viral supernatants were collected at 48 h, clarified by centrifugation, and applied to HT1080 cells. More in detail, HT1080 cells were plated at a density of 6×10^4 cells per well in 12-well plates containing complete DMEM F-12 medium. After 24 h, the culture medium was replaced with fresh complete medium supplemented with 8 $\mu\text{g/mL}$ polybrene (catalog# TR-1003-G, Sigma-Aldrich). Concentrated FUCCIplex lentiviral particles ($>10^8$ TU/mL, VectorBuilder; 10 μL resuspended in 500 μL of complete medium) were then added directly to the wells. Cells were incubated overnight at 37 °C in a humidified 5% CO₂ atmosphere. The following morning, the viral medium was aspirated and replaced with fresh complete medium. After 4 days, positively transduced cells were selected using Hygromycin B (Invitrogen, catalog# 10687010, 20 $\mu\text{L/mL}$ from a 50 mg/mL stock in PBS). Resistant populations were clonally seeded and expanded. PCR and confocal fluorescence imaging verified correct integration and reporter expression

PCR and RT-qPCR.

Genomic DNA was extracted from HT1080 EGFP LifeAct and FUCCIplex clones using the DNeasy Blood and Tissue Kit (Qiagen, catalog# 69504) according to the manufacturer's instructions. For genomic PCR, two different primer pairs were used to amplify the TUBA1B WT locus and the RFP-tagged gene. Specifically, primer pair 1-3 recognizes the WT locus, while primer pair 2-3 generates an amplicon only in the condition where the gene has been edited. PCR products were analyzed by agarose gel (1%) electrophoresis. PCR was carried out using Platinum PCR SuperMix, High Fidelity (Thermo Fisher Scientific, catalog# 12532016).

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, catalog# 15596026), treated with DNase I (Thermo Fisher Scientific, catalog# EN0521), and reverse-transcribed using SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific, catalog# 18090200). RT-qPCR was performed with primers specific for miRFP670, Hygromycin, and FUCCIplex. Relative expression levels were determined using the $\Delta\Delta C_t$ method, with HPRT as the internal control.

Cell culture

HT1080 CALIPERS cells were maintained in Dulbecco's Modified Eagle Medium / Ham's F-12 Nutrient Mixture without phenol red (DMEM F-12, Gibco, catalog# 21041-025), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, catalog# 10270-106) and 1% Penicillin-Streptomycin (HiMedia, catalog# A001-100ML). Cells were cultured at 37 °C in a humidified 5% CO₂ incubator and routinely split at 70% confluency. For experiment preparation, cells were detached using Trypsin/EDTA 0.25% (Thermo Fisher Scientific, catalog# 25200056), resuspended in complete DMEM F-12, and properly counted according to the experimental needs.

CC phase assignment with DNA-content profiling and FUCCIplex-based flow cytometry

To benchmark baseline DNA-content fractions and validate FUCCIplex-derived phase occupancy in multicolor-engineered HT1080 lines, flow cytometry was performed on three asynchronous cultures: parental HT1080 EGFP, a partially edited HT1080 EGFP LifeAct - Actin⁺/RFP - Tubulin⁺ line (FUCCIplex-negative control), and the fully edited HT1080 CALIPERS line (EGFP LifeAct - Actin, RFP-Tubulin, and FUCCIplex CFP/iRFP). Cells were harvested as single-cell suspensions and analyzed using sequential gating to exclude debris and doublets/aggregates, while retaining a single-cell population. For DNA-content analysis, samples were processed using a standard PI/RNase staining workflow, and PI DNA-content histograms were used to quantify the fractions of cells in G0/G1, S, and G2/M. Due to the multicolor nature of the edited lines and the observed sensitivity of PI readouts to instrument settings and spectral overlap with RFP in the PI

detection channel, PI-based DNA-content fractions are reported here only for the parental HT1080 EGFP line as a robust baseline reference (Fig. A2.2A). In parallel, FUCCIplex-based phase assignment in the HT1080 CALIPERS line was performed by bivariate analysis of CFP (G1) and iRFP (S/G2/M) fluorescence (Fig. A2.2B). CFP and iRFP thresholds were defined using the FUCCIplex-negative EGFP-LifeAct⁺/RFP-Tubulin⁺ control to set the negative baseline and account for autofluorescence and residual spillover/bleedthrough from structural reporters. Using these thresholds, the CALIPERS population was partitioned into CFP/iRFP quadrants to quantify reporter-positive fractions and derive the FUCCI-based phase distribution. All PI and FACS analyses were performed in FlowJo Software (FlowJo™ Software, v. 11)⁶⁰, as shown in Fig. A2.2A.

Cytoplasmic vs. nuclear CFP/iRFP evaluation

To evaluate CFP and iRFP signal expression and localization, HT1080 CALIPERS were screened with high-resolution widefield fluorescence microscopy with a Nikon CFI Plan Apo Lambda S 40× silicone oil objective (Silicone oil immersion, NA 1.25, WD 0.30 mm, catalog# MRD73400). See the microscopy acquisition section for detailed imaging settings.

Samples were prepared by seeding 5×10^4 cells on 35 mm #1.5 glass-bottom dishes (Ibidi, catalog# 81158) pre-coated with 20 µg/mL human plasma fibronectin (Sigma-Aldrich, catalog# F0895) in standard supplemented DMEM F-12 without phenol red. After 24 hours, the medium was refreshed, and the cells were imaged.

The dataset was manually curated to identify cells expressing either CFP or iRFP signals. For each population, signal localization was assessed to distinguish between exclusive nuclear versus cytoplasmic expression. GraphPad Prism (version 10.4.2) was used to generate the graphics of this panel set. Results are reported in bar graphs with mean ± SEM.

Scraping and clonal seeding

To enhance the yield of nuclear iRFP localization of HT1080 CALIPERS, a gentle scraping method was employed. A total of 5×10^4 cells were seeded on two 35 mm #1.5

glass-bottom dishes (Ibidi, catalog# 81158) pre-coated with 20 $\mu\text{g/mL}$ human plasma fibronectin (Sigma-Aldrich, catalog# F0895) in standard supplemented DMEM F-12 without phenol red medium.

After 24 hours, the medium (DMEM F-12, Gibco, catalog# 21041-025) was refreshed before the imaging session. Widefield fluorescence microscopy was used to manually annotate regions of interest (ROIs) corresponding to nuclear iRFP-positive cells directly on the dish.

Subsequently, adherent populations were gently detached using a sterile cell scraper. The scraped cell sample was resuspended in fresh medium and replated for expansion and subsequent clonal seeding. Once the population reached 70% confluency, cells were trypsinized with Trypsin/EDTA, resuspended in complete DMEM F-12 medium, counted, and diluted to a final concentration of ~ 0.8 cells per well. Clonal seeding was performed in a sterile 96-well plate (Ibidi, catalog# 89626), where 100 μL was dispensed per well to favor single-cell deposition. To ensure optimal fluorescence imaging, 48 wells were seeded, avoiding the outer rows/columns of the plate. The 96-well plate was incubated under standard culture conditions (37°C, 5% CO_2), and wells were monitored to confirm clonal origin. Of the 48 initially seeded wells, 8 remained empty, leaving 40 available clones for evaluation. The medium was refreshed every other day with standard supplemented DMEM F-12 until day 7 of clonal expansion.

Automated FUCCIplex clonal screening

A high-throughput live-cell imaging JOBS was developed using Nikon NIS-Elements AR software (version 5.42.03)⁶¹ to scan a multi-well plate through a multi-channel serial acquisition and large-image composition per well. The JOBS wizard enabled selection of wells of interest, definition of imaging channel stacks, and specification of the reference channel for automated focus adjustment, which was based on contrast optimization.

The implementation utilized 638 nm for iRFP (S/G2/M), 546 nm for RFP (Tubulin), 477 nm for EGFP LifeAct (Actin, used as the reference plane), 446 nm for CFP (G1), and brightfield acquisition. Each well is acquired as a large image of 6×6 fields within a Nikon CFI Plan Fluor DIC 10 $\times$ (air objective, NA 0.3, WD 16 mm, catalog# MRH00105). The entire well is then reconstructed by stitching with a 10% overlap and stored as a merged file.

FUCCIplex selector routine

An automated FUCCIplex selector routine was developed using the General Analysis 3 module in Nikon NIS-Elements AR software (version 5.42.03)⁶¹. The routine was based on tubulin and FUCCIplex nuclear signals. Specifically, FUCCIplex nuclear signals were combined into a DAPI-equivalent (DAPIEq) mask that captured both CFP and iRFP expression. The mask generated from CFP and iRFP signals was created within thresholds defined by object intensity and size to exclude cytoplasmic signals. CFP-positive nuclei were selected with a mean intensity > 800 and nuclear diameter $< 15 \mu\text{m}$. iRFP nuclear-localized expression was selected with intensity > 600 and a nuclear diameter $< 15 \mu\text{m}$. Using the tubulin and DAPIEq masks, the algorithm considered the number of cells detected by tubulin as the ground truth. As a selection criterion, the DAPIEq object count was required to reach $> 80\%$ of the tubulin-based cell count for a well to be classified as an HT1080 CALIPERS four-reporter clone. The threshold was relaxed to 80% because early G1 or M-phase cells may be missed by the FUCCIplex selector but still appear in tubulin-based counts, leading to slight mismatches.

HT1080 CALIPERS synchronization by nocodazole mitotic arrest

HT1080 CALIPERS cells were seeded and allowed to adhere for 8 h before synchronization. Cells were then treated overnight with nocodazole (Sigma-Aldrich, M1404-50MG, 100 ng/mL) to induce mitotic arrest. The following day, mitotic cells were collected by gentle shake-off, nocodazole was removed by washout, and the harvested cells were reseeded in an Ibidi 35 mm round glass-bottom dish in fresh complete medium⁶². Cells were allowed to re-adhere for 4 h before initiating live-cell imaging. Four-channel fluorescence time-lapse imaging was performed for 18 h at low magnification (large field of view) to enable tracking of the full population and quantification of cell-cycle progression and dynamics during release from nocodazole-induced arrest (Fig. A2.4 -A2.5).

Photofabrication

Substrate cleaning and passivation: 35 mm #1.5 glass-bottom dishes (Ibidi, catalog# 81158) were washed in 2% Hellmanex III solution (Hellma Analytics, catalog# 9-307-0110), rinsed with DI water, and dried. Dishes were exposed to UV-ozone for 30 minutes (BioForce Nanosciences ProCleaner). Substrates were coated with 200 μ L of 1 mg/mL Poly-L-Lysine (PLL, Sigma-Aldrich, catalog# P4707) for 30 minutes, rinsed with HEPES buffer (pH 8.3-8.6; Sigma-Aldrich, catalog# H3375), and incubated with freshly prepared 70 mg/mL PEG-SVA (Laysan Bio, catalog# SVA-PEG-5000) for one hour, followed by three DI water washes.

For #1.5 Ibidi 8-well glass plates (Ibidi, catalog# 80807), volumes were downscaled according to the single-well volume, and UV-ozone exposure was replaced with 200 μ L of 1M NaOH treatment for 30 minutes, to restrict substrate functionalization to individual wells.

ECM islands and validation designs: Square engineered ECM island designs of 625 μm^2 , 2500 μm^2 , and 10000 μm^2 were generated in Inkscape (version 1.3)⁶³ and converted into 8-bit grayscale format with a white square island foreground. Validation images to test the Fab2Mic routine were also generated to test both the small design with array repetition and a large photopatterned region. For validation, a large region was generated from an 8-bit grayscale .tif image of the “Ponte Coperto in Pavia” (Fig. A2.6A-B).

Photopatterning: 35 mm round substrates were coated with a photoinitiator solution consisting of 66 μ L ethanol (VWR, catalog# 20821.330) mixed with 4 μ L PLPP gel (Alvéole, catalog# LPPG-100) and dried in the dark. For #1.5 8-well glass Ibidi plates, a surfactant solution (Surfactant Mix, Alvéole) was required to allow the photoinitiator to spread evenly across the entire well bottom and prevent meniscus formation. The solution was therefore prepared by mixing 97% (v/v) DI water, 2.5% PLPP gel, and 0.5% Surfactant Mix. A volume of 150 μ L was dispensed into each well and allowed to dry completely.

Photopatterning was performed using the PRIMO system (Alvéole)²⁹ coupled to a Nikon Ti2 inverted epifluorescence microscope. The integrated 375 nm UV source and a Nikon CFI S Plan Fluor ELWD 20 $\times$ (air objective, NA 0.45, WD 6.9 - 8.2, catalog# MRH08230) provided illumination. Selected designs were projected through the optical path using a

Digital Micromirror Device (DMD), enabling the transfer of user-defined designs onto the substrate with a resolution of 0.28 $\mu\text{m}/\text{px}$. Fabrication layouts were tuned by selecting the array size (5×5 or 25×25) and inter-pattern spacing (50 μm or 150 μm). Pattern alignment and exposure control were managed through the Leonardo software package (Alvéole, v.5.2)⁶⁴.

ECM coating: Photopatterned substrates were incubated with 20 $\mu\text{g}/\text{mL}$ FITC-fibronectin (Sigma-Aldrich, catalog# FNR01-A) in PBS for 30 minutes for validation tests, or with human plasma fibronectin (Sigma-Aldrich, catalog# F0895) for 10 minutes. Three PBS washes removed excess coating.

Cell seeding on ECM photopatterned islands: HT1080 CALIPERS cells were seeded on pre-coated engineered ECM islands with human plasma fibronectin (Sigma-Aldrich, catalog# F0895) at a density adjusted for single-cell occupancy: $4 \times$ array size for 625/2500 μm^2 and $2 \times$ array size for 10000 μm^2 patterns (Fig. A2.6C-E). Cells were dispensed in complete DMEM F-12 filled photopatterned Ibidi 8-well glass plates (Ibidi, catalog# 80807) or 35 mm #1.5 glass-bottom dishes (Ibidi, catalog# 81158). After 15 minutes of settling under the hood, cells were incubated at 37 °C and 5% CO₂ for two hours before replacement with fresh phenol-red-free DMEM F-12 for imaging.

Fab2Mic - Correlative fabrication-to-microscopy pipeline

A Python pipeline (Fab2Mic) was developed to translate photofabrication templates directly into imaging coordinates. The routine accepts as input a binary fabrication mask (.tif) and a photofabrication report (.xml). The xml file encodes essential metadata, including the scale factor, substrate position, grid size, spacing, and fabrication objective specifications. These parameters were parsed to reconstruct the layout and to generate coordinate maps compatible with microscope control software.

For high-resolution acquisitions ($\leq 500 \mu\text{m}$ field of view), imaging positions were assigned to the centroid of each fabricated feature to preserve positional fidelity. For larger fields of view ($> 500 \mu\text{m}$), redundancy was minimized by computing a tiling grid, with fields automatically centered and spaced by their dimensions to ensure complete coverage without overlap (Fig. A2.6A-B). The pipeline, therefore, enables direct one-to-

one registration between fabrication and imaging, scaling seamlessly from single-feature to array-level designs without manual intervention.

Microscopy acquisitions

Acquisitions were performed on a widefield Nikon Ti2 inverted microscope with a CrestOptics X-Light V3 spinning disk confocal unit and an environmental chamber (Okolab Bold Line; 37 °C, 5% CO₂, humidity). The microscope is equipped with a Nikon linear-encoder motorized stage with a Mad City Labs 100 µm range Z-piezo insert (Mad City Labs, catalog# NI-2-C312). Illumination was provided by the Lumencor Celesta light engine (405, 446, 477, 520, 546, 638, 740 nm; up to 800 mW), routed through multiband dichroics and appropriate emission filters.

The dichroic mirror wheel was selectively positioned with either a hard-coated Full Multi Band Penta CELESTA -DA/FI/TR/Cy5/Cy7-A (Nikon, catalog# MXR00543) or a Full Multiband dual CELESTA -CFP/YFP-A (Nikon, catalog# MXR00544). Depending on the excitation line, the beam was subsequently filtered through either a Multiband Full Penta FF01-391/477/549/639/741 (Semrock, catalog# FL-416877) or a Full Multiband Dual FF01-449/520 (Semrock, catalog# FL-411981).

Emission was collected using single-band filters mounted in the wheel: FF01-484/561 (Semrock, catalog# FL-412124) for CFP, FF01-685/40-25 nm (Semrock, catalog# FL-011482) for the iRFP signal, FF01-595/31 (Semrock, catalog# FL-004391) for RFP, or FF01-511/20-25 (Semrock, catalog# FL-004306) for EGFP.

Fluorescence was captured with the same Teledyne Photometrics Kinetix CMOS camera (6.5 µm pixels; 16-bit; native resolution 3200 × 3200 pixels, cropped to 2700 × 2700 pixels). Acquisition and hardware synchronization were controlled through NIS-Elements AR (v.5.42.03)⁶¹. Data were stored as .nd2 files.

Low-resolution clone selection: Static widefield and brightfield acquisitions were performed using a CFI Plan Fluor DIC 10× (air objective, NA 0.3, WD 16 mm, catalog# MRH00105). Clone selection was run with the custom automated CALIPERS clonal screening JOBS in which each well was acquired as a large image of a 6 × 6 fields within

a CFI Plan Fluor DIC 10× (air objective, NA 0.3, WD 1.6 mm, catalog# MRH00105). Channel acquisition was sequentially ordered from 638 nm to 446 nm, with brightfield acquired last. The high-throughput JOBS covered 40 wells, with the shutter closed during stage movements. Illumination settings were:

- **iRFP (S/G2/M):** 638 nm, exposure 300 ms, laser power 28.71 ± 3 mW
- **RFP (Tubulin):** 546 nm, exposure 100 ms, laser power 15.79 ± 1.6 mW
- **EGFP (Actin):** 477 nm, exposure 100 ms, laser power 15.21 ± 1.5 mW
- **CFP (G1):** 446 nm, exposure 300 ms, laser power 3.12 ± 0.3 mW
- **Brightfield:** Dia lamp exposure 5 ms

Fab2Mic validation imaging: Fab2Mic validation was performed on square ECM designs of $625 \mu\text{m}^2$, $2500 \mu\text{m}^2$, and $10000 \mu\text{m}^2$, as well as on a photopatterned image of the “Ponte Coperto in Pavia”. Designs were photopatterned and FITC-fibronectin-coated. Widefield fluorescence microscopy was used to validate the algorithm using NIS-Elements software⁶¹. Imaging coordinates were imported from the xml file returned by Fab2Mic. Acquisitions of the photopatterned FITC-fibronectin square arrays were performed with a Nikon CFI Plan Fluor DIC 10× (air objective, NA 0.3, WD 16 mm, catalog# MRH00105) and the 477 nm line set at laser power 60.2 ± 6 mW with a 30 ms exposure. Acquisitions of the “Ponte Coperto in Pavia” pattern were performed with a Nikon CFI Plan Apo 20× (air objective, NA 0.75, WD 1 mm, catalog# MRD00205) and the 477 nm line set at laser power 27.8 ± 2.8 mW with a 300 ms exposure.

Low-resolution static live imaging: Static widefield acquisitions were performed using both Nikon CFI Plan Fluor DIC 10× (air objective, NA 0.3, WD 16 mm, catalog# MRH00105) and Nikon CFI Plan Apo 20× (air objective, NA 0.75, WD 1 mm, catalog# MRD00205). Multi-point acquisitions were conducted sequentially from 638 nm to 446 nm, with the shutter closed during stage movements.

For 10× imaging, sequential four-channel acquisitions were carried out with excitation settings as follows:

- **iRFP (S/G2/M):** 638 nm, 4x4 binning, exposure 100 ms, laser power 28.71 ± 3 mW

- **RFP (Tubulin):** 546 nm, 2x2 binning, exposure 50 ms, laser power 15.79 ± 1.6 mW
- **EGFP (Actin):** 477 nm, 2x2 binning, exposure 50 ms, laser power 15.21 ± 1.5 mW
- **CFP (G1):** 446 nm, 4x4 binning, exposure 30 ms, laser power 3.12 ± 0.3 mW

For 20× imaging:

- **iRFP (S/G2/M):** 638 nm, 4×4 binning, exposure 300 ms, laser power 21.4 ± 2.1 mW
- **RFP (Tubulin):** 546 nm, exposure 50 ms, laser power 20.0 ± 2 mW
- **EGFP (Actin):** 477 nm, exposure 50 ms, laser power 15.8 ± 1.6 mW
- **CFP (G1):** 446 nm, 4×4 binning, exposure 20 ms, laser power 3.1 ± 0.3 mW

High-resolution static confocal Z-stack: High-resolution static confocal imaging was performed with a Nikon CFI SR HP Plan Apo Lambda S 100× silicone oil objective (Silicone oil immersion, NA 1.35, WD 0.31 mm, catalog# MRD73950). Illumination settings were:

- **iRFP (S/G2/M):** 638 nm, 2×2 binning, exposure 2 s, laser power 5.61 ± 0.6 mW
- **RFP (Tubulin):** 546 nm, exposure 300 ms, laser power 1.8 ± 0.2 mW
- **EGFP (Actin):** 477 nm, exposure 300 ms, laser power 1.9 ± 0.2 mW
- **CFP (G1):** 446 nm, 2×2 binning, exposure 30 ms, laser power 1.4 ± 0.1 mW

Z-stacks were acquired using an MCL Piezo Z-device by capturing each channel across a 10 μm range within a step size of 0.3 μm .

High-resolution static widefield imaging: Static widefield imaging was performed with a Nikon CFI SR HP Plan Apo Lambda S 100× silicone oil objective (Silicone oil immersion, NA 1.35, WD 0.31 mm, catalog# MRD73950), Nikon CFI Plan Apo Lambda S 40× silicone oil objective (Silicone oil immersion, NA 1.25, WD 0.30 mm, catalog# MRD73400)

For 100× imaging, sequential four-channel acquisitions were carried out with the following settings:

- **iRFP (S/G2/M):** 638 nm, exposure 50 ms, laser power 14.8 ± 1.5 mW
- **RFP (Tubulin):** 546 nm, exposure 30 ms, laser power 6.7 ± 0.7 mW
- **EGFP (Actin):** 477 nm, exposure 30 ms, laser power 5.9 ± 0.6 mW
- **CFP (G1):** 446 nm, exposure 2 ms, laser power 0.8 ± 0.1 mW

For 40× imaging, acquisitions were performed with:

- **iRFP (S/G2/M):** 638 nm, exposure 100 ms, laser power 28.8 ± 3 mW
- **RFP (Tubulin):** 546 nm, exposure 40 ms, laser power 14.8 ± 1.5 mW
- **EGFP (Actin):** 477 nm, exposure 40 ms, laser power 13.5 ± 1.4 mW
- **CFP (G1):** 446 nm, exposure 60 ms, laser power 2.2 ± 0.2 mW

Low-resolution dynamic live imaging: Long-term (48h) widefield acquisitions were performed using a Nikon CFI Plan Fluor DIC 10× (air objective, NA 0.3, WD 16 mm, catalog# MRH00105). Multi-point time-lapse imaging was conducted sequentially from 638 nm to 446 nm, with the shutter closed during stage movements.

For 10× imaging, sequential four-channel acquisitions were carried out with excitation settings as follows:

- **iRFP (S/G2/M):** 638 nm, 4x4 binning, exposure 100 ms, laser power 28.71 ± 3 mW
- **RFP (Tubulin):** 546 nm, 2x2 binning, exposure 50 ms, laser power 15.79 ± 1.6 mW
- **EGFP (Actin):** 477 nm, 2x2 binning, exposure 50 ms, laser power 15.21 ± 1.5 mW
- **CFP (G1):** 446 nm, 4x4 binning, exposure 30 ms, laser power 3.12 ± 0.3 mW

Time series spanned 48 hours with a frame interval of 30 minutes. Stage movements followed an optimized multi-point path. Nikon Perfect Focus remained engaged throughout overnight acquisitions to minimize drift. Samples were maintained in phenol-red-free DMEM F-12 medium.

Low-resolution dynamic live imaging: Long-term widefield acquisitions were performed using a Nikon CFI Plan Apo 20× (air objective, NA 0.75, WD 1 mm, catalog# MRD00205). Multi-point time-lapse imaging was conducted sequentially from 638 nm to 446 nm, with the shutter closed during stage movements.

For 20× imaging, sequential four-channel acquisitions were carried out with excitation settings as follows:

- **iRFP (S/G2/M):** 638 nm, 4×4 binning, exposure 300 ms, laser power 21.4 ± 2.1 mW
- **RFP (Tubulin):** 546 nm, exposure 50 ms, laser power 20.0 ± 2 mW
- **EGFP (Actin):** 477 nm, exposure 50 ms, laser power 15.8 ± 1.6 mW
- **CFP (G1):** 446 nm, 4×4 binning, exposure 20 ms, laser power 3.1 ± 0.3 mW

Time series spanned 15 hours with a frame interval of 30 minutes. Stage movements followed an optimized multi-point path. Nikon Perfect Focus remained engaged throughout overnight acquisitions to minimize drift. Samples were maintained in phenol-red-free DMEM F-12 medium.

Image preprocessing and visualization: Raw acquisitions were denoised and deconvolved in NIS-Elements using the Richardson-Lucy algorithm (10 iterations). A custom Fiji⁶⁵ macro was applied for visualization, including a rolling-ball background subtraction (50 px radius) and channel-specific intensity level subtraction (446 nm = 50; 477 nm = 20; 546 nm = 20; 638 nm = 100). Scale bars were set at 25 μ m.

HT1080 CALIPERS CC-aware bioimage analysis

Static and dynamic microscopy datasets underwent a multi-step analysis pipeline to extract CC-aware dynamics, motility, and macro- and micro-scale morphology features.

Pre-processing: Low-resolution fluorescence acquisitions were first pre-processed by applying BaSiC flat-field correction⁶⁶ to compensate for non-uniform illumination and background shading, yielding homogeneous image intensity. Denoising was performed using a Butterworth filter implemented in the *dexp* library (Royer Lab, GitHub)^{67,68}. For all channels, parameters were calibrated on the first frame using

calibrate_denoise_butterworth, and the resulting function was applied across all time points. Channel indexing and metadata were provided via a configuration file. Processed stacks were saved as 16-bit OME-TIFFs using *AICSImageIO*⁶⁹. Image manipulations were performed with *scikit-image*⁷⁰.

Segmentation and tracking: Nuclear masks were generated with StarDist (version 0.8.3, pre-trained fluorescent nuclei model)³¹. Channel signals were merged into a single DAPI-equivalent image, from which segmentation masks were obtained. The resulting DAPIEq masks were used as labels for motility analysis. Cytoskeleton masks were generated with Cellpose v2.0³² applied to RFP (tubulin) and EGFP LifeAct (Actin) channels. The segmentation derived from cytoskeletal signals defined macro-scale morphological labels for HT1080 CALIPERS. Tubulin microstructures were labeled with Fiji Otsu-thresholding⁷¹ on background-subtracted (rolling ball 50 px) and Gaussian smoothed ($\sigma = 2$ px) RFP (tubulin) channel (Fig. A2.7).

FUCCIphase with morphology cues: Cell-cycle phase assignment was performed using FUCCIphase³⁴, with newly calibrated intensity curves of the HT1080 CALIPERS cells. For each tracked nucleus, normalized CFP and iRFP mean intensities were measured within the DAPIEq mask. Phase classification was performed using fixed intensity thresholds derived from 24-hour reference recordings, yielding G1 (CFP-high/iRFP-low) and S/G2/M (iRFP-high/CFP-low). Intermediate G1/S events were grouped with S/G2/M for the analyses. Time-resolved motility and morphology were computed by linking nuclear centroids in Fiji⁶⁵ using the TrackMate plugin³³ with LAP tracking.

The nuclear signal intensity of the two FUCCI channels was used for static and dynamic CC-phase assignment, and the centroid of the nuclear labels was used for cell motility parameter computation. Additionally, CC-related nuclear morphology features were directly computed from the nuclear labels (Fig. A2.7D-F, Fig. A2.10). In parallel, static or time-resolved cytoskeletal features were extracted from cytoskeleton mask centroids within the same pipeline. Additional morphological features, such as perimeter, circularity, and the ellipse aspect ratio (Ellipse AR), were evaluated (Fig. A2.7G-I, Fig. A2.11). The spread of tubulin microstructure was quantified through an automated pixel

count macro applied to tubulin masks, with measurements converted to physical area (μm^2).

For dynamic analyses, single-cell tubulin spread was computed per frame and paired with the concurrent FUCCIphase derived cell track. To link nuclear information with cytoskeletal information, a custom MATLAB script (FUCCIphase_morphology, version R2023b, 23.2)⁷² was developed to map each nuclear centroid to its enclosing cytoskeletal mask centroid within a 20 μm search radius, minimizing incorrect associations. The output was a unified FUCCIphase_morphology table (per-cell, per-frame for dynamic analyses) containing time stamps, positions, phase labels, cell-scale morphology, and tubulin spread. The same pipeline was applied to both static and dynamic datasets, acquired under both free space and confined conditions.

Static HT1080 CALIPERS CC-aware phenotyping

For free space condition, 5×10^4 cells were seeded on two 35 mm #1.5 glass-bottom dishes (Ibidi #81158) pre-coated with 20 $\mu\text{g}/\text{mL}$ human plasma fibronectin (Sigma-Aldrich, catalog# F0895) in standard supplemented DMEM F-12 without phenol red medium for Early (4-hour post seeding) vs Late (24-hours post seeding) time points evaluations. For confinement conditions, cells were seeded according to the engineered ECM island size and array dimension. See the photofabrication section for details on engineered ECM island seeding. In both conditions, the medium was refreshed two hours before the imaging session. Three independent high-resolution widefield fluorescence experiments were performed with a CFI SR HP Plan Apo Lambda S 100 $\times$ silicone oil objective (Silicone oil immersion, NA 1.35, WD 0.31 mm, catalog# MRD73950). Static datasets were analyzed with the HT1080 CALIPERS CC-aware bioimage analysis pipeline and stratified by cell-cycle phase (G1 = CFP; S/G2/M = iRFP) to extract principally, the average cell-cycle phase distribution, cell area, and tubulin spread per experiment (Fig. 3.2D-F, Fig. 3.4D-F). Additional information on nuclear morphology and cytoskeleton assembly is also quantified (Fig. A2.7).

Static HT1080 CALIPERS Population-scale CC phase distribution

To evaluate whether the FUCCIplex-defined cell-cycle phase distribution inferred from the baseline characterization is representative at the population level, a quantification of FUCCIplex phases in free-space conditions was performed. 10 independent FOVs were acquired using a 10× objective in fluorescence mode at low resolution across a large field of view (Fig

. A2.2A). A total of $n = 555$ cells were quantified by classifying each nucleus as G1 (CFP⁺) or S/G2/M (iRFP⁺). Images were processed in ARIVIS Pro (ARIVIS Pro, v. 4.40)⁷³, where nuclei were segmented and assigned to a FUCCIplex phase based on nuclear CFP and iRFP signal thresholds. The resulting phase fractions were aggregated across FOVs to yield a population-level estimate of the CC phase distribution (Fig. A2.2E).

Dynamic HT1080 CALIPERS CC-aware phenotyping

For free space condition, 5×10^4 cells were seeded on 35 mm #1.5 glass-bottom dishes (Ibidi #81158) pre-coated with 20 µg/mL human plasma fibronectin (Sigma-Aldrich, catalog# F0895) in standard supplemented DMEM F-12 without phenol red. For confinement conditions, cells were seeded according to the square ECM size and array dimension. See the photofabrication section for details on ECM island seeding. In both conditions, the medium was refreshed two hours before the imaging session in each case. Three independent dynamic low-resolution fluorescence experiments were performed with a CFI Plan Apo 20× (air objective, NA 0.75, WD 1 mm, catalog# MRD00205). Dynamic datasets were analyzed with the HT1080 CALIPERS CC-aware bioimage analysis pipeline.

CC-labeled tracks were computed using a custom MATLAB script (v.R2023b, 23.2)⁷² that generated color-coded CC on fluorescence images and extracted CC-aware motility metrics over time, including velocity, cell area, and tubulin spread (Fig. 3.2H-J, Fig. 3.4J-L). Comprehensive CC-related nuclear and cytoskeleton morphology features were evaluated (Figs. A2.10 – A2.11). Additionally, cell-cycle phase duration, total path length, and net displacement were quantified for each cell-cycle phase (G1 = CFP; S/G2/M = iRFP).

Dynamic HT1080 CALIPERS 48h CC-aware phenotyping at population level

To strengthen the time-dependent analysis with a larger sample size and capture dynamic CC progression, extended (48 h) low-magnification, multi-channel live imaging over a large field of view was performed. Cells were tracked, and a subset of trajectories with reliable single-cell segmentation and complete FUCCIplex phase transitions was retained for analysis. Using ARIVIS Pro, a large dataset of per-cell instantaneous velocity ($\mu\text{m}/\text{min}$) and projected area (μm^2) was extracted, cell-cycle-aligned profiles were computed on a common 0-100% average cell-cycle axis by normalizing each trajectory to its FUCCIplex-defined phase durations. This yielded population-level time-dependent curves computed over $n = 50$ complete cell-cycles; the data in Fig. A2.3D-E are reported as mean $\pm$ SEM across tracked cell profiles.

FUCCIplex cell-cycle-normalized population-level analysis

To compare motility and morphology across several cells with variable cycle lengths, a population-level analysis was performed using the same dynamic analysis's MATLAB script, in which trajectories were normalized to a common cell-cycle axis. For each trajectory, time was aligned to the first time point, and phase windows (G1 and S/G2/M) were identified to measure their respective durations. Population means $\pm$ SD of phase durations were computed, and a linear time-warping procedure was applied. The G1 and S/G2/M segments of each trajectory were independently rescaled to match phase-specific population windows defined as the mean + 2SD for each phase, used here as a robust inclusion window to accommodate inter-cell variability while limiting the influence of rare, outlier-long phases on the normalization axis. The resulting normalized axis was subsequently mapped back to an "average cell-cycle time" (minutes), defined as the sum of the mean G1 and mean S/G2/M durations. On this common axis, population-average instantaneous velocity ($\mu\text{m}/\text{min}$) and projected cell area (μm^2) were calculated, yielding phase-colored (G1 vs S/G2/M) profiles that represent the average phenotypic evolution across a FUCCIplex-defined cell-cycle (Fig. A2.3).

Abnormal cell-cycle events analysis

Cell-cycle abnormalities were manually annotated across the three analyzed experiments of dynamic HT1080 CALIPERS CC-aware phenotyping. Five categories were defined. To classify abnormal CC events, ‘Long G1’ (> 9 h) and ‘Long S/G2/M’ (> 10h) were pragmatically defined using a distribution-based threshold, set at two standard deviations above the mean of the free space distribution. This approach follows precedent from single-cell timing studies that applied distribution thresholds for outlier detection³⁷. Additional abnormal events included ‘Long Mitosis’, defined as persistence in M phase beyond the expected one-hour duration. Finally, rare events of direct S/G2/M-G1 re-entry without mitosis (‘slippage’) were identified exclusively under confinement. To minimize the risk of annotation errors, trajectories were manually curated, and only cases confirmed by visual inspection were retained.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v10.4.2⁷⁴ and SigmaPlot v14⁷⁵. Normality was assessed with the Shapiro-Wilk test, and equal variance with the Brown-Forsythe test. For parametric data, two-way ANOVA with Tukey’s post hoc correction was applied; for non-parametric distributions, Kruskal-Wallis tests were used. All static and dynamic phenotyping assays were performed in three independent experiments ($n = 3$), each derived from separate cell passages and sample/substrate preparations, representing both biological and technical replicates. Unless noted, values are reported as mean $\pm$ SD. Exceptionally, for cytoplasmic versus nuclear CFP/iRFP localization, values were averaged per field of view (FOV) and reported as mean $\pm$ SEM across FOVs; for population-level profiled plots, data are likewise displayed as mean $\pm$ SEM across cell tracks (e.g., Fig. A2.3). For abnormal CC event frequencies (Fig. 3.4I), percentages were calculated over the total number of tracked cells pooled within each confinement condition. A threshold of $p < 0.05$ was considered statistically significant. Graphical annotations (* $p < 0.05$).

3.8 Bibliography

1. Bray, F. *et al.* Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* 74, 229-263 (2024).
2. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9, 239-252 (2009).
3. Paul, C. D., Mistriotis, P. & Konstantopoulos, K. Cancer cell motility: lessons from migration in confined spaces. *Nat. Rev. Cancer* 17, 131-140 (2017).
4. Denais, C. M. *et al.* Nuclear envelope rupture and repair during cancer cell migration. *Science* 352, 353-358 (2016).
5. Raab, M. *et al.* ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. *Science* 352, 359-362 (2016).
6. Gupta, V. & Chaudhuri, O. Mechanical regulation of cell cycle progression and division. *Trends Cell Biol.* 32, 773-785 (2022).
7. Hunter, M. V. *et al.* Mechanical confinement governs phenotypic plasticity in melanoma. *Nature* 1-11 (2025) doi:10.1038/s41586-025-09445-6.
8. Justus, C. R., Marie, M. A., Sanderlin, E. J. & Yang, L. V. Transwell In Vitro Cell Migration and Invasion Assays. *Methods Mol. Biol. Clifton NJ* 2644, 349-359 (2023).
9. Riss, T. L. *et al.* Cell Viability Assays. in *Assay Guidance Manual* (eds Markossian, S. *et al.*) (Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD), 2004).
10. Liang, C.-C., Park, A. Y. & Guan, J.-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.* 2, 329-333 (2007).
11. Mark, C. *et al.* Collective forces of tumor spheroids in three-dimensional biopolymer networks. *eLife* (2020)
12. Salic, A. & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci.* 105, 2415-2420 (2008).
13. Pereira, P. D. *et al.* Quantification of cell cycle kinetics by EdU (5-ethynyl-2'-deoxyuridine)-coupled-fluorescence-intensity analysis. *Oncotarget* 8, 40514-40532 (2017).
14. Discher, D. E., Janmey, P. & Wang, Y.-L. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310, 1139-1143 (2005).
15. Janmey, P. A., Fletcher, D. A. & Reinhart-King, C. A. Stiffness Sensing by Cells. *Physiol. Rev.* 100, 695-724 (2020).
16. Vogel, V. & Sheetz, M. Local force and geometry sensing regulate cell functions. *Nat. Rev. Mol. Cell Biol.* 7, 265-275 (2006).
17. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, D. E. Geometric control of cell life and death. *Science* 276, 1425-1428 (1997).
18. Ingber, D. E. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J. Cell Sci.* 104 (Pt 3), 613-627 (1993).
19. Pathak, A. & Kumar, S. Independent regulation of tumor cell migration by matrix stiffness and confinement. *Proc. Natl. Acad. Sci.* 109, 10334-10339 (2012).

20. Wang, N., Butler, J. P. & Ingber, D. E. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260, 1124-1127 (1993).
21. Butcher, D. T., Alliston, T. & Weaver, V. M. A tense situation: forcing tumour progression. *Nat. Rev. Cancer* 9, 108-122 (2009).
22. Dike, L. E. *et al.* Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *Vitro Cell Dev Biol Anim* 35, 441-448 (1999).
23. Huang, S., Chen, C. S. & Ingber, D. E. Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol. Biol. Cell* 9, 3179-3193 (1998).
24. Desmaison, A., Frongia, C., Grenier, K., Ducommun, B. & Lobjois, V. Mechanical Stress Impairs Mitosis Progression in Multi-Cellular Tumor Spheroids. *PLOS ONE* 8, e80447 (2013).
25. Roca-Cusachs, P. *et al.* Micropatterning of Single Endothelial Cell Shape Reveals a Tight Coupling between Nuclear Volume in G1 and Proliferation. *Biophys. J.* 94, 4984-4995 (2008).
26. Versaevel, M., Grevesse, T. & Gabriele, S. Spatial coordination between cell and nuclear shape within micropatterned endothelial cells. *Nat. Commun.* 3, 671 (2012).
27. Sakaue-Sawano, A. *et al.* Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487-498 (2008).
28. Koh, S.-B. *et al.* A quantitative FastFUCCI assay defines cell cycle dynamics at a single-cell level. *J. Cell Sci.* 130, 512-520 (2017).
29. Melero, C. *et al.* Light-Induced Molecular Adsorption of Proteins Using the PRIMO System for Micro-Patterning to Study Cell Responses to Extracellular Matrix Proteins. *J. Vis. Exp. JoVE* (2019)
30. Toro-Nahuelpan, M. *et al.* Tailoring cryo-electron microscopy grids by photo-micropatterning for in-cell structural studies. *Nat. Methods* 17, 50-54 (2020).
31. Schmidt, U., Weigert, M., Broaddus, C. & Myers, G. Cell Detection with Star-Convex Polygons. in *Medical Image Computing and Computer Assisted Intervention - MICCAI 2018* (eds Frangi, A. F., Schnabel, J. A., Davatzikos, C., Alberola-López, C. & Fichtinger, G.) 265-273 (Springer International Publishing, Cham, 2018). doi:10.1007/978-3-030-00934-2_30.
32. Stringer, C., Wang, T., Michaelos, M. & Pachitariu, M. Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* 18, 100-106 (2021).
33. Tinevez, J.-Y. *et al.* TrackMate: An open and extensible platform for single-particle tracking. *Methods* 115, 80-90 (2017).
34. Sante, M. D. *et al.* CALIPERS: Cell cycle-aware live imaging for phenotyping experiments and regeneration studies. 2024.12.19.629259 (2025).
35. Chung, J., Khadka, P. & Chung, I. K. Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation. *J. Cell Sci.* 125, 2684-2697 (2012).
36. Skowicki, M., Tarvirdipour, S., Kraus, M., Schoenenberger, C.-A. & Palivan, C. G. Nanoassemblies designed for efficient nuclear targeting. *Adv. Drug Deliv. Rev.* 211, 115354 (2024).
37. Marcus, J. M., Burke, R. T., DeSisto, J. A., Landesman, Y. & Orth, J. D. Longitudinal tracking of single live cancer cells to understand cell cycle effects of the nuclear export inhibitor, selinexor. *Sci. Rep.* 5, 14391 (2015).

38. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. & Gardner, M. B. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* 33, 1027-1033 (1974).
39. Kagawa, Y. *et al.* Cell Cycle-Dependent Rho GTPase Activity Dynamically Regulates Cancer Cell Motility and Invasion In Vivo. *PLoS ONE* 8, e83629 (2013).
40. Vittadello, S. T., McCue, S. W., Gunasingh, G., Haass, N. K. & Simpson, M. J. Examining Go-or-Grow Using Fluorescent Cell-Cycle Indicators and Cell-Cycle-Inhibiting Drugs. *Biophys. J.* 118, 1243-1247 (2020).
41. Sinha, D., Duijf, P. H. G. & Khanna, K. K. Mitotic slippage: an old tale with a new twist. *Cell Cycle* 18, 7-15 (2019).
42. Xu, F. L. *et al.* Mitotic slippage in non-cancer cells induced by a microtubule disruptor, disorazole C1. *BMC Chem. Biol.* 10, 1 (2010).
43. Moriarty, R. A. & Stroka, K. M. Physical confinement alters sarcoma cell cycle progression and division. *Cell Cycle* 17, 2360-2373 (2018).
44. Barr, A. R. *et al.* DNA damage during S-phase mediates the proliferation-quiescence decision in the subsequent G1 via p21 expression. *Nat Commun* 8, 14728 (2017).
45. Raab, M. *et al.* Mitotic arrest and slippage induced by pharmacological inhibition of Polo-like kinase 1. *Mol. Oncol.* 9, 140-154 (2015).
46. The balance between mitotic death and mitotic slippage in acute leukemia: a new therapeutic window? | Journal of Hematology & Oncology
47. Gjorevski, N. *et al.* Designer matrices for intestinal stem cell and organoid culture. *Nature* 539, 560-564 (2016).
48. Haselgrübler, T. *et al.* High-throughput, multiparameter analysis of single cells. *Anal Bioanal Chem* 406, 3279-3296 (2014).
49. Chessel, A. & Carazo Salas, R. E. From observing to predicting single-cell structure and function with high-throughput/high-content microscopy. *Essays Biochem* 63, 197-208 (2019).
50. Neumann, B. *et al.* High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods* 3, 385-390 (2006).
51. Bray, M.-A. & Carpenter, A. Advanced Assay Development Guidelines for Image-Based High-content Screening and Analysis.
52. Qiu, M. *et al.* A cell-level quality control workflow for high-throughput image analysis. *BMC Bioinformatics* 21, 280 (2020).
53. Garay, T. *et al.* Cell migration or cytokinesis and proliferation? - Revisiting the “go or grow” hypothesis in cancer cells in vitro. *Exp. Cell Res.* 319, 3094-3103 (2013).
54. Akhmetkaliyev, A., Alibrahim, N., Shafiee, D. & Tulchinsky, E. EMT/MET plasticity in cancer and Go-or-Grow decisions in quiescence: the two sides of the same coin? *Mol. Cancer* 22, 90 (2023).
55. Claes, A., Idema, A. J. & Wesseling, P. Diffuse glioma growth: a guerilla war. *Acta Neuropathol. (Berl.)* 114, 443-458 (2007).
56. Purwada, A. *et al.* Self-assembly Protein Nanogels for Safer Cancer Immunotherapy. *Adv. Healthc. Mater.* 5, 1413-1419 (2016).
57. Azarin, S. M. *et al.* In vivo capture and label-free detection of early metastatic cells. *Nat. Commun.* 6, 8094 (2015).
58. Stephan, S. B. *et al.* Biopolymer implants enhance the efficacy of adoptive T-cell therapy. *Nat. Biotechnol.* 33, 97-101 (2015).
59. Invitrogen TrueDesign Genome Editor - IT.
60. FlowJo Portal | FlowJo, LLC. <https://flowjo.com/portal>.

61. NIS-Elements. v.5.42
https://www.microscope.healthcare.nikon.com/it_EU/products/software/nis-elements.
62. Zhong, L. *et al.* Phosphorylation of cGAS by CDK1 impairs self-DNA sensing in mitosis. *Cell Discov.* 6, 26 (2020).
63. Developers, I. W. Inkscape - Draw Freely. | Inkscape. <https://inkscape.org/>.
64. Photopatterning Software LEONARDO, better Micropatterning Experiments. *alvéole* <https://www.alveolelab.com/our-products/leonardo-photopatterning-software/>.
65. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676-682 (2012).
66. Peng, T. *et al.* A BaSiC tool for background and shading correction of optical microscopy images. *Nat. Commun.* 8, 14836 (2017).
67. Perfetto, S., Wilder, J. & Walther, D. B. Effects of Spatial Frequency Filtering Choices on the Perception of Filtered Images. *Vision* 4, 29 (2020).
68. royerlab/dexp: First zenodo release. <https://zenodo.org/records/7039059>.
69. AllenCellModeling/aicsimageio. Allen Institute for Cell Science - Modeling (2025).
70. Walt, S. van der *et al.* scikit-image: image processing in Python. *PeerJ* 2, e453 (2014).
71. Yang, X., Shen, X., Long, J. & Chen, H. An Improved Median-based Otsu Image Thresholding Algorithm. *AASRI Procedia* 3, 468-473 (2012).
72. MATLAB Documentation. <https://it.mathworks.com/help/matlab/index.html>.
73. ZEISS ARIVIS Pro Software. v.4.4
<https://www.zeiss.com/microscopy/en/products/software/ARIVIS-pro.html>.
74. GraphPad. v.5. <https://www.graphpad.com/>.
75. SigmaPlot.v14. *Grafiti LLC* <https://grafiti.com/sigmaplot-detail/>.

4

TE-DaXi: design a custom light-sheet microscope for tissue engineering applications

Parts of this chapter are adapted from the conference proceedings paper: *Pezzotti M., et al. TE-DaXi: LEGO®-like instructions for assembling a light-sheet microscope for tissue engineering applications*, included in the proceedings of the IX Congress of the National Group of Bioengineering (GNB 2025). The material has been revised and integrated into the present thesis to improve coherence with the overall methodological framework and chapter structure

Building on the vertically integrated HT/HC principles established in Chapters 2-3, this chapter shifts the engineering approach from assay and pipeline integration to instrument design. TE-DaXi is presented as a purpose-built light-sheet platform designed to enable dose-governed, multiplexed volumetric phenotyping, with acquisition modes tailored to tissue-engineering specimens. While the system remains under prototyping, the design choices and validation plan are set to support the experimental and analytical pipeline developed in Chapter 5.

Moving from 2.5 to 3D HT/HC phenotyping, we extend the structural-functional coupling framework to volumetric scales (e.g., cardioid architecture paired with beating/ Ca^{2+} dynamics and cell-cycle state) while maintaining quantitative stability over long time windows. As discussed in Chapter 1, this shift links the choice of imaging modality

directly to the phenotype being measured: tissue-engineering constructs require low-dose volumetric illumination, adaptability to heterogeneous 3D specimens, large fields of view, and sufficient speed and imaging depth to capture multiscale dynamics. These requirements are rarely satisfied simultaneously by a single commercial instrument. Chapter 4, therefore, introduces the design of TE-DaXi as an engineering response: a single-objective, oblique-plane light-sheet system intended to combine gentle volumetric acquisition with high-resolution planar compatibility, suggesting a practical route toward scalable, context-aware imaging in tissue engineering.

4.1 Introduction

Tissue engineering (TE) platforms have increased the availability of physiologically relevant models for studying development and disease¹. Three-dimensional organoids and hybrid organ-on-chip systems are now widely used investigative tools in the life sciences, enabling disease modeling and therapeutic testing²⁻⁴. These platforms range from 3D hiPSC-derived cardiac organoids, which support long-term studies of self-organizing tissue structures^{3,5,6}, to 2.5D microfluidic chips that provide precise control of the cellular microenvironment^{7,8}. Each type poses distinct imaging challenges. Three-dimensional organoids demand deep, volumetric imaging with minimal phototoxicity to capture complex morphologies over time. In contrast, flatter 2.5D platforms require fast, high-resolution imaging to observe cell-substrate interactions (as illustrated in Fig. 4.1A).

Light-sheet fluorescence microscopy (LSFM) has emerged as an imaging approach well-suited to these needs⁹⁻¹¹. By illuminating samples with a thin sheet of light, LSFM enables fast, gentle optical sectioning, achieving high resolution across large volumes while reducing photodamage^{9,12}. This makes it useful for long-term live imaging of organoids and other TE constructs. However, current commercial light-sheet microscopes are often optimized for specific applications and sample formats rather than broad versatility. For example, Luxendo's TruLive (Bruker)¹³ supports prolonged 3D organoid imaging¹⁴, but it cannot accommodate standard multi-well plates or flat substrates, which can limit its use for engineered tissue sheets. Conversely, lattice light-sheet microscopes can achieve high-resolution imaging for cells on coverslips (2.5D culture), but they are less practical

for thicker, more complex 3D samples¹⁵. As a result, a single off-the-shelf system does not consistently cover the range of imaging requirements across tissue engineering platforms.

This gap motivates the development of a more flexible light-sheet microscope intended for tissue engineering applications. We identified the custom DaXi microscope¹⁶ developed by Royer's lab^{16,17} as a suitable starting point to help address this gap. DaXi is an oblique plane light-sheet microscope that combines subcellular resolution with a large field of view, rapid volume acquisition, and low phototoxicity. Importantly, it uses single-objective geometry for illumination and detection, which, in principle, allows imaging samples in standard glass-bottom dishes or microfluidic devices. Building on this concept, we designed TE-DaXi, a tissue engineering-adapted DaXi microscope, as a versatile imaging platform intended to support both 3D and 2D TE models. In adapting DaXi to a typical bioengineering lab, a key challenge was to make an advanced optical system accessible to non-experts. Our approach emphasized a modular design and LEGO®-like assembly instructions, with the aim of allowing laboratories without specialized optical engineering expertise to build, operate, and upgrade the microscope. In the following sections, we describe the design, construction, and validation of TE-DaXi, and discuss how this custom light-sheet system addresses current challenges in tissue-engineering imaging.

Despite this need, existing microscope options do not consistently meet the range of imaging requirements encountered in tissue engineering labs in practice. TE researchers often lack the specialized optics training to modify complex commercial instruments or build experimental setups from scratch¹⁸. A practical challenge is therefore to develop a flexible light-sheet microscope that can support both large 3D organoids and high-resolution 2D culture imaging, while remaining user-friendly and affordable. Key technical requirements include compatibility with standard sample formats (such as multi-well plates and microfluidic chips), minimal phototoxicity for long-term live-cell imaging, and sufficient resolution to resolve fine cellular structures. At the same time, the system should be straightforward to assemble and align, so that a bioengineering lab can deploy it without extended specialist training. In summary, the unmet need is a versatile

light-sheet platform with documented interfaces and an alignment workflow intended to reduce the barrier for adoption in bioengineering labs.

In this chapter, we present TE-DaXi, a customizable oblique plane light-sheet microscope inspired by the Royer lab's DaXi system (Fig. 4.1C). TE-DaXi was designed with modular hardware and an intuitive assembly workflow to support high-resolution, large-volume fluorescence imaging across tissue engineering applications.

To implement TE-DaXi, we structured the project into three objectives, each addressing a core aspect of development. First, we focused on the microscope design by developing an optical and mechanical layout that targets tissue engineering imaging requirements (depth, resolution, speed) while relying on accessible, off-the-shelf components and a simplified single-objective architecture. Second, we assembled the microscope and established a straightforward alignment procedure. This included integrating control electronics and software and developing step-by-step alignment protocols usable by non-experts. Third, we performed validation and testing on the assembled system to evaluate imaging performance and versatility, including preliminary imaging experiments and optical alignment checks to assess whether TE-DaXi meets the intended functionality, such as fast volumetric imaging with minimal phototoxicity and multi-modal capability.

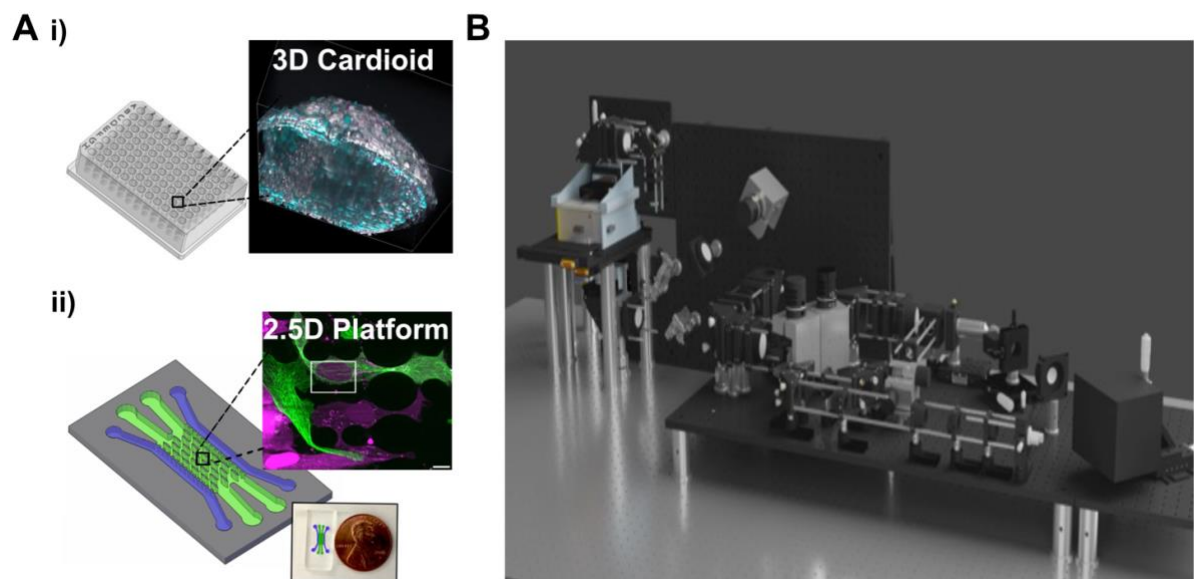


Figure 4.1. Tissue Engineered platform and cutting-edge DaXi light-sheet microscope

(A) i) 3D cardioid (heart organoid) imaged in a multi-well plate, illustrating gentle volumetric fluorescence acquisition of millimetre-scale tissues. ii) 2.5D micro-engineered platform with patterned ECM/microchannels guiding cell morphology and migration; inset shows device footprint (coin for scale). Representative multi-channel fluorescence highlights structural features at single-cell resolution, Courtesy of Sapoznik et al. (B) Render of the DaXi light-sheet microscope, developed by B. Yang et al. at CZ Biohub (San Francisco).

4.2 TE-DaXi microscope design

The goal of the design phase was to develop a light-sheet microscope concept optimized for tissue engineering needs while keeping the system as straightforward as possible. Advanced light-sheet designs, such as DaXi, can provide excellent imaging performance, but their complexity can limit adoption in non-optics labs. We therefore aimed to redesign the DaXi architecture into a simplified form that could be assembled without specialized expertise. Key design requirements were defined by the target applications: for thick 3D samples (e.g., organoids), the microscope should support deep low-phototoxic imaging; for planar 2.5D samples (e.g., organ-on-chip devices), it should provide high lateral resolution and remain compatible with standard glass substrates. To satisfy these requirements, we selected the Oblique Plane Microscopy (OPM) approach^{19,20}, which uses a single objective to both illuminate and collect fluorescence from a tilted light-sheet plane. OPM restricts illumination to a thin slice, reducing dose compared with widefield excitation, and its single-objective design permits imaging in conventional sample holders (e.g., multi-well plates) that are incompatible with dual-objective light-sheet setups. We also identified the need to image at different focal depths without moving heavy optics. To enable this, the design incorporates a remote focusing module²², which relays the image to an adjustable plane using secondary optics. This supports rapid refocusing within the volume. Overall, the design rationale was to balance imaging flexibility and performance (resolution, volume size, speed) with reduced system complexity. Each design choice was guided by this principle, from the selection of optical components to the design of the scanning mechanism.

We adopted Royer lab's DaXi (2022) as a blueprint and introduced targeted modifications to tailor it to TE applications. The core intent was to retain DaXi's high numerical aperture (NA) single-objective light-sheet geometry while simplifying alignment and ensuring compatibility with common lab materials. We selected optical components that are readily available and mechanically modular to support reproducibility and upgrades.

For instance, we chose a Nikon 25× silicone oil immersion objective as the primary lens (O1) because silicone oil's refractive index (~1.40) more closely matches that of thick

tissue specimens than water, which can reduce spherical aberrations and improve image quality deeper within samples²³. Inspired by the remote focusing technique of Botcherby et al. (2008)^{22,24}, we designed a relay optics system (secondary lens and objective) to project the intermediate image plane beyond the primary objective, preserving focus when imaging oblique planes. To support this design step, we consulted the Calico/York Lab open-source microscope design for OPM, using their published lens configuration²³ as a starting point for the detection path. The design was divided into modular sections: an illumination module, an imaging/detection module, and a sample-scanning unit, each of which could be prototyped and tested independently. In addition, we prioritized a stage-scanning strategy for volume acquisition, based on sample motion through a stationary light-sheet, to reduce moving parts and overall complexity. Finally, to aid non-expert builders, we decided to produce comprehensive design visualizations: a 2D optical schematic (Fig. 4.2) and an interactive 3D CAD model (using OnShape) to serve as a step-by-step assembly guide (Fig. 4.3).

The TE-DaXi design was intended to meet the targeted criteria by providing a blueprint for a high-resolution, accessible light-sheet microscope. By adopting the OPM geometry, the design supports compatibility with typical tissue engineering sample formats. The single-objective setup allows imaging samples directly on a glass coverslip or within a microfluidic device, which simplifies sample preparation compared to dual-objective light-sheet systems^{14,21,25}. The integration of a remote focusing relay (O2 and TL2) allows refocusing across Z without moving the heavy primary objective and provides a pathway for future fast 3D imaging implementations²⁶. Several key modifications to the original DaXi were implemented to tailor performance to TE needs. First, the use of the 25× silicone oil primary objective (instead of a water-immersion lens) can improve image quality inside organoids by reducing refractive index mismatch²⁷. Second, guided by the Calico/York design resources, we adjusted the spacing and focal lengths of the relay optics to reduce the risk that adding the remote focusing module would degrade optical performance (e.g., maintaining alignment of the pupil planes for diffraction-limited imaging). Third, in the light-sheet illumination path, we replaced the cylindrical lens with a Powell lens to generate a more uniform sheet. This supports more even illumination across a larger field of view and reduces the edge fall-off typical of Gaussian light-sheets. We also simplified the volume scanning mechanism: rather than synchronizing multiple

objective motions or using electrically tunable lenses, TE-DaXi performs volume acquisition by translating the sample stage (and thus the sample through the stationary light-sheet). This choice reduces mechanical complexity and potential points of failure at the cost of lower maximum speed than remote objective scanning. In this configuration, volumetric imaging remains compatible with the target application, assuming precise stage motion and software-synchronized exposure. Another outcome of the design phase was the creation of clear documentation and visualization of the microscope layout.

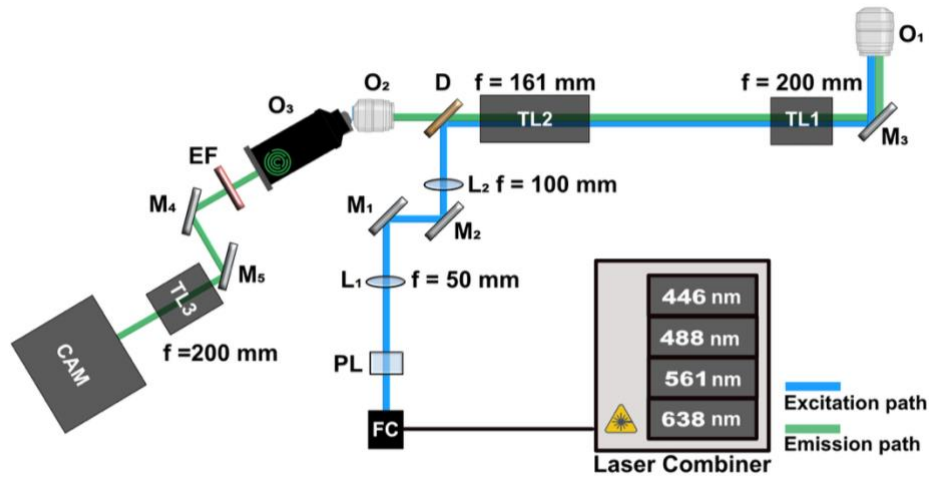
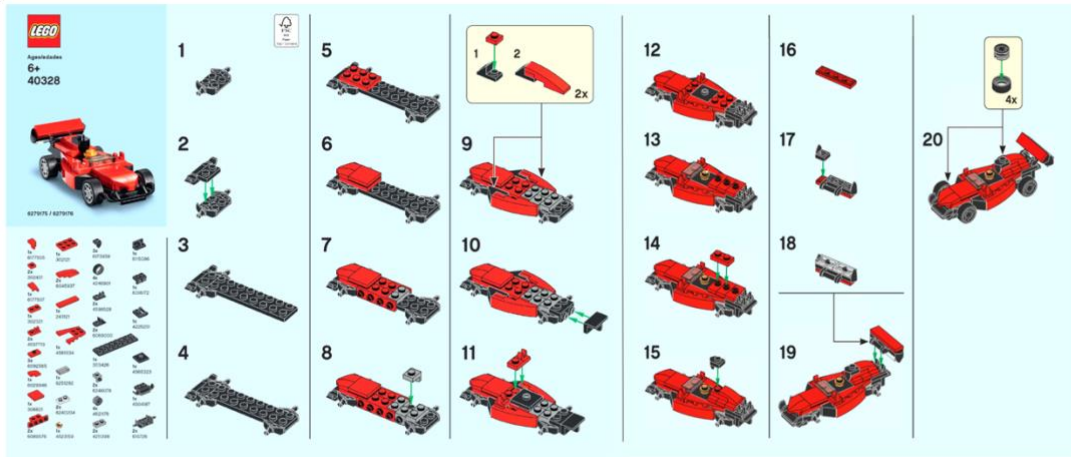


Figure 4.2. TE-DaXi optical layout Fiber-coupled (FC) laser combiner (446/488/561/638 nm) injects the excitation path (blue) and a beam-shaping/expansion relay (PL, L_1 $f=50$ mm, L_2 $f=100$ mm), steered by M_1 - M_2 , and combined at the dichroic D. The beam is relayed through TL2 ($f=161$ mm) and TL1 ($f=200$ mm) to the sample via O_1 . Fluorescence emission (green) is collected by O_1 , relayed back through TL1-TL2, passes the dichroic D to the detection arm (O_2 - O_3), filtered by EF, folded by M_4 - M_5 , imaged by TL3 ($f=200$ mm) onto CAM. Labels: O_1 - O_3 objectives; TL1-TL3 tube lenses; L_1 - L_2 lenses; M_1 - M_5 mirrors; D dichroic; EF emission filter; PL Powell/beam-shaping optic; FC fiber coupler; CAM sCMOS camera.

We produced a traditional 2D optical schematic of TE-DaXi (Fig. 4.2), highlighting all components in the optical train. Because schematics can be abstract for new users, we also constructed a detailed 3D CAD model of the microscope (Fig. 4.3B) using OnShape²⁸. This model was broken into an ordered assembly sequence, essentially forming a virtual instruction manual that mirrors a commercial step-by-step building guide (Fig. 4.3A). Users can inspect this model to understand how components stack and align in three dimensions, supporting both initial build and later modifications.

In the design phase, we established a practical foundation for TE-DaXi. The OPM-based architecture and component set were selected to support deep volumetric imaging and high resolution in a configuration intended to be more accessible than previous custom light-sheet setups. By incorporating features like remote focusing and stage-scanning, the design remains flexible for future enhancements (e.g., faster acquisition modes) while keeping the initial implementation straightforward. Overall, we produced a microscope design that balances performance and simplicity. TE-DaXi's design is modular and upgradable, and it is intended to support imaging of both 3D and 2.5D tissue engineering constructs with minimal phototoxicity and adequate image quality.

A



B

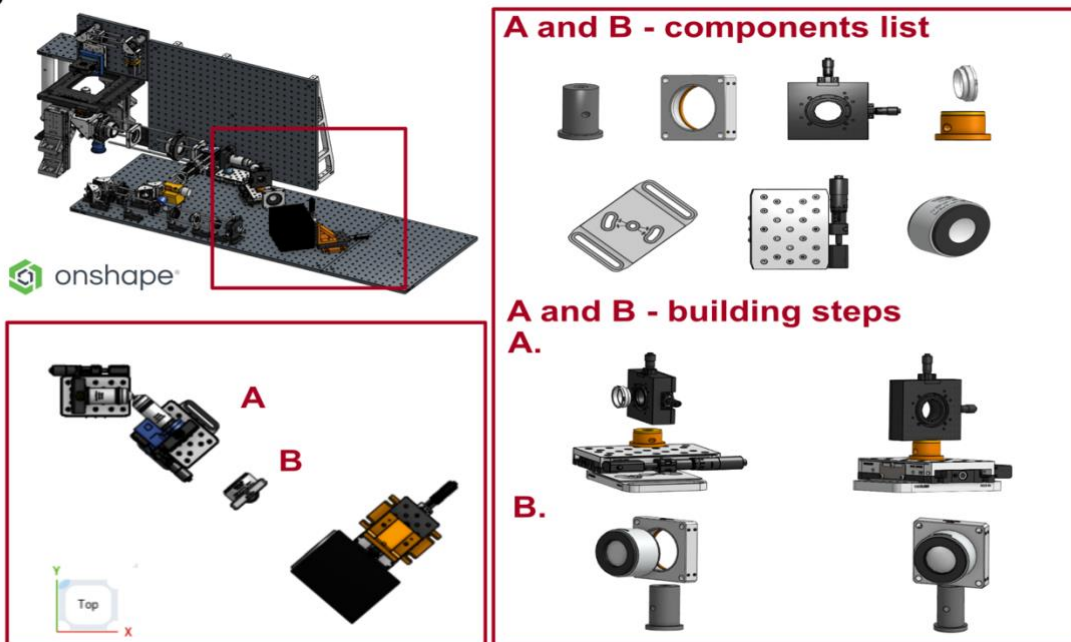


Figure 4.3. 3D TE-DaXi virtual assembly model. (A) Instruction-style storyboard illustrating stepwise assembly logic used to build TE-DaXi microscope modules (LEGO analogy for parts, order, and constraints). (B) 3D CAD/Onshape implementation: exploded view of the microscope with the targeted module, the remote refocus highlighted. On the right, component lists for sub-assemblies A and B, along with their corresponding build steps, document hardware choices and the order of operations for reproducible construction.

4.3 Construction and alignment

With the design finalized, the next goal was to build the TE-DaXi microscope and develop an alignment procedure suitable for a typical bioengineering lab. Constructing a complex optical system can be intimidating, so we aimed to translate the design into a clear, step-by-step assembly process, using the CAD model and schematic as guides. We also aimed to ensure that alignment, often the most challenging step and one that typically requires optical expertise, could be achieved using simple methods and tools. By planning the assembly sequence and incorporating visual alignment aids, we sought to reduce trial-and-error and limit common misalignments. In parallel, we integrated the control electronics and software alongside the physical assembly to ensure that all components (stages, lasers, camera) communicate effectively. We selected open-source, user-friendly software for microscope control, specifically Micro-Manager²⁹, instead of custom code or proprietary platforms to keep the system accessible. The available diSPIM plugin³⁰ (initially designed for dual-view SPIM) provided a practical starting point for managing our stage-scanning light-sheet setup. Overall, our goal was to make TE-DaXi building and alignment feasible for non-specialists by providing clear instructions and a reliable alignment strategy that does not rely on specialized equipment or advanced optical metrology skills.

The construction plan followed a modular assembly approach, dividing the build into logical stages. First, we set up the hardware infrastructure: mounting optical rails, stages, and breadboards, and connecting all devices (stage, lasers, camera, and controllers) to the workstation. This step would verify that motors and lasers can be controlled via software before optics are introduced. Next, we assembled and aligned the optical subsystems in sequence, using a 4f alignment technique for each conjugate lens pair. We identified critical alignments, including: (i) aligning the primary objective (O1) and primary tube lens (TL1) in a 4f configuration, (ii) aligning the secondary tube lens (TL2) with TL1 and

O2 for the remote focus module, and (iii) aligning the illumination path (Powell lens, beam expander, mirrors) through the dichroic and into O1 to form a light-sheet at the intended angle. For each of these, we outlined a method using a collimated low-power alignment laser and a shearing interferometer to check collimation and focus. The approach was to propagate the alignment laser backward from the O1 position through the system (via a mirror at the sample plane) to check that lenses share a common optical axis and the focal planes overlap. We also planned to use a simple 50:50 beam splitter to send the alignment beam into the detection path for checking focus at the camera plane. As each section was aligned, we secured the components before proceeding to the next step to limit cumulative errors.

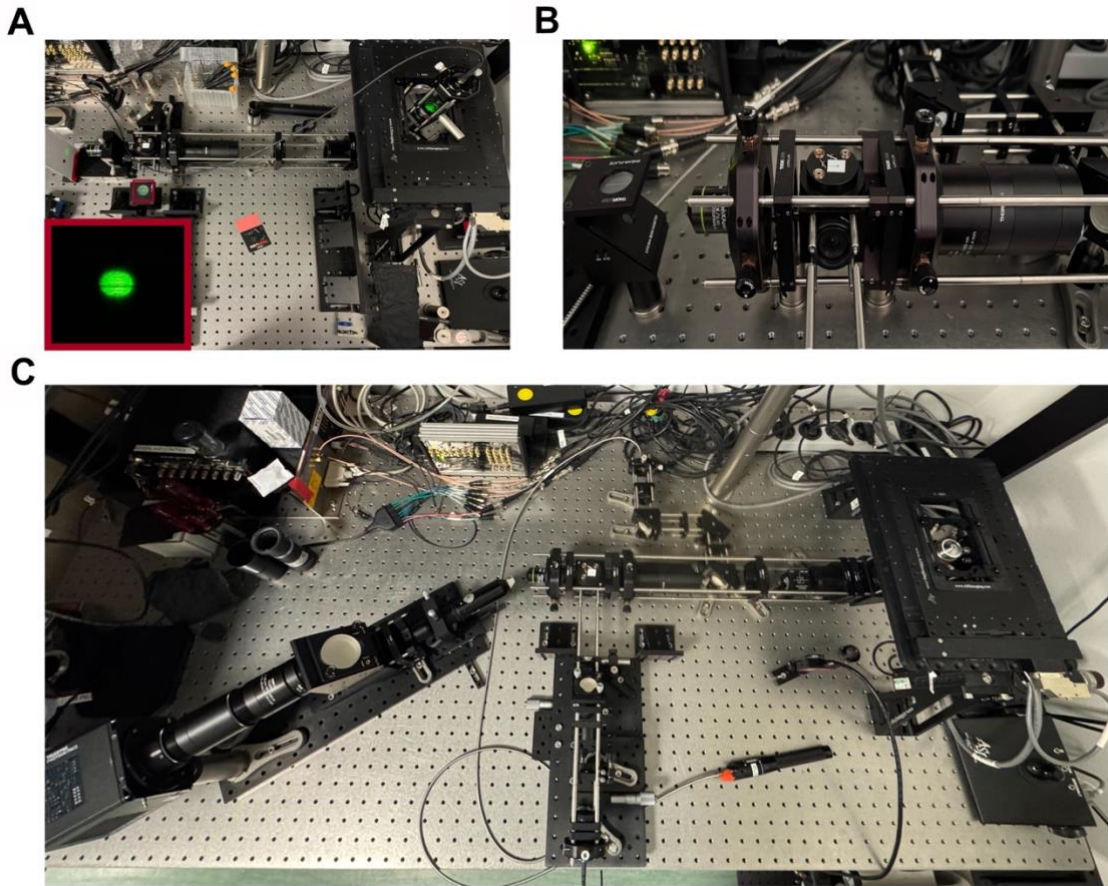


Figure 4.4. TE-DaXi construction and alignment (A) Excitation arm setup during alignment; inset shows the interferometer plate profile recorded in green laser emission to evaluate remote refocus $4f$ alignment. (B) Objective 2 (O2) relay with matching tube-lens assembly and intermediate dichroic filter cube mounted on precision rails for parfocal adjustment. (C) Top-down view of the complete instrument on the optical table,

from excitation input through remote refocus to the sample stage and detection camera, illustrating the final geometry.

By following the planned procedure, we assembled and aligned the TE-DaXi microscope. Major components were installed as expected, supported by the modular design and prior CAD visualization, which helped anticipate mechanical clearance and mounting requirements. The alignment protocol was executed using the shearing interferometer and the alignment laser. Using this approach, we achieved collimated 4f alignment for each lens pair across the optical train. For example, after aligning O1 with TL1 and TL2 with O2 as described, the system produced a sharply focused image on the camera at the expected plane without obvious astigmatism or distortion. We verified that the excitation light-sheet was formed and oriented by observing its reflection from the planar mirror at the sample plane (the sheet formed a tight line focus). Adjusting the dichroic position allowed control of the sheet tilt, and we aligned it so that it intersected the detection focal volume. Final alignment of the detection module (O3 and camera tilt) aimed to make the camera sensor parallel to the light-sheet plane; this was supported by a Z-scan test that produced minimal lateral shift of a focused spot, consistent with the intended geometry. Fig. 4.4 illustrates key steps of the alignment: aligning the tube lenses (Fig. 4.4A), positioning the remote focus objective O2 (Fig. 4.4B), and the completed TE-DaXi setup (Fig. 4.4C). A detailed alignment protocol for each module is described in the Materials and Methods section.

In parallel, the supporting systems were integrated and operational. The ASI stage and Tiger controller operated smoothly in Micro-Manager, and we established basic image acquisition protocols using the diSPIM plugin (repurposed for our single-objective setup). The 3D CAD guide and the systematic alignment method supported the goal of making assembly repeatable and user-friendly. Throughout construction, we documented each step with videos, photos, and notes, effectively creating a virtual handbook to follow.

We then performed a coarse alignment and defined clear benchmarks for verifying it. These benchmarks, refined during development (such as the interferometer fringe patterns and the Z-shift test for beam tilt), provide practical checks that a new user can apply to assess whether the system is aligned. In summary, TE-DaXi can be assembled

and aligned in a standard lab setting without sophisticated tools, supporting the practicality of the design for the intended user base.

4.4 Preliminary tests and validation

After assembling the microscope, we conducted preliminary tests to validate TE-DaXi's performance against the imaging requirements of tissue engineering applications. This preliminary validation serves two purposes: first, to confirm basic imaging capability by verifying fluorescence and transmitted-light operations and checking that the optics are correctly aligned. Second, to assess key optical performance parameters, including unintended beam tilt, aberrations, and light-sheet illumination behavior. These early checks establish whether the microscope can image representative biological samples and help identify adjustments needed before routine research use.

Specifically, our goal was to verify that the microscope can capture volumetric fluorescence data with minimal phototoxicity (as a preliminary assessment) and operate as a multimodal platform (e.g., providing widefield or brightfield imaging to facilitate sample positioning).

From an optical perspective, ensuring that the light-sheet is correctly formed, such as flat, uniform, and at the intended angle, is essential because misalignment would directly affect imaging quality. Therefore, our first validation strategy included performing a series of tests assessing both the imaging capability and the alignment accuracy of TE-DaXi.

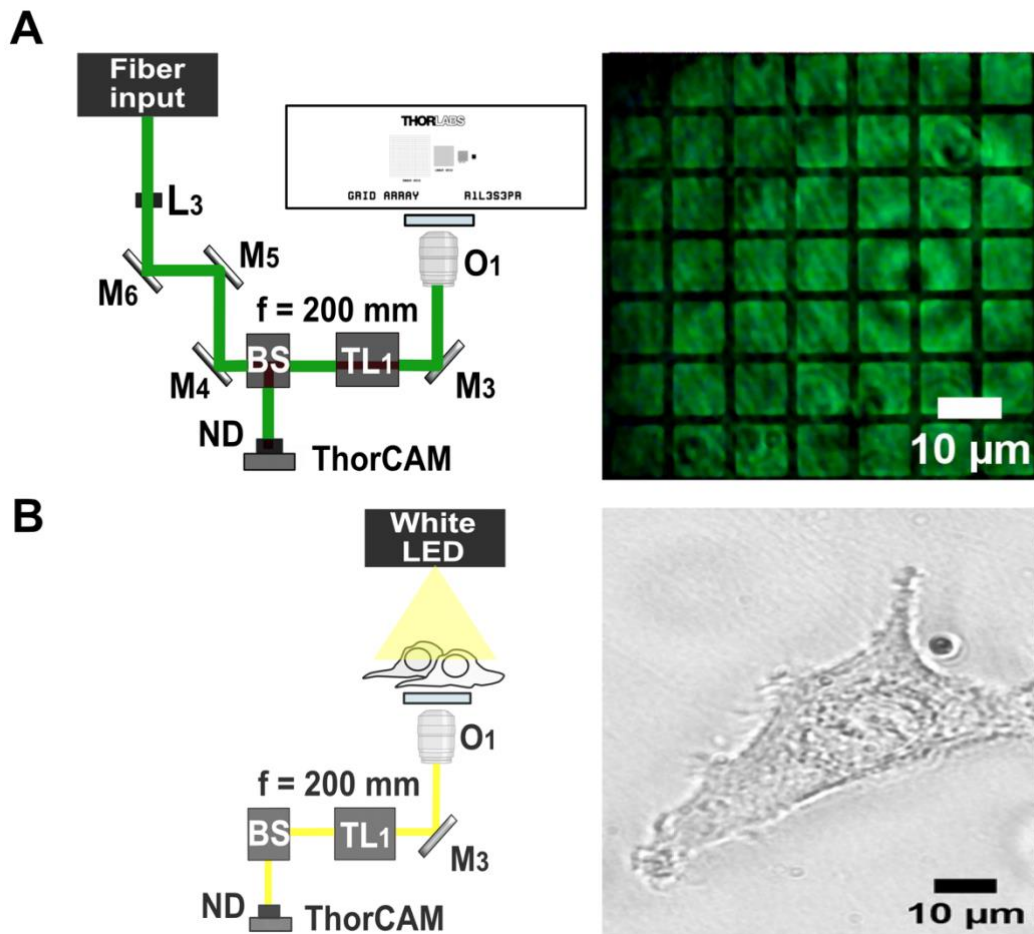


Figure 4.5. Test for detection-path alignment and imaging. (A) Fluorescence test layout: fiber-coupled input relayed by L_3 and mirrors M_6 - M_3 through TL_1 ($f = 200$ mm) and a beamsplitter (BS) to a calibration camera (ThorCAM); neutral density (ND) for power control. Right, image of a Thorlabs grid array acquired at the sample plane with O_1 , used to verify focus, parfocality, and pixel sampling (Scale bar, $10\ \mu\text{m}$). (B) Brightfield test layout: white-LED trans-illumination collected by O_1 , relayed by TL_1 /BS/ M_3 to ThorCAM for rapid visual alignment. Right, representative live-cell image with HT1080 cell line confirming contrast and imaging geometry. Scale bar, $10\ \mu\text{m}$.

Validation experiments indicated that TE-DaXi is functional and suitable for imaging tests. The microscope produced clear images in both fluorescence and brightfield modes. The widefield fluorescence image of the calibration grid showed sharp, evenly focused features across the field, indicating appropriate spacing of the detection optics and camera sensor (Fig. 4.5A). We measured the grid spacing in the image, and it matched the known value, confirming that the system's magnification was as expected (using the $25\times$ objective and tube lens combination). The brightfield image of the HT1080 cells (Fig. 4.5B) resolved individual cells and their morphology, demonstrating that TE-DaXi can

be used like a normal microscope for sample navigation and inspection. These tests confirm the microscope's basic imaging capabilities and highlight its multimodal flexibility: by simply adding a side camera and an LED, we achieved transmitted-light imaging, which is less commonly available in dedicated commercial light-sheet systems. This feature is practically useful for a TE lab, as it allows users to locate regions of interest in a sample and perform calibration without switching to a different microscope, demonstrating that TE-DaXi can be used for sample navigation and inspection.

The alignment verification test (zero-tilt test) suggested that the optical alignment was stable. With the Powell lens removed, the system produced a focused Gaussian excitation spot in the sample (Fig. 4.6A). When the focus was shifted axially from Z0 to Z1, the spot expanded (as it went out of focus) but crucially did not shift in the XY plane³¹. This indicates that the beam did not wander laterally and supports the interpretation that the illumination axis was orthogonal to the imaging plane. As depicted in Fig. 4.6A, the fluorescent spot remained centered as it defocused, yielding a symmetric “blur” above and below the focal plane. This result is consistent with the primary objective, the tube lens and camera being correctly aligned with no residual tilt, and the remote focusing module being calibrated so that refocusing does not introduce lateral offsets. This alignment is important for accurate volumetric imaging because residual tilt would introduce depth-dependent lateral shifts in the data.

The oblique light-sheet test demonstrated the expected behavior of OPM: as we shifted focus in Z, the portion of the light-sheet in focus moved laterally across the field, consistent with the sheet being tilted at approximately 45° (our chosen angle)³². Fig 4.6B shows that when the focal plane moved from Z0 to Z1, the illuminated region on the camera translated in XY, indicating we were capturing different parts of the tilted plane. Importantly, the light-sheet intensity was approximately uniform across a large central area. The Powell lens reduced the Gaussian intensity peaks³³. As evaluation criteria, we assessed that there were no pronounced hot spots or dim edges in the sheet: fluorescence excitation was evenly distributed, enabling quantitative imaging over large specimens without requiring intensity correction³³. We also noted that the sheet was thin and well confined in the Z direction, as expected for our optical parameters. The combination of a uniform sheet and proper alignment achieved in this preliminary test suggests that the

TE-DaXi system can deliver optical sectioning suitable for subsequent biological imaging.

Lastly, we performed a light-sheet tilt calibration by shifting the illumination plane in 1 mm steps from the zero position. At each offset, we captured three images at different focal planes ($Z = -10\text{ }\mu\text{m}$, $0\text{ }\mu\text{m}$, $+10\text{ }\mu\text{m}$). The lateral displacements between these Z planes were used to estimate the local tilt angle. A straightforward linear regression in Python relates the tilt to the axial stage movement. Acquisition controls were consistent with expectations: with the Powell lens inserted, the sheet aligns with the optical axis, whereas during Z motion, the lateral position remains stable in the aligned setup but shifts laterally in the intentionally tilted setup, as expected for an oblique sheet.

Across three replicated datasets, the pooled fit shows a linear dependence of tilt on stage shift: $\text{Tilt (}^\circ\text{)} = 14.36 \times \text{StageShift (mm)} + 4.73$, as shown in Fig. 4.6C. The slope ($14.36^\circ/\text{mm}$) is consistent with the designed obliqueness of the launcher: advancing the stage axially sweeps the tilted plane laterally in proportion to the travel, which is consistent with the remote-focus coupling. The intercept ($\sim 4.73^\circ$) indicates a baseline tilt at zero nominal position. However, given reduced precision near 0 mm, we treat this as an estimate rather than a definitive bias. Practically, this suggests a small residual misalignment that could be corrected through iterative zero-position alignment or re-zeroing the stage origin. Overall, the linear relationship and reproducibility across runs support correct oblique light-sheet formation and predictable scanning of the focal plane through the sample volume.

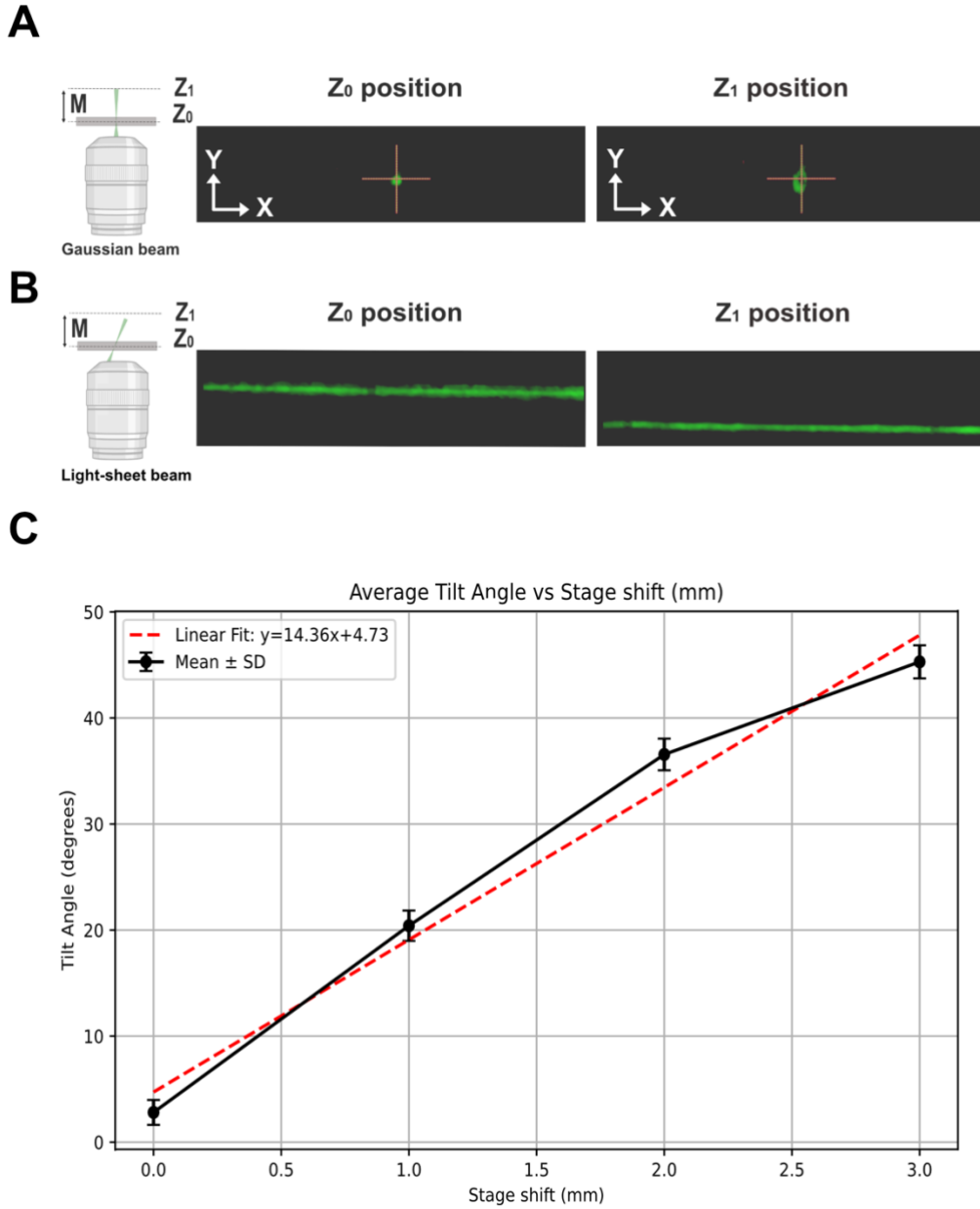


Figure 4.6. Illumination tilt from axial and lateral beam displacement. (A) Gaussian-beam test. A steering mirror (*M*) directs the beam through the detection objective. Images at two axial positions (Z_0 , Z_1) show no lateral shift of the focus, indicating no residual tilt. Axes: *X*-*Y* in the camera plane. (B) Light-sheet test. The sheet imaged at Z_0 and Z_1 exhibits *z*-dependent lateral displacement along *X*, used to estimate the effective tilt of the excitation plane relative to the detection axis. (C) Average tilt angle versus imposed stage shift (mm). Black markers show mean $\pm$ SD across repeats; red dashed line is a linear fit ($\theta = 14.36 \times \Delta_{\text{stage}} + 4.73$), confirming near-linear scaling and providing a calibration factor for setting the target tilt.

Together, these preliminary tests confirm TE-DaXi prototype alignment, image formation, multimodal sample inspection, and predictable oblique light-sheet behavior.

The results indicate that the microscope can acquire fluorescence and transmitted-light images, maintain appropriate detection-path alignment, generate an oblique illumination plane, and scan the tilted sheet through the sample volume. However, for complex 3D tissue-engineering applications, successful image formation is only the first validation step. 3D quantitative phenotyping requires a more complete performance evaluation, because volumetric datasets must preserve specimen geometry, channel correspondence, signal stability, and temporal consistency. This is particularly relevant for dense multicellular specimens, such as 3D organoids, where multiplexed structural analysis with cell-cycle state mapping and functional imaging depend on the reliability of the optical system across depth, time, wavelength, and reconstruction space.

We therefore define the next validation level for TE-DaXi linking microscope characterization to downstream 3D phenotyping requirements. In this view, we present a reporting framework organized around three main requirements. First, 3D spatial fidelity and resolution must be quantified to determine whether lateral and axial resolution, light-sheet geometry, and reconstruction accuracy are sufficient to preserve tissue morphology and support volumetric structural analysis. Second, multiplexed quantitative reliability must be evaluated to ensure that structural markers, cell-state reporters, and functional channels can be acquired and registered within a common 3D coordinate system. Third, multiscale imaging capability must be tested to determine whether the same imaging platform can support both fast volumetric bursts for functional readouts and long-term low-dose volumetric imaging for slower structural and cell-state dynamics. To make this transition from prototype validation to quantitative-use validation explicit, Fig. 4.7 summarizes the performance metrics and validation tests that should be investigated before routine use of TE-DaXi for complex 3D tissue-engineering imaging. These checks include optical, mechanical, photometric, environmental, and reconstruction-related parameters. Together, they define the operating conditions under which TE-DaXi can move from successful image acquisition toward reliable downstream quantitative analysis in 3D tissue-engineering models.

Validation axis	Performance check	Metrics and downstream relevance
A. 3D spatial fidelity & resolution	XY/Z resolution	PSF-derived lateral and axial FWHM; bead size; objective/relay configuration; voxel size used for reconstruction
A. 3D spatial fidelity & resolution	Depth-dependent PSF	PSF broadening and signal-to-background ratio at different depths in gel/sample-like phantoms
A. 3D spatial fidelity & resolution	Light-sheet geometry	Sheet waist/thickness; Rayleigh length; propagation uniformity; measured tilt angle relative to detection plane
A. 3D spatial fidelity & resolution	Deskew & reconstruction accuracy	Geometric transform used; scaling/shear error; axial compression/elongation; validation with bead grid or structured phantom
A. 3D spatial fidelity & resolution	Scan volume & distortion	Usable XY range; Z-equivalent scan depth; field distortion map; full-volume coverage for thick 3D specimens
B. Multiplexed quantitative reliability	Channel co-registration	X/Y/Z displacement between channels using multicolor beads; residual registration error after correction
B. Multiplexed quantitative reliability	Chromatic performance	Wavelength-dependent lateral shift, axial focal shift, and channel-specific magnification/scaling differences
B. Multiplexed quantitative reliability	Illumination uniformity	Flat-field or fluorescent phantom map; intensity variation across XY/Z; channel-specific uniformity correction
B. Multiplexed quantitative reliability	Photometric stability	Laser power at sample; exposure time; camera settings; intensity drift over repeated acquisitions; bleaching trend
B. Multiplexed quantitative reliability	Sample-induced aberration	Resolution and contrast loss in sample-like material; refractive-index mismatch; scattering-related depth limitation
C. Multiscale imaging capability	Fast volumetric bursts	Frame rate; plane rate; volume rate; planes/volume; exposure per plane; compatibility with functional readouts
C. Multiscale imaging capability	Long-term imaging stability	XY/Z drift over hours; focus stability; repositioning error; registration strategy for time-lapse volumes
C. Multiscale imaging capability	Stage/sample stability	Repeatability after stage motion; vibration sensitivity; sample immobilization/embedding condition; motion artifacts
C. Multiscale imaging capability	Environmental stability	Temperature, CO ₂ , humidity, medium evaporation control; equilibration time before acquisition; live-imaging limits
C. Multiscale imaging capability	Dose & operating envelope	Per-channel power/irradiance proxy; exposure budget; acquisition regime; safe operating conditions for live 3D samples
D. Reporting bundle	Performance summary	Single table linking validation metric, test sample, acceptance criterion, and affected downstream 3D phenotyping step

Figure 4.7. TE-DaXi performance-reporting framework for quantitative 3D tissue-engineering imaging. The table summarizes the main performance metrics and validation tests required to move from prototype-level image formation to quantitative 3D phenotyping. The checks are grouped according to the downstream requirements they support: 3D spatial fidelity and resolution, multiplexed quantitative reliability, and multiscale imaging capability.

4.5 Discussion

Light-sheet microscopy supports gentle, continuous 3D observation, which aligns with many tissue engineering needs, but adoption has been slowed by complex multi-objective geometries, rigid commercial platforms, and workflows that require labs to operate separate systems for organoids versus planar assays. TE-DaXi was designed to address these practical barriers. By implementing an oblique-plane light-sheet around a single objective, the design is compatible with common TE sample formats, such as organoids, multi-well plates, and microfluidic chips, supporting both long-term 3D imaging with high-NA 2D/2.5D imaging on a single optical axis. Our alignment checks (zero-tilt and oblique-sheet tests) and tilt-calibration analysis show that the sheet forms consistently and scans the volume with a linear, reproducible geometry. This supports treating reconstructed volumes as interpretable within the system's validated operating range, rather than only illustrative.

The first-generation build reflects the practical trade-offs of an open instrument. Stage-based volume scanning limits top-end frame rates compared with mirror scanners; single-view detection leaves anisotropy and can introduce shadowing in thicker tissues; and the sheet-to-camera tilt benefits from periodic checks as the lab environment drifts. User experience is simpler than many custom rigs, but careful immersion handling, objective hygiene, and basic alignment remain important. These limitations do not negate the central aim of the system: TE-DaXi is intended to support long-term, low-dose imaging of organoids and rapid phenotyping on the same platform, reducing the need to split workflow across multiple systems in day-to-day use.

The way forward is continued engineering. Future iterations could add image-based auto-alignment and autofocus to stabilize hands-off operation, implement adaptive illumination to manage dose budgets, and expand modalities while retaining the single objective architecture. One could envision the integration of phase/DIC for label-free structure, SPAD array³⁴, confocal³⁵, or two-photon³⁶ path adds-on for speed, contrast, or depth when needed. The same logic that motivates smart acquisition can also be extended

to HT/HC screening³⁷: low-dose widefield scouts can prioritize wells/fields with emerging events, followed by selective light-sheet stacks to record them^{38,39}. With these additions, TE-DaXi could move toward a more HT/HC phenotyping-oriented platform for capturing rare, 3D, cell-cycle phase-dependent phenomena at a practical cadence without sacrificing viability.

Taken together, TE-DaXi is presented as an attempt to translate the advantages of light-sheet microscopy into a more accessible, modular instrument that aligns with common tissue engineering workflows: standard culture-ware, multiple time scales, and illumination budgets that cells can tolerate. It aims to reduce the gap between concept and practice by supporting both quantitative 3D organoid imaging and high-resolution planar assays on a single platform. Looking ahead to automation, multimodality, and plate-scale scheduling, TE-DaXi is intended to support gentle imaging of engineered cell culture systems and to make those experiments easier to run in ways that are compatible with HT/HC phenotyping.

Although this work defines the architectural basis for volumetric imaging, it does not yet establish a complete 3D phenotyping workflow in a biological system. Chapter 5 develops such a workflow, integrating sample handling, volumetric acquisition, and quantitative analysis in 3D cardioid models.

4.6 Materials and Methods

Microscope optical components:

The microscope employs a stage-scanning oblique-plane illumination configuration. A motorized XYZ translation stage (ASI FTP-2000, controlled by an ASI Tiger controller) carries the sample and provides precise movement for focus and volume scanning. Four laser lines (446 nm, 488 nm, 561 nm, 638 nm) are combined in a commercial laser combiner (Vortran) and delivered via a single-mode optical fiber to the illumination path. The collimated output from the fiber (collimator: Thorlabs RC02APC-P01 off-axis parabolic mirror) passes through a neutral density filter (Thorlabs NE20B-A) for intensity tuning. Next, a Powell lens (Thorlabs LGL130) converts the Gaussian beam into a thin, uniformly illuminated sheet. The beam is expanded $2\times$ by a lens pair (L1: 50 mm focal length, Thorlabs AC254-050-A-ML; L2: 150 mm, AC254-150-A-ML) to increase the light-sheet width. Two mirrors (M1, M2, Thorlabs PF10-03-P01) then steer the expanded beam into a dichroic mirror (Chroma ZT405/488/561/640rpcv2-UF3). The dichroic reflects the excitation light toward the sample while transmitting emitted fluorescence to the detection path. Notably, this dichroic is mounted on a small linear translation stage (Thorlabs XR25P/M), allowing its lateral position to be adjusted. This enables fine-tuning of the light sheet's entry angle into the objective, effectively aligning the illuminated plane obliquely through the sample. The excitation light finally enters the primary objective (O1), a Nikon $25\times$ 1.05 NA silicone oil immersion lens (Nikon MRD77250). O1 focuses the light-sheet at an oblique angle within the sample and also collects the fluorescent emission from that illuminated plane.

On the detection side, TE-DaXi uses a multi-stage optical relay to achieve remote focusing and high NA imaging. Immediately above the primary objective, a small right-angle mirror (M3, Thorlabs PF20-03-P01) is placed to divert the emitted fluorescence 90° sideways. This light is first focused by a primary tube lens (TL1, Thorlabs TTL200MP, 200 mm focal length), producing an intermediate image. The intermediate image is directed into the remote refocus module ³²(implemented according to the 4f design in ³¹). The refocus module consists of a secondary tube lens (TL2, 161 mm focal length) paired with a secondary objective (O2, Olympus UPLXAPO $20\times$ Air, NA 0.8). The O2 lens features a thin coverslip permanently attached to its front, simulating the sample coverslip

for optimal aberration correction at the relay interface³⁴. The TL2-O2 pair forms a 4f system that projects the image to a remote plane while correcting optical aberrations, following the design guidelines from York Lab's open-source OPM implementation. The relayed image is then captured by the tertiary objective (O3), a custom high-NA lens (AMS-AGY v2, NA 1.0, from Calico Labs), designed to exploit the full numerical aperture of the system for high resolution²³. An emission filter (Chroma ZET405/488/561/640 multi-band filter) is placed either before O3 or immediately after it to block reflected excitation light and pass only fluorescence. Finally, a tertiary tube lens (TL3, another Thorlabs TTL200MP, 200 mm) focuses the light onto the sensor of a scientific CMOS camera (Teledyne Photometrics Kinetix). This detection path, consisting of three objectives (O1, O2, O3) and their corresponding tube lenses, is configured to preserve high resolution across a large field of view while allowing the detection plane to be physically shifted by adjusting the position of O2-TL2 (the remote focus) rather than moving O1. The entire assembly is mounted on an optical breadboard for stability, with standard cage-and-rail systems to maintain alignment. In addition to the optical hardware, we incorporated a fluorescence stage-top incubation chamber in the design to enable live-cell imaging under controlled environmental conditions for future long-term experiments.

Microscope assembly

Costruction began by mounting the ASI XYZ stage onto a reinforced optical table and attaching the primary objective (Nikon 25×, O1) on an adjustable holder beneath the stage. The ASI Tiger controller was connected to the stage motors and to the sCMOS camera and laser combiner via USB/Ethernet, and we installed Micro-Manager 1.4 on the control PC²⁹. Using Micro-Manager's device wizard, we added the stage (as a XYZ device), the Tiger controller's shutter/laser switches, and the camera. We then loaded the ASI diSPIM plugi³⁰, which we configured to treat our single light-sheet path as a single diSPIM arm. This allowed basic synchronous control of stage movement and camera acquisition using the 4-line laser combiner. With hardware communication confirmed, we proceeded with optical and mechanical assembly.

First, we would set up the hardware infrastructure, including mounting optical rails, stages, and breadboards, and connecting all devices (stages, lasers, camera, and

controllers) to the workstation. This step would verify that motors and lasers can be controlled via software before optics are introduced. Next, we planned to assemble and align the optical subsystems in sequence, using a 4f alignment technique for each conjugate lens pair. We identified critical alignments such as: (i) aligning the primary objective (O1) and primary tube lens (TL1) in a perfect 4f configuration, (ii) aligning the secondary tube lens (TL2) with TL1 and O2 for the remote focus module, and (iii) aligning the illumination path (Powell lens, beam expander, mirrors) through the dichroic and into O1 to form a light-sheet at the correct angle. For each of these, we outlined a method using a collimated low-power alignment laser and a shearing interferometer to check collimation and focus. The idea was to propagate the alignment laser backward through the system (via a mirror at the sample plane) to ensure all lenses are aligned on a common optical axis and focal planes overlap. We also planned to use a simple 50:50 beam splitter to send the alignment beam into the detection path for checking focus at the camera plane. As each section was aligned, we would secure the components, then proceed to the next, mitigating cumulative errors. Once the core microscope was assembled and aligned, we intended to test basic imaging (e.g. capturing an image of a microscope calibration target) to fine-tune focus and parfocality of the detection system. The final part of the plan involved installing any auxiliary modules (such as the side-view camera for widefield/brightfield imaging) and verifying their alignment relative to the main optical axis.

Alignment (Primary Optical Train)

We placed a mirror at the intended sample position (horizontally at the stage center) to serve as a temporary reflector. This mirror was used to align the primary detection 4f system: we directed a low-power collimated alignment laser (635 nm Thorlabs diode with collimator) into the detection path from the camera port, effectively sending it backward through TL3 and O3. We adjusted O3 and TL3 until the laser exiting O1 (through the mirror in place of the sample) was collimated, as observed with a shear plate interferometer (Thorlabs SI050) placed in front of O1. This ensured TL3-O3 and TL1-O1 were aligned for infinity focus. Next, we aligned TL1 and O1 precisely in a 4f configuration. We removed O3 (for direct access) and directed the alignment laser through TL1 toward O1. By observing the interference fringes on the shearing

interferometer placed after reflection from the mirror, we translated TL1 along the optical axis until collimated return beams indicated correct O1-TL1 spacing (i.e. the primary objective's focal plane coincident with the tube lens focus). We locked TL1 in this position. Then we inserted TL2 and O2 (the remote focus lenses) into the path. We sent the alignment laser through TL1 (with O1 still reflecting off the mirror) and adjusted TL2 position to find the point where it produced collimated light after O2 - confirming that TL2 was at the proper $4f$ distance from O2. For this step, we temporarily removed TL1 after the laser passed it, so that we could observe the beam coming through O2 without the first image formation, as suggested by the York design procedure. Fine adjustments of O2 (which was mounted on an XYZ micrometer stage) were made until the interferometer showed parallel fringes (collimated output) after O2, indicating O1-TL1-TL2-O2 were all aligned in series. At this stage, the detection path (including remote focus) was nearly aligned. We re-inserted O3 and verified that an object at the sample plane would be sharply imaged on the camera by doing a quick test image of the reflective target (placed at the sample position) using white-light illumination.

Alignment (Illumination Path)

We next assembled the illumination optics on a separate rail. The fibre collimator (FC), Powell lens (PL), and beam expander lenses (L1, L2) were mounted and roughly pre-aligned on a small optical breadboard. We adjusted distances to ensure the Powell lens was at the collimated beam output and L1-L2 were spaced for $2\times$ expansion. This sub-assembly was then positioned so that the expanded beam would strike the dichroic mirror at roughly its center. We installed the dichroic mirror on its translation stage at a 45° angle to the beam. With the alignment laser now sent through the fibre input, we fine-tuned the mirrors M1/M2 to center the beam on the dichroic and pass through the middle of the back aperture of O1 (visible by removing the sample mirror and looking at the back of O1 with an IR card). Once the beam was entering O1, we adjusted the micrometer translation stage holding the dichroic laterally (and locked its angle) to set the light-sheet angle: essentially, translating the dichroic introduced a slight offset so that the beam emerging from O1 was tilted relative to the horizontal focal plane. We calibrated this offset such that the light-sheet would coincide with the native focal plane of O1 when

remote focusing was at its zero position. With the excitation path aligned through the objective, we placed a fluorescent sample (a thin fluorescent slide) on the stage and observed the illumination on the side camera to ensure a proper sheet was forming.

Alignment (Detection and Camera)

Finally, we integrated the detection module. The tertiary objective (O3) and camera had been pre-mounted in a fixed relation (via a cage system) to maintain focus. We inserted this module such that O3 faced the secondary objective O2 across the intermediate image gap. Using a fluorescent bead sample, we adjusted the tilt and position of the O3-camera module to capture the oblique plane in focus on the camera sensor. This involved *tilting O3 and the camera* slightly to match the oblique angle of the light-sheet (since the image plane is tilted relative to the camera when using OPM). We iteratively adjusted the tilt and focus until fluorescent beads appeared sharp and not elongated, indicating the camera's sensor plane coincided with the oblique image plane. This completed the core alignment; the final assembly of TE-DaXi is shown in Fig. 4c.

Imaging functionality

We added two simple optical modules to TE-DaXi for this test. For widefield fluorescence, we used the main laser illumination but directed the emitted light to a side-mounted CMOS camera (Thorlabs CS165CU/M, termed the “side camera”) via a secondary tube lens. Essentially, this side camera looked at the sample from a different angle (orthogonal to the main detection path) capturing fluorescence in epifluorescence mode. For brightfield imaging, we introduced a white LED (Thorlabs MWWHL4 collimated LED) above the sample as a transmitted light source and again used the side camera to record the images. We first placed a reflective calibration grid (Thorlabs R1L3S3PR chrome grid target) on the sample stage. In widefield fluorescence mode (with appropriate excitation from the lasers and filters), we acquired images of the grid to check for distortion, focus across the field, and calibration (known spacing of the grid). Next, we cultured a sample of HT1080 fibrosarcoma cells expressing EGFP-actin (from Ibidi) on a 35 mm glass-bottom dish. For this test, the sample was imaged in brightfield by illuminating it with the LED and capturing the transmitted light using the side camera³⁰. This allowed us to observe cell morphology and confirm that the microscope could also function as a basic transmitted light microscope for locating and focusing on samples.

The use of the side camera in both cases means these imaging tests did not yet employ the oblique light-sheet; they were to validate the detection optics and general imaging capability in a simpler configuration.

Alignment validation (zero-tilt)

We switched back to the main camera on TE-DaXi for this and the following test. To isolate the effect of alignment, we temporarily removed the Powell lens from the excitation path, thus the system delivered a roughly collimated beam through O1 (or we replaced it with a lens that focuses the beam into the sample as a standard epifluorescent illumination). We placed a thin fluorescent film or a dilute fluorescence solution to visualize the excitation beam. We brought the beam into focus at a reference plane (Z0) near the center of the sample and captured an image (or observed the pattern of illumination). Then we moved the sample stage upward by a small amount (to plane Z1) so that the focus went slightly out of the original plane. We captured a second image. We compared the XY position of the fluorescence spot at Z0 vs Z1. In a well-aligned system, the out-of-focus spot at Z1 will appear expanded (blurred) but still centered at the same coordinates as at Z0. We quantitatively checked for lateral shift by measuring the centroid of the fluorescence spot between images.

Oblique light-sheet test

For the final test, we reinserted the Powell lens and set the system to its standard light-sheet configuration. We used a thin fluorescent sheet or a plane of fluorescent beads as a sample to see the illuminated plane. We again imaged the fluorescence with the main camera at two focal positions Z0 and Z1, separated by a known Z-step, e.g. 50 μm . Because the light-sheet is tilted, when the stage moves, the intersection of the sheet with the focal plane moves laterally. We recorded the images and live video to observe this lateral translation of the in-focus region. Additionally, we examined the intensity profile along the light-sheet in the image to check for uniformity. The Powell lens should produce a flat-top intensity profile, so we looked for even brightness across the central field of view without significant vignetting or Gaussian fall-off. Achieving a uniform sheet is important for quantitative imaging, as it ensures all parts of a large organoid receive similar excitation light. Throughout this process, we also monitored the system for any

signs of misalignment, such as astigmatism or coma in point spread functions, which might indicate a need to tweak lens positioning.

4.7 Bibliography

1. Wheeler, E. E. & Leach, J. K. Tissue-Engineered Three-Dimensional Platforms for Disease Modeling and Therapeutic Development. *Tissue Eng. Part B Rev.* ten.teb.2024.0212 (2024)
2. Pocock, M. W. *et al.* Maturation of human cardiac organoids enables complex disease modeling and drug discovery. *Nat. Cardiovasc. Res.* 4, 821-840 (2025).
3. Lewis-Israeli, Y. R. *et al.* Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021).
4. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 345, 1247125 (2014).
5. Hofbauer, P. *et al.* Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021).
7. Capulli, A. K. *et al.* JetValve: Rapid manufacturing of biohybrid scaffolds for biomimetic heart valve replacement. *Biomaterials* 133, 229-241 (2017).
8. Wang, G. *et al.* Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nat. Med.* 20, 616-623 (2014).
9. Reynaud, E. G., Kržič, U., Greger, K. & Stelzer, E. H. K. Light sheet-based fluorescence microscopy: More dimensions, more photons, and less photodamage. *HFSP J.* 2, 266-275 (2008).
10. Daetwyler, S. & Fiolka, R. P. Light-sheets and smart microscopy, an exciting future is dawning. *Commun. Biol.* 6, 1-11 (2023).
11. Stelzer, E. H. K. *et al.* Light sheet fluorescence microscopy. *Nat. Rev. Methods Primer* 1, 1-25 (2021).
12. Scherf, N. & Huisken, J. The smart and gentle microscope. *Nat. Biotechnol.* 33, 815-818 (2015).
13. Bruker Corporation. Light-Sheet Images of Organoids and Cell Cultures (Luxendo TruLive microscope web resource).
14. Krzic, U., Gunther, S., Saunders, T. E., Streichan, S. J. & Hufnagel, L. Multiview light-sheet microscope for rapid in toto imaging. *Nat. Methods* 9, 730-733 (2012).
15. Smart lattice light-sheet microscopy for imaging rare and complex cellular events | Nature Methods.
16. Yang, B. *et al.* DaXi-high-resolution, large imaging volume and multi-view single-objective light-sheet microscopy. *Nat. Methods* 19, 461-469 (2022).
17. Yang, B. *et al.* Epi-illumination SPIM for volumetric imaging with high spatial-temporal resolution. *Nat. Methods* 16, 501-504 (2019).
18. Girstmair, J. *et al.* Light-sheet microscopy for everyone? Experience of building an OpenSPIM to study flatworm development. *BMC Dev. Biol.* 16, 22 (2016).
19. Dunsby, C. Optically sectioned imaging by oblique plane microscopy. 11 (2008).
20. Sapoznik, E. *et al.* A Versatile Oblique Plane Microscope for Large-Scale and High-Resolution Imaging of Subcellular Dynamics. 78.

21. Kumar, A. *et al.* Dual-view plane illumination microscopy for rapid and spatially isotropic imaging. *Nat. Protoc.* 9, 2555-2573 (2014).
22. Botcherby, E. J., Juškaitis, R., Booth, M. J. & Wilson, T. An optical technique for remote focusing in microscopy. *Opt. Commun.* 281, 880-887 (2008).
23. Alfred Millett-Sikking & Andrew York. High NA single-objective light-sheet.
24. Botcherby, E. J. *et al.* Fast measurement of sarcomere length and cell orientation in langendorff-perfused hearts using remote focusing microscopy. *Circ. Res.* 113, 863-870 (2013).
25. Dualview Inverted Selective Plane Illumination Microscope (DISPIM) | National Institute of Biomedical Imaging and Bioengineering.
26. Dvinskikh, N *et al.* Volumetric light-sheet calcium mapping in 3D biological samples. *Front. Physiol.* (2023)
27. Boothe, T. *et al.* A tunable refractive index matching medium for live imaging cells, tissues and model organisms. *eLife* 6, e27240 (2017).
28. Business, O., a PTC. Onshape | Product Development Platform.
29. Micro-Manager.
31. Han, W., Cheng, W. & Zhan, Q. Design and alignment strategies of 4f systems used in the vectorial optical field generator. *Appl. Opt.* 54, 2275-2278 (2015).
32. Hong, W., Sparks, H. & Dunsby, C. Alignment and Characterisation of Remote-Refocusing Systems (2023).
33. Singh, R. *et al.* Oblique plane microscope for mesoscopic imaging of freely moving organisms with cellular resolution. *Opt. Express* 31, 2292 (2023).
34. Bruschini, C., Homulle, H., Antolovic, I. M., Burri, S. & Charbon, E. Single-photon avalanche diode imagers in biophotonics: review and outlook. *Light Sci. Appl.* 8, 87 (2019).
35. Confocal Microscopy: Principles and Modern Practices - Elliott - 2020 - Current Protocols in Cytometry - Wiley Online Library.
36. Truong, T. V., Supatto, W., Koos, D. S., Choi, J. M. & Fraser, S. E. Deep and fast live imaging with two-photon scanned light-sheet microscopy. *Nat. Methods* 8, 757-760 (2011).
37. André, O., Kumra Ahnslide, J., Norlin, N., Swaminathan, V. & Nordenfelt, P. Data-driven microscopy allows for automated context-specific acquisition of high-fidelity image data. *Cell Rep. Methods* 3, 100419 (2023).
38. Royer, L. A. *et al.* Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016).
39. Royer, L. A., Lemon, W. C., Chhetri, R. K. & Keller, P. J. A practical guide to adaptive light-sheet microscopy. *Nat. Protoc.* 13, 2462-2500 (2018).

5

Developing engineering tools for 3D HT/HC functional and structural phenotyping

Building on the HT/HC logic developed across Chapters 1 and 2, where phenotype definition is explicitly tied to acquisition design under dose constraints, and in Chapter 3 through vertically integrated readout and analysis, extending the same engineering framework to 3D self-assembling systems changes the nature of the problem: scaling from 2D to 3D models makes phenotype definition more tightly coupled to volumetric acquisition design and interpretability, and requires explicit choices about what constitutes the phenotype, how it can be measured, and how structural and functional descriptors should be paired. Accordingly, this final chapter establishes an end-to-end workflow for 3D HT/HC phenotyping, defining the acquisition and analysis requirements that will be transferred to the TE-DaXi platform described in Chapter 4. Here, we use self-organizing hiPSC-derived cardiac organoids (cardioids) that intrinsically specify, pattern, and morph into chamber-like structures containing a cavity as a reference platform. The workflow first establishes a stable, low-dose volumetric imaging pipeline, then a unified 3D analysis pipeline that links cardioid cavity architecture to proliferative

state through integrated structural-functional readouts, and finally interprets experimental datasets by adopting a multicellular phase-field model that captures two cavity morphogenesis regimes (spherical and branched) as distinct phenotypes.

5.1 Introduction

Cardiovascular disease remains a major global health burden, and the gap is increasingly one of prediction rather than awareness: the World Health Organization reports ~19.8 million CVD deaths in 2022, roughly one-third of global mortality, while large-scale epidemiologic syntheses continue to show persistent impact across risk factors and geographies¹⁻³. This context motivates tissue engineering and developmental bioengineering to prioritize human-relevant models that can resolve how cardiac structure emerges.

Human pluripotent stem cell-derived 3D cardiac systems address this need by embedding multicellular self-organization and morphogenesis into an experimentally accessible format, allowing developmental outcomes to be quantified as phenotypes rather than treated only as qualitative observations^{4,5}. However, turning these models into routine high-throughput/high-content (HT/HC) phenotyping, particularly when the goal is to couple structural state (e.g., cavity morphogenesis) with functional state (e.g., proliferation dynamics), remains challenging in practice.

A first bottleneck is experimental: organoid and cardiac differentiation workflows remain vulnerable to batch effects, variable maturation, and staging ambiguity; recent efforts emphasize benchmarked protocols and defined quality checkpoints precisely because uncontrolled process variance can dominate phenotype variance at scale^{6,7}. In parallel, while the community is actively developing automation-ready platforms for organoid HT/HC screening (multiwell formats, automated handling, standardized culture geometries), these solutions continue to highlight similar limitations, such as non-uniform sample mounting, inconsistent imaging conditions across wells, and fragile end-to-end data integration that complicates reliable cross-plate comparisons⁸⁻¹⁰. Long-term volumetric imaging introduces failure modes that are particularly damaging for

quantitative phenotyping, including motion and drift during acquisition, depth-dependent signal loss, and dose-driven physiological bias that can shift phenotypes long before overt damage is visible^{11,12}. In response, organoid imaging frameworks increasingly couple sample preparation, acquisition governance, and reconstruction/analysis into a single pipeline, reflecting that downstream image analysis performance is constrained by mounting stability, illumination control, and predictable acquisition geometry¹³.

These constraints feed into an analysis bottleneck that limits quantitative, predictive HT/HC outcomes: in many pipelines, structural descriptors (such as cavity number, cavity occupancy, topology, surface geometry) and functional descriptors (like resolved cell-cycle state distributions or dynamics), are not extracted and related under shared quality checks (QC), coordinate systems, and uncertainty tracking.

Instead, structure and function are typically processed using separate tools, assumptions, QC procedures, and summary statistics, and are then compared at the end. This separation is often difficult to reproduce and does not provide a unified representation that supports fitting, regime classification, or model-data mapping. As a result, analysis may not reliably distinguish biological variability from workflow-induced variability, and derived phenotypes can be difficult to generalize across plates, days, microscopes, or perturbations unless metadata and QC are managed as essential parts of the pipeline¹⁴⁻¹⁶.

A further bottleneck is interpretability: even when volumetric datasets and paired readouts are available, it can be difficult to summarize 3D phenotypes in a way that supports comparisons across conditions and developmental time points. This limitation is particularly consequential in self-assembling systems where outcome space is large, and where regime-like behavior suggests that a limited number of control variables can generate qualitatively distinct morphogenetic trajectories^{17,18}.

This chapter addresses these coupled bottlenecks by treating 3D HT/HC phenotyping as an engineering tools stack rather than as a sequence of disconnected techniques, using genetically edited hiPSC-derived cardioids¹⁹ as a reference platform to link structural and functional phenotyping in self-assembling systems. The chapter is organized around the development of engineering tools that support volumetric measurement, comprehensive 3D quantification, and model-based interpretation of experimental outcomes. First, we develop the experimental toolkit for an HT/HC imaging workflow,

including standardized sample generation supported by routine QC checks, optimized sample preparation, and light-sheet imaging protocols. Second, we implement a 3D phenotyping-oriented image analysis pipeline that quantifies cardioid structure and the organization of cavities across differentiation time and integrates these structural readouts with cell-cycle state to assess whether specific morphogenetic regimes are associated with shifts in proliferation dynamics. Third, to help interpret the experimental dataset, we adopt an existing phase-field model that describes cavity morphogenesis in organoids as a process balanced by proliferation, epithelial permeability/pressure, and interfacial tension, and use it as a framework to organize observed phenotypes and relate them to a set of parameters^{17,18,20}.

5.2 Experimental toolkits for volumetric HT/HC phenotyping workflow: from spheroids to cardioids

To improve the reliability of long-term imaging, we designed and implemented a staged HT/HC workflow for volumetric high-content phenotyping in 3D constructs. We first focused on sample preparation and mounting to limit sample motion during long acquisitions, then introduced HT plate-scale 2D acquisition JOBS as a front-end qualitative QC gate for primary differentiation success before committing to time- and data-intensive 3D imaging. Finally, we optimized dose-compatible volumetric acquisition while accounting for specimen-dependent illumination and artifacts that can complicate quantitative comparability across samples and imaging regimes. These practical constraints are common in live 3D fluorescence imaging, where phototoxicity can bias phenotypes before overt loss of viability^{12,21}, light-sheet alignment/aberrations can vary across specimens¹¹, and organoid-scale screening requires standardized QC gates to contain biological and technical variance²². To establish the workflow stepwise, we used a simple spheroid model to validate stable mounting and dose-compatible volumetric acquisition and then transferred these protocols to genome-edited hiPSC-derived cardioids.

5.2.1 Optimizing sample preparation and volumetric acquisition

First, we used a robust, low-cost 3D model (HaCaT spheroids)²³ to benchmark stabilization and acquisition regimes on a Luxendo TrueLive3D light-sheet microscope²⁴. To maintain continuity with the cell-cycle-aware phenotyping framework established earlier, we built the spheroid from the HaCaT FUCCIplex genome-edited line carrying the four fluorescent reporters described in Chapter 2, which, in principle, enable simultaneous structural and cell-state readouts in 3D. HaCaT Spheroids were generated using a standard self-assembly protocol in low-adhesion conditions to obtain multicellular aggregates suitable for volumetric imaging. After aggregation and compaction, we transferred the spheroids to the light-sheet workflow as a calibration model to establish stable sample preparation and dose-compatible imaging settings.

As described in Fig. 5.1A, we optimize spheroid mounting directly in the imaging chamber: spheroids were embedded in 0.5% (w/v) low-melting agarose (LMA), a classical mounting strategy for light-sheet microscopy²⁵. We let it equilibrate for approximately 10 min, and left it to gel at room temperature before adding maintenance medium as an overlay. Then, we evaluated this preparation as a mechanically stable mounting compatible with volumetric light-sheet imaging and supported both static stacks and long-term time-lapse acquisition.

In detail, as shown in Fig. 5.1B, representative optical sections acquired at increasing depths (30, 60, and 120 μm) indicate that 0.5% LMA does not introduce imaging artifacts and supports reproducible volumetric acquisition. However, as expected for dense 3D specimens, signal quality progressively decreased with depth, consistent with increased scattering and attenuation, which limited visualization of central regions beyond the first tens of micrometers. We next tested long-term feasibility under the same embedding condition to quantify stability over hours. Representative time points (0, 300, and 600 min) confirmed that 0.5% LMA stabilizes the spheroid, reducing motion-related blur and mitigating drift over prolonged imaging, thereby enabling repeated volumetric acquisition at 30 min intervals without overt structural degradation. Collectively, we established a baseline protocol for sample stability and viability under repeated illumination, providing a controlled reference before transferring the workflow to more biologically relevant models, the hiPSC-derived cardioids.

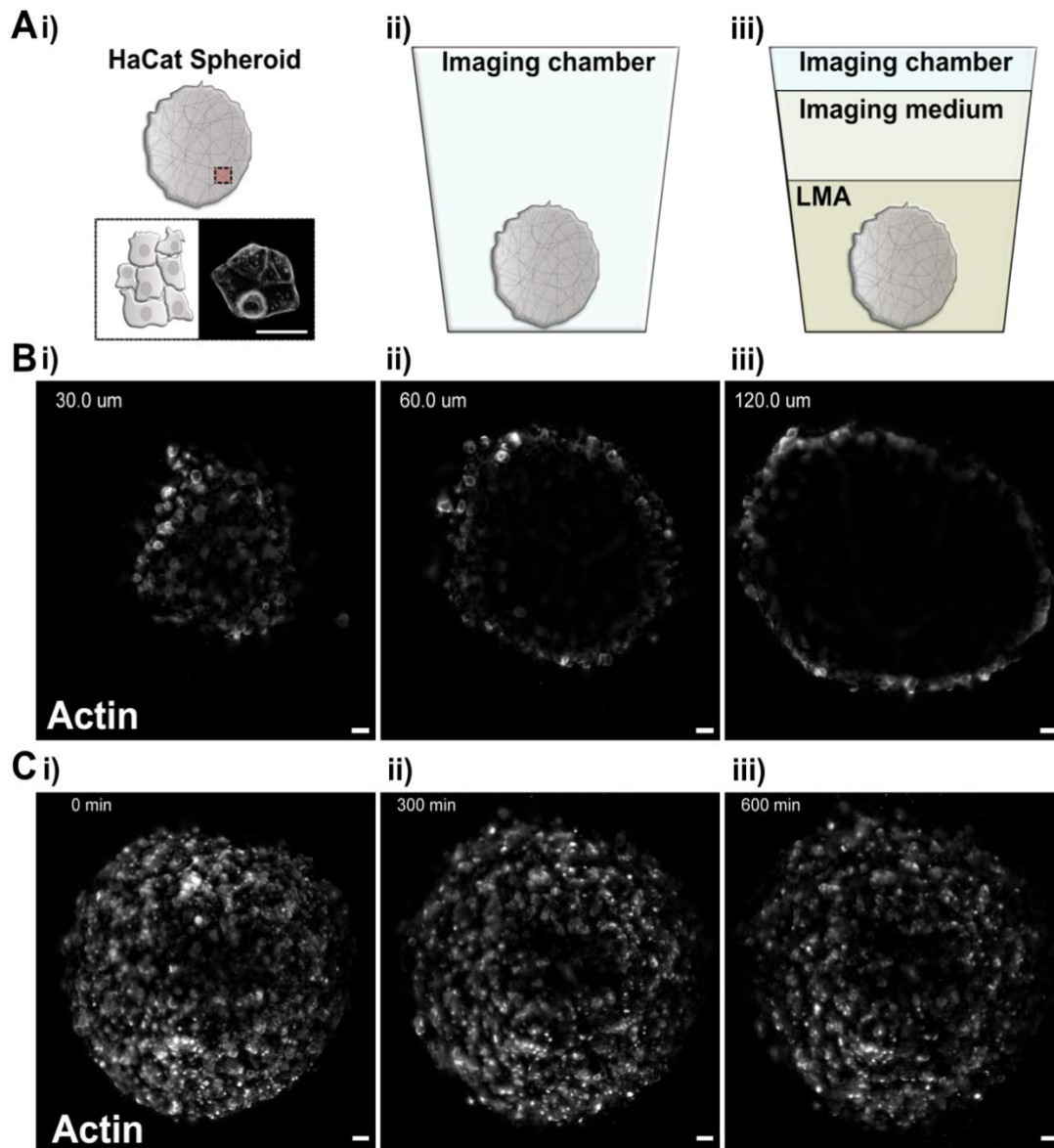


Figure 5.1. Low-melting agarose embedding enables stable static and long-term volumetric light-sheet imaging of HaCaT FUCCI Spheroids. (A) Workflow for spheroid preparation and chamber mounting. HaCaT spheroids are generated and transferred into the TruLive imaging chamber, positioned in the field of view, and embedded in 0.5% low-melting agarose (LMA) prepared in imaging medium. After gelation at room temperature, the chamber is filled with imaging medium to maintain hydration and viability during acquisition. (B) Static volumetric light-sheet fluorescence microscopy (LSFM) of an embedded HaCaT spheroid expressing RFP-LifeAct (actin). Representative optical sections at increasing depths (30, 60, and 120 μm) demonstrate that 0.5% LMA does not introduce obvious imaging artifacts and supports volumetric acquisition; as expected for dense 3D specimens, signal quality progressively decreases with depth due to scattering, limiting visualization of central regions beyond the first tens of micrometers. (C) Long-term LSFM feasibility test under the same embedding condition. Representative time points (0, 300, and 600 min) illustrate that 0.5% LMA stabilizes the spheroid, reducing

motion artifacts and mitigating drift over hours, supporting prolonged volumetric imaging. Scale bars, 25 μm .

5.2.2 High-throughput screening JOB for rapid morphological QC

With stable mounting and acquisition regimes established, we scaled the workflow to hiPSC-derived cardioids that were recently developed in our laboratory and reported in the CALIPERS work¹⁹. We genome-edited the GCaMP6 (a Ca^{2+} biosensor) hiPSC line with FUCCIplex to report cell-cycle status and RFP-LifeAct to visualize actin structure, to support integrated structural, functional, and proliferative phenotyping in 3D. Because transitioning from 2D maintenance to 3D self-assembly and differentiation introduces significant variability and increased experimental cost, we implemented a daily automated high-throughput (HT) 2D screening layer as a front-end QC step before volumetric imaging. As shown in Fig. 5.2A, we began with standardized hiPSC seeding in U-bottom wells (Day 0) and performed longitudinal, plate-wide monitoring starting on Day 2.5 using a commercial fluorescence microscope with a brightfield module to quickly capture cardioid morphology across all wells (Fig. 5.2A). We then applied a simple analysis algorithm to perform threshold-based segmentation on each brightfield image to create a binary mask (Fig. 5.2A, iii) and extract basic morphometrics, choosing projected area as the primary QC metric due to its robustness and straightforward interpretation as a growth indicator.

We used this screening layer to exclude non-conforming wells early on (e.g., abnormal size or shape trajectories) and to support more standardized downstream volumetric acquisitions by selecting cardioids that exhibit consistent growth behavior. Brightfield images illustrate the morphological progression from an early, compact, spheroid-like stage (Day 2.5) to more developed, structurally complex cardioids at later timepoints (Fig. 5.2B). Quantification of projected area as a fold change relative to Day 2.5 shows a steady increase, reaching nearly $2\times$ over the observed period (Fig. 5.2C). This provides a simple growth-rate metric to harmonize construct selection and timing for subsequent volumetric imaging.

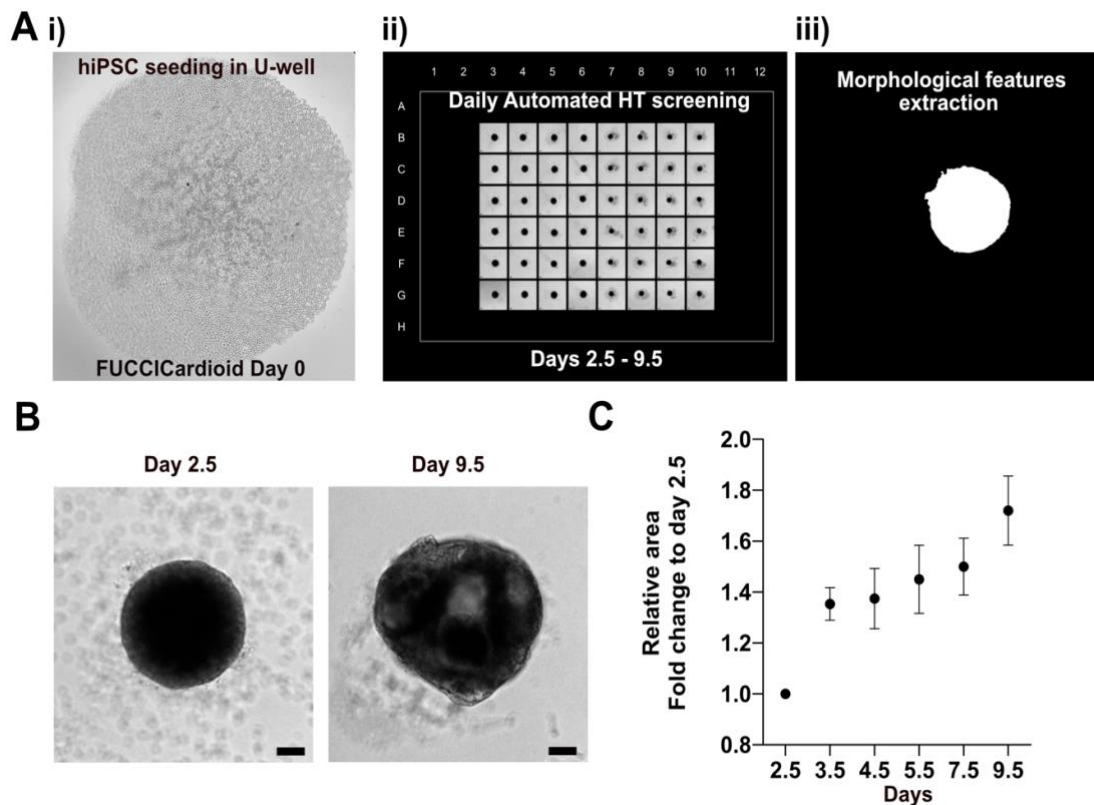


Figure 5.2. Daily High-throughput automated screening JOB for cardioids differentiation monitoring and rapid morphological QC. To enable routine, plate-wide monitoring of cardioids differentiation, we implemented a daily automated high-throughput workflow coupled to basic morphological feature extraction as an early quality-control (QC) readout of growth and developmental progression. (A) Overview of the screening pipeline. (i) hiPSC seeding in U-wells (Day 0). (ii) Automated High-throughput daily plate scan (Days 2.5 - 9.5) to capture brightfield cardioid morphology across wells. (iii) Segmentation-based feature extraction from each image (example binary mask) to compute basic morphometrics for QC. (B) Representative brightfield images illustrating morphological progression from Day 2.5 (compact spheroid-like stage) to Day 9.5 (expanded, structurally complex cardioid). Scale bars, 100 μ m. (C) Quantification of relative projected area (fold-change normalized to Day 2.5) across differentiation days, providing a simple growth-rate metric for early detection of abnormal trajectories and for standardizing downstream volumetric acquisitions.

5.2.3 Static and dynamic volumetric imaging of cardioids

Once we obtained quality-controlled cardioids using the HT 2D screening gate, we collected volumetric datasets as a static developmental series by sequentially imaging stacks of FUCCIplex nuclei and RFP-LifeAct actin. We imaged self-assembling hiPSC-derived cardioids at four key differentiation stages (Day 2.5, 3.5, 4.5, and 5.5) and performed serial acquisition of the RFP-actin, CFP-G1, and iRFP-S/G2/M channels. As shown in Fig. 5.3A, we created three-channel composite volumetric renderings of the bottom dome region for each stage as top-view projections of an approximately 150 μm -thick z-stack. When examining the actin channel alone (Fig. 5.3B), we observed day-to-day changes in overall cardioid shape and actin organization, suggesting progressive maturation of the self-assembling phenotype. When analyzing the FUCCIplex channels alone (Fig. 5.3C; G1 in cyan, S/G2/M in magenta), we observed increasing nuclear density over time and a noticeable accumulation of G1-labeled nuclei at later stages, consistent with a shift toward a G1-dominant state during differentiation.

During early volumetric acquisitions, we observed channel-dependent shadowing in the three channels, likely due to illumination-path misalignment and/or specimen-induced attenuation in thick, scattering samples. To reduce the impact of this artifact in routine runs, we introduce a rapid pre-acquisition light-sheet alignment check (~ 5 min) prior to each volumetric run, converting an otherwise hard-to-detect imaging artifact into a controlled acquisition variable.

After implementing this illumination governance step, we were able to perform long-term volumetric imaging of cardioids with sequential multi-channel stacks acquired over extended periods. We imaged a Day 3.5 self-assembling cardioids expressing RFP-LifeAct for 24 h at 30 min intervals, embedding the sample in 0.5% low-melting agarose (LMA) to minimize motion artifacts and long-term drift. At each time point, we acquired channels sequentially, using a consistent acquisition order to standardize illumination across the time series. Representative composite optical sections acquired at ~ 100 μm from the bottom of the cardioid illustrate simultaneous capture of tissue structure (actin) and cell-cycle state (FUCCIplex) within the same illumination window (Fig. 5.4A). Over time, actin-focused views of the internal region highlight cavity emergence, expansion, and remodeling across the 24 h window (Fig. 5.4B), while the FUCCIplex channels reveal

nuclei undergoing cell-cycle progression during the long-term acquisition (Fig. 5.4C). Collectively, these results suggest experimental feasibility of long-term longitudinal phenotyping in live cardioids, coupling 3D morphogenesis (including early cavity formation and remodeling) with continuous cell-cycle tracking under controlled illumination quality.

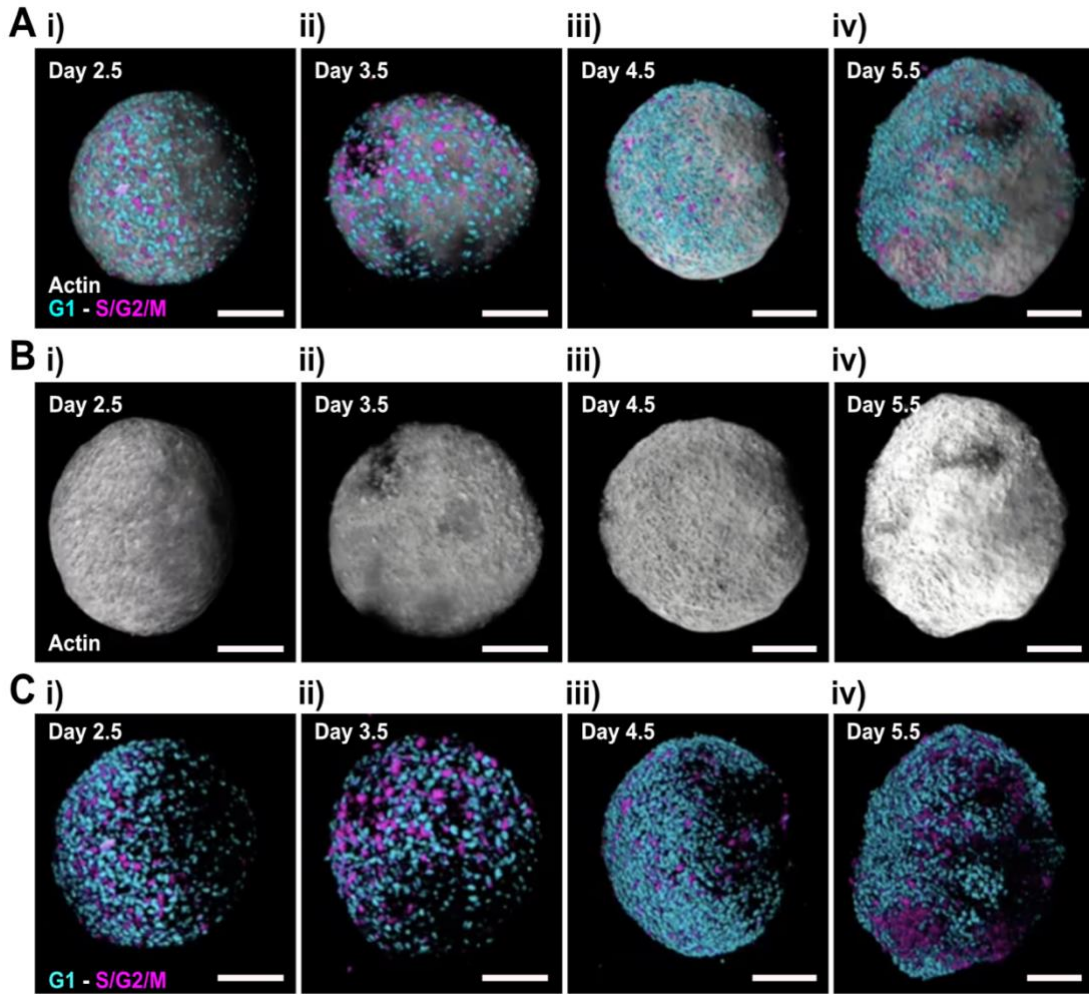


Figure 5.3. Static volumetric light-sheet imaging of FUCCIplex hiPSC-derived cardioids across differentiation (Day 2.5-5.5). Static light-sheet fluorescence microscopy (LSFM) volumes were acquired from self-assembling FUCCIplex edited hiPSC-derived cardioids expressing RFP-LifeAct (actin) together with the FUCCIplex cell-cycle reporters. G1 nuclei are shown in cyan, and S/G2/M nuclei in magenta. Four representative differentiation stages are shown (Day 2.5, 3.5, 4.5, and 5.5). (A) Three-channel composite volumetric renderings of the bottom dome region for each differentiation day (serial acquisition of actin, G1, and S/G2/M channels). Each view represents a top-view rendering of a $\sim 150 \mu\text{m}$ -thick z-stack, capturing tissue-scale structure and spatial cell-cycle distributions. (B) Structural channel alone (RFP-LifeAct actin), highlighting day-dependent changes in overall cardioid morphology and actin

organization across the same stages. (C) FUCCIplex nuclear channels alone (G1 in cyan; S/G2/M in magenta) visualizing the spatial distribution of cell-cycle states within each cardioid. Qualitatively, later stages exhibit increased nuclear density and an apparent enrichment of G1-labeled nuclei consistent with progressive maturation of the self-assembling phenotype. Scale bar, 250 μm .

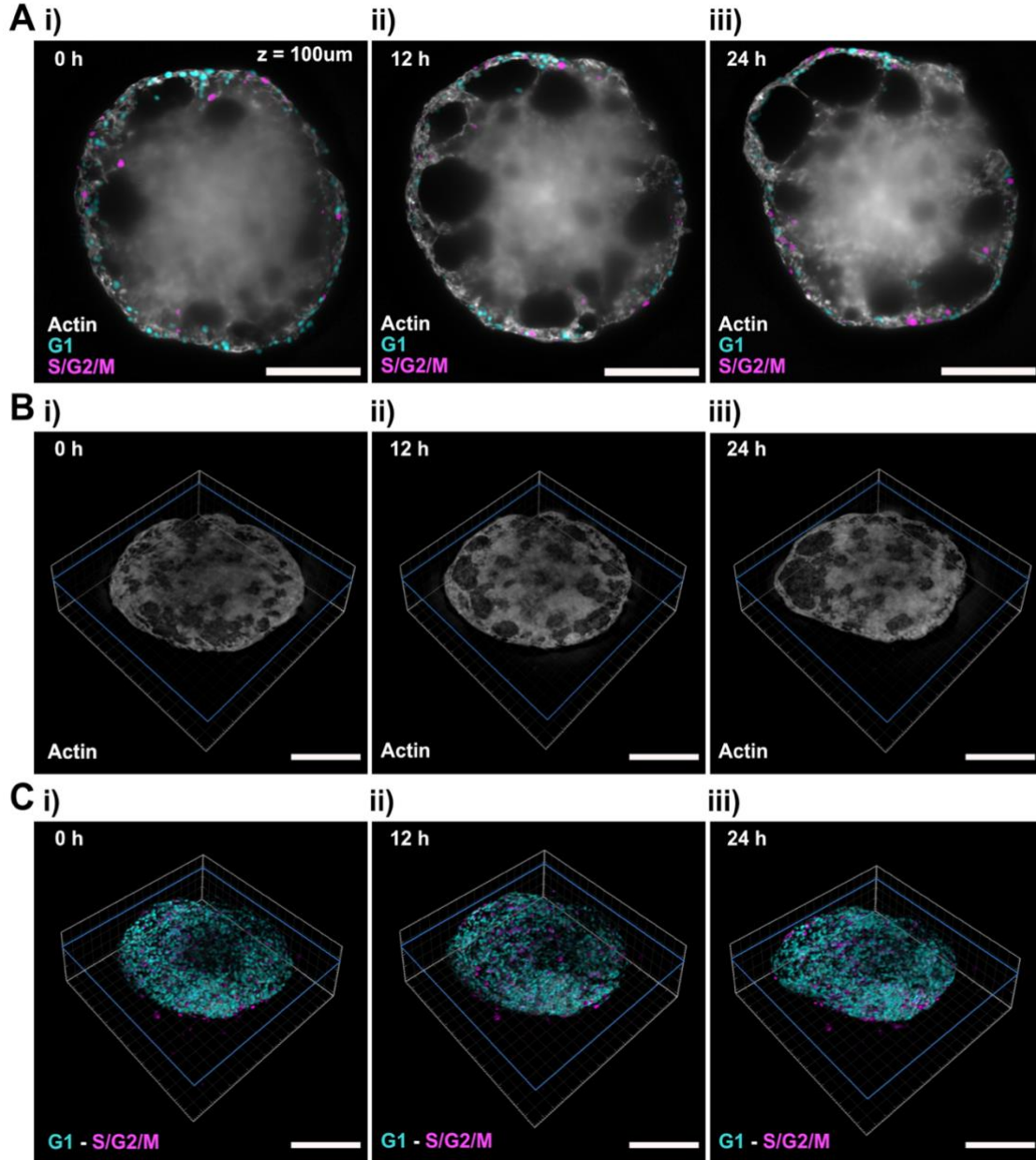


Figure 5.4. Long-term volumetric light-sheet time-lapse imaging of a FUCCIplex hiPSC-derived Cardioid at Day 3.5. A self-assembling FUCCIplex hiPSC-derived cardioid (Day 3.5) expressing RFP-LifeAct (actin) and FUCCIplex nuclear reporters was imaged by volumetric light-sheet fluorescence microscopy (LSFM) for 24 h at 30 min intervals. The sample was embedded in 0.5% low-melting agarose (LMA) to minimize

motion artifacts and long-term drift. At each time point, channels were acquired sequentially, with the laser line order decreasing to standardize illumination across the time series. G1 nuclei are shown in cyan and S/G2/M nuclei in magenta. (A) Representative three-channel composite single optical section acquired at $\sim 100\ \mu\text{m}$ from the bottom of the cardioid, illustrating simultaneous capture of tissue structure (actin) and cell-cycle state (FUCCIplex). (B) Time-course visualization of the RFP-LifeAct actin channel with the volume cropped/sectioned to emphasize the internal region, highlighting cavity emergence, expansion, and remodeling over the 24 h window. (C) FUCCIplex nuclear reporters over time, showing nuclei undergoing cell-cycle progression and mitotic events within the illumination window during long-term volumetric acquisition. Scale bar, $250\ \mu\text{m}$.

5.3 Image analysis toolkits for 3D structural and functional phenotyping

Building on the optimized HT/HC workflow for phenotyping cardioids, this section aims to address the remaining need: a robust 3D image analysis toolkit that transforms light-sheet datasets into more reproducible, interpretable phenotypes. Here, we develop a unified analysis pipeline that extracts two linked readout axes from the same 3D volumes: a functional axis indicating proliferative state via FUCCIplex, and a structural axis representing cavity architecture within the reconstructed cardioid volume.

We first focus on the functional axis to quantify proliferative states across differentiation stages in cardioids, providing a time-resolved readout of cell-cycle distribution in 3D. Then, we move to the structural axis, evaluating cardioids' morphology and self-assembling cavity architecture.

5.3.1 Unified 3D analysis pipeline for coupled functional-structural phenotyping

Once volumetric imaging datasets were collected, we processed them within a unified 3D analysis pipeline to couple functional and structural 3D phenotyping.

Before launching the pipeline, light-sheet data require preparation steps, such as dual-view fusion, timepoint/slice registration, and binning, to reduce dataset size and support sharing within software architectures that can work online, such as ARIVIS (version

4.4.0)²⁶ with its cloud module, ARIVIS Cloud. The prepared volumetric light-sheet datasets of cardioids were then loaded into ARIVIS software and analyzed using a unified workflow to extract functional (cell-cycle) and structural (cardioids morphology and cavities) readouts from the same reconstructed 3D volumes (Fig. 5.5). After collecting each dataset, we applied preprocessing to improve signal consistency and enable batch comparison across differentiation time points.

For the functional axis, we built a nuclei-centric FUCCIplex workflow, following the analysis guidelines reported in FUCCIphase, as part of the CALIPERS work¹⁹. We denoised the nuclear channels and enhanced particle-like features to improve nuclear detectability in 3D. We then generated a “DAPI-equivalent” detection channel by combining the FUCCIplex nuclear reporters (G1 and S/G2/M) and detected nuclei using a blob-finder module. Next, we classified each detected nucleus using an intensity-based decision step applied to the CFP fluorescence signal (G1, cyan). If the CFP signal of a detected nucleus exceeds an intensity threshold determined by experimental calibration, the pipeline assigns the object to the G1 class; otherwise, it belongs to the S/G2/M class. This step produced a 3D nuclear object set with cell-cycle labels that we used for both quantitative summaries and 3D renderings of the proliferative state.

For the structural axis, we analyzed the actin volume to segment the cardioid tissue envelope and internal cavities. We first used intensity-based processing to define the global structural volume. To support cavity segmentation across heterogeneous actin contrast and multicavity morphologies, we developed a dedicated cavity model using ARIVIS Cloud (Fig. 5.6). We manually annotated representative cavity regions to build a training set, trained the model in an iterative loop, and inspected predictions to identify typical failure modes (boundary ambiguity, small cavities, low-contrast interiors) before refining annotations and retraining. After convergence, we deployed the trained model to run inference on the full dataset and generate 3D cavity masks that were consistent across time points, whereas threshold-based methods showed instability. We retained the resulting cardioids' envelope and cavity masks as 3D objects suitable for morphometric readouts, including total structural volume, cavity number, cavity size distribution, and cavity volume fraction.

Finally, we consolidated the FUCCIplex-classified nuclei, cardioids' volume, and cavity objects into a shared analysis object, through which we generated matched 3D renderings of functional state, structural organization, and combined overlays. This unified output enabled direct, time-matched comparisons across differentiation stages.

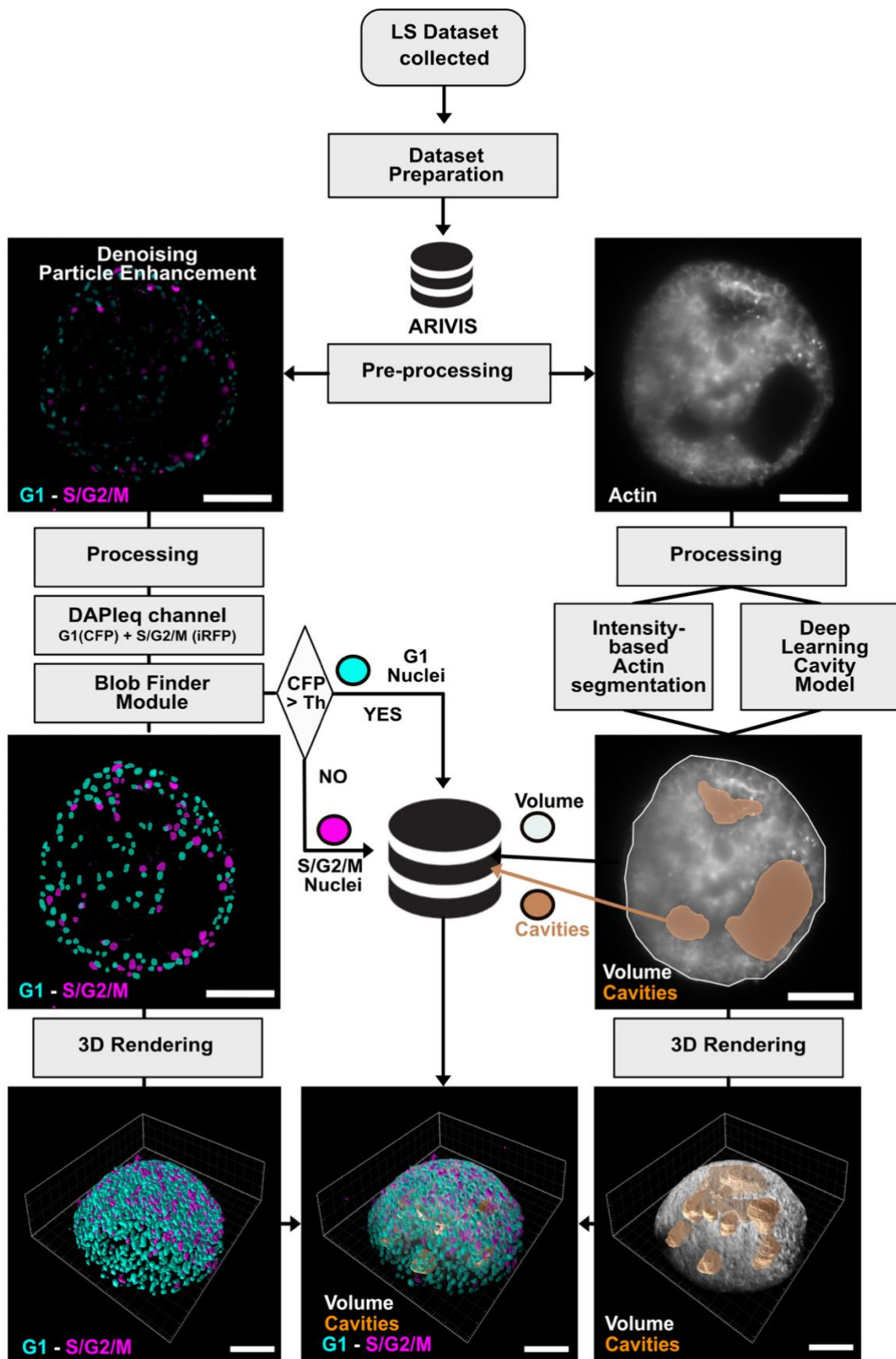


Figure 5.5. Unified 3D analysis pipeline for coupled functional-structural phenotyping
Light-sheet datasets are prepared and imported into ARIVIS Software (version 4.4.0) for pre-processing and processing. The pipeline branches into two analysis tracks: FUCCIplex nuclei classification and actin-based total morphology with cavity detection. Extracted outputs are consolidated into a shared data object to support 3D rendering and downstream quantification of functional (cell-cycle state) and structural (cavity geometry/topology) descriptors. Scale bar, 100 μm .

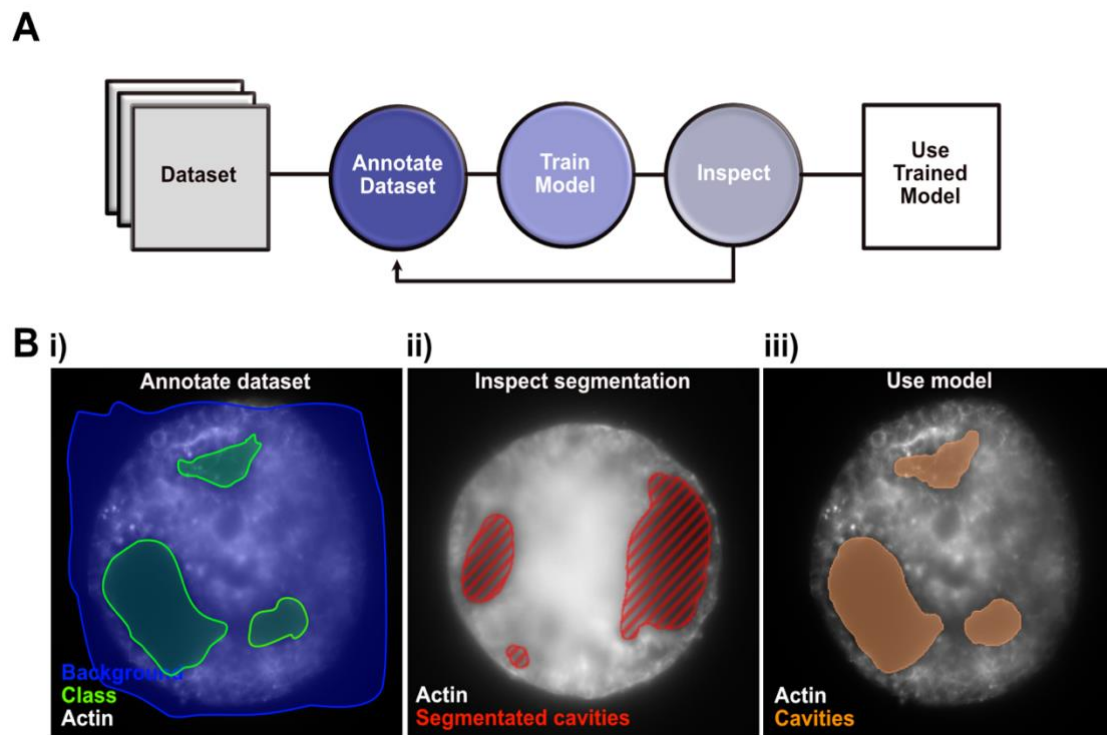


Figure 5.6. Training an ARIVIS Cloud deep-learning model for cavity segmentation.
(A) ARIVIS Cloud model-building workflow: an input dataset is manually annotated, used to train an instance-segmentation model, iteratively inspected for failure modes, and then deployed to segment new volumes. (B) Representative steps on an actin (RFP-LifeAct) slice. (i) Annotation of background and cavity classes to generate training labels. (ii) Inspection of model output during validation (example segmentation overlaid in red) to identify under/over-segmentation and refine annotations if needed. (iii) Application of the trained model to unseen cardioid images, producing cavity masks (orange) over the actin channel.

5.3.2 Functional 3D readout: cardioids' proliferative state at different stages

Thanks to the unified 3D analysis pipeline, we quantified the proliferative state in cardioids during differentiation (Day 2.5-5.5) by classifying nuclei into G1 versus S/G2/M phases while maintaining their spatial relationship with the reconstructed actin volume (Fig. 5.7A-B). Whole-volume renderings revealed that FUCCI-labeled nuclei occupied the entire 3D tissue structure at each stage, while orthogonal views showed how nuclear distributions aligned with the cardioid geometry and internal organization (Fig. 5.7B). We then summarized the per-timepoint nuclear counts and derived the cell-cycle distribution to highlight a 3D functional readout and evaluate how the proliferative state changes throughout differentiation to a mature phenotype.

Across time points, the cell-cycle composition remained close to balance at early stages and shifted at mid-differentiation. At Day 2.5, we detected (1822 G1, 1607 S/G2/M, n=3429) 53.1% G1 and 46.9% S/G2/M of nuclei, (1822 G1, 1607 S/G2/M, n=3429). Day 3.5 showed a highly similar distribution (900 G1, 812 S/G2/M, n=1712), with 52.6% G1 and 47.4% S/G2/M, representing the highest S/G2/M fraction in this series. In contrast, Day 4.5 exhibited a clear G1-skew (1350 G1, 922 S/G2/M, n=2272), with 59.4% G1 and 40.6% S/G2/M, marking the lowest S/G2/M fraction and a ~6-7 percentage-point decrease relative to Day 2.5-3.5. By Day 5.5, the distribution partially rebounded (3377 G1, 2598 S/G2/M, n=5975), yielding 56.5% G1 and 43.5% S/G2/M, but it did not return to the early-stage S/G2/M level.

We acknowledge that total nuclear count varies across time points (n=1712-5975), likely reflecting a combination of biological growth, stage-dependent tissue compaction, and differences in nuclear detectability in dense 3D volumes. However, the magnitude and direction of the Day 4.5 shift remain evident in the normalized distributions.

Overall, these data suggest that Days 2.5 and 3.5 maintain a comparable proliferative balance, followed by a transient shift toward G1 at Day 4.5, and a partial recovery by Day 5.5 (Fig. 5.7D).

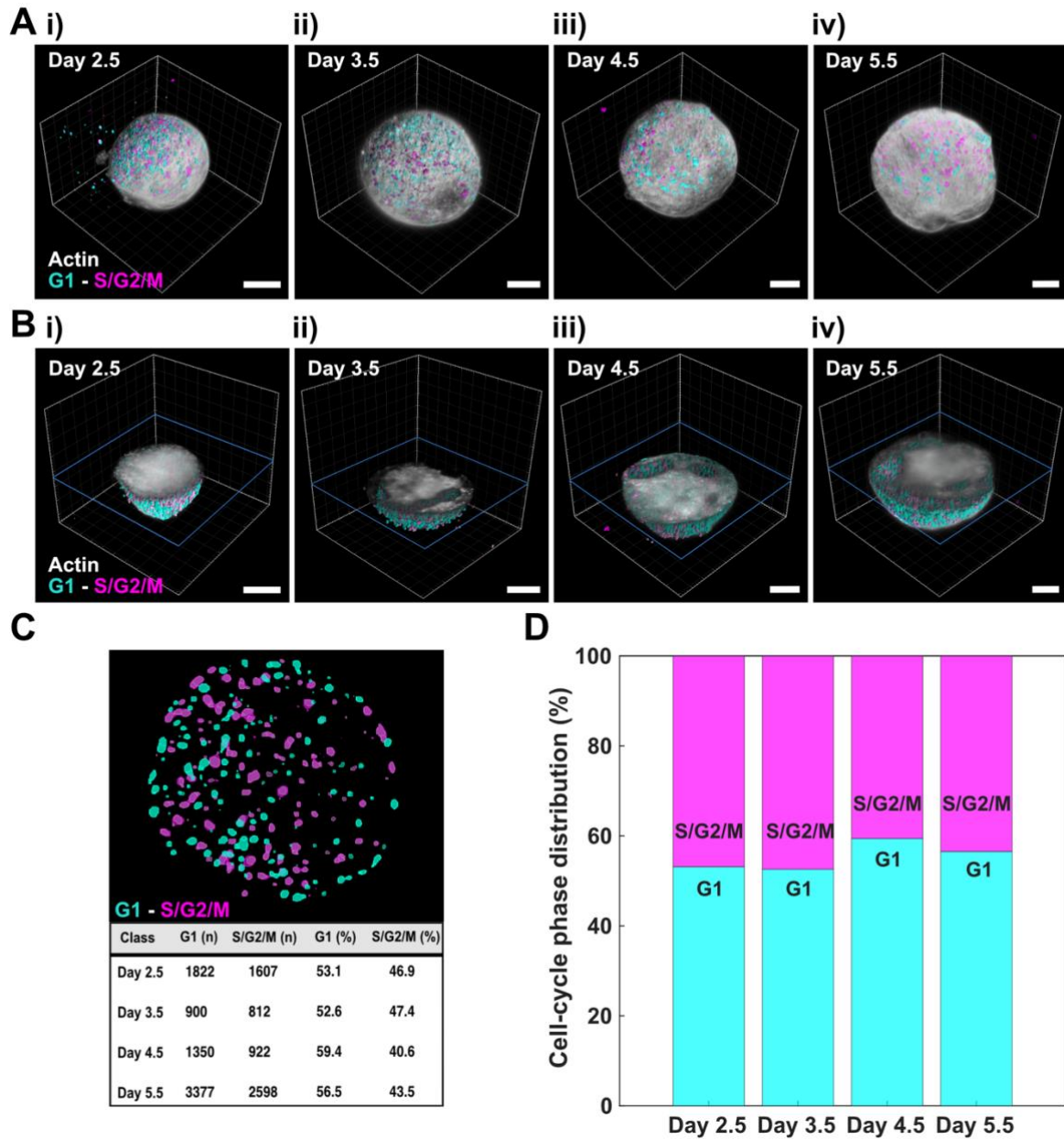


Figure 5.7. FUCCIplex cell-cycle state mapping in 3D cardioids across differentiation (Day 2.5-5.5). FUCCIplex hiPSC-derived cardioids were imaged by static LSFM and reconstructed in 3D to map cell-cycle phase distributions within the developing tissue. Actin structure is shown in gray (RFP-LifeAct). FUCCIplex nuclei are classified as G1 (cyan) or S/G2/M (magenta). (A) Full-volume 3D renderings showing actin morphology with phase-assigned nuclei overlaid for Day 2.5, 3.5, 4.5, and 5.5 (i-iv). (B) Cropped “bottom-dome” views (blue plane indicates the cutting plane), highlighting local nuclear distributions at the tissue-cavity interface across the same stages (i-iv). (C) Representative nuclei detection and classification output with the corresponding counts and percentages per day (G1 and S/G2/M). (D) Population-level stacked bar plot summarizing the FUCCIplex phase distribution across differentiation. Across days, the population remains near-balanced but shows an overall enrichment of G1 at later stages

(notably Day 4.5-5.5), consistent with progressive maturation of the cardioid phenotype. Scale bar, 100 μm .

5.3.3 Structural 3D readout: cardioids' volumetric growth and multicavity morphologies across differentiation

We measured cardioids' growth and cavities development by segmenting the actin-defined structural volume and internal cavities in 3D at matched differentiation stages (Day 2.5-5.5; Fig. 5.8A-C), and we summarized the resulting morphometrics over time (Fig. 5.9A-D). The total structural volume increased from 0.150 mm³ (Day 2.5) to 0.312 mm³ (Day 3.5), then remained in a similar range (0.263 mm³ at Day 4.5; 0.324 mm³ at Day 5.5), corresponding to an approximate spherical diameter of 0.66-0.85 mm. This size range aligns with the levels reported for heart organoid/cardioid systems used for chamber-level imaging and volumetric phenotyping^{4,5,27}

Cavity segmentation showed stage-dependent changes in cavity structures during development (Fig. 5.9C-D). At Day 2.5, we found no cavities, consistent with an early, largely compact internal organization. By Day 3.5, cardioids presented multiple cavities ($N_{cav} = 96$) and a small cavity occupancy ($V_{cav,tot}/V_{card} = 0.158$; 15.8%). At Day 4.5, the system still appeared fragmented, with similar occupancy (0.144; 14.4%), but the number of cavities decreased ($N_{cav} = 54$), which is consistent with remodeling rather than steady cavity formation. By Day 5.5, the phenotype shifted toward a cavity-dominant state, with $V_{cav,tot}/V_{card} = 0.747$ (74.7%) and 59 cavities, indicating that the luminal space had become the main structural component despite a cavity count similar to that on Day 4.5.

Cavity size statistics indicated a shift from “many small” to “fewer larger” cavities (Fig. 5.9D). In fact, the average cavity volume increased from 5.14×10^{-4} mm³ (Day 3.5) to 7.04×10^{-4} mm³ (Day 4.5), then rose to 4.10×10^{-3} mm³ (Day 5.5), roughly an eightfold increase relative to Day 3.5, consistent with late-stage cavity coalescence and enlargement. Significantly, the maximum cavity volume at Day 5.5 reached 0.0853 mm³, closely matching OCT-based 3D segmentation findings in human heart organoids, which reported the largest cavity as 0.086 mm³ during the late remodeling stage²⁷. Meanwhile, the largest cavity made up only about 35% of the total cavity volume at Day 5.5. This is

representative of a multicavity structure with multiple large cavities and smaller satellite cavities, rather than a single-cavity outcome.

Notably, the magnitude of the Day 5.5 occupancy increase aligns with models in which cavity morphology shows threshold-like changes driven by competing influences of proliferation versus pressure and permeability-driven cavity expansion¹⁷. Because cavity occupancy is also influenced by 3D segmentation definitions, we interpret this metric cautiously as an indicator of the overall regime.

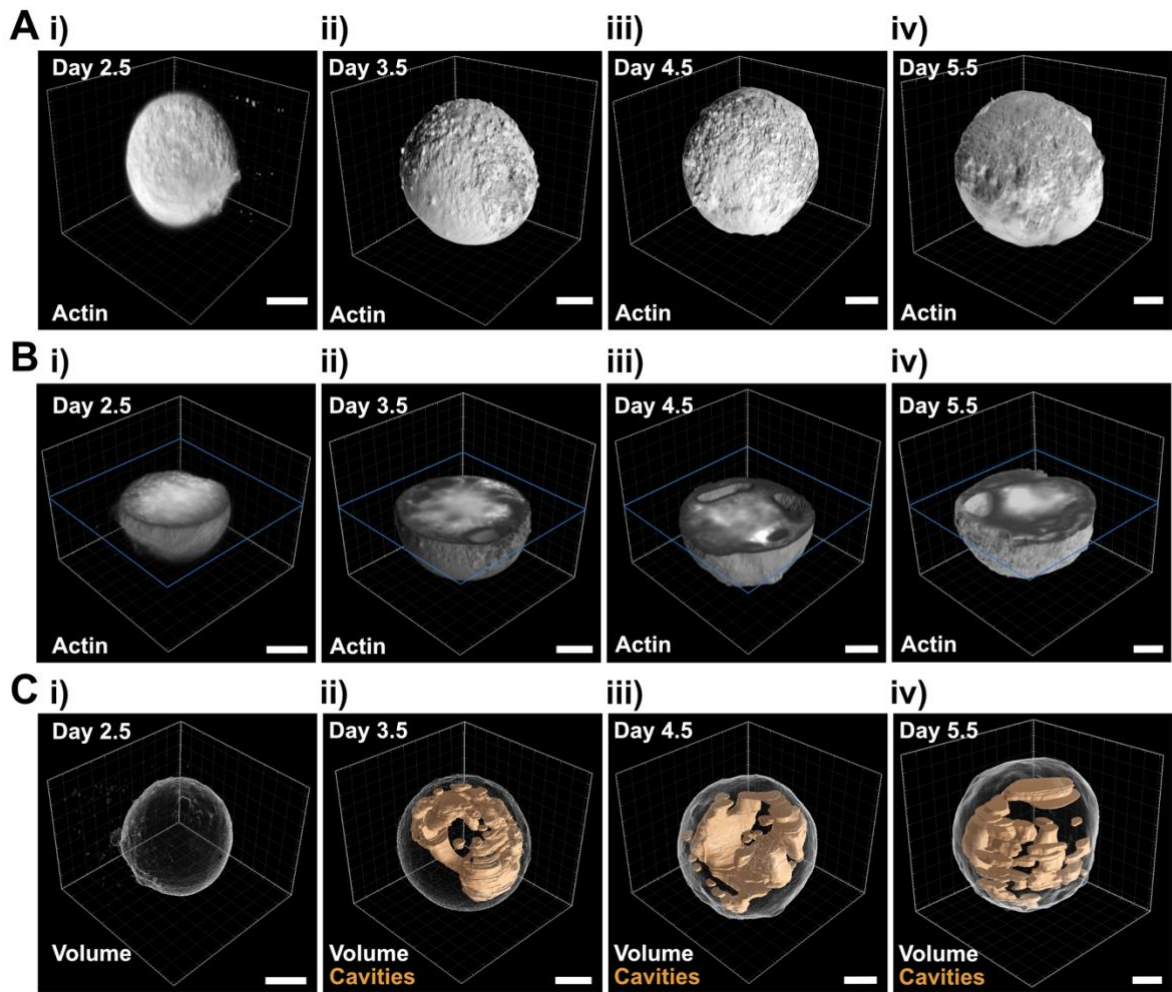


Figure 5.8. 3D reconstruction of cardioids' morphology and cavity architecture across differentiation (Day 2.5 - 5.5). Static LSM volumes of FUCCIplex hiPSC-derived cardioids were reconstructed in 3D across differentiation days (Day 2.5, 3.5, 4.5, 5.5). (A) 3D renderings of the full RFP-LifeAct actin volume for each time point. (B) Cropped renderings of the bottom-dome region (blue plane indicates the cutting plane) to expose internal organization and facilitate cavity inspection. (C) Combined volumetric reconstructions showing the segmented total cardioid volume (gray) and extracted

internal cavities (orange), highlighting the emergence and increase in cavity complexity across differentiation. Scale bar, 100 μm .

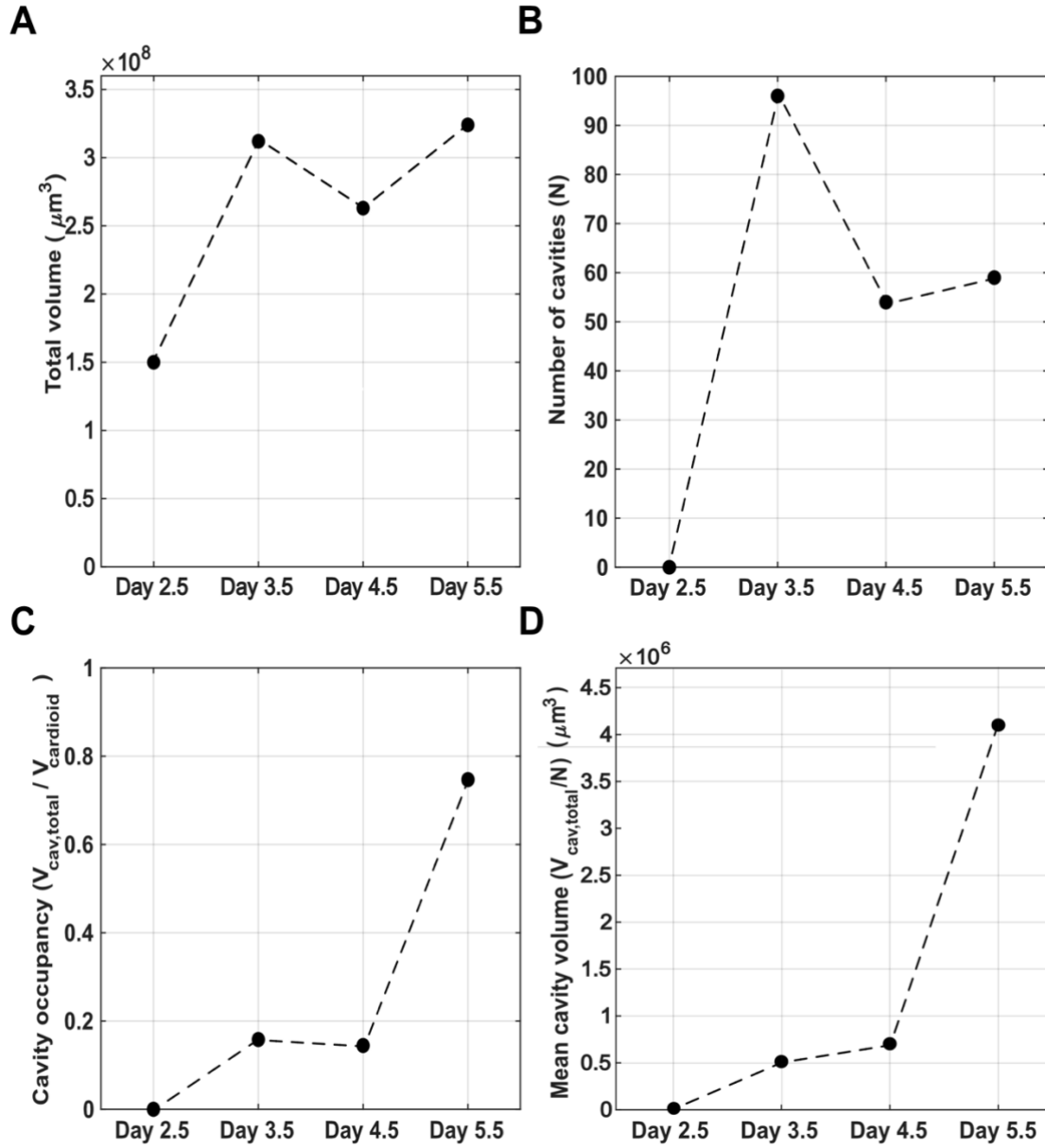


Figure 5.9. Quantitative evolution of cardioid volume and cavity architecture across differentiation (Day 2.5-5.5). Morphometric features were extracted from static LSFM reconstructions using actin-based cardioid segmentation and cavity instance segmentation. Across development, cardioid volume increases early, cavities appear abruptly by Day 3.5, and later stages shift toward fewer but larger cavities, driving a strong increase in cavity occupancy. (A) Total cardioid volume (μm^3) increases from Day 2.5 to Day 3.5 and remains in a comparable range at Days 4.5-5.5. (B) Number of cavities (N) is near zero at Day 2.5, peaks at Day 3.5, then decreases at Days 4.5 and 5.5, consistent with cavity consolidation. (C) Cavity occupancy is minimal at Day 2.5, modest at Days 3.5-4.5, and rises sharply by Day 5.5, indicating a cavity-rich mature state. (D) Mean cavity volume remains low at Days 3.5-4.5 but increases substantially at Day 5.5, consistent with coarsening into larger cavities.

5.4 Multicellular Phase-field Model of lumen morphogenesis

Three-dimensional organoids provide an experimentally tractable way to study how controlled cellular programs relate to organoid-scale morphology, but it remains difficult to translate simple, measurable cell behaviors into quantitative expectations for whole-organoid structure across systems. To address this gap in a minimal and interpretable way, we adopted a two-dimensional multicellular phase-field framework and used it as a mechanistic model for lumen morphogenesis²⁰. In this formulation, we represent each epithelial cell as an individual phase field and the cavity as a distinct phase field, such as distinct but continuous scalar fields whose smooth boundaries define cell-cell and cell-cavity interfaces, and we let the system evolve via energy-minimizing dynamics. This supports stable interfaces while allowing remodeling events such as cavity initiation, growth, fusion, and branching.

By adopting MCPFM, we could implement a minimal simulation of cavity morphogenesis and generate representative outcomes that resemble two commonly observed qualitative scenarios: a single, compact cavity versus a more complex, multi-cavity/branched organization (Fig. 5.10A). In Fig. 5.10A, the “spherical” example on the left side shows a dominant cavity bounded by an epithelial shell, whereas the “branched/complex” example, right side of Fig. 5.10A, shows a dominant cavity accompanied by distributed satellite cavities, consistent with incomplete coarsening and fusion. Within the MCPFM framework, the spherical morphology supports regimes where pressure/permeability-driven lumen expansion dominates over growth, whereas the branched/complex morphology is more consistent with regimes where rapid proliferation outpaces cavity coarsening and fusion. To describe these outcomes consistently throughout the chapter, we refer to an outcome as spherical when the cavity remains a single connected component with a smooth, approximately isotropic boundary, and as branched/complex when the cavity displays topological complexity (≥ 1 branch point) and/or maintains persistent multi-cavity organization (> 1 connected component) rather than coalescing into a single cavity. The time-resolved simulations in Fig. 5.10B-C provide a mechanistic view of how these structures can emerge within MCPFM. In the spherical trajectory (Fig. 5.10B), a cavity nucleates early and then inflates and smooths

as the tissue grows, yielding a stable single-cavity architecture by 900 min. In contrast, in the branched/complex trajectory (Fig. 5.10C), repeated micro-cavity formation persists during growth; cavities remain distributed and only partially merge, resulting in a heterogeneous multi-cavities topology at late times.

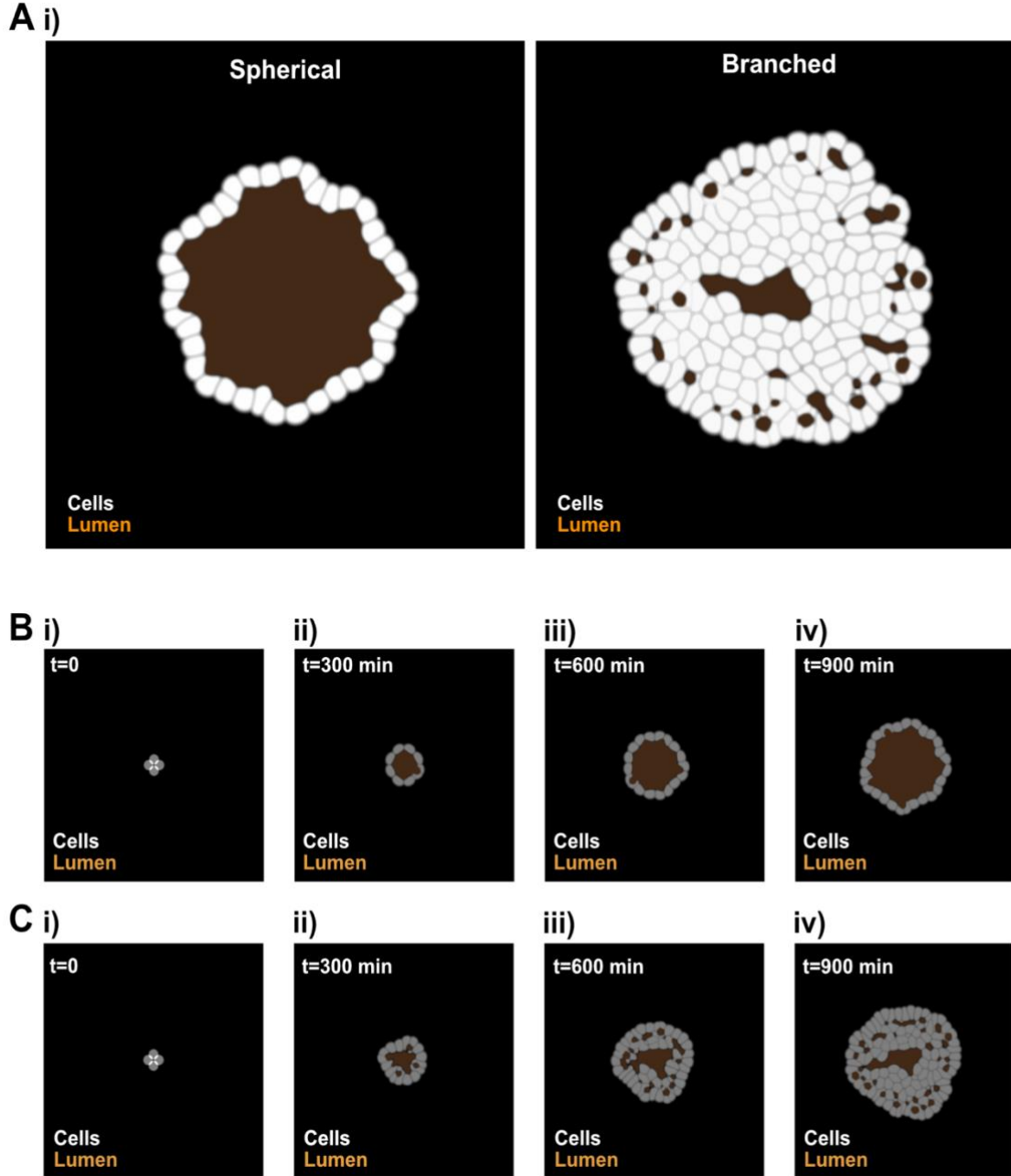


Figure 5.10. Representative MCPFM morphologies and temporal trajectories defining spherical versus branched/complex regimes. (A) Endpoint morphologies from the multicellular phase-field model (MCPFM) illustrating the two operational classes used in the $\tau_V - \xi$ phase diagram: spherical (left), characterized by a single connected cavity

with approximately isotropic boundary, and branched/complex (right), characterized by a persistent multi-cavity structure and/or non-isotropic cavity organization. Cells are shown in grey and cavity in brown. (B) Time-resolved simulation snapshots ($t = 0, 300, 600, 900$ min) for a parameter set that converges to the spherical regime, showing early cavity nucleation followed by progressive cavity inflation and boundary smoothing. (C) Time-resolved simulation snapshots ($t = 0, 300, 600, 900$ min) for a parameter set that converges to the branched/complex regime, showing repeated cavity nucleation and persistence of multiple lumina alongside incomplete coarsening/fusion during tissue growth

We then introduce how these phenotype scenarios can be mapped onto an interpretable parameter space and summarized using quantitative order parameters (Fig. 5.11). First, we repeated the array of simulations represented in the Lee et al.'s work¹⁷ to reproduce key results of MCPFM outcomes, organized as a parameter sweep (Fig. 5.11A). In this *in silico* view, each snapshot is a model endpoint in which epithelial cells and the cavities are represented by phase fields so morphology and topology can remodel over time (e.g., cavity initiation, growth, fusion, and branching) without imposing hard geometric constraints. Within this framework, the sweep varies two control terms that are experimentally interpretable^{17,20}. First, we encode a growth-division timescale by relaxing each cell's target volume toward a division threshold, and we summarize this control using τ_V as a proxy for the proliferation timescale. Second, we introduce a luminal driving term, ξ , that acts as a pressure-like energetic bias favoring cavity expansion and coarsening/fusion¹⁸. The grid in Fig. 5.11A therefore provides a direct visual mapping between parameter settings (τ_V, ξ) and representative cavity morphologies across the explored space. To convert these outcomes mapping into a comparable summary across conditions, Fig. 5.11B reports a phase diagram of cavity occupancy, computed over the same τ_V - ξ sweep. Cavity occupancy is defined as:

$$\frac{V_l}{V_l + \sum_m V_m}$$

where V_l denotes the effective cavity area (2D model) computed from the cavity phase field, and V_m , which denotes the effective area measure of cell m computed from its corresponding phase field.

The cavity occupancy phase diagram (Fig. 5.11B) shows a monotonic increase with ξ , supporting the interpretation that stronger luminal driving stabilizes and inflates cavity volume fraction and promotes fusion/coarsening relative to persistent fragmentation.

Taken together, Fig. 5.11B provides a regime map: low ξ with rapid growth (small τ_V) favors low fractional occupancy, whereas increasing ξ and/or slowing growth shifts the system toward higher occupancy, consistent with the emergence of a dominant cavity.

In this chapter, we refer to the combined view as a regime map: it links a compact control space (τ_V, ξ) to measurable descriptors of cavity architecture, enabling model outcomes to be compared systematically and later related to experimental measurements extracted from cardioids.

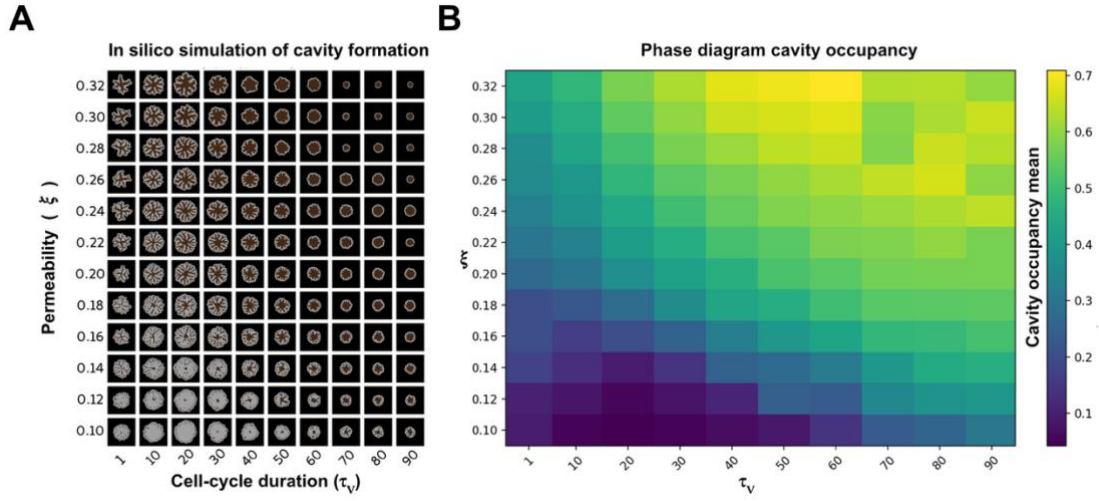


Figure 5.11. MCPFM parameter sweep and cavity-occupancy regime map. (A) *In silico* parameter sweep of cavity morphogenesis outcomes across the control space spanned by cell-cycle duration (τ_V) and luminal driving (ξ), shown as representative simulation endpoints organized on a τ_V - ξ grid. Each snapshot reports the simulated tissue configuration with epithelial cells and cavity rendered as phase-field components. (B) Phase diagram of cavity occupancy (mean) computed over the same τ_V - ξ sweep. Color encodes the mean cavity occupancy for each parameter pair.

Finally, we use this regime-level framework to guide a qualitative comparison between experimental and simulated cavity morphologies. We qualitatively compare the experimental static time-course dataset from differentiation day 2.5 to 5.5 with MCPFM time-course simulations covering both spherical and branched phenotypes, to assess whether we can achieve an agreement between observed cavity morphologies and model-mapped regimes. Experimental actin-based visualizations showed a developmental transition from relatively compact internal organization to increasingly complex cavity topologies at later stages, including multicavity structures and enlarged cavity

compartments (Fig. 5.12A). MCPFM snapshots showed a qualitatively similar progression, moving from an initially compact state to multicavity configurations through cavity nucleation, coarsening, and remodeling (Fig. 5.12B). This regime-level similarity supports subsequent mapping between the model and data by suggesting that the experimental phenotypes are compatible with the range of morphologies produced by MCPFM. At the same time, this qualitative compatibility could suggest a trend toward effective growth/proliferation control in parameter regions associated with higher cavity occupancy and, depending on the regime, higher cavity multiplicity.

In our Cardioid dataset, however, the proliferative state measured alongside the structural phenotype does not show a straightforward correspondence with the model's growth-timescale proxy at late stages: Specifically, at Day 5.5 we observed a morphology consistent with highly developed cavity, with high cavity count and high cavity occupancy, while our proliferative state quantification shows a reduction in the S/G2/M phase (section 5.3.2). In other words, the structural phenotype appears to converge toward morphologies associated with regimes where growth/proliferation would be expected to dominate, yet the coupled functional readout indicates a shift toward a more G1-biased, proliferation-slowness state. This mismatch suggests that the experimental regime position may be shaped by additional biological or physical drivers not captured by a single growth-timescale proxy, and it also underscores the limits of interpreting 3D experimental phenotypes using an intentionally simplified 2D model.

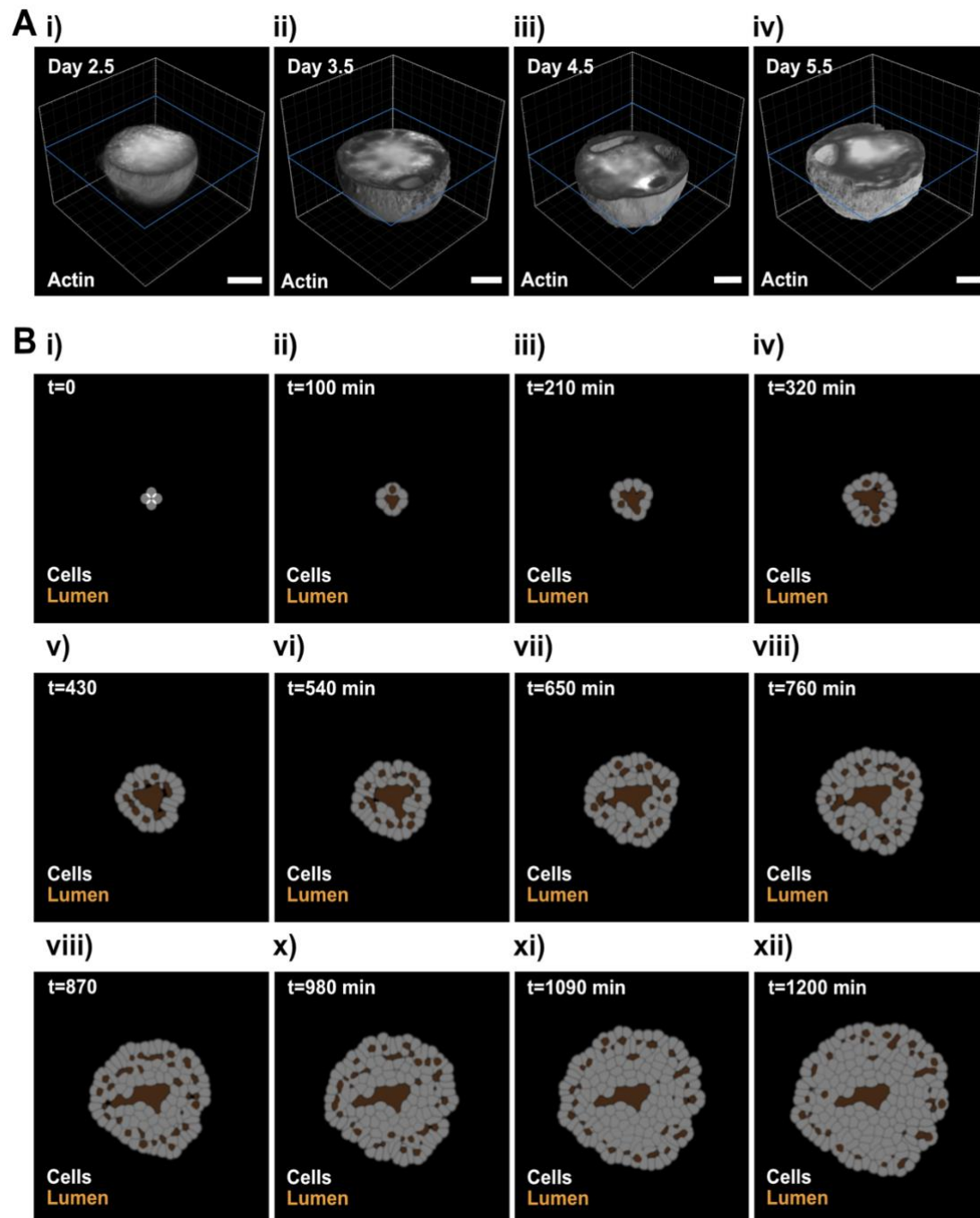


Figure 5.12. Experiments to MCPFM simulations: qualitative regime comparison for branched/multicavity cavity formation. Qualitative correspondence between experimental 3D cavity morphologies and MCPFM regime behavior. (A) Experimental actin-based renderings illustrating the emergence of complex cavity topologies at later differentiation stages. Scale bar, 100 μm . (B) Representative MCPFM snapshots showing time evolution from an initial compact state toward multicavity/branched cavity configurations.

5.5 Discussion

This chapter develops an engineering stack to support 3D HT/HC phenotyping in self-assembling cardioids, by integrating three components: an optimized workflow for volumetric imaging intended to improve reliability and scalability, a unified analysis pipeline that extracts paired structural and functional phenotypes from the same 3D dataset, and an interpretive regime model used to contextualize these measurements within an explicit parameter space.

We first aimed to operationalize volumetric HT/HC phenotyping by addressing three bottlenecks that commonly limit 3D acquisition in self-assembling systems: mechanical stability during imaging, early standardization of differentiation outcomes, and optical robustness across static and long-duration acquisitions. Embedding calibration spheroids in 0.5% low-melting agarose provides a straightforward, reproducible stabilization method widely adopted for LSMF immobilization with minimal disturbance²⁵, and it supports the interpretation that depth-dependent attenuation in our measurements is mainly driven by scattering in dense 3D samples rather than by embedding artifacts. Staging the workflow with keratinocyte spheroids before hiPSC-derived tissues is technically practical and consistent with the literature, given their robustness and scalability for controlled 3D self-assembly^{23,28}. In addition, the daily 2D HT screening layer serves as an early, cost-effective quality control (QC) checkpoint that reduces variability as samples move from 2D to 3D differentiation and can limit volumetric imaging of outlier constructs, aligning with organoid-screening approaches that emphasize front-end standardization using minimal descriptors²². Lastly, volumetric illumination control addresses a known LSMF failure mode, shadowing and channel-dependent attenuation in thick, heterogeneous samples, where alignment and illumination geometry interact with sample-induced artifacts¹¹. Treating this as a controllable variable through fast pre-acquisition checks is consistent with dose and alignment-aware imaging strategies intended to reduce the risk that subtle phototoxic or optical biases are misinterpreted as biological effects¹².

We then developed an analysis toolkit that converts volumetric datasets into paired functional-structural phenotypes, so that measurements used for biological interpretation are extracted from the same registered 3D volumes. Structurally, cavity self-assembly and remodeling represent key phenotypes in cardioids and heart organoids^{4,5,29}. The pipeline extracts cavity count, occupancy, and cavity size distributions, matching the order-parameter family used later for qualitative regime matching. Implementing a deep-learning cavity model, trained and deployed within the ARIVIS system, addresses a throughput and robustness constraint, since intensity-threshold segmentation can become fragile when cavity topology fragments and optical contrast vary across specimens. Functionally, FUCCIplex readouts provide a cell-cycle-resolved layer that can be interpreted alongside cavity architecture. This is important because organoid morphology alone does not indicate whether a structure is actively proliferating, slowing down, or entering a different developmental state. By coupling cavity morphometrics with G1 and S/G2/M distributions, the pipeline provides a more informative description of cardioid development than either structural or functional readouts alone. Quantitative anchoring to OCT-based heart organoid cavity segmentation further supports the use of cavity-scale descriptors as comparable 3D readouts across different modalities^{27,30}. The structural analysis indicates that cardioid cavity architecture changes substantially across differentiation. Early time points show compact morphologies with limited or absent visible cavities, whereas later stages display fragmented, multicavity structures and increased cavity occupancy. These measurements provide a quantitative description of the transition from compact organization to more complex lumen-like architectures. At the same time, the observed morphologies remain heterogeneous, and the extracted cavity descriptors are sensitive to imaging depth, actin signal quality, segmentation stability, and partial-volume effects. For this reason, cavity metrics should be interpreted as operational descriptors of the imaged volumes rather than absolute ground-truth measurements of lumen architecture.

Finally, we use the multicellular phase-field model (MCPFM) that provides a useful regime-level scaffold for interpreting these structural descriptors^{17,18,20}. The model is useful in this context because it provides an explicit mapping between a compact control space and measurable outputs, rather than requiring each dataset to be interpreted purely

from morphology. In our model adoption (τ_V , ξ) sweeps generate a regime map summarized by cavity occupancy, as measurable output from volumetric datasets once segmentation is stable (connected components, cavity fraction, and topology-aware morphology class). This regime framing was adopted from organoid studies that link growth and permeability/pressure-like forces to transitions between fragmented multi-lumen morphologies and expanded lumen state^{17,18,20}, and it is applied here with experimental constraints in mind. At late stages of Cardioid self-assembling (Day 5.5), the structural phenotype aligns with morphologies often associated with more growth-dominated regimes, while the proliferative readout points toward a G1-biased slowdown. The comparison between experimental cardioids and MCPFM simulations should therefore be understood as qualitative regime matching, not as model validation in the strict sense. Experimental actin-based renderings show a developmental progression toward more complex cavity topologies, and MCPFM simulations can reproduce qualitatively similar transitions from compact states to multicavity configurations. This divergence suggests that the experimental regime position may be modulated by additional biological or physical drivers beyond those captured by permeability vs growth-timescale proxies. In addition, the model is intentionally simplified, two-dimensional, and based on a reduced set of control parameters; the cardioid experiments are three-dimensional, multicellular, and shaped by additional biological and physical processes, including tissue mechanics, differentiation state, matrix interactions, pressure regulation, and transport constraints. These considerations motivate caution when extrapolating 3D developmental phenotypes from an intentionally simplified 2D model.

Three limitations remain explicit in this engineering tools stack, and they motivate the next engineering moves. First, the structural phenotypes rely on stable 3D segmentation and topology definitions. In thick samples, cavity fragmentation can be inflated by partial-volume effects and depth-dependent contrast loss, which can bias cavity count and connectivity metrics; this makes acquisition governance and analysis QC important for HT/HC use. Second, FUCCIplex captures proliferative state but not Cardioid's core physiological function: beating and calcium handling. Adding this functional layer introduces a multiplexing and multiscale imaging problem, including more channels, faster volumetric rates, different acquisition settings, larger data sizes, and an additional

integration branch in the analysis pipeline^{13,31,32}. Third, MCPFM is an intentionally simplified 2D model applied to 3D experimental phenotypes; the regime map therefore serves as an interpretation scaffold rather than a complete description of 3D development, and mapping should be treated cautiously when 3D topology is sensitive to segmentation and projection artifacts.

By this stage, the thesis has addressed four linked dimensions of the same measurement problem: temporal control of acquisition, spatial registration of engineered assays, volumetric imaging design, and extraction of coupled 3D phenotypes. Chapter 6 draws these contributions together and evaluates their significance as parts of a single framework for controlled live-cell phenotyping in tissue engineering.

5.6 Materials and Methods

FUCCIplex-HaCaT line generation

The generation of HaCaT FUCCIplex-prototype clonal cell line consisted of 3 different and sequential steps. First, HaCaT cells were infected with the FUCCIplex lentivirus and positively selected with antibiotics. A second lentiviral infection with a genetically encoded LifeAct-ACTB-RFP probe created a double reporter cell line that has the (F-)actin filaments tagged with a red fluorescent reporter in addition to the cell-cycle indicator FUCCIplex. After antibiotic selection, cells were genome-edited to tag alpha-tubulin with an EGFP fluorophore at its N-terminus; the resulting 3-reporter cell line was then clonally expanded and selected. In more detail, HaCaT cells were plated at a density of 6×10^4 cells per well in a 12-well plate in complete DMEM/F12 (catalog # 21041-025, Gibco). The following day, the medium from the tissue culture plate was removed by aspiration and replaced with a fresh complete medium containing 8 $\mu\text{g/ml}$ polybrene (VectorBuilder). Next, 10 μL of concentrated FUCCIplex lentivirus particles (>108 TU/ml, VectorBuilder) were resuspended in 500 μL of complete medium and added directly into the well. Cells were incubated overnight at 37 °C and 5% CO₂, and the next morning the transduction medium was aspirated and replaced with a fresh complete medium. After 4 days, positively transduced cells were selected with 20 $\mu\text{L/ml}$ of Hygromycin B (50 mg/ml in PBS) (catalog # 10687010, Invitrogen). HaCaT FUCCIplex cells were then clonally seeded and expanded. Positive cells were evaluated by PCR and confocal imaging to assess the correct expression of the different reporters. For the Lifeact-ACTB-RFP lentivirus infection (rLV Ubi-LifeAct-TagRFP, 1×10^7 TU/ml, 10 μL), cells underwent the same procedure with the only difference that 0.62 $\mu\text{L/ml}$ of Puromycin (1 $\mu\text{g}/\mu\text{L}$) was used to select positive cells. Finally, HaCaT cells were genome-edited using the Thermo Fisher Scientific TrueTag system. The donor sequence and primers with specific homology arms for GFP-tagging the N terminus of alpha-tubulin (TUBA1B, NM006082.3) were designed online with the assistance of the ThermoFisher Scientific TrueDesign Genome Editor tool. Briefly, the linear TrueTag dsDNA donor oligo with the TUBA1A homology arms was amplified by PCR using Phusion Flash

High-Fidelity PCR Master Mix, TrueTag Donor DNA Kit, GFP (catalog# A42992, Thermo). The TrueTag homology arm primer forward sequence is:

TCCTGTCGCCTTCGCCTCCTAATCCCTAGCCACTATGGGAGGTAAGCCCTTG
CATTCG;

The reverse is:

CCTGAAAGCAGCCGGGAGCCGCACGGCTTACTCACACCGCTTCCACTACCT
GAACC. Next, the TrueGuide Synthetic gRNA (sgRNA) (ThermoFisher Scientific, sequence: GCACGGCTTACTCACCATAG) and True cut CAS9 Protein V2 (catalog# A36496, ThermoFisher Scientific) were combined to form the ribonucleoprotein (RNP) complex (CAS9+gRNA). True cut CAS9 Protein V2 (2 µg, 12 pmol), sgRNA (400 ng, 12 pmol), TrueTag donor (500 ng) were resuspended in 10 µL of Resuspension Buffer R (catalog# MPK1025, ThermoFisher Scientific) and then combined with 8 x 10⁴ cells resuspended in 8 µL of Resuspension Buffer R for a total final volume of 18 µL. 10 µL of this mix was used to electroporate the cells with the Neon transfection system (catalog# MPK5000, ThermoFisher Scientific). The conditions for electroporation were the following: 1200 V / 20 ms / 4 pulses. Electroporated cells were seeded in a 24-well plate filled with 0.5 ml of DMEM/F12 medium containing 10% FBS without antibiotics. The plate was incubated at 37 °C in a humidified CO₂ incubator. Positive cells were then clonally plated and expanded.

FUCCIplex HaCaT cell culture

HaCaT cells were kindly provided by Professor Hugo de Jonge (Department of Molecular Medicine, University of Pavia) and cultured in DMEM/F12 no phenol red (catalog# 21041-025, Gibco) with 10% Fetal Bovine Serum (FBS) (catalog # 10270-106, Gibco) and 1% Penicillin/Streptomycin (P/S) (catalog# A001, HiMedia). Cells were never allowed to reach full confluency and were split at 70% of confluence.

HaCaT FUCCISpheroid generation

HaCaT cells were seeded for spheroid formation using a 96-well plate with ultra-low attachment (ULA) round-bottom wells. A single-cell suspension was prepared and

adjusted to a final concentration of 15000 cells per 100 μL (i.e., 1.5×10^5 cells/mL). 100 μL of the suspension was dispensed into each well (15000 cells/well), seeding 32 wells located in the central region of the plate to minimize edge-related variability. Immediately after seeding, the plate was centrifuged at $300\times g$ for 5 min to promote rapid aggregation. Spheroid formation and homogeneity were verified by brightfield inspection and plates were incubated overnight at 37°C , 5% CO_2 prior to downstream imaging and experimental use.

For spheroid maintenance, culture medium was refreshed every other day using a gentle partial medium exchange to avoid disrupting spheroid integrity.

HaCaT FUCCISpheroid embedding in 0.5% Low Melting Agarose (LMA)

For motion-stabilized live imaging, HaCaT spheroids were embedded in 0.5% (w/v) low-gelling-temperature agarose (catalog #A9414, Sigma-Aldrich). A 2% (w/v) agarose stock was prepared and subsequently diluted in pre-warmed DMEM/F-12 spheroid culture medium to obtain the final 0.5% working solution. The diluted agarose was maintained in the liquid phase and allowed to equilibrate for 30 min prior to embedding. Spheroids were transferred into the imaging chamber and immediately overlaid with 0.5% agarose to fully cover the sample. The agarose was left to gel at room temperature, allowing the hydrogel to stabilize spheroid positioning. After gelation, the chamber was filled with DMEM/F-12 medium to keep the agarose hydrated throughout the imaging session.

hiPSCs maintenance

The GCaMP6 (WTC11-AAV-CAG-GCaMP6) hiPSC lines were kindly provided by Dr. Bruce Conklin at the Gladstone Institute of Cardiovascular Research. Cells were maintained in complete Essential 8 Flex Medium (catalog # A2858501, ThermoFisher Scientific) supplemented with Penicillin/Streptomycin (P/S) (catalog # A001, HiMedia) on 1:100 diluted Growth Factor Reduced (GFR) Matrigel (7-10 mg/ml, depending on the lot), phenol red-free (catalog # 356231, Corning). When they reached 70% confluency (usually in 3-4 days), cells were detached using 0.5 mM EDTA (Ethylenediaminetetraacetic acid, Life Technologies) in Dulbecco's PBS (DPBS) without Ca^{2+} or Mg^{2+} (catalog # C-40232, PromoCell) for 5 minutes at 37°C in a 5% CO_2

environment. Subsequently, cells were expanded in 6-well plates by passaging 1:10 in the presence of RevitaCell (catalog # A26445-01, Life Technologies). 24 hours after plating, the medium was replaced with complete Essential 8 Flex Medium (without RevitaCell) and refreshed every other day.

Generation of the hiPS R26-CAG-FUCCIplex clonal cell line

To generate the single reporter, 2-color hiPS R26-CAG-FUCCIplex clonal cell line, WTC-11 (UCSFi001-A-1) hiPS cells were genome-edited to specifically insert the FUCCIplex cassette into the ROSA26 locus THUMPD3-AS1 (PMID: 18037879) using a CRISPR/Cas9n-based gene-trap strategy. Unless otherwise noted, genome editing was performed as described in Bertero et al. (PMID: 27899508). First, the FUCCIplex cDNA fragment, flanked by the ROSA26 homology arms, was amplified by PCR from the original FUCCIplex plasmid (pLV-Hygro-EF1a-FUCCIplex) using primers fwd: CATCATTTTGGCAAAGAATTAATTTCGGATCCgccaccatggtgagcaagg and rev: CGAGGCTGATCAGCGAGCTACGCGTttacagggccttcgcgcg. Meanwhile, the pR26-Bsd_CAG-EGFPd2 plasmid (kindly provided by Professor Alessandro Bertero, Department of Molecular Biotechnology and Health Sciences, University of Turin) was digested with BamHI and MluI (catalog # R3136S and R3198S, respectively, New England Biolabs) to remove the EGFPd2 fragment. The DNA was then ligated via NEBuilder HiFi DNA assembly (catalog# E5520S, New England Biolabs) with the FUCCIplex DNA fragment to create the pR26-Bsd_CAG-FUCCIplex construct. Next, hiPSCs were electroporated (1200V/30ms/1 pulse) along with two other plasmids, pSpCas9n(BB)_R26-L and pSpCas9n(BB)_R26-R (kindly provided by Professor Alessandro Bertero), to induce a targeted double-strand break in the intron between exons 1 and 2 of THUMPD3-AS1 on chromosome 3 (ROSA26 locus). Following this, cells were seeded into a 24-well plate without antibiotics and incubated at 37°C in a humidified CO₂ incubator. Positive cells were then clonally plated and expanded. The strategy for generating the triple-reporter, four-color R26-CAG-FUCCIplex clonal cell line followed the same approach as for the single reporter, with the modification that the WTC-11 double reporter (GCaMP6f and RFP-LifeAct) was used.

Gene-targeted hPSC clonal lines were screened by genomic PCR to verify site-specific targeting using the following primers:

Locus	PCR type	Primer	Sequence
ROSA	WT (locus)	<u>1</u> <u>FW</u>	GAGAAGAGGCTGTGCTTCGG
		<u>2</u> <u>REV</u>	ACAGTACAAGCCAGTAATGGAG
ROSA	5' INT	<u>3</u> <u>FW</u>	GAGAAGAGGCTGTGCTTCGG
		<u>4</u> <u>REV</u>	AAGACCGCGAAGAGTTTGTCC
ROSA	3' INT	<u>5</u> <u>FW</u>	GAGAATAGCAGGCATGCTG
		<u>6</u> <u>REV</u>	ACAGTACAAGCCAGTAATGGAG
ROSA	5' BB	<u>7</u> <u>FW</u>	CGTTGTAAAACGACGGCCAG
		<u>8</u> <u>REV</u>	AAGACCGCGAAGAGTTTGTCC
ROSA	3' BB	<u>9</u> <u>FW</u>	GAGAATAGCAGGCATGCTG
		<u>10</u> <u>REV</u>	TGACCATGATTACGCCAAGC

Wild Type (WT) ROSA 26 locus is detected by primers number 1 and 2. Site-specific cassette integration (INT) is detected by 5'INT and 3'INT PCRs (primers 3-4 and 5-6). Unintentional off-target random integrations were evaluated by two additional backbone (BB) PCRs: 5'BB and 3'BB (primers 7-8 and 9-10). PCRs were performed with Platinum PCR SuperMix High Fidelity (catalog # 12532016, Thermo), according to manufacturer instructions.

Generation of 3D self-assembling human heart organoids (cardioids)

To generate cardiac organoids (cardioids) from FUCCIplex hiPS cells, we followed the protocol described by Mendjan et al. (PMID: 38029745, <http://dx.doi.org/10.2139/ssrn.4854180>) with minor modifications. Briefly, for anterior primitive streak (APS) induction (day -1), FUCCIplex hiPSC cells were seeded at 5.000 cells per well in a Matrigel-coated 24-well plate with Essential 8 Flex Plus 5 μ M Y-27632 dihydrochloride (catalog # 004CA16644, Cayman Chemicals). After 24 hours, the supernatant was removed and replaced with 1 ml APS-induction

medium per well (see table below), and the cells were incubated at 37 °C with 5% CO₂ for 36-40 hours. On day 1.5, cells were detached, counted, and resuspended in CardMeso-Rocki (see table below) to a final concentration of 15.000 cells per well in a Nunclon Sphera 96-well U-shaped-bottom microplate (catalog#174929, Thermo). Cells were centrifuged at 140 xg for 4 minutes and incubated at 37 °C with 5% CO₂ for 24 hours. From day 2.5 to 4.5, the medium was changed daily, and wells were filled with CardMeso-induction medium without ROCKi. From day 5.5 to 6.5, the medium was replaced with CardMyo-induction medium (see table below). Organoids began beating at this stage. From day 7.5 onward, the supernatant was replaced with CardMaint medium (see table below) every 48 hours.

Reagent name	Supplier and catalog number #
Bovine serum albumin (BSA)	Europa Biosciences, #EQBAH70-1000, Lot #190618-0170
Optiferrin	Invitria, #777TRF029
Human FGF2-G3	Qkine, #Qk053
Activin A1	Qkine, #Qk001
BMP4	Qkine, #Qk038
Insulin	Thermo Scientific, #A11382II
Y-27632 dihydrochloride (ROCK inhibitor, ROCKi)	Cayman Chemicals, #004CA16644
LY 294002 hydrochloride	Selleckchem, #1105
CHIR99021	Cayman Chemicals, #004CA13122
C59	Cayman Chemicals, #16644
Retinoic acid	Sigma Aldrich, #R2625
F-12	Thermo Scientific, #31765068
IMDM	Thermo Scientific, #21980032
Chemically defined lipid concentrate	Thermo Scientific, #11905031
Mono-Thio-Glycerol (MTG)	Sigma Aldrich, #M6145

Medium	Reagent	Final Concentration
CDM	BSA IMDM F-12 Conc. lipids MTG Optiferrin	0.3 % ~50% ~50% 1% 0.004% 15µg/ml
APS - Induction (CDM-FLyABCh) 200µL/96w, 20ml/plate	CDM FGF2-G3 LY 294002 Activin A1 BMP4 CHIR99021	6ng/ml 5µM 5ng/ml 10ng/ml 5µM
CardMeso - Induction (CDM-BFIC59Ra) 200µL/96w, 80ml/96wp	CDM BMP4 FGF2-G3 Insulin C59 RA Y-27632 Day 1.5 only	10ng/ml 1.6ng/ml 10µg/ml 2µM 50nM 5µM
CardMyo - Induction (CDM-BFI) 200µL/96w, 40ml/96wp	CDM BMP4 FGF2-G3 Insulin	10ng/ml 1.6ng/ml 10µg/ml
CardMaintenance (CDM-I) 200µL/96w, 20ml/96wp	CDM Insulin	10µg/ml

High-throughput cardioids' screening JOBS

A HT-2D screening JOB was implemented to perform automated high-throughput imaging of U-bottom 96-well plates for routine monitoring of cardioids' differentiation. Screening automation was executed on a widefield Nikon Ti2 inverted microscope with a CrestOptics X-Light V3 spinning disk confocal and brightfield units within an environmental chamber (Okolab Bold Line; 37 °C, 5% CO₂, humidity). The JOBS was developed NIS-Elements AR (v.5.42.03)³³. Within the JOBS routine, the plate type was defined, wells of interest were selected, and brightfield acquisition parameters were specified together with a z-stack configuration designed to capture edge-localized cardioids. For each well, a 250 µm brightfield z-stack was acquired using 25 µm z-steps (11 planes per well). All outputs were saved automatically using a plate-coordinate naming convention (Row A-H, Column 1-12) to ensure traceability across days and wells. Acquired stacks were processed using the Stack-Focuser plug-in³⁴ to generate a single extended-depth-of-field (fully focused) image per well. Focused images were assembled using a validated full-plate reconstruction routine and were collected longitudinally from Day 3.5 to Day 9.5.

High-throughput cardioids' morphometrical analysis

Following focus projection, batch image analysis was performed to extract basic morphological QC descriptors. Fully focused brightfield images were segmented in batch using Otsu thresholding³⁵ to generate binary masks for each well. Masks were cleaned using standard morphological operations (e.g., hole filling and small-object removal) to reduce segmentation noise at well edges. From each mask, morphometric readouts were extracted, including projected area (primary metric for growth tracking), and, when required for additional QC, perimeter, circularity/solidity, and equivalent diameter. Morphometric outputs were aggregated across differentiation days to compute fold-change trajectories normalized to the earliest analyzed time point, enabling rapid identification of off-trend wells and standardization of construct selection prior to downstream volumetric imaging.

hiPSC-derived cardioids embedding in 0.5% Low-Melting-Agarose (LMA)

Cardioids were embedded for light-sheet microscopy using the same low-melting agarose strategy optimized with HaCaT FUCCISpheroids to maximize positional stability and minimize motion drift during long-term acquisition. On the imaging day (day-matched to the differentiation stage), constructs were immobilized in 0.5% (w/v) low-gelling-temperature agarose (catalog #A9414, Sigma-Aldrich) equilibrated in the same cardioid maintenance medium used at that stage. A sterile 2% (w/v) agarose stock was prepared and diluted in pre-warmed, day-matched maintenance medium to achieve the final 0.5% working solution, which was maintained in the liquid phase at ~37-40°C to reduce thermal stress during mounting. Embedding was performed directly in the imaging chamber to minimize handling and repositioning artifacts: cardioids were gently transferred and positioned with minimal manipulation, then covered with a small volume of agarose to mechanically constrain the construct while preserving optical access. Samples were allowed to equilibrate in-chamber for ~10 min, for the agarose gelation at room temperature. After gelation, the chamber was carefully filled with the same maintenance medium (added gently along the chamber wall to avoid shear and air bubbles) to keep the gel hydrated throughout imaging, and acquisition was initiated only after confirming stable immobilization and absence of visible drift.

Light-sheet imaging

Light-sheet imaging was conducted using the commercial Luxendo TrueLive3D, a high-resolution water-based illumination light-sheet microscope. The system utilizes dual-scanned Gaussian beam illumination with three discrete laser lines (642-561-446 nm), selecting a light-sheet width of 2 μ m. Detection is achieved with a 25x NA 1.1 water immersion objective and two Hamamatsu sCMOS Orca Flash 4.0 V3 cameras.

Static volumetric imaging employed laser lines at 446 nm, 561 nm, and 642 nm to observe different stages of cardioid self-assembly. The detectors captured emitted filtered light using matching filters in the wheel: Band-pass 418-462 nm for mTurquoise2 emission, Band-pass 655-704 nm for the Geminin signal, and Band-pass 580-627 nm for RFP.

Signals from each channel were acquired sequentially, following a far-red (638 nm) to cyan (488 nm) laser line order. Image acquisition was controlled via LuxBundle software (version 5). Data were processed with registration and fusion within the same platform and saved as H5 and TIF files.

Based on the sample preparation, all experiments were performed under live imaging conditions using a phenol red-free imaging medium. Samples were mounted in the appropriate Luxendo TruLive3D sample holder. The microscope has an enclosed chamber to maintain physiological conditions during imaging. The temperature was maintained at 37°C with 5% CO₂ and active humidification.

Volumetric datasets were collected with a step size of 1 µm over a total z-range of 500-1000 µm, depending on the full volume of the 3D model, enabling high-resolution, multi-channel visualization of cardiac morphogenesis stages. In dynamic long-term acquisition, the full stack is acquired every 30 minutes for a total duration of 24 hours; extended acquisitions proved viability up to 48 hours of consecutive light exposure.

Unified Image Analysis pipeline

Volumetric light-sheet datasets were prepared in LuxBundle software (version 5), the Luxendo acquisition software, and processed with standard light-sheet post-acquisition steps: registration, fusion, and binning to reduce data size.. Multi-channel stacks (actin structural channel and FUCCIplex nuclear reporters) were imported as 3D volumes and standardized during dataset preparation by assigning voxel size, channel identity, and timepoint labels. All downstream processing was performed on full 3D volumes to preserve spatial correspondence between functional and structural readouts. The datasets are then loaded into ARIVIS Software (Vision4D, version 4.4.0) for pre-processing, processing, 3D visualization, and batch quantification.

Pre-processing and channel standardization

Preprocessing was applied to stabilize downstream object detection and segmentation under intensity variability across depth and time. Nuclear channels were background-subtracted and denoised with a particle enhancement module applied to improve nuclear

detectability in 3D. A “DAPI-equivalent” detection channel was generated by combining the FUCCIplex nuclear reporters (G1 and S/G2/M) to provide a single detection channel independent of cell-cycle state. For the actin channel, Richardson-Lucy deconvolution (5 iterations) was applied to improve robust segmentation of the tissue envelope and reduce sensitivity to local contrast fluctuations. Parameter settings were held constant across timepoints within a dataset and across datasets acquired under matched imaging conditions.

FUCCIplex-based nuclear detection and cell-cycle classification

Proliferative state was quantified by detecting nuclei in 3D and classifying each nucleus as G1 or S/G2/M using FUCCIplex reporter intensities. Nuclei were detected on the “DAPI-equivalent” channel using a 3D blob-finder module with size constraints matched to nuclear dimensions (max object diameter: 25 μm) and with splitting of touching objects enabled. Spurious detections were excluded using morphological and intensity filters (e.g., minimum object volume $> 1000\mu\text{m}^3$)

Cell-cycle classification was performed by extracting per-object intensity features from the FUCCIplex channels and applying an intensity decision rule on the G1 reporter (CFP thresholding). Nuclei were labeled as G1 when the G1 reporter exceeded the classification threshold and as S/G2/M otherwise, with a corresponding signal expected in the S/G2/M reporter. Per-timepoint raw counts and within-class fractions (G1 vs S/G2/M) were computed and retained, and the classified nuclear objects were stored as a 3D object/point layer for visualization and downstream correlation with structural metrics.

Actin-based tissue segmentation (structural volume)

The cardioid structural volume was segmented from the actin channel to quantify total tissue volume and to provide a geometric reference for cavity localization. A tissue mask was generated using intensity-based segmentation (global thresholding), followed by morphological operations (Fill holes) to obtain a contiguous tissue envelope.

Segmentations were reviewed in orthogonal planes to confirm that tissue boundaries were captured across depth.

Deep-learning cavity segmentation using ARIVIS Cloud

Internal cavities were segmented using a deep-learning model trained and deployed with ARIVIS Cloud to improve robustness in heterogeneous actin contrast and complex multicavity morphologies. Manual annotations of cavity regions and background were generated to build the training dataset. Model training was performed iteratively, with prediction outputs inspected on held-out regions and annotations refined to address common failure modes until stable performance was obtained on validation examples. The trained model was applied to full dataset series to generate cavity binary cavity masks.

Structural morphometrics and derived order parameters

Structural descriptors were computed per timepoint from the tissue and cavity masks with a custom MATLAB script. These included total cardioid volume (V_{card}), cavity count (N_{cav}), total cavity volume ($V_{cav,tot}$), maximal cavity volume (V_{max}), and cavity volume fraction (occupancy; $V_{cav,tot}/V_{card}$). Cavity size distribution statistics were also computed (e.g., mean cavity volume ($V_{cav,tot}/N_{cav}$)) to summarize coarsening/enlargement trends across development. Summary tables were exported for each dataset.

Integration of functional and structural readouts

Functional and structural outputs were consolidated into a shared analysis object for each dataset. 3D renderings were generated by overlaying classified nuclei, tissue volume, and cavity masks. These co-registered renderings were generated to preserve spatial correspondence between FUCCIplex-classified nuclei and cavity architecture within the actin-defined tissue volume.

Multicellular Phase-Field Model (MCFPM)

Cavity morphogenesis was simulated using a two-dimensional multicellular phase-field model (MCPFM) adapted from published work. Each epithelial cell $m=1, \dots, M$ is represented by an individual phase field $u_m(\mathbf{r}, t) \in [0, 1]$ and the cavity by a distinct phase field $s(\mathbf{r}, t) \in [0, 1]$. Model evolution follows Allen - Cahn (gradient-descent) dynamics that minimize a total free-energy functional comprising interface regularization terms, cell size control via quadratic penalties to time-dependent target areas, cell-cell adhesion, excluded-volume repulsion between cells and between cells and cavity, a cortical-tension/surface regularization term, and a pressure-like cavity driving term $\bar{\xi}$ that biases cavity expansion. Cell growth is implemented by relaxing each cell's target area toward a division threshold with timescale $\bar{\tau}_v$ (a proliferation timescale proxy); division is triggered when the target criterion is met, and it produces two daughter phase-fields via field partitioning. Cavity nucleation is enabled by division-coupled micro-cavity seeding. The coupled PDEs are discretized on a uniform Cartesian grid and integrated using explicit finite differences and forward-Euler time stepping. Across parameter sweeps in $(\bar{\tau}_v, \bar{\xi})$ simulations were summarized by cavity count, computed as the number of connected components of the thresholded cavity field, and cavity occupancy, defined as $V_l/(V_l + \sum_m V_m)$, where V_l and V_m are effective cavity and cell areas computed from the corresponding phase fields using a smooth indicator function. Detailed equations, parameter values, numerical settings, and the ξ -to-pressure calibration are reported in the Appendix section.

5.7 Bibliography

1. Roth, G. A. *et al.* Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. *J. Am. Coll. Cardiol.* 76, 2982-3021 (2020).
2. Martin, S. S. *et al.* 2024 Heart Disease and Stroke Statistics: A Report of US and Global Data From the American Heart Association. *Circulation* 149, e347-e913 (2024).
3. Xiao, Y., Zhuang, Z., Shi, X. & Wu, F. Challenges and opportunities for cardiac organoids: A comprehensive review. *Regeneration Repair Rehabil.* (2025)
4. Hofbauer, P. *et al.* Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021).
5. Lewis-Israeli, Y. R. *et al.* Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021).
6. Prondzynski, M. *et al.* Efficient and reproducible generation of human iPSC-derived cardiomyocytes and cardiac organoids in stirred suspension systems. *Nat. Commun.* 15, 5929 (2024).
7. Kim, J., Koo, B.-K. & Knoblich, J. A. Human organoids: model systems for human biology and medicine. *Nat. Rev. Mol. Cell Biol.* 21, 571-584 (2020).
8. Bozal, S. B. *et al.* Development of an automated 3D high-content cell screening platform for organoid phenotyping. *SLAS Discov.* 29, 100182 (2024).
9. Louey, A., Hernández, D., Pébay, A. & Daniszewski, M. Automation of Organoid Cultures: Current Protocols and Applications. *SLAS Discov.* 26, 1138-1147 (2021).
10. Lampart, F. L., Iber, D. & Doumpas, N. Organoids in high-throughput and high-content screenings. *Front. Chem. Eng.* 5, (2023).
11. Royer, L. A. *et al.* Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016).
12. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017).
13. de Medeiros, G. *et al.* Multiscale light-sheet organoid imaging framework. *Nat Commun* 13, 4864 (2022).
14. Linkert, M. *et al.* Metadata matters: access to image data in the real world. *J. Cell Biol.* 189, 777-782 (2010).
15. Boehm, U. *et al.* QUAREP-LiMi: a community endeavor to advance quality assessment and reproducibility in light microscopy. *Nat. Methods* 18, 1423-1426 (2021).
16. Sarkans, U. *et al.* REMBI: Recommended Metadata for Biological Images-enabling reuse of microscopy data in biology. *Nat. Methods* 18, 1418-1422 (2021).
17. Lee, B.H *et al.* Permeability-driven pressure and cell proliferation control lumen morphogenesis in pancreatic organoids. *Nat Cell Biol* 28, 113–124 (2026)
18. Lu, L *et al.* Generic comparison of lumen nucleation and fusion in epithelial organoids with and without hydrostatic pressure. *Nat Commun* 16, 6307 (2025)
19. Di Sante, M. *et al.* CALIPERS: Cell cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* 2024.12.19.629259 (2024)
20. Tanida, S. *et al.* Predicting organoid morphology through a phase field model: Insights into cell division and luminal pressure. *PLoS Comput Biol.* 2025 .

21. Kiepas, A., Voorand, E., Mubaid, F., Siegel, P. M. & Brown, C. M. Optimizing live-cell fluorescence imaging conditions to minimize phototoxicity. *J. Cell Sci.* 133, jcs242834 (2020).
22. Lukonin, I., Zinner, M. & Liberali, P. Organoids in image-based phenotypic chemical screens. *Exp. Mol. Med.* 53, 1495-1502 (2021).
23. Nie, Y., Xu, X., Wang, W., Ma, N. & Lendlein, A. Spheroid formation of human keratinocyte: Balancing between cell-substrate and cell-cell interaction. *Clin. Hemorheol. Microcirc.* 76, 329-340 (2020).
24. Bruker Corporation. Light-Sheet Images of Organoids and Cell Cultures
25. Dyer, L. et al. Lightsheet Microscopy. Wiley Online Library. (2022).
26. ZEISS ARIVIS Pro Software. v.4.4
<https://www.zeiss.com/microscopy/en/products/software/ARIVIS-pro.html>.
27. Ming, Y. et al. Longitudinal morphological and functional characterization of human heart organoids using optical coherence tomography. *Biosens. Bioelectron.* 207, 114136 (2022).
28. Woappi, Y., Altomare, D., Creek, K. E. & Pirisi, L. Self-assembling 3D spheroid cultures of human neonatal keratinocytes have enhanced regenerative properties. *Stem Cell Res.* 49, 102048 (2020).
29. Cho, J., Lee, H., Rah, W., Chang, H. J. & Yoon, Y.-S. From engineered heart tissue to cardiac organoid. *Theranostics* 12, 2758-2772 (2022).
30. Hofbauer, P. et al. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021).
31. Ong, H. T. et al. Digitalized organoids: integrated pipeline for high-speed 3D analysis of organoid structures using multilevel segmentation and cellular topology. *Nat. Methods* 22, 1343-1354 (2025).
32. Brezak, M., Khan, A. A., Jechlinger, M. & Sumbalova Koledova, Z. Live-cell imaging of mammary organoids using light sheet microscopy. *J. Mammary Gland Biol. Neoplasia* 30, 12 (2025).
33. NIS-Elements.v.5.42
<https://www.microscope.healthcare.nikon.com/NISelements>.
34. Stack Focuser. <https://imagej.net/ij/plugins/stack-focuser.html>.
35. Yang, X., Shen, X., Long, J. & Chen, H. An Improved Median-based Otsu Image Thresholding Algorithm. *AASRI Procedia* 3, 468-473 (2012).

6

Conclusions

This thesis develops an integrated framework for live-cell fluorescence phenotyping in tissue engineering. Its main contribution lies in the coordination of biological state, acquisition logic, engineered microenvironments, and quantitative analysis within workflows that make major sources of measurement variability more explicit. Across the chapters, the same principle has guided the work: measurements should be concentrated where biological information is highest, while sources of technical variation should be made explicit enough to support reliable comparison across conditions and platforms.

Key contributions and validation

Four experimental constraints motivated the thesis. The first was photon burden during long-term live imaging, particularly when the events of interest are sparse and transient. The second was the loss of throughput and comparability that arises when photofabricated layouts are not translated into microscopy coordinates. The third was the lack of acquisition architectures suited to both rapid volumetric imaging and prolonged low-dose observation in three-dimensional specimens. The fourth was the difficulty of extracting structural and functional phenotypes from the same registered volumetric dataset in organoid systems. The chapters addressed these constraints through four linked contributions: FUCCIsmart, Fab2Mic, TE-DaXi, and a three-dimensional phenotyping workflow for cardioids.

FUCCIsmart shows that cell state can be used as an acquisition control signal to improve information-per-photon in long-term, mitosis-focused imaging, as assessed by mitosis

capture rate and phototoxic event rate. The Fab2Mic pipeline enables engineered microenvironments as scalable, reproducible assays by converting fabrication layouts into imaging coordinates and embedding multiplexed cell-cycle states into longitudinal single-cell phenotyping. At the 3D level, the constraints revealed by complex tissue-engineered models motivated the development of TE-DaXi as a platform concept for multi-timescale acquisition governance, designed to support fast volumetric bursts together with long-term low-dose imaging of function, morphology, and cell-cycle state. In parallel, the thesis establishes an experimental-analytical 3D framework built on three linked toolkits: a light-sheet imaging protocol for stable volumetric acquisition, a quantitative 3D structural and proliferative phenotyping pipeline, and a mechanistic multi-component model used as an interpretive layer for experimental phenotypes. This framework not only enables the study of cardioids as 3D complex models in the present work but also provides the practical testbed through which TE-DaXi will be evaluated once fully calibrated.

Adaptive imaging, micropatterning, and light-sheet microscopy each have strong precedents relative to the prior art reviewed in Chapter 1. The novelty here lies in the engineered coupling between modules, with measurement comparability, control of avoidable technical variation, and biological interpretability treated as primary design constraints. Specifically, cell-cycle state is used as an acquisition control signal, fabrication coordinates are translated into imaging coordinates as a throughput primitive, and 3D phenotypes are extracted as paired structural-functional descriptors from the same registered volumes and interpreted within a compact regime map rather than treated as morphology-only endpoints. In combination, these choices shift the workflow from locally optimized tools toward a governed measurement framework, helping to reduce the risk that instrument settings, fabrication mismatch, or analysis choices are misinterpreted as biological differences. Importantly, this integration also changes what can be measured biologically: mitosis can be sampled as a state-dependent event, confinement can be evaluated as a regulator of cell-cycle fidelity rather than only as a geometric migration cue, and cardioid cavity formation can be interpreted as a structural-functional phenotype rather than as an endpoint morphology alone.

Among the key achievements of this thesis, we first demonstrated that cell state can be translated into acquisition control in a setting where phototoxicity is a persistent limitation for long-term, high-resolution imaging. Mitotic imaging provides a stringent test case because it requires repeated high-NA, multi-plane acquisitions over extended periods, during which photodamage can accumulate and bias sensitive phenotypes. FUCCIsmart implements a state-aware trigger that concentrates high-dose acquisition into the narrow window in which mitotic information peaks and reduces illumination outside that window. In 24-hour tests with the same optics and illumination levels, FUCCIsmart captured $66.67 \pm 11.50\%$ of mitoses while keeping phototoxic events at $7.33 \pm 8.74\%$. Hourly Z-stack automation achieved a similar capture rate but with substantially higher phototoxic events ($60.33 \pm 6.43\%$). In a mitosis-focused mode tuned to collect a short time series around single events to monitor spindle dynamics, FUCCIsmart captured $3.3\times$ more mitoses than a sparse 6-hour schedule while maintaining phototoxicity close to the 6-hour baseline. Together, these results support the thesis claim that event-driven, state-aware acquisition can outperform fixed schedules by allocating photons when information density is highest and withholding them when it is low, improving information-per-photon in long-term imaging scenarios. Biologically, this means that mitotic spindle imaging no longer needs to be treated as a random event captured by oversampling; instead, it can be targeted as a predictable cell-cycle transition, enabling 3D mitotic information to be collected with less perturbation of the same biological process being measured.

Building on governance in time and state, the next scaling barrier is governance in space and in the experimental context. Engineered microenvironments, such as geometric confinement, can, in principle, convert cell behavior into measurable rules for migration and proliferation. In practice, these experiments can become fragile at scale when fabrication and imaging are not interoperable and when cell-state readouts are not embedded into analysis. To address these constraints, this work develops an integrated platform that combines multiplexed cell-cycle reporters, engineered ECM islands, and correlative high-content imaging to probe the relationship between cell-cycle progression and migration under geometric constraints. Three technical choices are central: spectral reallocation via FUCCIplex; Fab2Mic, which bridges the registration-throughput gap by translating fabrication coordinates into imaging coordinates; and longitudinal single-cell

tracking, which converts confinement from a static condition into a measurable, time-resolved parameter. Incorporating cell-cycle analysis directly into migration and morphology phenotyping provides a systematic framework for studying migration and proliferation under constraints while extending established assays of adhesion and geometry-driven regulation. Importantly, live-cell imaging resolves single-cell behaviors that are difficult to infer from static snapshots, including abnormal cell-cycle phenotypes consistent with evidence that mechanical stress can hinder mitotic progression and promote abnormal outcomes under constraint. Overall, these findings indicate that confinement phenotyping can become more reproducible and interpretable when coordinate correlation, multiplexed readouts, and longitudinal imaging are integrated as explicit workflow components. Biologically, this shifts the interpretation of confinement from a primarily morphological or migratory perturbation toward a condition that can alter the coordination between cell shape, migration, and cell-cycle progression. This is important because abnormal or prolonged cell-cycle trajectories under constraint would likely remain hidden in endpoint assays, population averages, or imaging workflows that do not preserve the link between single-cell history and microenvironment geometry.

After establishing dose and coordinate-guided phenotyping in 2D, the thesis extends the framework to 3D biological models, which are important in regenerative medicine and disease modeling. In 3D, the constraints intensify: first, differentiation is expensive and difficult to standardize. Second, volumetric imaging is required, yet phototoxicity and specimen motion frequently compromise long acquisitions. Third, quantitative, cell-cycle-aware structural and functional 3D phenotyping remains underdeveloped, limiting scalable HT/HC phenotyping screening. Chapter 4 introduces the next iteration of the HT/HC journey at the instrument architecture level. Building on the requirements revealed by dose-governed phenotyping and coordinate interoperability in earlier chapters, this chapter focuses on hardware design and initial fabrication of TE-DaXi, an oblique-plane light-sheet microscope designed to better match tissue-engineering constraints. The goal of this imaging platform is to remove recurring bottlenecks that limit volumetric screening, such as throughput limitations, stability during long acquisitions, and insufficient governance of the photon budget. TE-DaXi is therefore presented as a prototype architecture intended to support quantitative 3D phenotyping after further

calibration, benchmarking, and validation against downstream biological and analytical requirements.

Chapter 5 then extends the framework to volumetric HT/HC phenotyping in 3D by establishing an experimental, imaging, and analysis workflow on a commercial light-sheet system. The workflow is first optimized on HaCaT spheroids to define stable sample-handling and embedding conditions that reduce drift and enable robust 3D reconstruction in both static and long-term settings. It is then adapted to hiPSC-derived cardioids, where variability across a multi-day differentiation protocol motivates a high-throughput daily screening routine: plate-scale imaging extracts 2D morphological QC metrics to gate which specimens proceed to costly volumetric acquisition. Using multiplexed light-sheet imaging of FUCCIplex cardioids, a quantitative pipeline links cell-cycle distributions with cavity morphometrics throughout differentiation. Across time, the cell-cycle distribution remains broadly balanced between G1 and S/G2/M, with Day 4.5 showing the most G1-skewed state (G1 59.4%, S/G2/M 40.6%). In parallel, cavity morphometrics indicate a transition from no visible cavities at Day 2.5 to a fragmented multi-cavity structure at Days 3.5-4.5 with modest cavity occupancy (~15%). By Day 5.5, cavity occupancy increases sharply (~74.7%), accompanied by an approximately eightfold rise in mean cavity volume relative to Day 3.5, while the largest cavity fraction remains ~0.35, suggesting luminal space distributed across multiple large cavities rather than dominated by a single chamber. To interpret these trends within a compact mechanistic framework, the chapter finally introduces a two-phase model as an interpretation layer for lumen formation, linking proliferation-related growth and interface transport/permeability mechanisms to cavity outcomes and enabling qualitative regime-based comparison with the experimental phenotypes. Biologically, this moves cavity analysis beyond a structural endpoint: lumen organization, cavity occupancy, and tissue growth are interpreted together with proliferative-state distributions, suggesting that cavity phenotypes may reflect coupled growth, transport, and interface-remodeling regimes rather than morphology alone. Taken together, the final two chapters close the thesis by separating volumetric hardware development from end-to-end 3D phenotyping implementation: Chapter 4 defines the instrument path through TE-DaXi, while Chapter 5 establishes the biological and analytical testbed through which that path can be evaluated in practice.

Beyond the individual technical modules, this thesis also contributed to reproducibility at the level of experimental practice. A recurring effort throughout the work was to reduce hidden variability in live-cell imaging by formalizing minimal operational standards for dataset acquisition (the **dataset passport**, Chapter 1) and routine measurement procedures, acquisition checks, and instrument-performance monitoring (Appendix B4). These activities included standardized live-cell imaging protocols, routine laser power measurements and calibration checks, and the documentation of practical quality-control steps needed to maintain comparability across imaging sessions, samples, and instruments. Although less visible than the biological case studies, this operational layer was essential to ensure that measured differences could be interpreted as biological rather than as consequences of drift in imaging conditions, setup variability, or inconsistent acquisition practice. In this sense, the thesis contributes not only new tools for phenotype measurement, but also to a more reproducible measurement framework for deploying them in the lab.

Limitations and future directions

Several limitations follow from the scope of this thesis. First, state-aware acquisition improves information-per-photon under the tested reporters, thresholds, and imaging conditions, but it does not remove phototoxicity as a confound. Instead, it shifts the critical requirement toward calibration, dose reporting, and monitoring of biological failure sentinels when the approach is transferred across cell types, reporter-expression regimes, optical configurations, or noise conditions. Second, although the confinement workflow improves scalability and interpretability by making fabrication-to-microscopy coordinate correlation and state readouts explicit, confinement phenotypes remain dependent on assay operating ranges, including seeding density, boundary effects, ECM presentation, cell-cell interactions, and pattern occupancy. These variables must be controlled or reported to avoid context-dependent conclusions. Third, in 3D cardioids, cavity morphometrics remain sensitive to imaging physics and segmentation stability, including depth-dependent attenuation, shadowing, partial-volume effects, and heterogeneous reporter signal. These factors can propagate into topology, occupancy, and cavity-size estimates, particularly in fragmented or multicavity regimes. As a result, computational analysis can become a practical bottleneck, requiring dataset-specific

preprocessing, segmentation validation, manual quality control, and version-controlled analysis pipelines before large-scale deployment. Fourth, the custom instrumentation work, particularly TE-DaXi, demonstrates a feasible design logic for tissue-engineering-oriented light-sheet microscopy, but its broader adoption remains constrained by optical alignment complexity, calibration requirements, component availability, user expertise, and cross-laboratory standardization. Finally, the two-phase model is used as an interpretation layer, which qualitatively maps experimental phenotypes onto its regime space. The observed trends motivate extending the model-data interface with additional measured biological and physical drivers beyond proliferative state alone, including transport, pressure, matrix properties, contractility, and interfacial mechanics.

Future work is most usefully organized around three priorities. The first is multi-timescale acquisition governance in 3D cardioids: integrating fast functional readouts, such as calcium handling, with long-term low-dose volumetric monitoring of morphology and cell-cycle state within a single workflow, so that function and morphogenesis remain comparable outputs rather than separate experiments. The second is strengthening the model-data interface by using the multicellular phase-field model as a state-aware feedback layer during HT/HC screening. In this scenario, early image-derived descriptors, such as organoid size, cavity topology/occupancy, and cell-cycle-state distributions, could be compared with precomputed model regimes to estimate whether a specimen is evolving toward a transport/permeability-dominated or proliferation/growth-dominated morphogenetic state. This would allow the model to support acquisition decisions rather than only post hoc interpretation, for example, by identifying the most informative window, adjusting the experiment sampling/duration, or by prioritizing specimens for structural-functional readouts based on their self-assembly stage. Such a workflow would be particularly useful in 3D systems, where fixed imaging schedules may miss the relevant morphodynamical window or collect large volumes of weakly informative data. The third priority is coupling adaptive, cell-cycle-aware imaging with spatial provenance by integrating fabrication-to-microscopy coordinate correlation as an explicit workflow layer. This would enable targeted acquisition across engineered geometries while preserving registration and comparability as constructs increase in size and architectural complexity. In this context, TE-DaXi provides the natural platform for the next step: implementing these workflow principles in a custom system explicitly

designed for tissue-engineering constraints. Its intended use across two acquisition regimes, such as fast volumetric bursts over short windows and long-term low-dose volumetric imaging for FUCCIplex state and morphology, positions it as the hardware architecture for integrating multi-timescale phenotyping once calibration is complete. The commercial-system workflow established in Chapter 5 therefore serves as a transferable reference implementation against which TE-DaXi can be validated and extended.

Taken together, these contributions define a measurement framework in which acquisition timing, spatial targeting, instrument design, and phenotypic extraction are treated as interdependent parts of the same experiment, as summarized in Fig. 6.1 . Cell-cycle state is used to govern imaging windows, fabrication coordinates are translated into microscopy coordinates, volumetric imaging is structured around distinct operational regimes, and 3D phenotypes are extracted as coupled structural and functional descriptors. This framework strengthens the conditions under which measured differences can be interpreted as biological rather than procedural. However, broader causal interpretation, phenotype fidelity across platforms, and generalized HT/HC scalability remain future-oriented goals that will require further validation across additional biological systems, perturbations, instruments, and analysis pipelines.

HT/HC bottlenecks	Contribution	Validation	Metric improved
Long-term high-resolution live imaging is dose-limited	FUCCIsmart cell-cycle/event-driven 3d imaging that concentrates dose into information-dense mitotic windows	Chapter 2: 24h benchmark vs fixed schedules, matched optics/illumination	Capture-damage trade-off improved: $66.67 \pm 11.50\%$ mitoses with $7.33 \pm 8.74\%$ phototoxic events
Phenotyping becomes fragile at scale without fabrication-imaging interoperability	Fab2Mic with Multiplexed CC-readouts: fabrication-to-microscopy coordinate correlation with FUCCIplex-enabled longitudinal phenotyping	Chapter 3: Fab2Mic coordinate mapping with longitudinal single-cell tracking under geometric constraints	scalable targeting of patterned regions and time-resolved CC-aware motility/morphology, enabling detection of abnormal cc phenotypes under constraint
Need an instrument architecture that supports both fast functional and long-term low-dose volumetric readouts	TE-DaXi Single-objective oblique-plane LSMF with designed for TE constraints	Chapter 4: Design, building and alignment strategy with preliminary tests for a TE-adapted light-sheet microscope	Provides the platform-level path to multiscale governance (3D fast + long-term monitoring) in 3D HT/HC phenotyping setting
3D TE models require structural-functional phenotyping but motion/phototoxicity and analysis challenges limit quantitative readouts and interpretation	3D HT/HC imaging workflow Link 3D functional-structural readouts, quantification and interpretation	Chapter 5: 3D HT/HC imaging workflow applied to hiPSC-cardioids for 3D functional-structural readouts , interpreted within MCPFM regime mapping	Quantitative 3D developmental mapping with CC distribution and cavity morphometrics tracked across days. MCPFM improves interpretability with regime-framed anchored to a compact control space

Figure 6.1. Key thesis contributions. Summary of the main HT/HC bottlenecks, the corresponding engineering contribution, where each module is validated, and the primary metric or capability improved.

Annotated bibliography

1 Chapter: Engineering tools for high-throughput and high-content phenotyping

1. Caicedo, J.C. et al. Data-analysis strategies for image-based cell profiling. *Nat. Methods* 14, 849-863 (2017). It lays out the analytical steps needed to turn image-based profiling into robust and comparable measurements. This matters for the thesis because it supports the thesis view that HT/HC phenotyping is an end-to-end workflow, not just an imaging modality.
2. Chandrasekaran, S.N. et al. Three million images and morphological profiles of cells treated with matched chemical and genetic perturbations. *Nat. Methods* 21, 1114-1121 (2024). It provides a very large matched perturbation resource showing how morphology can support scalable phenotypic mapping across chemical and genetic space. For the thesis, this is useful because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
3. Hofer, M. et al. Engineering organoids. *Nat. Rev. Mater.* 6, 402-420 (2021). It explains how organoids can be engineered as controllable biological systems rather than treated as purely self-organized models. Within the thesis framework, this helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
4. Seal, S. et al. Integrating image-based profiling with cell health data as a foundation for interpretable prediction of biological phenotypes. *Mol. Biol. Cell* 35, ar15 (2024). It shows that morphological profiles become more interpretable when they are linked to orthogonal measures of cell health. In the thesis context, this supports the argument that it positions profiling as a systems problem linking sample design, imaging, and downstream interpretation.

5. Way, G.P. et al. Evolution and impact of high content imaging. *SLAS Discov.* 28, 292-305 (2023). It traces how high-content imaging matured into a scalable approach for multiparametric phenotyping. This supports the thesis because it informs the thesis argument that richer phenotypes require coordinated experimental and computational infrastructure.
6. Stossi, F. et al. High throughput microscopy and single cell phenotypic image-based analysis in toxicology and drug discovery. *Biochem. Pharmacol.* 216, 115770 (2023). It shows how microscopy and single-cell image analysis can be coupled for scalable phenotypic screening in applied settings. For this thesis, it is important because it supports the move from simple 2D screens toward more informative but more technically constrained phenotyping workflows.
7. Lukonin, I. et al. Organoids in image-based phenotypic chemical screens. *Exp. Mol. Med.* 53, 1495–1502 (2021). It shows that organoids expand phenotypic screening into more physiologically complex 3D systems, but with added experimental and analytical constraints. This is valuable to the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
8. Arevalo, J. et al. Evaluating batch correction methods for image-based cell profiling. *Nat. Commun.* 15, 6516 (2024). It demonstrates that batch correction can strongly alter image-based profiling results and therefore must be validated rather than assumed. In this dissertation, it helps because it anchors the thesis emphasis on scalable, interpretable phenotyping across acquisition, analysis, and data handling.
9. Eliceiri, K.W. et al. Biological imaging software tools. *Nat. Methods* 9, 697-710 (2012). It maps the software ecosystem that underpins modern biological image analysis and data handling. This strengthens the thesis argument because it helps justify why reproducibility and comparability must be designed into HT/HC assays from the start.
10. Laissue, P. et al. Assessing phototoxicity in live fluorescence imaging. *Nat. Methods* 14, 657-661 (2017). It establishes that phototoxicity must be measured through biological readouts rather than inferred from bleaching alone. For the

broader thesis logic, this helps because it defines photon dose as an experimental variable that can reshape the phenotype being measured.

11. Bray, M.-A. et al. Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. *Nat. Protoc.* 11, 1757-1774 (2016). It describes a multiplexed dye-based assay that converts morphology into rich phenotypic fingerprints at scale. This matters for the thesis because it informs the thesis argument that richer phenotypes require coordinated experimental and computational infrastructure.
12. Wan, Y. et al. Light-Sheet Microscopy and Its Potential for Understanding Developmental Processes. *Annu. Rev. Cell Dev. Biol.* 35, 655-681 (2019). It explains why light-sheet imaging is especially powerful for long-term volumetric observation of developing systems. For the thesis, this is useful because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
13. Durkee, M.S. et al. Artificial Intelligence and Cellular Segmentation in Tissue Microscopy Images. *Am. J. Pathol.* 191, 1693-1701 (2021). It reviews how AI-based segmentation expands the extraction of cellular measurements from microscopy datasets. Within the thesis framework, this helps because it supports the thesis view that HT/HC phenotyping is an end-to-end workflow, not just an imaging modality.
14. Kasprzycka, W. et al. New approaches for low phototoxicity imaging of living cells and tissues. *BioEssays* 46, e2300122 (2024). It surveys practical ways to reduce light burden while preserving usable information in live imaging. In the thesis context, this supports the argument that it defines photon dose as an experimental variable that can reshape the phenotype being measured.
15. Lampart, F.L. et al. Organoids in high-throughput and high-content screenings. *Front. Chem. Eng.* 5, 1120348 (2023). It outlines how organoid screening combines greater physiological relevance with harder imaging, standardization, and analysis problems. This supports the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.

16. Berg, S. et al. ilastik: interactive machine learning for (bio)image analysis. *Nat. Methods* 16, 1226-1232 (2019). It presents an interactive machine-learning approach that lowers the barrier to segmentation and classification in bioimage analysis. For this thesis, it is important because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
17. Carpenter, A.E. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100 (2006). It introduced a modular analysis environment for extracting quantitative cell features from microscopy images. This is valuable to the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
18. Ellenberg, J. et al. A call for public archives for biological image data. *Nat. Methods* 15, 849-854 (2018). It argues that large-scale image science depends on public archives that preserve data and metadata together. In this dissertation, it helps because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
19. Greenwald, N.F. et al. Whole-cell segmentation of tissue images with human-level performance using large-scale data annotation and deep learning. *Nat. Biotechnol.* 40, 555–565 (2022). It shows that large annotated datasets and deep learning can achieve high-quality whole-cell segmentation in complex tissue images. This strengthens the thesis argument because it supports the thesis view that HT/HC phenotyping is an end-to-end workflow, not just an imaging modality.
20. Moore, J. et al. OME-NGFF: a next-generation file format for expanding bioimaging data-access strategies. *Nat. Methods* 18, 1496–1498 (2021). It proposes a cloud-ready image format designed to improve access, scalability, and interoperability for large bioimaging datasets. For the broader thesis logic, this helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
21. Sofroniew, N. et al. napari: a multi-dimensional image viewer for Python. <https://doi.org/10.5281/ZENODO.15421167> (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-

- based biology. This matters for the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
22. Kemmer, I. et al. Building a FAIR image data ecosystem for microscopy communities. *Nat. Methods* 20, 1712–1718 (2023). It argues that image data remain interpretable only when metadata, standards, and reuse infrastructure are built in. For the thesis, this is useful because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
 23. Schmidt, C. et al. Research data management for bioimaging: the 2021 NFDI4BIOIMAGE community survey. *F1000Research* 11, 638 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs the thesis argument that richer phenotypes require coordinated experimental and computational infrastructure.
 24. Sivagurunathan, S. et al. Bridging imaging users to imaging analysis – A community survey. *J. Microsc.* 1–15 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this supports the argument that it supports the move from simple 2D screens toward more informative but more technically constrained phenotyping workflows.
 25. Swedlow, J.R. et al. A global view of standards for open image data formats and repositories. *Nat. Methods* 18, 1440-1446 (2021). It argues that image data remain interpretable only when metadata, standards, and reuse infrastructure are built in. This supports the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
 26. Williams, E. et al. Image Data Resource: a bioimage data integration and publication platform. *Nat. Methods* 14, 775-781 (2017). It argues that image data remain interpretable only when metadata, standards, and reuse infrastructure are built in. For this thesis, it is important because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.

27. Mattiazzi Usaj, M. et al. Single-cell image analysis to explore cell-to-cell heterogeneity in isogenic populations. *Cell Syst.* 12, 608-621 (2021). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it helps justify why reproducibility and comparability must be designed into HT/HC assays from the start.
28. Menduti, G. et al. Recent Advances in High-Content Imaging and Analysis in iPSC-Based Modelling of Neurodegenerative Diseases. *Int. J. Mol. Sci.* 24, 14689 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it positions profiling as a systems problem linking sample design, imaging, and downstream interpretation.
29. Salic, A. et al. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci.* 105, 2415-2420 (2008). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This strengthens the thesis argument because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
30. Scholzen, T. et al. The Ki-67 protein: from the known and the unknown. *J. Cell. Physiol.* 182, 311-322 (2000). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
31. Held, M.W. et al. CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging. *Nat. Methods* 7, 747–754 (2010). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it the thesis treats cell-cycle state as a core axis that must be resolved to interpret live phenotypes correctly.
32. Wang, J. et al. A concise guide to fluorescent cell cycle reporters for live-cell imaging. *Front. Cell Dev. Biol.* 13, 1702230 (2025). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For

the thesis, this is useful because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.

33. Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487-498 (2008). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. Within the thesis framework, this helps because it underpins the use of cell-cycle reporters to align morphology, migration, and state transitions over time.
34. Bajar, B.T. et al. Fluorescent indicators for simultaneous reporting of all four cell cycle phases. *Nat. Methods* 13, 993-996 (2016). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. In the thesis context, this supports the argument that it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
35. Grant, G.D. et al. Accurate delineation of cell cycle phase transitions in living cells with PIP-FUCCI. *Cell Cycle* 17, 2496-2516 (2018). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. This supports the thesis because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
36. Oki, T. et al. A novel cell-cycle-indicator, mVenus-p27K-, identifies quiescent cells and visualizes G0-G1 transition. *Sci. Rep.* 4, 4012 (2014). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For this thesis, it is important because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
37. Neumann, B. et al. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature* 464, 721-727 (2010). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports phase-aware phenotyping rather than averaging behavior across asynchronous cell states.
38. Pargett, M. et al. Live-cell imaging and analysis with multiple genetically encoded reporters. *Curr. Protoc. Cell Biol.* 78, 4.36.1–4.36.19 (2018). It expands the molecular labeling toolbox used to make specific biological processes visible in

- live cells. In this dissertation, it helps because it underpins the use of cell-cycle reporters to align morphology, migration, and state transitions over time.
39. Specht, E.A. et al. A critical and comparative review of fluorescent tools for live-cell imaging. *Annu. Rev. Physiol.* 79, 93–117 (2017). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. This strengthens the thesis argument because it justifies assigning temporal state explicitly when studying dynamic phenotypes in live imaging.
 40. Tosheva, K.L. et al. Between life and death: strategies to reduce phototoxicity in super-resolution microscopy. *J. Phys. D Appl. Phys.* 53, 163001 (2020). It shows that light exposure must be treated as a biological constraint, not just an imaging parameter. For the broader thesis logic, this helps because it defines photon dose as an experimental variable that can reshape the phenotype being measured.
 41. Yang, M. et al. To see and to know: the power of live imaging in illuminating and decoding biological complexity. *J. Genet. Genomics* (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it the thesis treats cell-cycle state as a core axis that must be resolved to interpret live phenotypes correctly.
 42. Gardel, M.L. et al. Mechanical integration of actin and adhesion dynamics in cell migration. *Annu. Rev. Cell Dev. Biol.* 26, 315–333 (2010). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. For the thesis, this is useful because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
 43. Jones, M.C. et al. Connections between the cell cycle, cell adhesion and the cytoskeleton. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20180227 (2019). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. Within the thesis framework, this helps because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
 44. Conboy, J.P. et al. How cytoskeletal crosstalk makes cells move. *Biophys. Rev.* 5, 021307 (2024). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this

supports the argument that it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.

45. Godard, B.G. et al. Combined effect of cell geometry and polarity domains determines the orientation of unequal division. *eLife* 10, e75639 (2021). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This supports the thesis because it supports using cytoskeletal and physiological signals as informative phenotypes rather than decorative channels.
46. Etienne-Manneville, S. et al. Microtubules in cell migration. *Annu. Rev. Cell Dev. Biol.* 29, 471–499 (2013). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. For this thesis, it is important because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
47. Sallee, M.D. et al. Apical PAR complex proteins protect against programmed epithelial assaults to create a continuous and functional intestinal lumen. *eLife* 10, e64437 (2021). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. This is valuable to the thesis because it supports the thesis focus on coupling state, structure, and function in the same imaging workflow.
48. Boutros, M. et al. Microscopy-based high-content screening. *Cell* 163, 1314–1325 (2015). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it helps justify multiplexed readouts when morphology alone is insufficient to define tissue behavior.
49. Scheeder, C. et al. Machine learning and image-based profiling in drug discovery. *Curr. Opin. Syst. Biol.* 10, 43–52 (2018). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. This strengthens the thesis argument because it connects reporter choice to the biological processes the thesis aims to quantify across scales.
50. Dugina, V. et al. Interaction of microtubules with F-actin in cell protrusions depends on F-actin bundling by fascin. *J. Cell Sci.* 122, 2791–2801 (2009). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. For the broader thesis logic, this helps because it

links the measured signal to interpretable structure or function, which is central to the thesis readout framework.

51. Lukinavičius, G. et al. Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat. Methods* 11, 731-733 (2014). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. This matters for the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
52. Akbarzadeh, M. et al. Morphological profiling by means of the Cell Painting assay enables identification of tubulin-targeting compounds. *Cell Chem. Biol.* 29, 1053–1064.e3 (2022). It describes a multiplexed dye-based assay that converts morphology into rich phenotypic fingerprints at scale. For the thesis, this is useful because it supports the thesis focus on coupling state, structure, and function in the same imaging workflow.
53. Hofbauer, P. et al. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. Within the thesis framework, this helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
54. Lewis-Israeli, Y.R. et al. Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. In the thesis context, this supports the argument that it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
55. Dalangin, R. et al. Far-red fluorescent genetically encoded calcium ion indicators. *Nat. Commun.* 16, 3318 (2025). It makes dynamic functional behavior measurable in living cells or tissues rather than inferring state from morphology alone. This supports the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
56. Mills, R.J. et al. Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. *Proc. Natl. Acad. Sci.*

- U. S. A. 114, E8372-E8381 (2017). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For this thesis, it is important because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
57. Zhang, Y. et al. Fast and sensitive GCaMP calcium indicators for imaging neural populations. *Nature* 615, 884-891 (2023). It makes dynamic functional behavior measurable in living cells or tissues rather than inferring state from morphology alone. This is valuable to the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
58. Dvinskikh, L. et al. Remote-refocusing light-sheet fluorescence microscopy enables 3D imaging of electromechanical coupling of hiPSC-derived and adult cardiomyocytes in co-culture. *Sci. Rep.* 13, 3342 (2023). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. In this dissertation, it helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
59. Hochbaum, D.R. et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat. Methods* 11, 825-833 (2014). It makes dynamic functional behavior measurable in living cells or tissues rather than inferring state from morphology alone. This strengthens the thesis argument because it connects reporter choice to the biological processes the thesis aims to quantify across scales.
60. Milosevic, M.M. et al. In Vitro Testing of Voltage Indicators: Archon1, ArcLightD, ASAP1, ASAP2s, ASAP3b, Bongwoori-Pos6, BeRST1, FlicR1, and Chi-VSFP-Butterfly. *eNeuro* 7, ENEURO.0060-20.2020 (2020). It makes dynamic functional behavior measurable in living cells or tissues rather than inferring state from morphology alone. For the broader thesis logic, this helps because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
61. Boutin, M.E. et al. A multiparametric calcium signal screening platform using iPSC-derived cortical neural spheroids. *SLAS Discov.* 27, 209–218 (2022). It uses engineered 3D models to show how tissue-level phenotype emerges under more

- realistic biological constraints. This matters for the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
62. Skardal, A. et al. Drug compound screening in single and integrated multi-organoid body-on-a-chip systems. *Biofabrication* 12, 025017 (2020). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. For the thesis, this is useful because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
63. Pocock, M.W. et al. Maturation of human cardiac organoids enables complex disease modeling and drug discovery. *Nat. Cardiovasc. Res.* 4, 821-840 (2025). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. Within the thesis framework, this helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
64. Tang, Q. et al. Morphological profiling for drug discovery in the era of deep learning. *Brief. Bioinform.* 25, bbae284 (2024). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. In the thesis context, this supports the argument that it supports choosing labels not only for brightness but also for biological fidelity over time.
65. Way, G.P. et al. Morphology and gene expression profiling provide complementary information for mapping cell state. *Cell Syst.* 13, 911–923.e9 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
66. Sen, O. et al. The live cell DNA stain SiR-Hoechst induces DNA damage responses and impairs cell cycle progression. *Sci. Rep.* 8, 7898 (2018). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For this thesis, it is important because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.

67. Tovar-Ferrero, O. et al. Measuring mitochondrial membrane potential. *EMBO J.* 44, 7334–7345 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it informs the thesis argument that signal design upstream of imaging strongly conditions what can be measured reliably.
68. Albert, L. et al. Nanobodies equipped with HaloTag variants enable rapid and straightforward one-step immunofluorescence lifetime multiplexing. *bioRxiv [Preprint]* (2026). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. In this dissertation, it helps because it helps compare trade-offs among dyes, genetic reporters, endogenous tagging, and delivery strategies.
69. Grimm, J.B. et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat. Methods* 14, 987-994 (2017). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. This strengthens the thesis argument because it supports choosing labels not only for brightness but also for biological fidelity over time.
70. Los, G.V. et al. HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem. Biol.* 3, 373-382 (2008). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. For the broader thesis logic, this helps because it frames reporter engineering as part of phenotype design rather than a downstream technical detail.
71. Keppler, A. et al. Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc. Natl. Acad. Sci.* 101, 9955-9959 (2004). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. This matters for the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
72. Kühn, S. et al. SNAP-tag2 for faster and brighter protein labeling. *Nat. Chem. Biol.* 21, 1754-1761 (2025). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. For the thesis, this is useful because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.

73. Cong, L. et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819-823 (2013). It advances genetic engineering strategies for building more precise and informative reporter systems. Within the thesis framework, this helps because it helps compare trade-offs among dyes, genetic reporters, endogenous tagging, and delivery strategies.
74. Nguyen, D.N. et al. Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. *Nat. Biotechnol.* 38, 44-49 (2020). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. In the thesis context, this supports the argument that it supports choosing labels not only for brightness but also for biological fidelity over time.
75. Cho, N.H. et al. OpenCell: Endogenous tagging for the cartography of human cellular organization. *Science* 375, eabi6983 (2022). It advances genetic engineering strategies for building more precise and informative reporter systems. This supports the thesis because it frames reporter engineering as part of phenotype design rather than a downstream technical detail.
76. Schmid-Burgk, J.L. et al. CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat. Commun.* 7, 12338 (2016). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. For this thesis, it is important because it shows that reporter and labeling design determine specificity, perturbation burden, and live-cell compatibility.
77. Willems, J. et al. ORANGE: A CRISPR/Cas9-based genome editing toolbox for epitope tagging of endogenous proteins in neurons. *PLOS Biol.* 18, e3000665 (2020). It advances genetic engineering strategies for building more precise and informative reporter systems. This is valuable to the thesis because it informs the thesis argument that signal design upstream of imaging strongly conditions what can be measured reliably.
78. Shalem, O. et al. Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. *Science* 343, 84-87 (2014). It advances genetic engineering strategies for building more precise and informative reporter systems. In this dissertation, it

- helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
79. Cockrell, A.S. et al. Gene delivery by lentivirus vectors. *Mol. Biotechnol.* 36, 184-204 (2007). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This strengthens the thesis argument because it supports choosing labels not only for brightness but also for biological fidelity over time.
 80. De Wannemaeker, L. et al. Standardization of Fluorescent Reporter Assays in Synthetic Biology across the Visible Light Spectrum. *ACS Synth. Biol.* 12, 3591-3607 (2023). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. For the broader thesis logic, this helps because it frames reporter engineering as part of phenotype design rather than a downstream technical detail.
 81. Naldini, L. et al. In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. *Science* 272, 263-267 (1996). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
 82. Pannell, D. et al. Silencing of gene expression: implications for design of retrovirus vectors. *Rev. Med. Virol.* 11, 205-217 (2001). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it informs the thesis argument that signal design upstream of imaging strongly conditions what can be measured reliably.
 83. Sadelain, M. et al. Safe harbours for the integration of new DNA in the human genome. *Nat. Rev. Cancer* 12, 51-58 (2012). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it helps compare trade-offs among dyes, genetic reporters, endogenous tagging, and delivery strategies.
 84. Awada, B. et al. Phenotypic screening with primary and human iPSC-derived neurons. In: Markossian, S., Grossman, A., Baskir, H., et al., editors. *Assay*

- Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this supports the argument that it informs the trade-off between throughput and physiological relevance that runs through the thesis.
85. Zingales, V. et al. The growing importance of three-dimensional models and microphysiological systems in the assessment of mycotoxin toxicity. *Toxins* 15, 422 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
86. Boukamp, P. et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106, 761-771 (1988). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For this thesis, it is important because it model choice sets the balance between biological realism, assay tractability, and experimental variability.
87. Rasheed, S. et al. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* 33, 1027-1033 (1974). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports the thesis discussion of why more predictive systems also create greater imaging and analysis demands.
88. Harper, J.M. et al. Primary Cell Culture as a Model System for Evolutionary Molecular Physiology. *Int. J. Mol. Sci.* 25, 7905 (2024). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it helps justify comparing robust cell lines with primary, iPSC-derived, and organoid models.
89. Lancaster, M.A. et al. Generation of cerebral organoids from human pluripotent stem cells. *Nat. Protoc.* 9, 2329-2340 (2014). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. This strengthens the thesis argument because it contributes practical

- infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
90. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872 (2007). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
 91. Gintant, G. et al. Use of human induced pluripotent stem cell-derived cardiomyocytes in preclinical cancer drug cardiotoxicity testing: a scientific statement from the American Heart Association. *Circ. Res.* 125, e75–e92 (2019). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it model choice sets the balance between biological realism, assay tractability, and experimental variability.
 92. Karakikes, I. et al. Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes: Insights Into Molecular, Cellular, and Functional Phenotypes. *Circ. Res.* 117, 80-88 (2015). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it supports the thesis discussion of why more predictive systems also create greater imaging and analysis demands.
 93. Zhao, Z. et al. Organoids. *Nat. Rev. Methods Primers* 2, 94 (2022). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. Within the thesis framework, this helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
 94. Cho, J. et al. From engineered heart tissue to cardiac organoid. *Theranostics* 12, 2758-2772 (2022). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. In the thesis context, this supports the argument that it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.

95. Gjorevski, N. et al. Bioengineering approaches to guide stem cell-based organogenesis. *Dev. Camb. Engl.* 141, 1794-1804 (2014). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it grounds the thesis move from simplified models toward more informative tissue-engineering systems.
96. Garreta, E. et al. Rethinking organoid technology through bioengineering. *Nat. Mater.* 20, 145–155 (2021). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. For this thesis, it is important because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
97. Joddar, B. et al. Engineering approaches for cardiac organoid formation and their characterization. *Transl. Res., Special Issue: Organoid Models in Translational Research* 250, 46-67 (2022). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. This is valuable to the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
98. Stirling, D.R. et al. CellProfiler Analyst 3.0: accessible data exploration and machine learning for image analysis. *Bioinformatics* 37, 3992-3994 (2021). It introduced a modular analysis environment for extracting quantitative cell features from microscopy images. In this dissertation, it helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
99. Serrano, E. et al. Reproducible image-based profiling with Pycytominer. *Nat. Methods* 22, 677-680 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This strengthens the thesis argument because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
100. Pearson, Y.E. et al. A statistical framework for high-content phenotypic profiling using cellular feature distributions. *Commun. Biol.* 5, 1409 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps

because it supports treating analysis choices as experimental variables that must remain visible and auditable.

101. Corbe, M. et al. Transfer learning for versatile and training free high content screening analyses. *Sci. Rep.* 13, 22599 (2023). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. This matters for the thesis because it robust segmentation, tracking, and feature extraction are prerequisites for comparable HT/HC phenotyping.
102. Seal, S. et al. Cell Painting: a decade of discovery and innovation in cellular imaging. *Nat. Methods* 22, 254-268 (2025). It describes a multiplexed dye-based assay that converts morphology into rich phenotypic fingerprints at scale. For the thesis, this is useful because it supports the thesis argument that quantitative extraction is core measurement infrastructure, not post-processing.
103. Ashesh, A. et al. MicroSplit: semantic unmixing of fluorescent microscopy data. *Nat. Methods* (2026). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. Within the thesis framework, this helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
104. Huang, X. et al. BINGO: a blind unmixing algorithm for ultra-multiplexing fluorescence images. *Bioinformatics* 40, btae052 (2024). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. In the thesis context, this supports the argument that it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
105. Ong, H.T. et al. Digitalized organoids: integrated pipeline for high-speed 3D analysis of organoid structures using multilevel segmentation and cellular topology. *Nat. Methods* 22, 1343-1354 (2025). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. This supports the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
106. Mukashyaka, P. et al. High-throughput deconvolution of 3D organoid dynamics at cellular resolution for cancer pharmacology with Cellos. *Nat.*

- Commun. 14, 8406 (2023). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. For this thesis, it is important because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
107. Andrés-San Román, J.A. et al. CartoCell, a high-content pipeline for 3D image analysis, unveils cell morphology patterns in epithelia. *Cell Rep. Methods* 3, 100597 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
108. Bozal, S.B. et al. Development of an automated 3D high content cell screening platform for organoid phenotyping. *SLAS Discov.* 29, 100182 (2024). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. In this dissertation, it helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
109. Albanese, A. et al. Multiscale 3D phenotyping of human cerebral organoids. *Sci. Rep.* 10, 21487 (2020). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. This strengthens the thesis argument because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
110. Schmidt, U. et al. Cell Detection with Star-Convex Polygons, in: Frangi, A.F., Schnabel, J.A., Davatzikos, C., Alberola-López, C., Fichtinger, G. (Eds.), *Medical Image Computing and Computer Assisted Intervention-MICCAI 2018*. Springer International Publishing, Cham, pp. 265-273 (2018). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it supports treating analysis choices as experimental variables that must remain visible and auditable.

111. Weigert, M. et al. Star-convex Polyhedra for 3D Object Detection and Segmentation in Microscopy, in: 2020 IEEE Winter Conference on Applications of Computer Vision (WACV). Presented at the 2020 IEEE Winter Conference on Applications of Computer Vision (WACV), pp. 3655-3662 (2020). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. This matters for the thesis because it robust segmentation, tracking, and feature extraction are prerequisites for comparable HT/HC phenotyping.
112. Stringer, C. et al. Cellpose3: one-click image restoration for improved cellular segmentation. *Nat. Methods* 22, 592-599 (2025). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. For the thesis, this is useful because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
113. Isensee, F. et al. nnU-Net: a self-configuring method for deep learning-based biomedical image segmentation. *Nat. Methods* 18, 203-211 (2021). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. Within the thesis framework, this helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
114. Ershov, D. et al. TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nat. Methods* 19, 829-832 (2022). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. In the thesis context, this supports the argument that it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
115. Bragantini, J. et al. Ultrack: pushing the limits of cell tracking across biological scales. *Nat. Methods* 22, 2423-2436 (2025). It develops tracking strategies that preserve temporal continuity and enable trajectory-level analysis. This supports the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.

116. Sugawara, K. et al. Tracking cell lineages in 3D by incremental deep learning. *eLife* 11, e69380 (2022). It develops tracking strategies that preserve temporal continuity and enable trajectory-level analysis. For this thesis, it is important because it robust segmentation, tracking, and feature extraction are prerequisites for comparable HT/HC phenotyping.
117. Booth, M.J. et al. Adaptive optical microscopy: The ongoing quest for a perfect image. *Light Sci. Appl.* 3 (2014). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports choosing imaging regimes by biological requirement rather than by nominal instrument performance.
118. Icha, J. et al. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017). It shows that light exposure must be treated as a biological constraint, not just an imaging parameter. In this dissertation, it helps because it defines photon dose as an experimental variable that can reshape the phenotype being measured.
119. Royer, L.A. et al. Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This strengthens the thesis argument because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
120. Power, R.M. et al. A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nat. Methods* 14, 360-373 (2017). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. For the broader thesis logic, this helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
121. Reynaud, E.G. et al. Guide to light-sheet microscopy for adventurous biologists. *Nat. Methods* 12, 30-34 (2015). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This matters for the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.

122. Sakaue-Sawano, A. et al. Genetically Encoded Tools for Optical Dissection of the Mammalian Cell Cycle. *Mol. Cell* 68, 626-640.e5 (2017). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For the thesis, this is useful because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
123. Treppe, X. et al. Physical forces during collective cell migration. *Nat. Phys.* 5, 426-430 (2009). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. Within the thesis framework, this helps because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
124. Lancaster, M.A. et al. Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373-379 (2013). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. In the thesis context, this supports the argument that it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
125. Galland, R. et al. 3D high-and super-resolution imaging using single-objective SPIM. *Nat. Methods* 12, 641-644 (2015). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This supports the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
126. Gustafsson, M.G.L. et al. Three-Dimensional Resolution Doubling in Wide-Field Fluorescence Microscopy by Structured Illumination. *Biophys. J.* 94, 4957-4970 (2008). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. For this thesis, it is important because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
127. York, A.G. et al. Instant super-resolution imaging in live cells and embryos via analog image processing. *Nat. Methods* 10, 1122-1126 (2013). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it contributes

practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.

128. Cabillic, M. et al. In-depth single molecule localization microscopy using adaptive optics and single objective light-sheet microscopy. *Nat. Commun.* 16, 8362 (2025). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. In this dissertation, it helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
129. Weigert, M. et al. Content-aware image restoration: pushing the limits of fluorescence microscopy. *Nat. Methods* 15, 1090-1097 (2018). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. This strengthens the thesis argument because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
130. Koh, S.-B. et al. A quantitative FastFUCCI assay defines cell cycle dynamics at a single-cell level. *J. Cell Sci.* 130, 512-520 (2017). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For the broader thesis logic, this helps because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
131. Zielke, N. et al. FUCCI sensors: powerful new tools for analysis of cell proliferation. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 469-487 (2015). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. This matters for the thesis because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
132. Gross, S.M. et al. Analysis and modeling of cancer drug responses using cell cycle phase-specific rate effects. *Nat. Commun.* 14, 3450 (2023). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For the thesis, this is useful because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
133. Hunter, M.V. et al. Mechanical confinement governs phenotypic plasticity in melanoma. *Nature* 1-11 (2025). It reports a study that sharpens how phenotype

can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs the thesis emphasis on photon budget, live compatibility, and adaptive acquisition in long-term imaging.

134. Barry, D.J. et al. Open source software for quantification of cell migration, protrusions, and fluorescence intensities. *J. Cell Biol.* 209, 163-180 (2015). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. In the thesis context, this supports the argument that it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
135. Bolado-Carrancio, A. et al. Periodic propagating waves coordinate RhoGTPase network dynamics at the leading and trailing edges during cell migration. *eLife* 9, e58165 (2020). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. This supports the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
136. Hino, N. et al. ERK-Mediated Mechanochemical Waves Direct Collective Cell Polarization. *Dev. Cell* 53, 646-660.e8 (2020). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For this thesis, it is important because it the thesis treats imaging design as a trade-off among dose, speed, depth, field of view, and information content.
137. Pittman, M. et al. Membrane ruffling is a mechanosensor of extracellular fluid viscosity. *Nat. Phys.* 18, 1112-1121 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports choosing imaging regimes by biological requirement rather than by nominal instrument performance.
138. Azzam, S. et al. A high-precision wound healing assay based on photosensitized culture substrates. *Sci. Rep.* 14, 9103 (2024). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it informs the thesis emphasis on photon budget, live compatibility, and adaptive acquisition in long-term imaging.

139. Hamazaki, N. et al. Retinoic acid induces human gastruloids with posterior embryo-like structures. *Nat. Cell Biol.* 26, 1790-1803 (2024). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. This strengthens the thesis argument because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
140. Mitrofanova, O. et al. Bioengineered human colon organoids with in vivo-like cellular complexity and function. *Cell Stem Cell* 31, 1175-1186.e7 (2024). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. For the broader thesis logic, this helps because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
141. Beghin, A. et al. Automated high-speed 3D imaging of organoid cultures with multi-scale phenotypic quantification. *Nat. Methods* 19, 881-892 (2022). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. This matters for the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
142. Moshksayan, K. et al. A microfluidic platform for culturing and high-content imaging of adult stem cell-derived organoids. *Sci. Rep.* 15, 40162 (2025). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. For the thesis, this is useful because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
143. Huisken, J. et al. Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305, 1007-1009 (2004). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs the thesis emphasis on photon budget, live compatibility, and adaptive acquisition in long-term imaging.
144. Yang, B. et al. DaXi-high-resolution, large imaging volume and multi-view single-objective light-sheet microscopy. *Nat. Methods* 19, 461-469 (2022).

It advances low-perturbation volumetric imaging for dynamic 3D biological systems. In the thesis context, this supports the argument that it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.

145. De Medeiros, G. et al. Multiscale light-sheet organoid imaging framework. *Nat. Commun.* 13, 4864 (2022). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This supports the thesis because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
146. Malin-Mayor, C. et al. Automated reconstruction of whole-embryo cell lineages by learning from sparse annotations. *Nat. Biotechnol.* 41, 44-49 (2023). It develops tracking strategies that preserve temporal continuity and enable trajectory-level analysis. For this thesis, it is important because it the thesis treats imaging design as a trade-off among dose, speed, depth, field of view, and information content.
147. Mau, A. et al. Fast widefield scan provides tunable and uniform illumination optimizing super-resolution microscopy on large fields. *Nat. Commun.* 12, 3077 (2021). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
148. Ronceray, N. et al. Wide-field fluorescence lifetime imaging of single molecules with a gated single-photon camera. *Light Sci. Appl.* 14, 258 (2025). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. In this dissertation, it helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
149. Laine, R. F. et al. High-fidelity 3D live-cell nanoscopy through data-driven enhanced super-resolution radial fluctuation. *Nature Methods* 20, 1949–1956 (2023). It reports a computational super-resolution strategy that improves how live-cell structures can be resolved from fluorescence image sequences under photon-limited conditions. This strengthens the thesis argument because it shows

how imaging regime and computational reconstruction can be matched to phenotype when live-cell assays are constrained by dose, speed, resolution, and volumetric sampling.

150. Elliott, A.D. et al. Confocal Microscopy: Principles and Modern Practices. Curr. Protoc. Cytom. 92, e68 (2020). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
151. Shimozawa, T. et al. Improving spinning disk confocal microscopy by preventing pinhole cross-talk for intravital imaging. Proc. Natl. Acad. Sci. U. S. A. 110, 3399-3404 (2013). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
152. Goswami, M. et al. simpleISM —A straight forward guide to upgrade from confocal to ISM. PLOS ONE 17, e0279378 (2022). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. For the thesis, this is useful because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
153. Schulz, O. et al. Resolution doubling in fluorescence microscopy with confocal spinning-disk image scanning microscopy. Proc. Natl. Acad. Sci. U. S. A. 110, 21000-21005 (2013). It expands the molecular labeling toolbox used to make specific biological processes visible in live cells. Within the thesis framework, this helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
154. Liang, Q. et al. High-fidelity tissue super-resolution imaging achieved with confocal2 spinning-disk image scanning microscopy. Light Sci. Appl. 14, 260 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this

- supports the argument that it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
155. Zunino, A. et al. Structured detection for simultaneous super-resolution and optical sectioning in laser scanning microscopy. *Nat. Photonics* 19, 888-897 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
 156. Bouchard, M.B. et al. Swept confocally-aligned planar excitation (SCAPE) microscopy for high-speed volumetric imaging of behaving organisms. *Nat. Photonics* 9, 113-119 (2015). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. For this thesis, it is important because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
 157. Sapoznik, E. et al. A versatile oblique plane microscope for large-scale and high-resolution imaging of subcellular dynamics. *eLife* 9, e57681 (2020). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This is valuable to the thesis because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
 158. Chang, B.-J. et al. Real-time multi-angle projection imaging of biological dynamics. *Nat. Methods* 18, 829-834 (2021). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it informs the thesis emphasis on photon budget, live compatibility, and adaptive acquisition in long-term imaging.
 159. Chen, B. et al. Resolution doubling in light-sheet microscopy via oblique plane structured illumination. *Nat. Methods* 19, 1419-1426 (2022). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. This strengthens the thesis argument because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.

160. Fadero, T.C. et al. LITE microscopy: Tilted light-sheet excitation of model organisms offers high resolution and low photobleaching. *J. Cell. Biol.* 217, 1869-1882 (2018). It shows that light exposure must be treated as a biological constraint, not just an imaging parameter. For the broader thesis logic, this helps because it defines photon dose as an experimental variable that can reshape the phenotype being measured.
161. Del Rosario, M. et al. PhotoFiTT: a quantitative framework for assessing phototoxicity in live-cell microscopy experiments. *Nat. Commun.* 16, 11401 (2025). It shows that light exposure must be treated as a biological constraint, not just an imaging parameter. This matters for the thesis because it defines photon dose as an experimental variable that can reshape the phenotype being measured.
162. Madhusoodanan, J. et al. Smart microscopes spot fleeting biology. *Nature* 614, 378-380 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it supports choosing imaging regimes by biological requirement rather than by nominal instrument performance.
163. Daetwyler, S. et al. Light-sheets and smart microscopy, an exciting future is dawning. *Commun. Biol.* 6, 1-11 (2023). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. Within the thesis framework, this helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
164. André, O. et al. Data-driven microscopy allows for automated context-specific acquisition of high-fidelity image data. *Cell Rep. Methods* 3 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this supports the argument that it helps justify matching modality to phenotype rather than seeking one universally optimal microscope.
165. Mahečić, D. et al. Event-driven acquisition for content-enriched microscopy. *Nat. Methods* 19, 1262-1267 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it informs how the thesis matches

- imaging regime to phenotype under constraints of dose, speed, depth, and duration.
166. Alvelid, J. et al. Event-triggered STED imaging. *Nat. Methods* 19, 1268-1275 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For this thesis, it is important because the thesis treats imaging design as a trade-off among dose, speed, depth, field of view, and information content.
 167. Ibrahim, K.A. et al. Self-driving microscopy detects the onset of protein aggregation and enables intelligent Brillouin imaging. *Nat. Commun.* 16, 6699 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports choosing imaging regimes by biological requirement rather than by nominal instrument performance.
 168. Shi, Y. et al. Smart lattice light-sheet microscopy for imaging rare and complex cellular events. *Nat. Methods* 21, 301-310 (2024). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. In this dissertation, it helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
 169. Lu, Z. et al. Virtual-scanning light-field microscopy for robust snapshot high-resolution volumetric imaging. *Nat. Methods* 20, 735-746 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This strengthens the thesis argument because it helps justify matching modality to phenotype rather than seeking one universally optimal microscope.
 170. Belthangady, C. et al. Applications, promises, and pitfalls of deep learning for fluorescence image reconstruction. *Nat. Methods* 16, 1215-1225 (2019). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. For the broader thesis logic, this helps because it supports the argument that acquisition strategy is part of phenotype design in live-cell assays.
 171. Engler, A.J. et al. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* 126, 677-689 (2006). It develops tracking strategies that

preserve temporal continuity and enable trajectory-level analysis. This matters for the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.

172. Hannezo, E. et al. Mechanochemical Feedback Loops in Development and Disease. *Cell* 178, 12-25 (2019). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it supports the thesis view of microenvironments as programmable control layers rather than passive culture settings.
173. Sonn, A. et al. 3D fabrication of artificial cell microenvironments for mechanobiology. *Curr. Opin. Biomed. Eng.* 33, 100574 (2025). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. Within the thesis framework, this helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
174. Barthes, J. et al. Cell microenvironment engineering and monitoring for tissue engineering and regenerative medicine: the recent advances. *BioMed Res. Int.* 2014, 921905 (2014). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In the thesis context, this supports the argument that it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
175. Torchia, E. et al. Fabrication of cell culture hydrogels by robotic liquid handling automation for high-throughput drug testing. *Commun. Eng.* 4, 222 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
176. Enrico, A. et al. LigHTS: Massively Parallel Biomimetic Photo-Functionalization for Imaging-Based Ultra-High-Throughput Screening. *bioRxiv [Preprint]* (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For this thesis, it is important because it engineered boundary conditions shape phenotype and enable causal perturbation in tissue-engineering assays.

177. Ho, C.M.B. et al. Femtosecond-Laser-Based 3D Printing for Tissue Engineering and Cell Biology Applications. *ACS Biomater. Sci. Eng.* 3, 2198-2214 (2017). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports the thesis view of microenvironments as programmable control layers rather than passive culture settings.
178. Qin, D. et al. Soft lithography for micro-and nanoscale patterning. *Nat. Protoc.* 5, 491-502 (2010). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In this dissertation, it helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
179. Théry, M. et al. Micropatterning as a tool to decipher cell morphogenesis and functions. *J. Cell Sci.* 123, 4201-4213 (2010). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This strengthens the thesis argument because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
180. Piunti, C. et al. Microfluidic loading of verteporfin into extracellular vesicles for neuroblastoma therapy. *Lab. Chip* 25, 1718-1727 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
181. Bugaj, L.J. et al. High-throughput multicolor optogenetics in microwell plates. *Nat. Protoc.* 14, 2205-2228 (2019). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
182. Supatto, W. et al. Advances in multiphoton microscopy for imaging embryos. *Curr. Opin. Genet. Dev.* 21, 538-548 (2011). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it supports the thesis view of

microenvironments as programmable control layers rather than passive culture settings.

183. Trepap, X. et al. Mesoscale physical principles of collective cell organization. *Nat. Phys.* 14, 671-682 (2018). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
184. Bera, K. et al. Extracellular fluid viscosity enhances cell migration and cancer dissemination. *Nature* 611, 365-373 (2022). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. In the thesis context, this supports the argument that it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
185. Chen, C.S. et al. Geometric control of cell life and death. *Science* 276, 1425-1428 (1997). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it supports causal phenotyping by linking external control to measurable biological response.
186. Dike, L.E. et al. Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro Cell. Dev. Biol. Anim.* 35, 441-448 (1999). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For this thesis, it is important because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
187. Mooney, D.J. et al. Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix. *J. Cell Sci.* 108, 2311-2320 (1995). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This is valuable to the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
188. Zhu, X. et al. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the

- retinoblastoma protein. *J. Cell Biol.* 133, 391-403 (1996). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. In this dissertation, it helps because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
189. Théry, M. et al. The extracellular matrix guides the orientation of the cell division axis. *Nat. Cell Biol.* 7, 947-953 (2005). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This strengthens the thesis argument because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
190. Heinrich, M.A. et al. Size-dependent patterns of cell proliferation and migration in freely-expanding epithelia. *eLife* 9, e58945 (2020). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. For the broader thesis logic, this helps because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
191. Devany, J. et al. Epithelial tissue confinement inhibits cell growth and leads to volume-reducing divisions. *Dev. Cell* 58, 1462-1476.e8 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it engineered boundary conditions shape phenotype and enable causal perturbation in tissue-engineering assays.
192. Isenberg, B.C. et al. Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. *Biophys. J.* 97, 1313-1322 (2009). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For the thesis, this is useful because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
193. Hartman, C.D. et al. Vascular smooth muscle cell durotaxis depends on extracellular matrix composition. *Proc. Natl. Acad. Sci.* 113, 11190-11195 (2016). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. Within the thesis framework, this helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.

194. Vassaux, M. et al. A Biophysical Model for Curvature-Guided Cell Migration. *Biophys. J.* 117, 1136-1144 (2019). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In the thesis context, this supports the argument that it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
195. Luciano, M. et al. Cell monolayers sense curvature by exploiting active mechanics and nuclear mechanoadaptation. *Nat. Phys.* 17, 1382-1390 (2021). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This supports the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
196. Leclech, C. et al. Topography-induced large-scale antiparallel collective migration in vascular endothelium. *Nat. Commun.* 13, 2797 (2022). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. For this thesis, it is important because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
197. Kalukula, Y. et al. The actin cortex acts as a mechanical memory of morphology in confined migrating cells. *Nat. Phys.* 21, 1451-1461 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
198. Di Meglio, I. et al. Pressure and curvature control of the cell cycle in epithelia growing under spherical confinement. *Cell Rep.* 40, 111227 (2022). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. In this dissertation, it helps because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.
199. Karzbrun, E. et al. Human neural tube morphogenesis in vitro by geometric constraints. *Nature* 599, 268-272 (2021). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated.

This strengthens the thesis argument because it helps connect fabrication choices to interpretable changes in cell state, migration, or morphogenesis.

200. De Medeiros, G. et al. Cell and tissue manipulation with ultrashort infrared laser pulses in light-sheet microscopy. *Sci. Rep.* 10, 1942 (2020). It advances low-perturbation volumetric imaging for dynamic 3D biological systems. For the broader thesis logic, this helps because it informs how the thesis matches imaging regime to phenotype under constraints of dose, speed, depth, and duration.
201. Guglielmi, G. et al. An Optogenetic Method to Modulate Cell Contractility during Tissue Morphogenesis. *Dev. Cell* 35, 646-660 (2015). It makes dynamic functional behavior measurable in living cells or tissues rather than inferring state from morphology alone. This matters for the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
202. Izquierdo, E. et al. Guided morphogenesis through optogenetic activation of Rho signalling during early *Drosophila* embryogenesis. *Nat. Commun.* 9, 2366 (2018). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
203. Marshall, A.R. et al. Two-photon cell and tissue level laser ablation methods to study morphogenetic biomechanics. *Methods Mol. Biol.* 2438, 217-230 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
204. Hu, X. et al. Advances in micropatterning technology for mechanotransduction research. *Mechanobiol. Med.* 2, 100066 (2024). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In the thesis context, this supports the argument that it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.

205. Miklosic, G. et al. Engineering complex tissue-like microenvironments with biomaterials and biofabrication. *Trends Biotechnol.* 42, 1241–1257 (2024). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This supports the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
206. Truong, H.D. et al. Strategies to control cellular spatial organization in microphysiological systems. *Microsyst. Nanoeng.* 12, 85 (2026). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For this thesis, it is important because it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
207. McBeath, R. et al. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* 6, 483–495 (2004). It develops tracking strategies that preserve temporal continuity and enable trajectory-level analysis. This is valuable to the thesis because it supports the thesis view of microenvironments as programmable control layers rather than passive culture settings.
208. Li, Y. et al. Reciprocity of cell mechanics with extracellular stimuli: emerging opportunities for translational medicine. *Small* 18, e2107305 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
209. Wang, H. et al. Patterning meets gels: advances in engineering functional gels at micro/nanoscales for soft devices. *J. Polym. Sci.* 60, 2679–2700 (2022). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This strengthens the thesis argument because it helps connect fabrication choices to interpretable changes in cell state, migration, or morphogenesis.
210. Bril, M. et al. Stimuli-responsive materials: a smart way to study dynamic cell responses. *Smart Mater. Med.* 3, 257–273 (2022). It reports a study that

sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it supports causal phenotyping by linking external control to measurable biological response.

211. Davletshin, E. et al. Optogenetic approaches for neural tissue regeneration: a review of basic optogenetic principles and target cells for therapy. *Neural Regen. Res.* 21, 521–533 (2026). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This matters for the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
212. Obenaus, A.M. et al. (De)form and function: measuring cellular forces with deformable materials and deformable structures. *Adv. Healthc. Mater.* 9, 1901454 (2020). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the thesis, this is useful because it supports the thesis view of microenvironments as programmable control layers rather than passive culture settings.
213. Fu, J. et al. Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nat. Methods* 7, 733–736 (2010). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
214. Oakes, P.W. et al. Geometry regulates traction stresses in adherent cells. *Biophys. J.* 107, 825–833 (2014). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In the thesis context, this supports the argument that it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
215. Melero, C. et al. Light-Induced Molecular Adsorption of Proteins Using the PRIMO System for Micro-Patterning to Study Cell Responses to Extracellular Matrix Proteins. *JoVE* (2019). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This supports the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.

216. Van der Putten, C. et al. Protein Micropatterning in 2.5D: An Approach to Investigate Cellular Responses in Multi-Cue Environments. *ACS Appl. Mater. Interfaces* 13, 25589-25598 (2021). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For this thesis, it is important because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
217. Batalov, I. et al. Photopatterned biomolecule immobilization to guide three-dimensional cell fate in natural protein-based hydrogels. *Proc. Natl. Acad. Sci.* 118, e2014194118 (2021). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
218. Bretherton, R.C. et al. The art of engineering biomimetic cellular microenvironments. *ACS Biomater. Sci. Eng.* 7, 3997–4008 (2021). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. In this dissertation, it helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
219. Tian, C. et al. Light controlled biomaterials for regulating cell migration and differentiation. *Smart Mater. Med.* 3, 209–216 (2022). It resolves migration as a dynamic, mechanically regulated phenotype rather than a simple endpoint displacement. This strengthens the thesis argument because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
220. Enrico, A. et al. 3D Microvascularized Tissue Models by Laser-Based Cavitation Molding of Collagen. *Adv. Mater.* 34, 2109823 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. For the broader thesis logic, this helps because it supports causal phenotyping by linking external control to measurable biological response.
221. Martinelli, C. et al. A 3D millifluidic model of a dermal perivascular microenvironment on a chip. *Lab. Chip* 25, 423-439 (2025). It shows that externally imposed geometry or mechanics can actively shape cell behavior and

- state. This matters for the thesis because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
222. Kumar, S. et al. Image-guided optogenetic spatiotemporal tissue patterning using μ PatternScope. *Nat. Commun.* 15, 10469 (2024). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For the thesis, this is useful because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
223. Pinheiro, D. et al. Mechanical force-driven adherens junction remodeling and epithelial dynamics. *Dev. Cell* 47, 3–19 (2018). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
224. Fox, Z.R. et al. Enabling reactive microscopy with MicroMator. *Nat. Commun.* 13, 2199 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In the thesis context, this supports the argument that it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
225. Crossley, R.M. et al. Modeling the extracellular matrix in cell migration and morphogenesis: a guide for the curious biologist. *Front. Cell Dev. Biol.* 12, 1354132 (2024). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This supports the thesis because it links the measured signal to interpretable structure or function, which is central to the thesis readout framework.
226. Di Gravina, G.M. et al. Computer-aided engineering and additive manufacturing for bioreactors in tissue engineering: State of the art and perspectives. *Biophys. Rev.* 4, 031303 (2023). It shows how learning-based analysis can extend what is measurable from microscopy data when properly validated. For this thesis, it is important because it engineered boundary conditions shape phenotype and enable causal perturbation in tissue-engineering assays.
227. Keshavanarayana, P. et al. Leveraging computational modeling to explore epithelial and endothelial cell monolayer mechanobiology. *Trends Cell Biol.* 35,

- 799-813 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This is valuable to the thesis because it supports the thesis view of microenvironments as programmable control layers rather than passive culture settings.
228. Su, W. et al. Numerical models for organ-on-a-chip: A systematic review and analyses. *Biomicrofluidics* 19, 041501 (2025). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. In this dissertation, it helps because it informs how geometry, mechanics, and external modulation can be integrated with imaging readouts.
229. Doyle, A.D. et al. Cell-extracellular matrix dynamics. *Phys. Biol.* 19, 021002 (2022). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This strengthens the thesis argument because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
230. Li, Y. et al. 3D micropattern force triggers YAP nuclear entry by transport across nuclear pores and modulates stem cells paracrine. *Natl. Sci. Rev.* 10, nwad165 (2023). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For the broader thesis logic, this helps because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
231. Wang, T.-C. et al. Matrix stiffness drives drop like nuclear deformation and lamin A/C tension-dependent YAP nuclear localization. *Nat. Commun.* 15, 10151 (2024). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. This matters for the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
232. Toro-Nahuelpan, M. et al. Tailoring cryo-electron microscopy grids by photo-micropatterning for in-cell structural studies. *Nat. Methods* 17, 50-54 (2020). It shows that externally imposed geometry or mechanics can actively shape cell behavior and state. For the thesis, this is useful because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.

233. Bril, M. et al. Shape-Morphing Photoresponsive Hydrogels Reveal Dynamic Topographical Conditioning of Fibroblasts. *Adv. Sci.* 10, 2303136 (2023). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. Within the thesis framework, this helps because it shows how engineered boundary conditions can be used to provoke and interpret phenotype in a controlled way.
234. Legnini, I. et al. Spatiotemporal, optogenetic control of gene expression in organoids. *Nat. Methods* 20, 1544-1552 (2023). It uses engineered 3D models to show how tissue-level phenotype emerges under more realistic biological constraints. In the thesis context, this supports the argument that it grounds the thesis move toward complex 3D tissue-engineering models where imaging and analysis constraints become central.
235. Boehm, U. et al. QUAREP-LiMi: a community endeavor to advance quality assessment and reproducibility in light microscopy. *Nat. Methods* 18, 1423-1426 (2021). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This supports the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
236. Kiepas, A. et al. Optimizing live-cell fluorescence imaging conditions to minimize phototoxicity. *J. Cell Sci.* 133, jcs242834 (2020). It shows that light exposure must be treated as a biological constraint, not just an imaging parameter. For this thesis, it is important because it defines photon dose as an experimental variable that can reshape the phenotype being measured.
237. Linkert, M. et al. Metadata matters: access to image data in the real world. *J. Cell Biol.* 189, 777-782 (2010). It argues that image data remain interpretable only when metadata, standards, and reuse infrastructure are built in. This is valuable to the thesis because it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.
238. Sarkans, U. et al. REMBI: Recommended Metadata for Biological Images—enabling reuse of microscopy data in biology. *Nat. Methods* 18, 1418-1422 (2021). It argues that image data remain interpretable only when metadata, standards, and reuse infrastructure are built in. In this dissertation, it helps because

it supports the thesis claim that interoperable formats, metadata, and provenance are required for reusable phenotyping data.

239. Chiron, L. et al. CyberSco.Py an open-source software for event-based, conditional microscopy. *Sci. Rep.* 12, 11579 (2022). It reports a study that sharpens how phenotype can be measured, interpreted, or controlled in imaging-based biology. This strengthens the thesis argument because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
240. Di Sante, M. et al. CALIPERS: Cell-cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* [Preprint] (2025). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For the broader thesis logic, this helps because it supports minimum operational standards for transferable phenotyping workflows.
241. Stringer, C. et al. Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* 18, 100-106 (2021). It advances segmentation as a prerequisite for extracting consistent measurements from microscopy data. This matters for the thesis because it contributes practical infrastructure for extracting, organizing, or validating comparable phenotypes at scale.
242. Zimmermann, J. et al. Bioimage analysis for multiplexed FUCCI acquisitions powered by deep learning. *npj Imaging* 4, 27 (2026). It makes cell-cycle state optically tractable so temporal behavior can be aligned to phase transitions. For the thesis, this is useful because it supports phase-resolved phenotyping and the explicit assignment of temporal state in live imaging.

2 Chapter: FUCCIsmart

1. Théry, M. *et al.* The extracellular matrix guides the orientation of the cell division axis. *Nat. Cell Biol.* 7, 947-953 (2005). Establishes that ECM geometry can instruct the division axis, linking external constraints to mitotic mechanics. Provides a concrete mechanistic bridge between engineered microenvironments

- and tissue-scale architecture. Motivates treating mitosis as an information-dense window rather than a background event.
2. Lechler, T. & Mapelli, M. Spindle positioning and its impact on vertebrate tissue architecture and cell fate. *Nat. Rev. Mol. Cell Biol.* 22, 691-708 (2021). Synthesizes vertebrate spindle-positioning mechanisms and their regulation by epithelial/extracellular cues. Frames spindle positioning as a determinant of tissue cohesion, organization, and fate decisions. Supports prioritizing division readouts when interpreting phenotypes in engineered models.
 3. Morin, X. & Bellaïche, Y. Mitotic spindle orientation in asymmetric and symmetric cell divisions during animal development. *Dev. Cell* 21, 102-119 (2011). Reviews how spindle orientation interfaces with polarity and developmental programs across models. Clarifies why orientation errors can propagate into altered morphogenesis and fate outcomes. Reinforces the need for imaging strategies that resolve mitotic geometry rather than only phase timing.
 4. Noatynska, A., Gotta, M. & Meraldi, P. Mitotic spindle (DIS)orientation and DISease: cause or consequence? *J. Cell Biol.* 199, 1025-1035 (2012). Connects spindle misorientation to disease phenotypes while critically discussing causality and pleiotropy. Emphasizes that “spindle defects” often sit at the intersection of multiple cellular processes. Grounds the biomedical relevance of accurate mitotic phenotyping.
 5. Kline-Smith, S. L. & Walczak, C. E. Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. *Mol. Cell* 15, 317-327 (2004). Highlight microtubule dynamics as the organizing principle behind spindle formation and function. Supports the view of the spindle as transient, highly structured, and inherently 3D. Justifies volumetric sampling during mitosis when tubulin architecture is the endpoint.
 6. Siller, K. H. & Doe, C. Q. Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* 11, 365-374 (2009). Summarizes conserved pathways that couple polarity cues to spindle orientation in asymmetric division. Establishes that orientation is regulated and interpretable, not merely stochastic variability. Provides mechanistic context for using spindle organization/positioning as a phenotyping readout.

7. Fucci (Fluorescent Ubiquitination-based Cell-cycle Indicator). Provides a practical overview of FUCCI constructs, components, and usage. Useful for defining the tool family at an implementation level (reagents/variants). Not a primary source for mechanistic claims, but appropriate for technology description.
8. Zielke, N. & Edgar, B. A. FUCCI sensors: powerful new tools for analysis of cell proliferation. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 469-487 (2015). Reviews FUCCI as a broadly deployable approach for live cell-cycle state annotation. Discusses interpretability limits and design constraints relevant to imaging-based phenotyping. Supports cell-cycle context as a key covariate in high-content measurements.
9. Koh, S.-B. *et al.* A quantitative FastFUCCI assay defines cell-cycle dynamics at a single-cell level. *J. Cell Sci.* 130, 512-520 (2017). Demonstrates a quantitative FUCCI-based workflow for resolving single-cell-cycle dynamics under perturbation. Shows that FUCCI readouts can be operationalized for automated, phase-resolved decisions. Supports building threshold/gate logic on top of cell-cycle reporters.
10. Sakaue-Sawano, A. *et al.* Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487-498 (2008). Foundational work establishing live cell-cycle visualization via regulated degradation tags. Defines the canonical mapping between fluorescent state and cycle progression. Serves as the baseline against which newer spectral or control-oriented variants can be positioned.
11. Marcus, J. M., Burke, R. T., DeSisto, J. A., Landesman, Y. & Orth, J. D. Longitudinal tracking of single live cancer cells to understand cell-cycle effects of the nuclear export inhibitor, selinexor. *Sci. Rep.* 5, 14391 (2015). Illustrates phase-dependent responses and fates under drug treatment using longitudinal single-cell tracking. Demonstrates how mixing phases can confound phenotypic interpretation in perturbation experiments. Reinforces cell-cycle-aware readouts as essential for causal inference.
12. Chai, F., Cheng, D., Nasu, Y., Terai, T. & Campbell, R. E. Maximizing the performance of protein-based fluorescent biosensors. *Biochem. Soc. Trans.* 51, 1585-1595 (2023). Reviews strategies to improve biosensor performance including brightness, kinetics, photostability, and multiplexing compatibility.

Frames spectral crowding as a systems-level constraint in modern fluorescence experiments. Supports “spectral economy” as a legitimate engineering design objective.

13. Qian, Y., Celiker, O. T., Wang, Z., Guner-Ataman, B. & Boyden, E. S. Temporally multiplexed imaging of dynamic signaling networks in living cells. *Cell* 186, 5656-5672.e21 (2023). Introduces temporally multiplexed imaging to expand multiplex capacity using time-domain encoding. Demonstrates a credible alternative route around spectral bottlenecks. Provides contrast for approaches that instead reallocate channels to reduce integration overhead.
14. Di Sante, M. *et al.* CALIPERS: Cell-cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* (2024). Presents a framework that integrates cell-cycle sensing with acquisition strategy and analysis for phenotyping. Positions cell-cycle awareness as an organizing principle for experimental design rather than a post hoc annotation. Provides the context for CC-aware multiplexing and adaptive imaging logic.
15. Petry, S. Mechanisms of mitotic spindle assembly. *Annu. Rev. Biochem.* 85, 659-683 (2016) Provides a mechanistic taxonomy of spindle assembly modules and their biophysical roles. Supports spindle morphology as a meaningful endpoint tightly coupled to function. Reinforces mitosis as a high-value interval for concentrated high-resolution imaging.
16. Oreopoulos, J., Berman, R. & Browne, M. Spinning-disk confocal microscopy: present technology and future trends. In *Methods in Cell Biology* 123, 153-175 Summarizes spinning-disk confocal principles and performance trade-offs for live imaging. Provides the technical basis for choosing SD when short, high-information volumetric bursts are needed. Anchors the rationale for confocal z-stacks as a targeted modality rather than a continuous schedule.
17. Chen, B. *et al.* Resolution doubling in light-sheet microscopy via oblique plane structured illumination. *Nat. Methods* 19, 1419-1426 (2022). Shows how advanced volumetric imaging can improve resolution while controlling dose via structured illumination and reconstruction. Exemplifies the general principle that information efficiency, not raw frame rate, governs long-term live imaging feasibility. Supports photon-budgeted strategies for 3D observation.

18. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017). Defines mechanisms and practical mitigations for phototoxicity in live fluorescence experiments. Establishes illumination as a biological perturbation that must be managed as a first-class experimental variable. Supports adaptive acquisition concepts that minimize dose outside critical windows.
19. GraphPad Software. GraphPad Prism. Software reference documenting the environment used for statistics and plotting. Improves reproducibility by specifying analysis tooling. Appropriate for methods-level attribution rather than biological justification.

3 Chapter: A Vertically Integrated System for Tracking and Assessing CC-Aware Phenotypes

1. Bray, F. et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* 74, 229-263 (2024). Global epidemiology report quantifying worldwide cancer incidence and mortality. Cited to substantiate the clinical-scale burden motivating metastasis-focused phenotyping
2. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9, 239-252 (2009). Review outlining how stromal, immune, and ECM contexts shape metastatic progression. Leveraged to anchor the argument that dissemination is microenvironment-regulated beyond genetics.
3. Paul, C. D., Mistriotis, P. & Konstantopoulos, K. Cancer cell motility: lessons from migration in confined spaces. *Nat. Rev. Cancer* 17, 131-140 (2017). Synthesis of migration modes and constraints in physically confined microenvironments. It invokes confinement as a primary control axis for motility phenotypes.
4. Denais, C. M. et al. Nuclear envelope rupture and repair during cancer cell migration. *Science* 352, 353-358 (2016). Experimental evidence that confined migration can rupture the nuclear envelope. We referenced it to motivate nuclear integrity as a mechanistic vulnerability under confinement.

5. Raab, M. et al. ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. *Science* 352, 359-362 (2016). Shows ESCRT-III-mediated repair of nuclear envelope ruptures during migration. Drawn upon to contextualize repair pathways that limit damage during confined transit.
6. Gupta, V. & Chaudhuri, O. Mechanical regulation of cell-cycle progression and division. *Trends Cell Biol.* 32, 773-785 (2022). Review summarizing how forces and mechanics regulate checkpoints and division. Applied to support the premise that mechanics can gate cell-cycle progression.
7. Hunter, M. V. et al. Mechanical confinement governs phenotypic plasticity in melanoma. *Nature* (2025). Demonstrates confinement-driven phenotypic plasticity and state remodeling in melanoma. Cited to connect confinement to durable state changes relevant to drug response and fate.
8. Justus, C. R. et al. Transwell In Vitro Cell Migration and Invasion Assays. *Methods Mol. Biol.* 2644, 349-359 (2023). Protocol-focused chapter describing transwell migration and invasion assays. Included to position transwells as standard migration readouts typically lacking CC dynamics.
9. Riss, T. L. et al. Cell Viability Assays. In *Assay Guidance Manual* (2004). Reference overview of viability endpoints and assay design considerations. Cited to frame how common endpoints compress dynamic biology into snapshots.
10. Liang, C.-C., Park, A. Y. & Guan, J.-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.* 2, 329-333 (2007). Canonical protocol for the scratch/wound-healing migration assay. We referenced to illustrate the migration-proliferation decoupling in standard 2D assays.
11. Mark, C. et al. Collective forces of tumor spheroids in three-dimensional biopolymer networks. *eLife* (2020). Quantifies forces and mechanics of tumor spheroids embedded in 3D matrices. Employed to motivate 3D relevance while noting that CC staging is rarely tracked live.
12. Salic, A. & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci.* 105, 2415-2420 (2008).

- Introduces EdU chemistry for labeling nascent DNA synthesis. Cited to establish EdU as an endpoint S-phase proxy rather than a dynamic CC reporter.
13. Pereira, P. D. et al. Quantification of cell-cycle kinetics by EdU-coupled-fluorescence-intensity analysis. *Oncotarget* 8, 40514-40532 (2017). EdU intensity-based approach to infer cell-cycle kinetics from fixed samples. We used it emphasize kinetic inference without longitudinal single-cell tracking.
 14. Discher, D. E., Janmey, P. & Wang, Y.-L. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310, 1139-1143 (2005). Seminal work establishing stiffness as a regulator of cell behavior. Cited to ground stiffness as a canonical mechanical variable shaping phenotype.
 15. Janmey, P. A. et al. Stiffness sensing by cells. *Physiol. Rev.* 100, 695-724 (2020). Comprehensive review of mechanosensing pathways and stiffness response. Drawn on to support mechanistic plausibility for stiffness-linked proliferation/migration programs.
 16. Vogel, V. & Sheetz, M. Local force and geometry sensing regulate cell functions. *Nat. Rev. Mol. Cell Biol.* 7, 265-275 (2006). Review describing force/geometry sensing and downstream cellular responses. It positions geometry as an instructive cue rather than a boundary artifact.
 17. Chen, C. S. et al. Geometric control of cell life and death. *Science* 276, 1425-1428 (1997).
Classic demonstration that adhesion geometry regulates survival and proliferation decisions.
Cited to establish historical precedent for geometry-controlled fate logic.
 18. Ingber, D. E. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J. Cell Sci.* 104, 613-627 (1993). Conceptual framework linking cytoskeletal prestress to mechano-regulated function, the mechanistic worldview underpinning geometry-tension coupling.
 19. Pathak, A. & Kumar, S. Independent regulation of tumor cell migration by matrix stiffness and confinement. *Proc. Natl. Acad. Sci.* 109, 10334-10339 (2012). Separates stiffness-driven and confinement-driven control of migration. Cited to justify treating stiffness and confinement as distinct experimental axes.

20. Wang, N., Butler, J. P. & Ingber, D. E. Mechano-transduction across the cell surface and through the cytoskeleton. *Science* 260, 1124-1127 (1993). Foundational synthesis of how forces are transmitted across the cell surface and the cytoskeleton. It supports the physical pathway from ECM constraints to intracellular responses.
21. Butcher, D. T. et al. A tense situation: forcing tumor progression. *Nat. Rev. Cancer* 9, 108-122 (2009). Review on how tissue mechanics and ECM remodeling promote tumor progression. Cited to connect physical microenvironments to malignant phenotypes.
22. Dike, L. E. et al. Geometric control of switching between growth, apoptosis, and differentiation... *Vitro Cell Dev Biol Anim* 35, 441-448 (1999). Micropatterning study showing geometry-driven switching among fate programs. We referenced to motivate patterned substrates as tunable fate-selection tools.
23. Huang, S. et al. Control of cyclin D1, p27(Kip1), and cell-cycle progression... *Mol. Biol. Cell* 9, 3179-3193 (1998). Links shape/tension to cell-cycle regulators in endothelial cells. Cited to connect geometry to canonical CC machinery through cytoskeletal tension.
24. Desmaison, A. et al. Mechanical stress impairs mitosis progression in multicellular tumor spheroids. *PLOS ONE* 8, e80447 (2013). Shows that stress in spheroids perturbs mitotic progression. Cited to motivate mitotic disruption as a plausible confinement-associated outcome.
25. Roca-Cusachs, P. et al. Micropatterning... coupling between nuclear volume in G1 and proliferation. *Biophys. J.* 94, 4984-4995 (2008). Micropatterning reveals nuclear volume scaling with G1 and proliferation competence. In this view, nuclear morphology is seen as a CC-linked readout under geometry control.
26. Versaevel, M. et al. Spatial coordination between cell and nuclear shape within micropatterned endothelial cells. *Nat. Commun.* 3, 671 (2012). Quantifies coordinated cell-nucleus shape remodeling on patterns. Cited to justify nucleus-aware morphology metrics as mechanistically meaningful.
27. Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487-498 (2008). Introduces FUCCI for live,

- phase-resolved cell-cycle visualization. It grounds live CC reporting as the enabling layer for phase-aware phenotyping.
28. Koh, S.-B. et al. A quantitative FastFUCCI assay defines cell-cycle dynamics at a single-cell level. *J. Cell Sci.* 130, 512-520 (2017). Presents quantitative FUCCI readouts for single-cell-cycle timing. Cited to support quantitative phase calling and CC timing extraction.
 29. Melero, C. et al. Light-induced molecular adsorption of proteins using the PRIMO system... *J. Vis. Exp.* (2019). Protocol for PRIMO-based protein micropatterning to study ECM responses. It justifies photopatterning as a reproducible route to engineered islands.
 30. Toro-Nahuelpan, M. et al. Tailoring cryo-EM grids by photo-micropatterning... *Nat. Methods* 17, 50-54 (2020). Applies photopatterning to enable precise spatial control for in-cell studies. Cited to reinforce that micropatterning demands reliable registration workflows.
 31. Schmidt, U. et al. Cell detection with star-convex polygons. In *MICCAI* (2018) (pp. 265-273). Springer International Publishing. Introduces StarDist segmentation using star-convex polygon representations. Cited to support modern segmentation foundations for scalable quantitative imaging.
 32. Stringer, C. et al. Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* 18, 100-106 (2021). Generalist segmentation method designed to reduce retraining burden. It supports algorithmic options for robust segmentation across conditions.
 33. Tinevez, J.-Y. et al. TrackMate: an open and extensible platform for single-particle tracking. *Methods* 115, 80-90 (2017). Open tracking framework broadly used for particle and cell tracking. Cited to justify standard tracking infrastructure for longitudinal motility analysis.
 34. Sante, M. D. et al. CALIPERS: Cell-cycle-aware live imaging for phenotyping experiments and regeneration studies. *Preprint* (2025). Describes the CC-aware imaging framework underpinning CALIPERS. It establishes the platform baseline that is extended toward confinement assays.
 35. Chung, J. et al. Nuclear import of hTERT requires... *J. Cell Sci.* 125, 2684-2697 (2012). Mechanistic study of nuclear import requirements and regulation. Cited

- to contextualize nuclear localization variability as a biologically plausible bottleneck.
36. Skowicki, M. et al. Nanoassemblies designed for efficient nuclear targeting. *Adv. Drug Deliv. Rev.* 211, 115354 (2024). Review on engineered strategies and constraints for nuclear targeting. Used to support the broader context that nuclear delivery/localization is nontrivial.
 37. Marcus, J. M. et al. Longitudinal tracking... selinexor. *Sci. Rep.* 5, 14391 (2015). Single-cell longitudinal imaging to resolve drug effects across the cell-cycle. Cited to support time-resolved CC-aware tracking as an established experimental strategy.
 38. Rasheed, S. et al. Characterization of... HT-1080. *Cancer* 33, 1027-1033 (1974). Original characterization of the HT1080 human fibrosarcoma cell line. Cited to anchor model provenance and baseline properties.
 39. Kagawa, Y. et al. Cell-cycle-dependent Rho GTPase activity... *PLoS ONE* 8, e83629 (2013). In vivo study linking CC-dependent signaling to motility/invasion. It supports biological plausibility for CC-motility coupling.
 40. Vittadello, S. T. et al. Examining go-or-grow... *Biophys. J.* 118, 1243-1247 (2020). Tests go-or-grow behavior using FUCCI and CC-inhibiting drugs. Cited to contextualize the go-or-grow framework and its experimental interrogation.
 41. Sinha, D. et al. Mitotic slippage: an old tale with a new twist. *Cell-cycle* 18, 7-15 (2019). Review defining mitotic slippage and its consequences. It standardizes terminology and interpretation of slippage-like events.
 42. Xu, F. L. et al. Mitotic slippage... disorazole C1. *BMC Chem. Biol.* 10, 1 (2010). Demonstrates pharmacologically induced mitotic slippage in non-cancer cells. Cited to support slippage as a classifiable stress response, not exclusive to cancers.
 43. Moriarty, R. A. & Stroka, K. M. Physical confinement alters sarcoma cell-cycle progression and division. *Cell-cycle* 17, 2360-2373 (2018). Directly tests confinement effects on CC progression and division outcomes. Cited to provide close precedent for confinement-modulated CC phenotypes.

44. Barr, A. R. et al. DNA damage during S-phase mediates... *Nat. Commun.* 8, 14728 (2017).
Links S-phase damage to p21-driven decisions in the subsequent G1. We cited to support mechanistic routes to prolonged G1 under stress.
45. Raab, M. et al. Mitotic arrest and slippage induced by... PLK1 inhibition. *Mol. Oncol.* 9, 140-154 (2015). Shows arrest/slippage outcomes under mitotic control perturbation.
Cited to contextualize mitotic failure modes relevant to prolonged confinement.
46. The balance between mitotic death and mitotic slippage in acute leukemia: a new therapeutic window? *J. Hematol. Oncol.* (2019). Perspective on how arrest outcomes partition into death versus slippage with therapeutic implications. It frames slippage as clinically consequential rather than merely descriptive.
47. Gjorevski, N. et al. Designer matrices for intestinal stem cell and organoid culture. *Nature* 539, 560-564 (2016). Demonstrates engineered matrices as state-controlling, reproducible organoid substrates. Cited to motivate microenvironment design as a lever for phenotypic control.
48. Haselgrübler, T. et al. High-throughput, multiparameter analysis of single cells. *Anal Bioanal Chem* 406, 3279-3296 (2014). Overview of multiparametric single-cell analysis in HT contexts. It justifies multi-metric phenotyping as feasible and informative.
49. Chessel, A. & Carazo Salas, R. E. From observing to predicting... *Essays Biochem* 63, 197-208 (2019). Perspective on predictive single-cell phenotyping with HT/HC microscopy.
Cited to support the conceptual move from observation to prediction-ready phenoprints.
50. Neumann, B. et al. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods* 3, 385-390 (2006). Early precedent for time-lapse HT screening with live cells. It validates the practicality and value of temporal phenotyping at scale.
51. Bray, M.A. & Carpenter, A. Advanced assay development guidelines for image-based high-content screening and analysis. 2017 Jul 8. Guidelines emphasizing

- assay design, artifacts, and QC in image-based screening. Cited to justify the QC-first framing for scalable, reliable phenotyping.
52. Qiu, M. et al. A cell-level quality control workflow for high-throughput image analysis. *BMC Bioinformatics* 21, 280 (2020). Introduces cell-level QC strategies to detect segmentation artifacts in HT pipelines. It motivated automated QC as essential for scaling CC-aware assays.
53. Garay, T. et al. Revisiting the “go or grow” hypothesis in cancer cells in vitro. *Exp. Cell Res.* 319, 3094-3103 (2013). Re-evaluates go-or-grow trade-offs across in vitro cancer models. Cited to support nuance and boundary conditions of the go-or-grow framing.
54. Akhmetkaliyev, A. et al. EMT/MET plasticity in cancer and Go-or-Grow decisions in quiescence... *Mol. Cancer* 22, 90 (2023). Connects plasticity programs to quiescence and go-or-grow decisions. It integrates state plasticity into interpretation of slow-cycling invasive behaviors.
55. Claes, A. et al. Diffuse glioma growth: a guerilla war. *Acta Neuropathol.* 114, 443-458 (2007). Pathology perspective on diffuse invasion patterns versus proliferative expansion. Cited to support spatial separation of invasion and proliferation in vivo.
56. Purwada, A. et al. Self-assembly protein nanogels for safer cancer immunotherapy. *Adv. Healthc. Mater.* 5, 1413-1420 (2016). Biomaterial platform that modulates immune function through engineered matrices. It viewed biomaterials as intervention platforms that shape cellular states.
57. Azarin, S. M. et al. In vivo capture and label-free detection of early metastatic cells. *Nat. Commun.* 6, 8094 (2015). Demonstrates biomaterial-based capture of early metastatic cells in vivo. Cited to motivate the “capture-site” analogy for engineered confinement concepts.
58. Stephan, S. B. et al. Biopolymer implants enhance the efficacy of adoptive T-cell therapy. *Nat. Biotechnol.* 33, 97-101 (2015). Shows implants can localize and amplify therapeutic cell function in vivo. It supports the broader notion that materials can be designed to steer cell behavior.

59. Invitrogen TrueDesign Genome Editor. Vendor resource describing a CRISPR guide/RNP design ecosystem. Cited to document tooling context for genome editing workflow components.
60. FlowJo Portal. Software portal for flow cytometry analysis and gating workflows. It documents the analysis environment supporting population-level validation.
61. NIS-Elements. Microscope control and acquisition software commonly used for multipoint imaging. Cited to document the acquisition ecosystem interfacing with automated imaging routines.
62. Zhong, L. et al. Phosphorylation of cGAS by CDK1 impairs self-DNA sensing in mitosis. *Cell Discov.* 6, 26 (2020). Demonstrates mitosis-linked regulation of innate sensing machinery. It highlights that cell-cycle phase modulates broader cellular programs beyond morphology.
63. Inkscape. An open-source vector graphics editor for figure assembly. Cited to document figure-preparation tooling.
64. Alvéole Leonardo Photopatterning Software. Software environment for PRIMO photopatterning experiment design and execution. It documents the patterning workflow layer used in micropattern fabrication.
65. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676-682 (2012). General-purpose bioimage analysis platform supporting reproducible workflows. Cited to document core image processing infrastructure.
66. Peng, T. et al. A BaSiC tool for background and shading correction of optical microscopy images. *Nat. Commun.* 8, 14836 (2017). Algorithm for illumination/background correction in microscopy images. Referenced to justify intensity correction as a prerequisite for quantitative analysis.
67. Perfetto, S. et al. Effects of spatial frequency filtering choices on the perception of filtered images. *Vision* 4, 29 (2020). Shows that filtering choices can alter perceived content and interpretation. Cited to motivate conservative filtering and explicit reporting of preprocessing steps.
68. royerlab/dexp (Zenodo software release). Open software for large microscopy data processing and visualization, supporting computational tooling for scalable image handling.

69. AllenCellModeling/aicsimageio Library for standardized reading/writing of microscopy image formats. Cited to document reproducible IO and metadata handling.
70. van der Walt, S. et al. scikit-image: image processing in Python. *PeerJ* 2, e453 (2014). Core Python library implementing standard image processing algorithms. Referenced to document the computational foundation for custom analysis pipelines.
71. Yang, X. et al. An improved median-based Otsu image thresholding algorithm. *AASRI Procedia* 3, 468-473 (2012). Variant thresholding approach for robust binarization under noise. Cited to justify segmentation primitives when intensity distributions are imperfect.
72. MATLAB Documentation. Reference documentation for MATLAB functions and workflows. Cited to document the computational environment for analysis and plotting.
73. ZEISS ARIVIS Pro Software. Commercial software for 3D visualization, segmentation, and analysis. Cited to document volumetric analysis tooling used in the workflow.
74. GraphPad. Statistical analysis and plotting environment commonly used in biosciences. Cited to document the statistics/plotting toolchain.
75. SigmaPlot. Plotting and statistics software used for publication figures. An alternative plotting environment used in reporting.

4 Chapter: TE-DaXi, a custom oblique-plane light-sheet microscope for tissue engineering applications

1. Wheeler, E. E. & Leach, J. K. Tissue-Engineered Three-Dimensional Platforms for Disease Modeling and Therapeutic Development. *Tissue Eng. Part B Rev.* (2024).

A field-mapping review of modern 3D tissue-engineered disease platforms and the translational logic connecting model design to therapeutic development. This work supports the opening positioning of TE platforms as mainstream biomedical research infrastructure. This reference supports the breadth claim that TE models span multiple formats with distinct constraints.

2. Pocock, M. W. et al. Maturation of human cardiac organoids enables complex disease modeling and drug discovery. *Nat. Cardiovasc. Res.* 4, 821-840 (2025).

A cardiac organoid study linking maturation state to disease-relevant phenotypes and screening-readiness. This reference supports the statement that human cardiac organoids can enable complex disease modeling and drug discovery when maturation is addressed. This reference supports the motivation for gentle volumetric imaging over long time windows.

3. Lewis-Israeli, Y. R. et al. Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021). A heart organoid system demonstrating self-assembly with developmental and congenital heart disease relevance. This reference emphasizes the claim that organoids can recapitulate developmental programs and disease-relevant phenotypes beyond 2D assays. Here we want to support the need for deep, volumetric imaging to capture evolving 3D morphology.

4. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 345, 1247125 (2014). A foundational perspective establishing organoids as self-organizing systems for development and disease modeling. This reference claims that the field is shifting toward complex 3D models as standard experimental substrates. This supports the rationale that model complexity increases imaging demands and standardization pressure.

5. Hofbauer, P. et al. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021). A cardioid framework paper connecting self-organization to interpretable principles of human cardiogenesis. This reference links to the statement that hiPSC-derived cardiac models can encode structured morphogenetic programs over time. It support cardioids as a primary TE use case, motivating low-dose 3D time-lapse imaging.
6. Capulli, A. K. et al. JetValve: Rapid manufacturing of biohybrid scaffolds for biomimetic heart valve replacement. *Biomaterials* 133, 229-241 (2017). A rapid-manufacturing approach for biohybrid heart valve scaffolds emphasizing geometry/material constraints and biomimetic performance. This reference helps claiming that TE platforms include engineered constructs whose form factor drives imaging requirements, such as the need for microscope versatility across heterogeneous sample geometries.
7. Wang, G. et al. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nat. Med.* 20, 616-623 (2014). A disease modeling example integrating iPSC-derived cardiac biology with heart-on-chip microphysiological control. This reference is used to support the statement that organ-on-chip systems enable controlled microenvironments for disease-relevant assays. Used to support the 2.5D platform category that prioritizes fast, high-resolution imaging of cell-substrate interactions.
8. Reynaud, E. G., Kržič, U., Greger, K. & Stelzer, E. H. K. Light-sheet-based fluorescence microscopy: More dimensions, more photons, and less photodamage. *HFSP J.* 2, 266-275 (2008). An early articulation of LSFM's core advantage: volumetric imaging with reduced photodamage through plane illumination. Researchers state that LSFM is intrinsically aligned with long-term live imaging in thick samples. This work emphasizes the mechanistic rationale for reduced phototoxicity via restricted excitation volume.
9. Daetwyler, S. & Fiolka, R. P. Light-sheets and smart microscopy, an exciting future is dawning. *Commun. Biol.* 6, 1-11 (2023). A perspective linking light-sheet microscopy to adaptive and automated acquisition paradigms ("smart microscopy"). This reference supports the transition from LSFM as an instrument

- to LSFM as a programmable platform. This reference supports the forward-looking framing that TE imaging will rely on adaptive sampling rather than uniform high-dose acquisition.
10. Stelzer, E. H. K. et al. Light-sheet fluorescence microscopy. *Nat. Rev. Methods Primer* 1, 1-25 (2021). A consolidated primer covering LSFM principles, implementations, and practical limits across sample types. It includes statements about LSFM's capability envelope for large-volume, low-photodamage imaging. Here, it supports the claim that LSFM is a leading modality for 3D TE constructs requiring prolonged observation.
 11. Scherf, N. & Huiskens, J. The smart and gentle microscope. *Nat. Biotechnol.* 33, 815-818 (2015). A conceptual framing of "gentle" imaging as an engineering constraint and a case for intelligent photon allocation. Phototoxicity must be treated as a primary experimental variable. We support the rationale for adaptive/low-dose acquisition strategies as central design requirements.
 12. Bruker Corporation. Light-Sheet Images of Organoids and Cell Cultures (Luxendo TruLive microscope web resource). A vendor documentation resource illustrating a commercial system optimized for long-term organoid imaging. This reference supports the concrete example that commercial LSFM systems are often optimized for specific sample formats. This reference supports the limitation claim that plate and flat-substrate compatibility remains a bottleneck for many commercial systems.
 13. Krzic, U., Gunther, S., Saunders, T. E., Streichan, S. J. & Hufnagel, L. Multiview light-sheet microscope for rapid in toto imaging. *Nat. Methods* 9, 730-733 (2012). A multi-view LSFM implementation enabling fast in toto imaging with improved reconstruction quality. This reference supports the claim that LSFM can scale to whole-sample volumetric imaging when multi-view geometry is engineered. Used for multi-view capability as a relevant design axis for thick TE samples.
 14. Shi, Y. et al. Smart lattice light-sheet microscopy for imaging rare and complex cellular events. *Nat. Methods* (2023). A lattice LSFM advance emphasizing high spatiotemporal resolution and reduced phototoxicity for rare event capture. This reference highlights that lattice LSFM excels for near-coverslip/high-resolution

- 2.5D contexts. This reference supports the claim that such systems can be impractical for thick, diverse 3D TE samples at scale.
15. Yang, B. et al. DaXi-high-resolution, large imaging volume and multi-view single-objective light-sheet microscopy. *Nat. Methods* 19, 461-469 (2022). A single-objective light-sheet architecture combining high resolution with large imaging volume and multi-view acquisition. This reference supports the selection of DaXi as the technical blueprint for TE-DaXi. This reference highlights the feasibility of single-objective geometry for format flexibility in TE contexts.
 16. Yang, B. et al. Epi-illumination SPIM for volumetric imaging with high spatial-temporal resolution. *Nat. Methods* 16, 501-504 (2019). An epi-illumination SPIM approach expanding sample access while maintaining volumetric performance. This reference supports the claim that single-objective/epi-illumination geometries broaden compatibility with standard sample holders. This supports the architectural motivation for TE-DaXi's plate- and device-oriented design goals.
 17. Girstmair, J. et al. Light-sheet microscopy for everyone? Experience of building an OpenSPIM to study flatworm development. *BMC Dev. Biol.* 16, 22 (2016). A practical build-and-use account emphasizing the real barriers to adoption for custom light-sheet systems. This reference supports the statement that adoption is limited by training and assembly/alignment complexity, not solely by performance. This reference supports the need for simplified, instruction-like documentation for non-expert labs.
 18. Dunsby, C. Optically sectioned imaging by oblique plane microscopy. (2008). The original introduction of oblique plane microscopy as a route to optical sectioning with single-objective access. This reference supports the selection of OPM as the enabling architecture for TE-DaXi. This reference supports the claim that single-objective imaging can be compatible with conventional sample holders.
 19. Sapoznik, E. et al. A Versatile Oblique Plane Microscope for Large-Scale and High Resolution Imaging of Subcellular Dynamics. A demonstration of OPM as a scalable platform for large fields and high-resolution dynamics. This reference supports the statement that OPM is a practical, validated approach to combining

scale and resolution. This reference supports the versatility claim required for TE sample diversity.

20. Kumar, A. et al. Dual-view plane illumination microscopy for rapid and spatially isotropic imaging. *Nat. Protoc.* 9, 2555-2573 (2014). A dual-view SPIM protocol establishes methods to improve isotropy and reconstruction quality in volumetric imaging. This reference supports the comparison between dual-objective multi-view systems and single-objective architectures regarding sample compatibility, it motivates TE-DaXi's design choices.
21. Botcherby, E. J., Juškaitis, R., Booth, M. J. & Wilson, T. An optical technique for remote focusing in microscopy. *Opt. Commun.* 281, 880-887 (2008). A foundational remote focusing method enabling axial refocus without moving heavy optics. Here, they introduce the remote focusing as the mechanism enabling fast refocusing for volumetric imaging. This supports the design rationale that refocusing should not introduce lateral offsets or mechanical instability.
22. Millett-Sikking, A. & York, A. High NA single-objective light-sheet. Zenodo (2019).
An open design resource detailing a high-NA single-objective light-sheet configuration and component logic. This reference supports the claim that open hardware resources accelerate adoption beyond optics-specialist labs.
23. Botcherby, E. J. et al. Fast measurement of sarcomere length and cell orientation in Langendorff-perfused hearts using remote focusing microscopy. *Circ. Res.* 113, 863-870 (2013). A cardiac-tissue demonstration of remote focusing applied to fast measurement under challenging imaging conditions. This reference supports remote focusing as a proven strategy in cardiac-relevant samples, not merely a theoretical add-on. This reference supports the rationale that TE-DaXi's design choices align with cardiac tissue-engineering needs.
24. Dualview Inverted Selective Plane Illumination Microscope (diSPIM). A web resource describing diSPIM concepts and implementation context for dual-view inverted SPIM systems. Researchers support the provenance of the diSPIM ecosystem referenced for control and workflow inspiration. In TE-DaXi work, we referenced it to support reuse of established control paradigms for stage-scanned light-sheet acquisition.

25. Dvinskikh, N. et al. Volumetric light-sheet calcium mapping in 3D biological samples. *Front. Physiol.* (2023). A functional LSFM example for mapping calcium dynamics in 3D samples under realistic constraints. LSFM is suited to functional readouts in thick living specimens where dose and speed are limiting. This reference support the screening logic that combines low-dose scouting with selective volumetric acquisition.
26. Boothe, T. et al. A tunable refractive index matching medium for live imaging cells, tissues and model organisms. *eLife* 6, e27240 (2017). A refractive index matching strategy for live imaging to reduce aberrations and improve depth performance. Deep imaging quality depends on refractive index management; we claim that this is the rationale for silicone-oil objectives and aberration mitigation in thick TE samples.
27. Onshape. A collaborative CAD environment enabling shareable 3D designs and assembly sequences. An interactive 3D documentation can reduce assembly and maintenance barriers for non-experts. Our step-by-step instruction approach is presented as a reproducibility tool, not only a convenience.
28. (Micro-Manager). Micro-Manager open-source microscope control software. An open-source control layer enabling hardware integration and reproducible acquisition across systems. This is used to support the choice of accessible, non-proprietary software for TE-DaXi control. It supports the statement that reproducibility and adoption improve when control stacks are standardized and community-supported.
29. (diSPIM Wiki). start - diSPIM Wiki. A community documentation hub for diSPIM workflows, control logic, and practical implementation notes. This reference is useful for existing light-sheet control paradigms as a starting point for TE-DaXi. It supports the feasibility of adapting established workflows to a stage-scanned OPM-like configuration.
30. Han, W., Cheng, W. & Zhan, Q. Design and alignment strategies of 4f systems used in the vectorial optical field generator. *Appl. Opt.* 54, 2275-2278 (2015). A technical alignment reference for 4f relay systems and how misalignment propagates into field errors. This reference supports the alignment rationale for

- the “zero-tilt” verification logic. This reference supports the claim that stable refocusing without XY drift indicates correct relay alignment.
31. Hong, W., Sparks, H. & Dunsby, C. Alignment and Characterisation of Remote-Refocusing Systems. *bioRxiv* (2023). A modern characterization framework for remote refocusing modules, including alignment checks and measurable failure modes. This reference helps to claim that interpreting refocus-without-lateral-shift as evidence of correct calibration. In addition, it states that remote refocusing requires explicit characterization rather than qualitative inspection.
 32. Singh, R. et al. Oblique plane microscope for mesoscopic imaging of freely moving organisms with cellular resolution. *Opt. Express* 31, 2292 (2023). An OPM implementation demonstrating mesoscopic-scale imaging with cellular-resolution performance. It claims that OPM can scale across size regimes while maintaining useful resolution. Supports TE-DaXi’s intended balance of field of view, speed, and resolution.
 33. Bruschini, C., Homulle, H., Antolovic, I. M., Burri, S. & Charbon, E. Single-photon avalanche diode imagers in bio-photonics: review and outlook. *Light Sci. Appl.* 8, 87 (2019). A review of SPAD arrays and their value for fast, low-light detection regimes relevant to live imaging. Used for the claim that performance upgrades can be pursued without abandoning the single-objective platform concept.
 34. (Elliott, 2020 Current Protocols). Confocal Microscopy: Principles and Modern Practices. *Curr. Protoc. Cytom.* (2020). A practical confocal reference establishing standard performance trade-offs in contrast, resolution, and dose. This reference supports the statement that alternate modalities may be integrated when contrast requirements exceed the limits of widefield/LSFM performance. This reference supports modality expansion as a controlled, modular engineering choice.
 35. Truong, T. V., Supatto, W., Koos, D. S., Choi, J. M. & Fraser, S. E. Deep and fast live imaging with two-photon scanned light-sheet microscopy. *Nat. Methods* 8, 757-760 (2011). A two-photon light-sheet approach improves depth performance in scattering samples while maintaining speed. Researchers demonstrated the proposed

pathway for deeper imaging in thick TE constructs when scattering becomes limiting. In this view, two-photon add-ons can be seen as an engineering extension rather than a full platform replacement.

36. André, O., Kumra Ahnslide, J., Norlin, N., Swaminathan, V. & Nordenfelt, P. Data-driven microscopy allows for automated context-specific acquisition of high-fidelity image data. *Cell Rep. Methods* 3, 100419 (2023). A data-driven acquisition paradigm demonstrating context-specific imaging rather than fixed, uniform sampling. This reference supports the screening logic that allows scouts to prioritize informative wells/fields before committing to high-content volumetric acquisition. This reference supports the claim that throughput and viability constraints can be reconciled by adaptive sampling policies.
37. Royer, L. A. et al. Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016).
An adaptive LSFM approach demonstrating long-term imaging with active correction to preserve quality and viability. This reference supports the forward-looking claim that stable, hands-off long-term imaging requires adaptation, not just hardware stability. Therefore, adaptive elements (alignment, illumination control) can be credible next steps in engineering.
38. Royer, L. A., Lemon, W. C., Chhetri, R. K. & Keller, P. J. A practical guide to adaptive light-sheet microscopy. *Nat. Protoc.* 13, 2462-2500 (2018).
A protocol-level operationalization of adaptive LSFM, including implementation steps and calibration logic. Researchers claim that “smart/gentle” imaging can be converted into reproducible procedures rather than lab-specific craftsmanship. Therefore, adaptive acquisition represents a practical pathway toward HT/HC screening behavior in TE contexts

5 Chapter: Engineering tools for 3D HT/HC structural and functional phenotyping

1. Roth, G. A. et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. *J. Am. Coll. Cardiol.* 76, 2982-

- 3021 (2020). We reference this work to substantiate the disease-scale motivation for quantitative, translatable cardiac models.
2. Martin, S. S. et al. 2024 Heart Disease and Stroke Statistics: A Report of US and Global Data From the American Heart Association. *Circulation* 149, e347-e913 (2024). This report is cited to anchor the manuscript's urgency claims with current epidemiological baselines.
 3. Xiao, Y., Zhuang, Z., Shi, X. & Wu, F. Challenges and opportunities for cardiac organoids: A comprehensive review. *Regeneration Repair Rehabil.* (2025). This review surveys cardiac organoid capabilities, limitations, and emerging opportunities across engineering and biology. This synthesis is drawn upon to frame the field-level gaps that the proposed framework targets.
 4. Hofbauer, P. et al. cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184, 3299-3317.e22 (2021). This study demonstrates self-assembling cardioids as a tractable model of human cardiogenesis with emergent tissue organization. This work justifies cardioids as an appropriate experimental substrate for morphogenesis-focused phenotyping.
 5. Lewis-Israeli, Y. R. et al. Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* 12, 5142 (2021). This paper introduces self-assembling human heart organoids for developmental modeling and the generation of disease-relevant phenotypes. This work supports the manuscript's use of organoid systems as experimentally accessible developmental proxies.
 6. Prondzynski, M. et al. Efficient and reproducible generation of human iPSC-derived cardiomyocytes and cardiac organoids in stirred suspension systems. *Nat. Commun.* 15, 5929 (2024). This study presents a scalable stirred-suspension method for reproducible generation of iPSC-derived cardiomyocytes and cardiac organoids. This work motivates throughput-compatible organoid production enabling systematic perturbation studies.
 7. Kim, J., Koo, B.-K. & Knoblich, J. A. Human organoids: model systems for human biology and medicine. *Nat. Rev. Mol. Cell Biol.* 21, 571-584 (2020). This review establishes organoids as model systems for human biology and medicine

while outlining strengths and limitations. This work legitimizes organoids as translational platforms requiring quantitative, standardized phenotyping.

8. Bozal, S. B. et al. Development of an automated 3D high-content cell screening platform for organoid phenotyping. *SLAS Discov.* 29, 100182 (2024). This paper describes an automated 3D high-content screening platform tailored for organoid phenotyping workflows. This work reinforces the feasibility of automation as a prerequisite for scalable 3D phenotyping.
9. Louey, A., Hernández, D., Pébay, A. & Daniszewski, M. Automation of Organoid Cultures: Current Protocols and Applications. *SLAS Discov.* 26, 1138-1147 (2021).

This article reviews protocols and applications for automating organoid culture handling and workflows. It justifies automation as an enabling layer for reproducible, multi-condition organoid studies.

10. Lampart, F. L., Iber, D. & Doumpas, N. Organoids in high-throughput and high-content screenings. *Front. Chem. Eng.* 5, (2023). This review discusses how organoids can be integrated into high-throughput and high-content screening paradigms. This work positions the manuscript within the HT/HC screening trajectory while emphasizing remaining engineering bottlenecks.
11. Royer, L. A. et al. Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat. Biotechnol.* 34, 1267-1278 (2016). This work introduces adaptive light-sheet strategies that stabilize image quality during long-term live imaging. It supports the argument that feedback control is necessary for reliable 3D/4D live phenotyping.
12. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* 39, 1700003 (2017). This paper explains mechanisms of phototoxicity and practical mitigation strategies in live fluorescence microscopy. This work justifies dose-governed acquisition constraints for longitudinal fluorescence phenotyping.
13. de Medeiros, G. et al. Multiscale light-sheet organoid imaging framework. *Nat Commun* 13, 4864 (2022). This work presents a multiscale framework for light-sheet imaging of organoids across spatial scales. This supports the manuscript's emphasis on scalable volumetric imaging across specimen sizes.

14. Linkert, M. et al. Metadata matters: access to image data in the real world. *J. Cell Biol.* 189, 777-782 (2010). This paper argues that standardized metadata is essential for access, interoperability, and reuse of bioimaging data. This emphasizes the manuscript's focus on metadata completeness as a reproducibility control.
15. Boehm, U. et al. QUAREP-LiMi: a community endeavor to advance quality assessment and reproducibility in light microscopy. *Nat. Methods* 18, 1423-1426 (2021).
This article describes a community initiative to standardize quality assessment and reproducibility practices in light microscopy. This work explicates QC gates and reporting standards in the imaging workflow.
16. Sarkans, U. et al. REMBI: Recommended Metadata for Biological Images-enabling reuse of microscopy data in biology. *Nat. Methods* 18, 1418-1422 (2021). This work proposes recommended metadata elements to enable reuse and interpretation of biological image datasets. This supports the manuscript's argument for standardized acquisition descriptors in HT/HC settings.
17. Lee, B.H et al. Permeability-driven pressure and cell proliferation control lumen morphogenesis in pancreatic organoids. *Nat Cell Biol* 28, 113–124 (2026). This work links epithelial permeability, hydrostatic pressure, and proliferation to emergent lumen morphogenesis regimes in organoids. This motivates pressure-growth coupling as a mechanistic axis for regime mapping in lumen outcomes.
18. Lu, L. et al. Generic comparison of lumen nucleation and fusion in epithelial organoids with and without hydrostatic pressure. *Nat Commun* 16, 6307 (2025). This work compares lumen nucleation and fusion dynamics under conditions with and without hydrostatic pressure in epithelial organoids. This contextualizes lumen topology changes as pressure-dependent morphogenetic processes.
19. Di Sante, M. et al. CALIPERS: Cell-cycle-aware live imaging for phenotyping experiments and regeneration studies. *bioRxiv* 2024.12.19.629259 (2024). This preprint introduces a cell-cycle-aware live-imaging strategy for phenotyping and regeneration-focused measurements. This justifies cell-cycle stratification as a necessary layer for reducing hidden heterogeneity in readouts.

20. Tanida, S. et al. Predicting organoid morphology through a phase field model: Insights into cell division and luminal pressure. *PLoS Comput Biol.* 2025. This work models organoid morphology using a phase-field approach that incorporates cell division and luminal pressure. This work supports the mechanistic framing that coupled growth, and pressure can predict organoid-scale structure.
21. Kiepas, A., Voorand, E., Mubaid, F., Siegel, P. M. & Brown, C. M. Optimizing live-cell fluorescence imaging conditions to minimize phototoxicity. *J. Cell Sci.* 133, jcs242834 (2020). This study provides experimental guidance for minimizing phototoxicity via optimization of fluorescence imaging conditions. This work justifies practical acquisition parameter tuning as part of dose governance.
22. Lukonin, I., Zinner, M. & Liberali, P. Organoids in image-based phenotypic chemical screens. *Exp. Mol. Med.* 53, 1495-1502 (2021). This review outlines how organoids are used in image-based phenotypic chemical screens and the challenges associated with them. We referenced this to support the manuscript's positioning of organoid phenotyping as a screening-ready but analysis-limited domain.
23. Nie, Y., Xu, X., Wang, W., Ma, N. & Lendlein, A. Spheroid formation of human keratinocyte: Balancing between cell-substrate and cell-cell interaction. *Clin. Hemorheol. Microcirc.* 76, 329-340 (2020). This paper analyzes how keratinocyte spheroid formation depends on the balance between cell-substrate and cell-cell interactions. This work highlights the rationale for spheroid-based benchmarking as a controlled 3D testbed.
24. Bruker Corporation. Light-Sheet Images of Organoids and Cell Cultures (Luxendo TruLive microscope web resource). This web resource documents light-sheet imaging examples and capabilities for organoids and cell cultures on the Luxendo TruLive platform. This resource supports instrument-specific feasibility claims for light-sheet acquisition in similar specimens.
25. Dyer L. et al. Lightsheet Microscopy. Wiley Online Library.(2022). This protocol resource describes practical implementation details for light-sheet microscopy workflows. This resource is leveraged to support methodological choices that follow established community protocols for LSM.

26. ZEISS ARIVIS Pro Software. This software provides tools for 3D visualization, segmentation, and quantitative analysis of volumetric microscopy datasets. This software is included to justify the feasibility of standardized 3D morphometry extraction from light-sheet data.
27. Ming, Y. et al. Longitudinal morphological and functional characterization of human heart organoids using optical coherence tomography. *Biosens. Bioelectron.* 207, 114136 (2022). This study applies optical coherence tomography to longitudinally quantify morphological and functional features in human heart organoids. We support that longitudinal functional-structural readouts are achievable with appropriate imaging modalities.
28. Woappi, Y., Altomare, D., Creek, K. E. & Pirisi, L. Self-assembling 3D spheroid cultures of human neonatal keratinocytes have enhanced regenerative properties. *Stem Cell Res.* 49, 102048 (2020). This study reports that self-assembling neonatal keratinocyte spheroids exhibit enhanced regenerative properties relative to simpler culture formats. This work claims the biological relevance of spheroid models used as validation specimens.
29. Cho, J., Lee, H., Rah, W., Chang, H. J. & Yoon, Y.-S. From engineered heart tissue to cardiac organoid. *Theranostics* 12, 2758-2772 (2022). This review connects engineered heart tissues and cardiac organoids, outlining design principles and application space. This work situates cardiac organoids within the continuum of engineered cardiac model systems.
30. Ong, H. T. et al. Digitalized organoids: integrated pipeline for high-speed 3D analysis of organoid structures using multilevel segmentation and cellular topology. *Nat. Methods* 22, 1343-1354 (2025). This work presents an integrated pipeline for high-speed 3D organoid analysis using multilevel segmentation and cellular topology metrics.
This work supports the manuscript's argument that analysis throughput must match imaging throughput in 3D phenotyping.
31. Brezak, M., Khan, A. A., Jechlinger, M. & Sumbalova Koledova, Z. Live-cell imaging of mammary organoids using light-sheet microscopy. *J. Mammary Gland Biol. Neoplasia* 30, 12 (2025). This paper describes live-cell light-sheet imaging

strategies tailored to mammary organoids. We used this work to support feasibility of long-term LSM live imaging in sensitive organoid specimens.

32. NIS-Elements. This software provides microscope control, automation, and orchestration of acquisition for high-throughput imaging workflows. This software is used to support the manuscript's automation implementation within standardized imaging routines.
33. Stack Focuser. This tool performs stack-based focusing to generate in-focus images from z-stacks for downstream analysis. This tool strengthens the manuscript's approach to plate-scale focus stabilization prior to segmentation.
34. Yang, X., Shen, X., Long, J. & Chen, H. An Improved Median-based Otsu Image Thresholding Algorithm. *AASRI Procedia* 3, 468-473 (2012). This work proposes an improved Otsu-thresholding variant that incorporates median-based preprocessing for robustness. We use it to support the manuscript's use of automated thresholding as a reproducible segmentation baseline.

A

Appendix A Additional figures

A1 Chapter 2 - FUCCIsmart performance evaluation

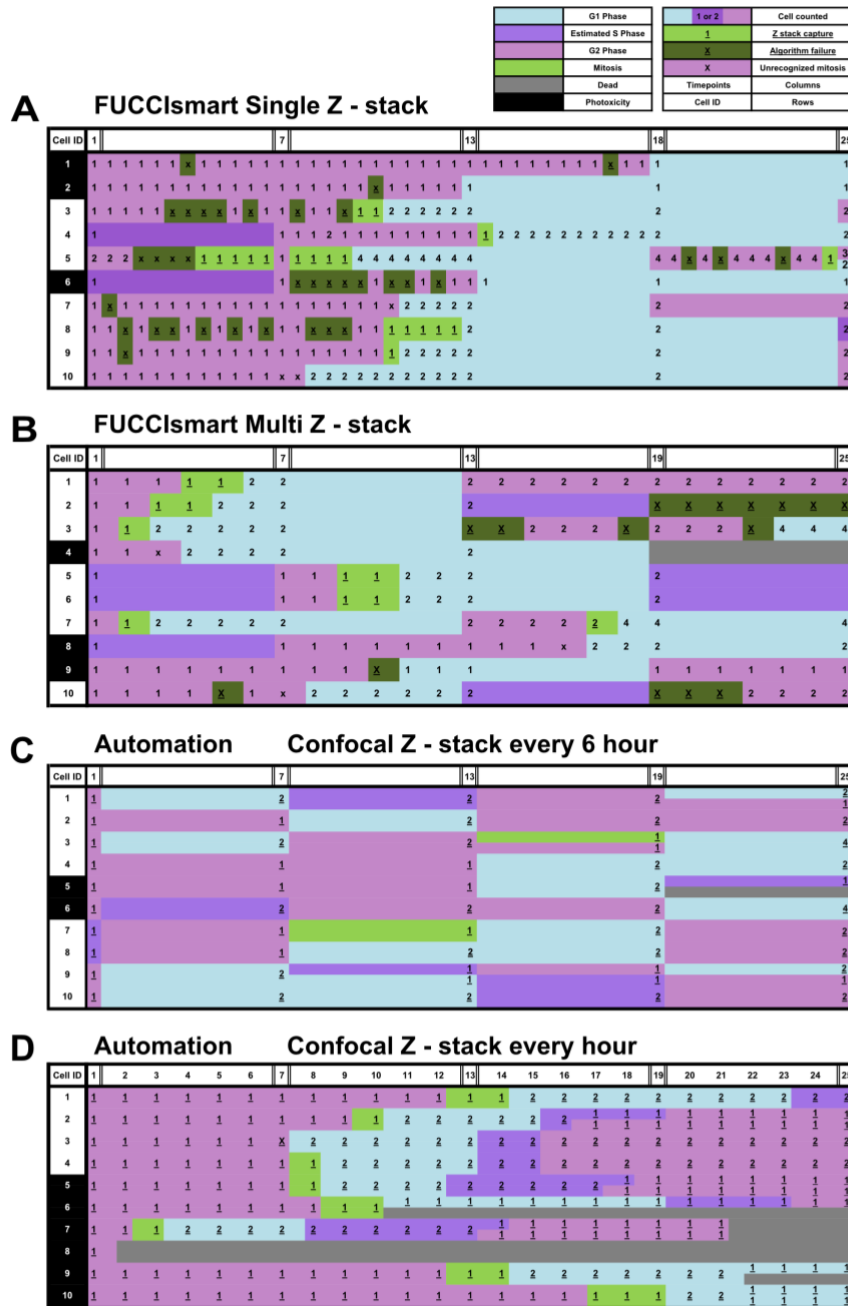


Figure A1. FUCCIsmart performance evaluation tables. (A-D) Data acquisition sequences in FUCCIsmart routines for single (A) and multiple (B) acquisitions compared to confocal automation every six hours (C) and one hour (D). Rows represent individual cell recordings over 24 hours; columns correspond to the acquisition time points. CC phases are color-coded according to FUCCIplex labeling. The FUCCIsmart performance was evaluated across three independent experiments.

A2 Chapter 3 - FUCCIplex cells, Fab2Mic, and 2D CC-aware motility and morphology

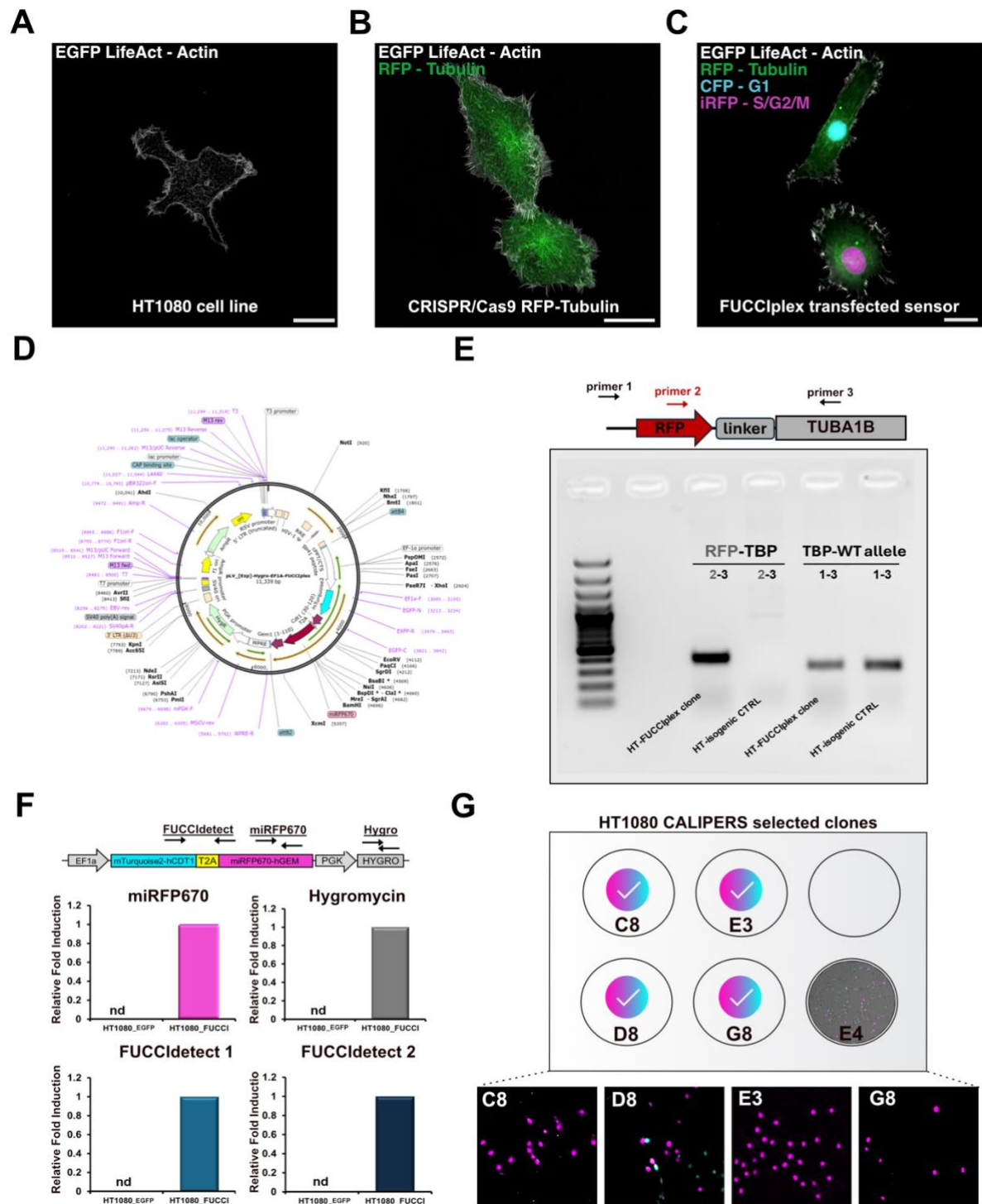


Figure A2.1 HT1080 CALIPERS four fluorophores generation.

(A-C) Representative fluorescence micrographs showing (A) HT1080 cells expressing EGFP LifeAct to visualize actin cytoskeleton, (B) CRISPR/Cas9-mediated knock-in of RFP-tubulin, and (C) multiplexed FUCCIplex reporters integrated with the RFP-tubulin line to monitor cytoskeletal structure and cell-cycle phase (CFP for G1, iRFP for S/G2/M). Scale bars, 25 μ m. (D) Schematic plasmid map of FUCCIplex construct highlighting insertion sites, promoters, and selection cassettes. (E) Genomic PCR shows successful integration of RFP at the TUBA1B locus in HT1080 CALIPERS clones (primers 2-3), with HT1080 EGFP LifeAct (WT) alleles detected using primers 1-3. CTRL = non-edited HT1080 DNA (HT1080_EGFP). (F) RT-qPCR detects miRFP670, Hygromycin, and FUCCIplex-specific transcripts in HT1080 CALIPERS clone E4 but not in HT1080 EGFP LifeAct cells (nd, not detected). (G) Four additional stable reference lines for HT1080 CALIPERS: C8, D8, E3, G8, representative imaging under expansion condition within the selected clone E4, as the HT1080 CALIPERS line used for downstream experiments. Scale bars, 100 μ m.

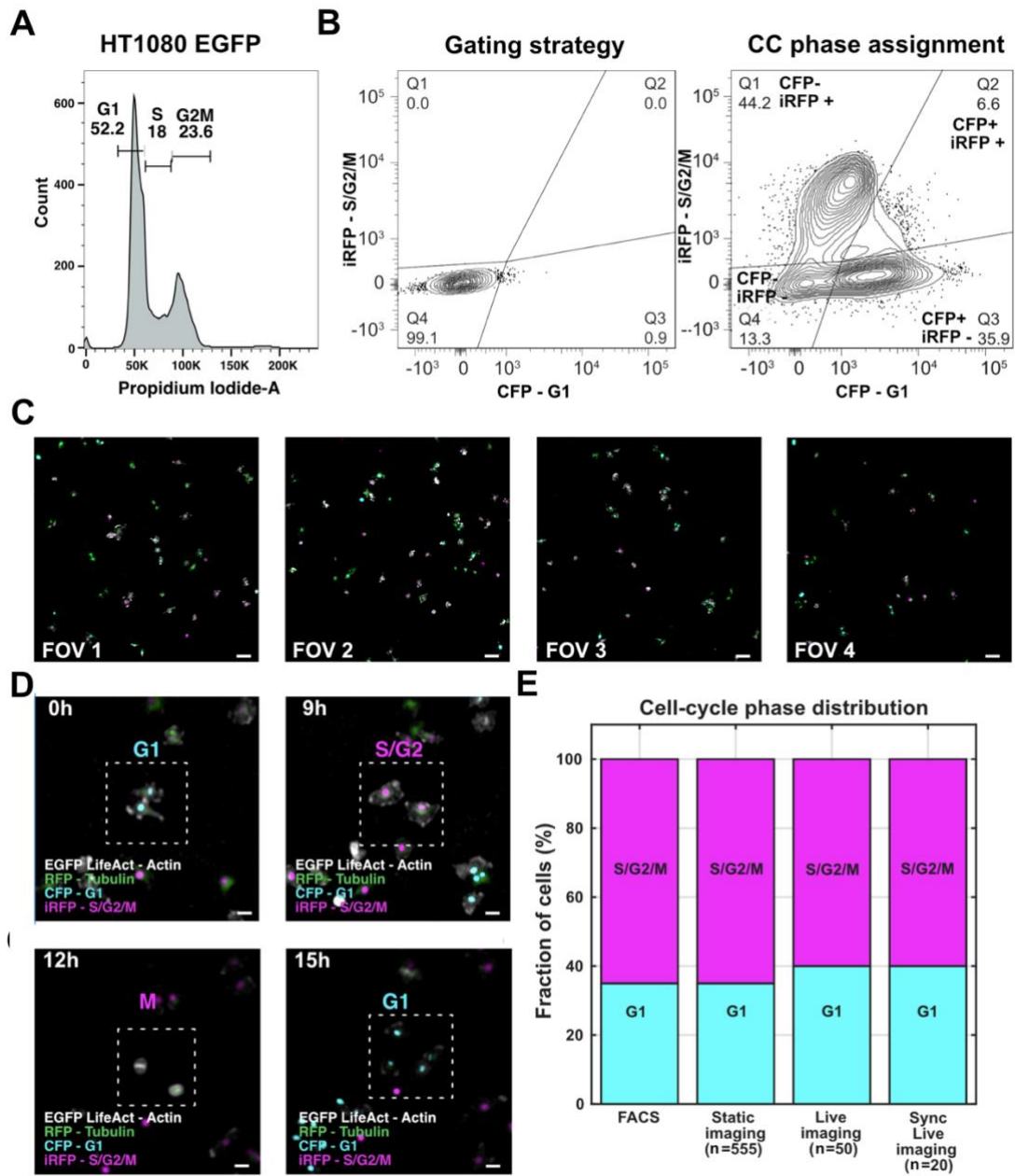


Figure A2.2. Validation of FUCCIplex phase assignment and timing consistency.

(A) Propidium iodide (PI) DNA-content histogram of the parental HT1080 EGFP line, with gates used to quantify the fraction of cells in G1 (52.2%), S (18.0%), and G2/M (23.6%). (B) FUCCIplex flow-cytometry gating and phase assignment using CFP (G1 phase) versus iRFP (S/G2/M phase): left, background population (HT1080 EGFP+/RFP+) used to define quadrant gates; right, application of the same gates to FUCCIplex-positive cells to assign CFP⁺iRFP⁻ (G1), CFP⁺iRFP⁺ (G1/S transition), and CFP⁻iRFP⁺ (S/G2/M). (C) Representative low-magnification fields of view (4 FOVs shown over 10 acquired) showing HT1080 CALIPERS for population-level CC phase distribution by static imaging. Scale bar, 100 μ m. (D) Representative time-lapse sequence illustrating a single-cell progression through phases (annotated G1 $\rightarrow$ S/G2 $\rightarrow$ M $\rightarrow$ 2 $\times$ G1) with multiplexed structural (Actin/Tubulin) and FUCCIplex (CFP/iRFP) readouts.

Dashed boxes indicate the region shown. Scale bar, 25 μm (E) Comparison of G1 vs S/G2/M fractions measured by FACS, static imaging ($n = 555$ cells), live imaging ($n = 50$ tracked cells), and synchronized live imaging ($n = 20$ tracked cells), showing consistent phase distributions across readouts.

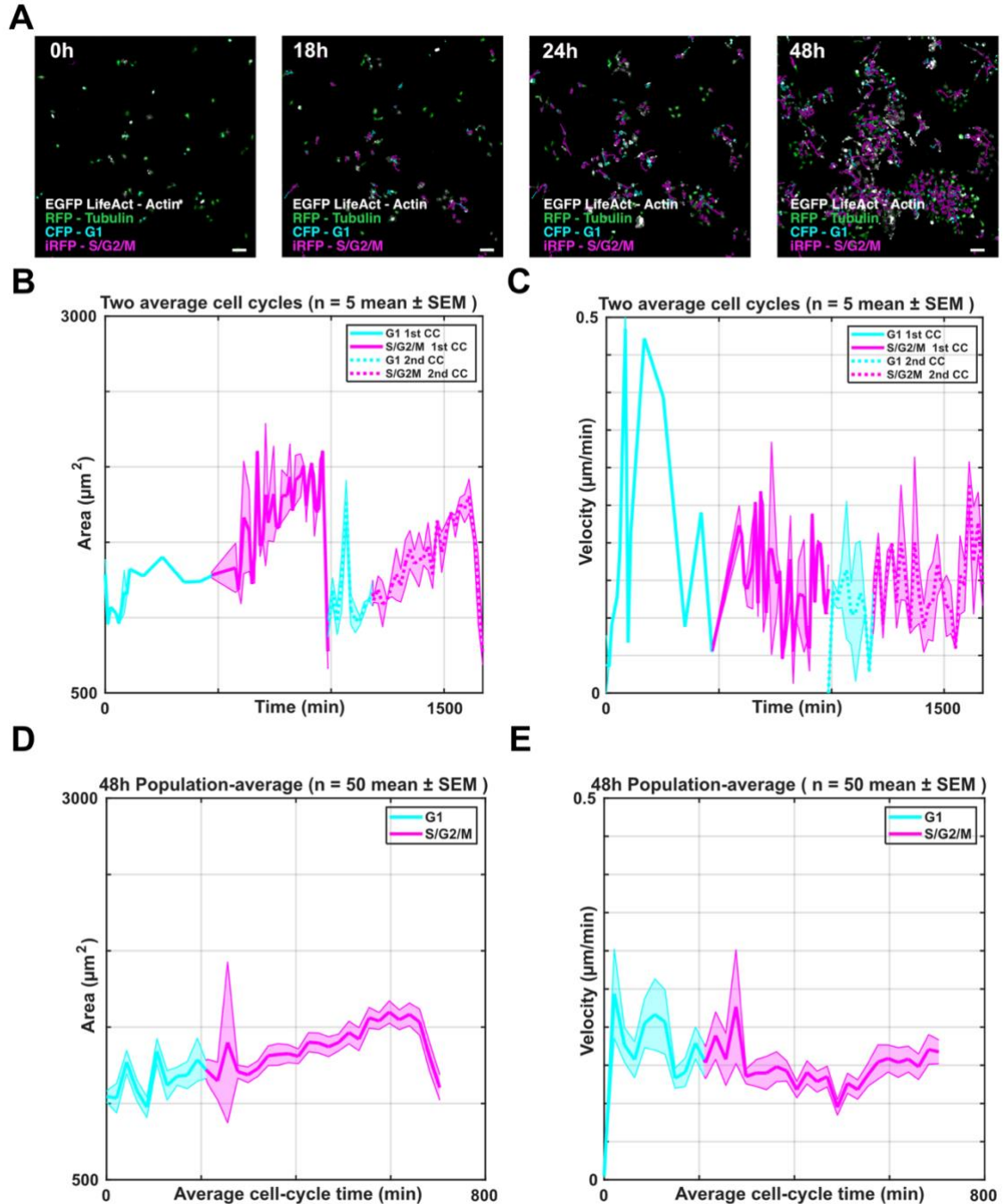


Figure A2.3. Free space 48h long-term validation at the population level.

(A) Representative snapshots (0 h, 18 h, 24 h, 48 h) from a 48 h four-channel fluorescence acquisition enabling tracking across multiple divisions in a large FOV. Scale bar, 100 μm . Two cell-cycle subset ($n = 5$ cells): phase-resolved cell area (B) and instantaneous velocity (C) mean $\pm$ SEM plotted over two consecutive cycles. Population-level analysis

across $n = 50$ complete cell-cycles: phase-aligned average projected area (D) and velocity (E) $\pm$ SEM.

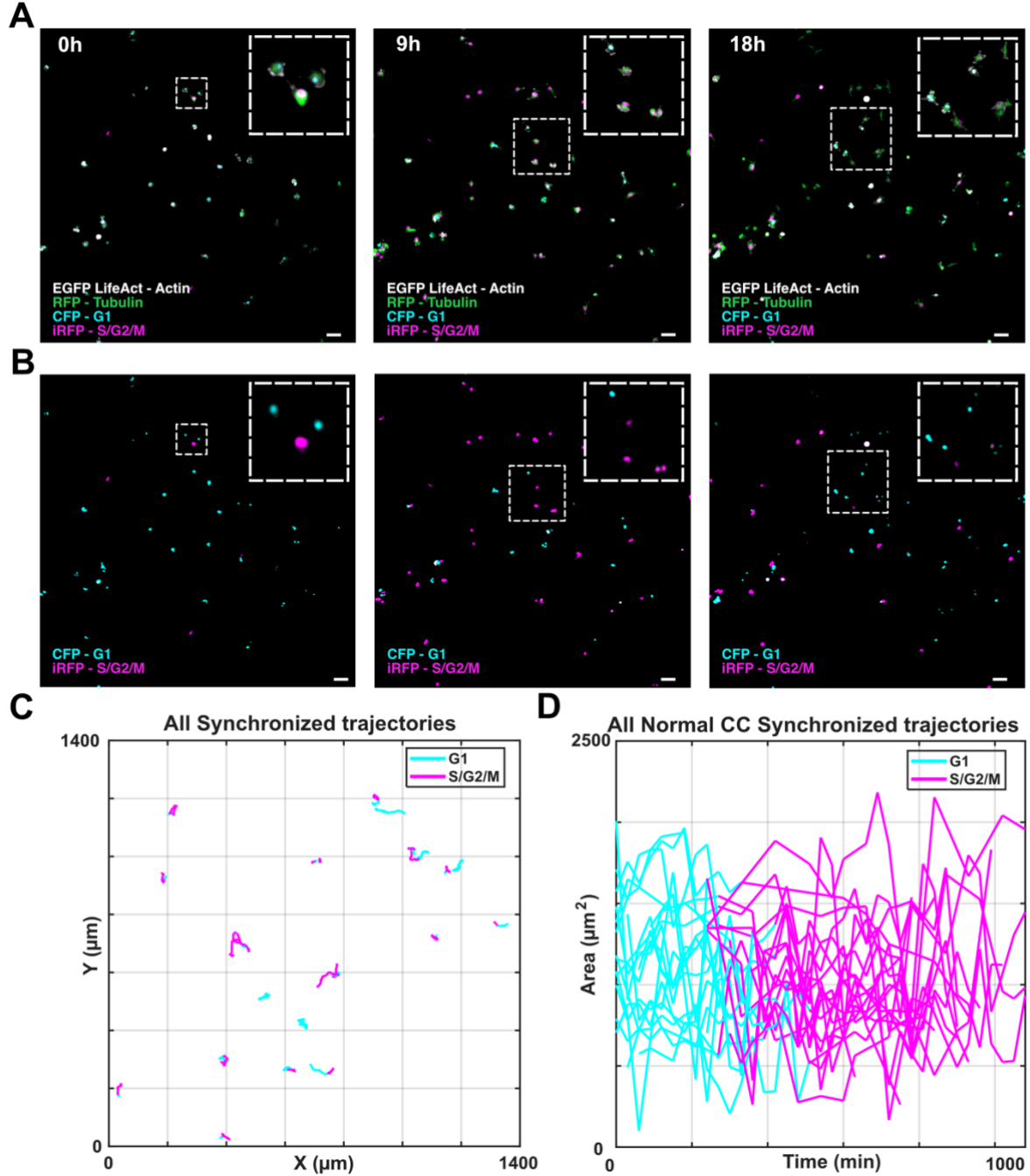


Figure A2.4. Synchronization time-dependent analysis in HT1080 CALIPERS cells.

(A) Representative 4-channel snapshots at 0 h, 9 h, and 18 h of live-cell fluorescence imaging. Scale bar, 100 μ m. (B) CFP/iRFP nuclear state highlighting initial enrichment in G1 phase after mitotic collection and reseeded, followed by progression into S/G2/M phase over time. (C) All XY trajectories of tracked cells ($n = 20$), color-coded by FUCCIplex phase. (D) Single-cell projected area trajectories over time for normal cycling cells.

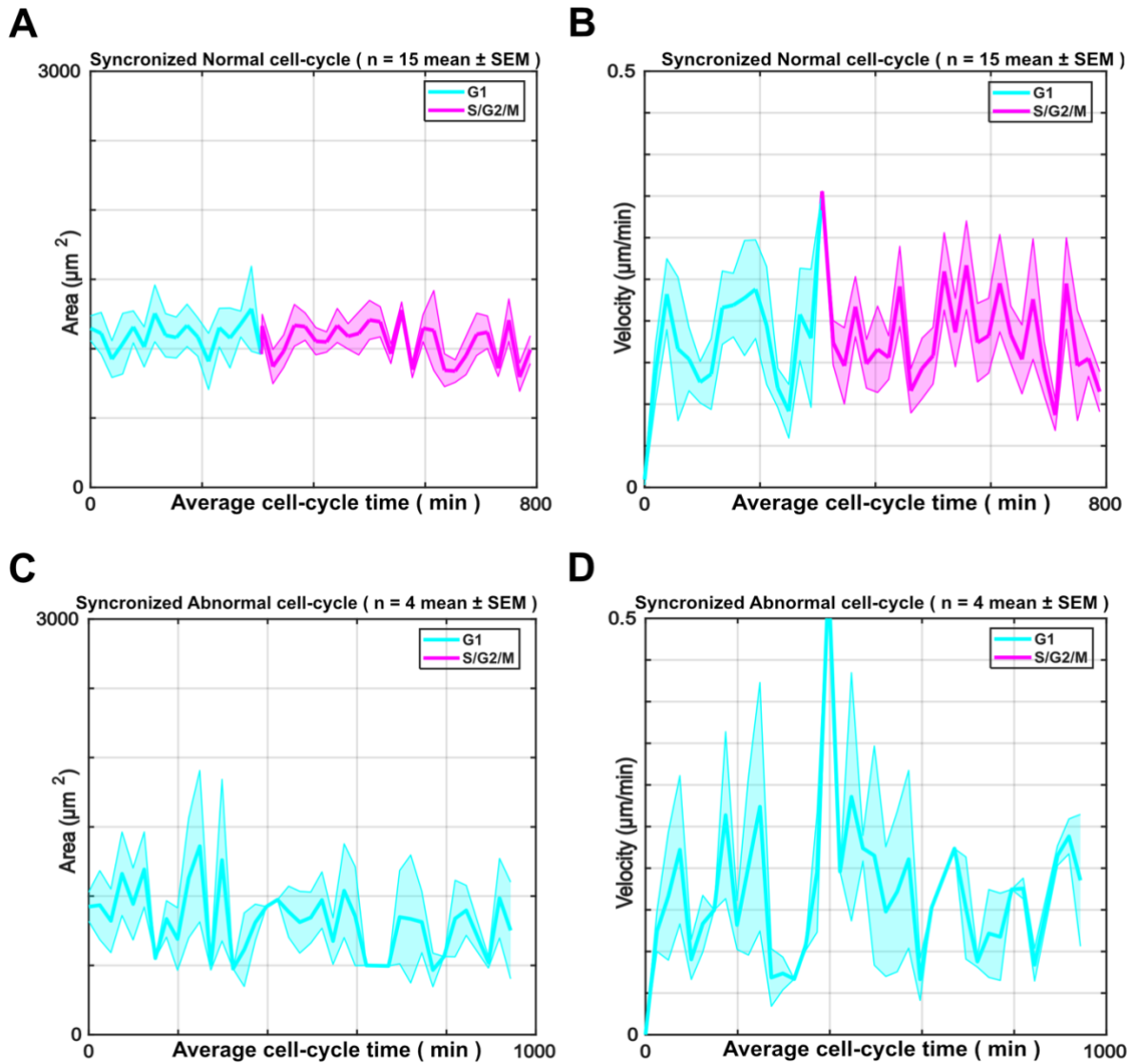


Figure A2.5. Population-average time-dependent profiles in normal vs. abnormal synchronized HT1080 CALIPERS cells.

(A-B). Population-average projected area vs normalized cell-cycle time for normal (A; n = 16) and abnormal (B; n = 4) trajectories. (C-D) Population-average instantaneous velocity vs normalized cell-cycle time for normal (C) and abnormal (D) trajectories.

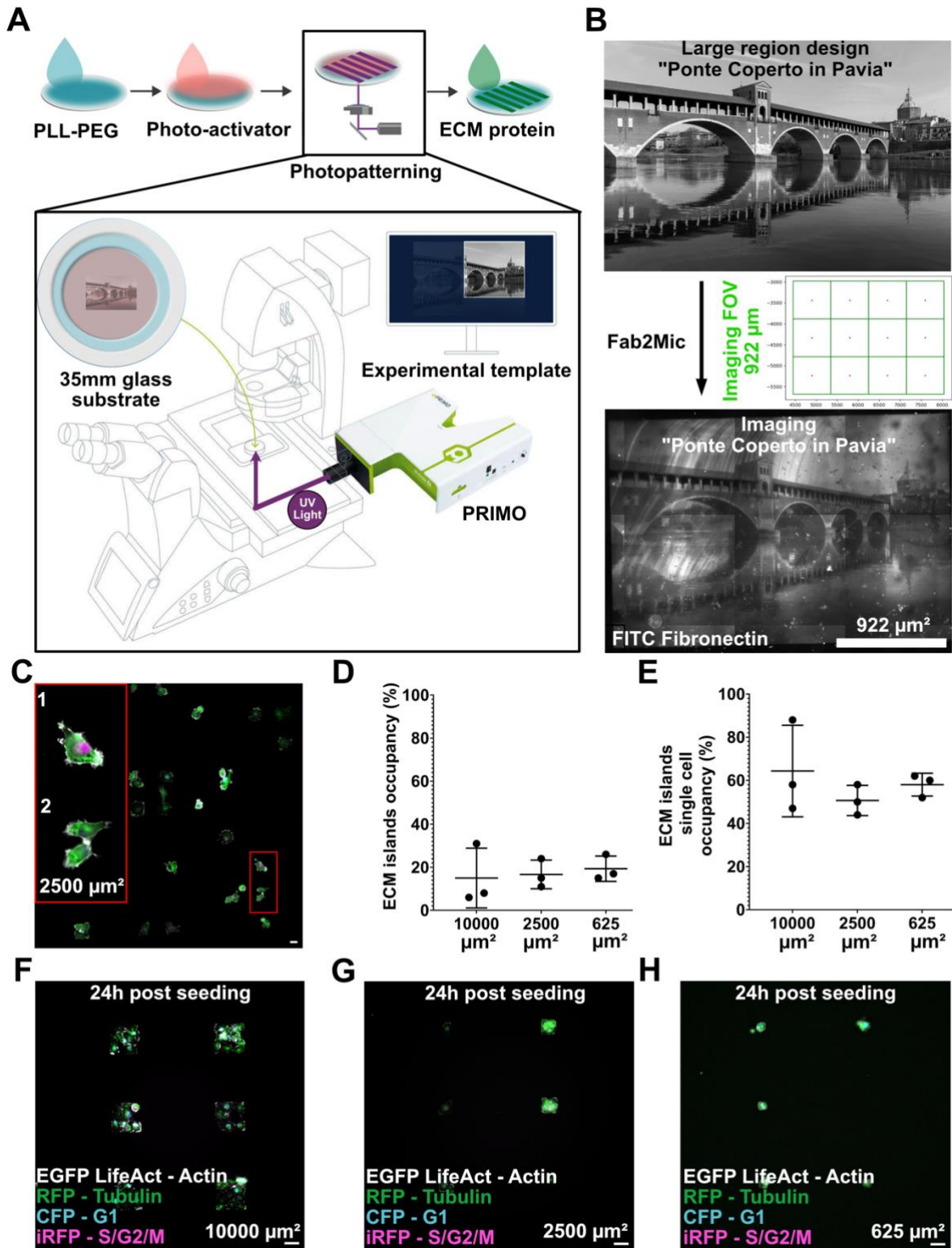


Figure A2.6. Fab2Mic validation and single-cell occupancy evaluation in square ECM islands.

(A) Schematic of the photopatterning protocol. Glass substrates were coated with PLL-PEG, exposed to photoactivator through the PRIMO UV system, and functionalized with ECM protein. Additionally, the developed Fab2Mic routine aligned fabrication templates

with imaging fields of view (FOVs). (B) Example of a large-region design patterned and coated with FITC-fibronectin, representing the “Ponte Coperto in Pavia.” The Fab2Mic algorithm generated imaging coordinates (922 μm FOV) for tiled acquisition. (C) Representative fluorescence images of patterned engineered ECM islands (2500 μm^2). Insets highlight poor single-cell occupancy and clusters adhering to individual islands. Scale bar, 25 μm . (D) Quantification of the overall engineered ECM island occupancy percentage across designs (10000, 2500, 625 μm^2) within the defined seeding strategy (E). Single-cell occupancy percentage per island. Data show mean $\pm$ SD. (F-H) Representative live-cell imaging of HT1080 CALIPERS cells seeded on ECM islands of (F) 10000 μm^2 , (G) 2500 μm^2 , and (H) 625 μm^2 . Actin (EGFP LifeAct), microtubules (RFP-Tubulin), and cell-cycle reporters (CFP-G1, iRFP-S/G2/M) are shown at 24 hours post-seeding, highlighting overcrowded environments. Scale bars, 25 μm .

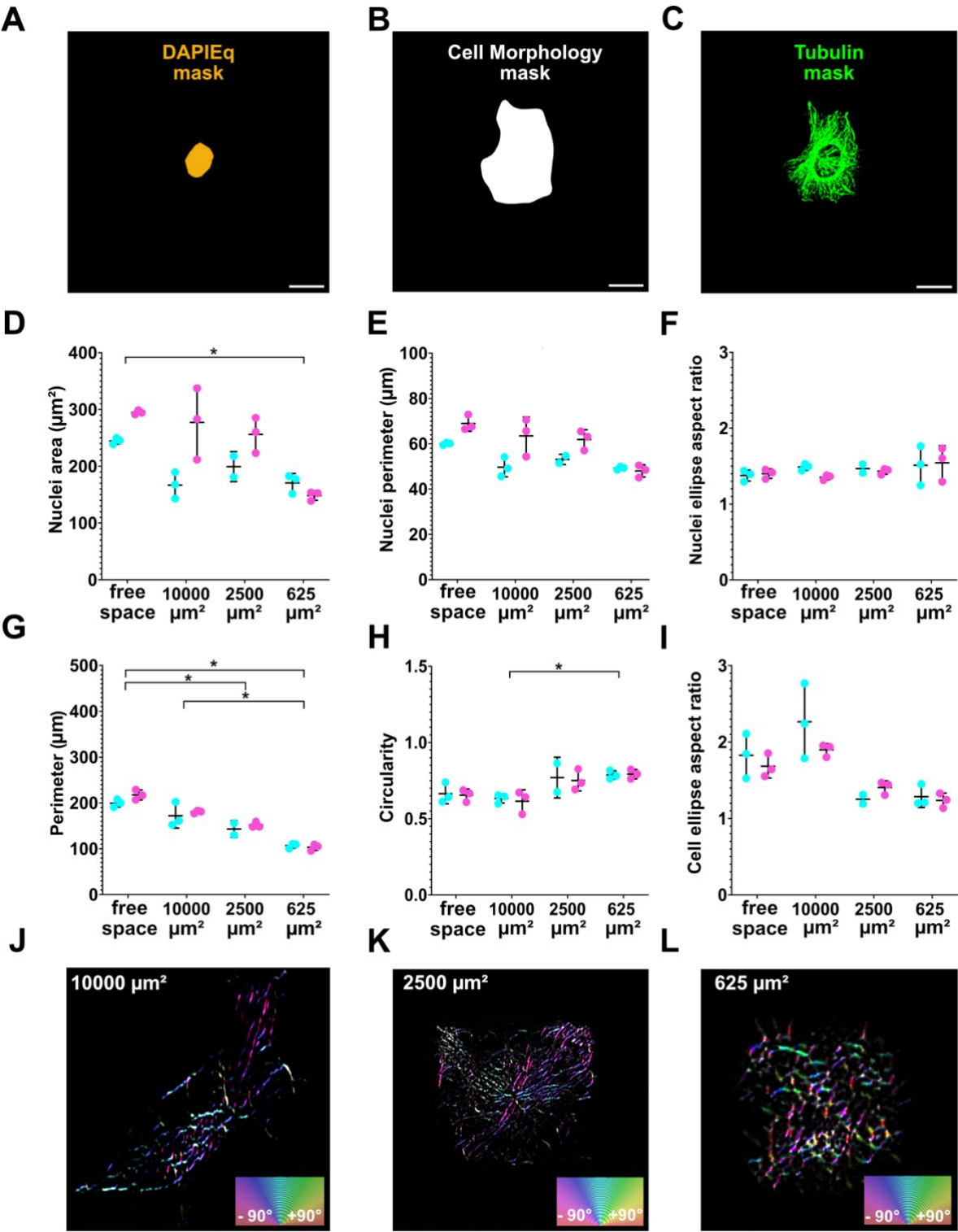


Figure A2.7. Additional nuclear and cytoskeletal morphology features across ECM confinements.

(A-C) Representative segmentation masks used for quantitative analysis: (A) nuclear mask (DAPIEq), (B) whole-cell morphology mask, and (C) tubulin cytoskeleton mask. Scale bars, 25 μm . (D-F) Nuclear morphology static metrics across free space and confined geometries (10000, 2500, 625 μm^2 islands) in three independent experiments: (D) nuclear area, (E) nuclear perimeter, and (F) nuclear ellipse aspect ratio. Data show mean $\pm$ SD; (* $p < 0.05$). (G-I) Whole-cell morphological descriptors across conditions: (G) cell perimeter, (H) circularity, and (I) ellipse aspect ratio. Data show mean $\pm$ SD; (* $p < 0.05$). (J-L) Orientation maps⁷³ of tubulin filaments in cells confined on ECM islands of (J) 10000, (K) 2500, and (L) 625 μm^2 . Orientation vectors are color-coded according to the indicated scale bars (-90° to $+90^\circ$). Insets show color scales.

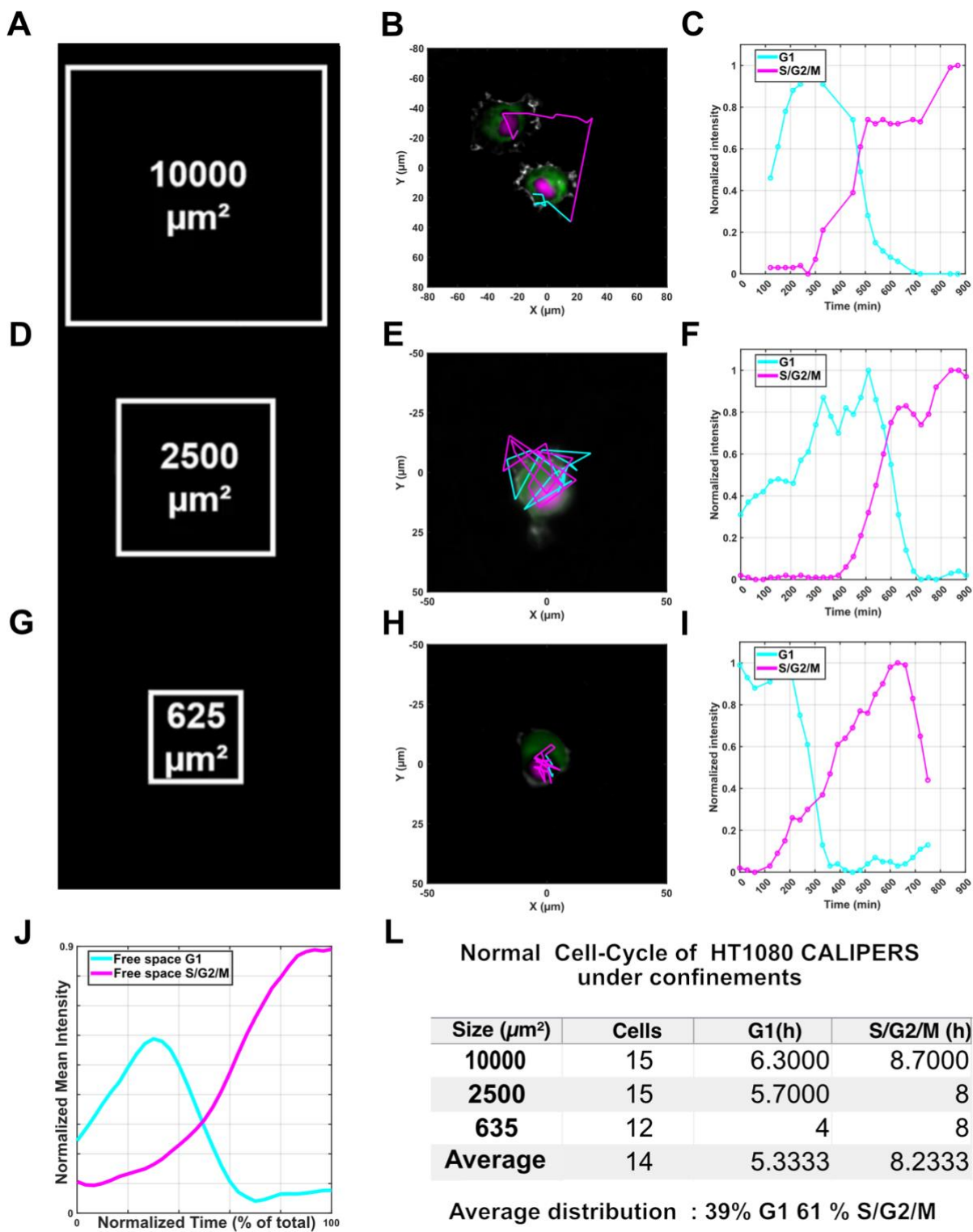


Figure A2.8. Normal cell-cycle dynamics of HT1080 CALIPERS cells under ECM confinement.

(A, D, G) Representative ECM confinement geometries of $10000\ \mu\text{m}^2$ (A), $2500\ \mu\text{m}^2$ (D), and $625\ \mu\text{m}^2$ (G) patterned islands used for analysis. (B, E, H) Trajectories of representative HT1080 CALIPERS cells undergoing normal cell-cycle in each confinement, showing actin (EGFP LifeAct), tubulin (RFP), and FUCCIplex reporters (CFP for G1, iRFP for S/G2/M). Migration paths are overlaid. (C, F, I) Time-resolved FUCCIplex intensity profiles (CFP = cyan, iRFP = magenta) corresponding to the cells shown in (B, E, H), with sequential progression through G1 and S/G2/M phases. (J) Average normalized FUCCIplex intensity traces of HT1080 CALIPERS cells in free space, showing mean temporal dynamics of G1 and S/G2/M phases. (L) Summary table of normal cell-cycle duration under confinement, reporting average times spent in G1 and S/G2/M phases across 10000 , 2500 , and $625\ \mu\text{m}^2$ ECM-islands. The overall distribution across all conditions was 39% G1 and 61% S/G2/M.

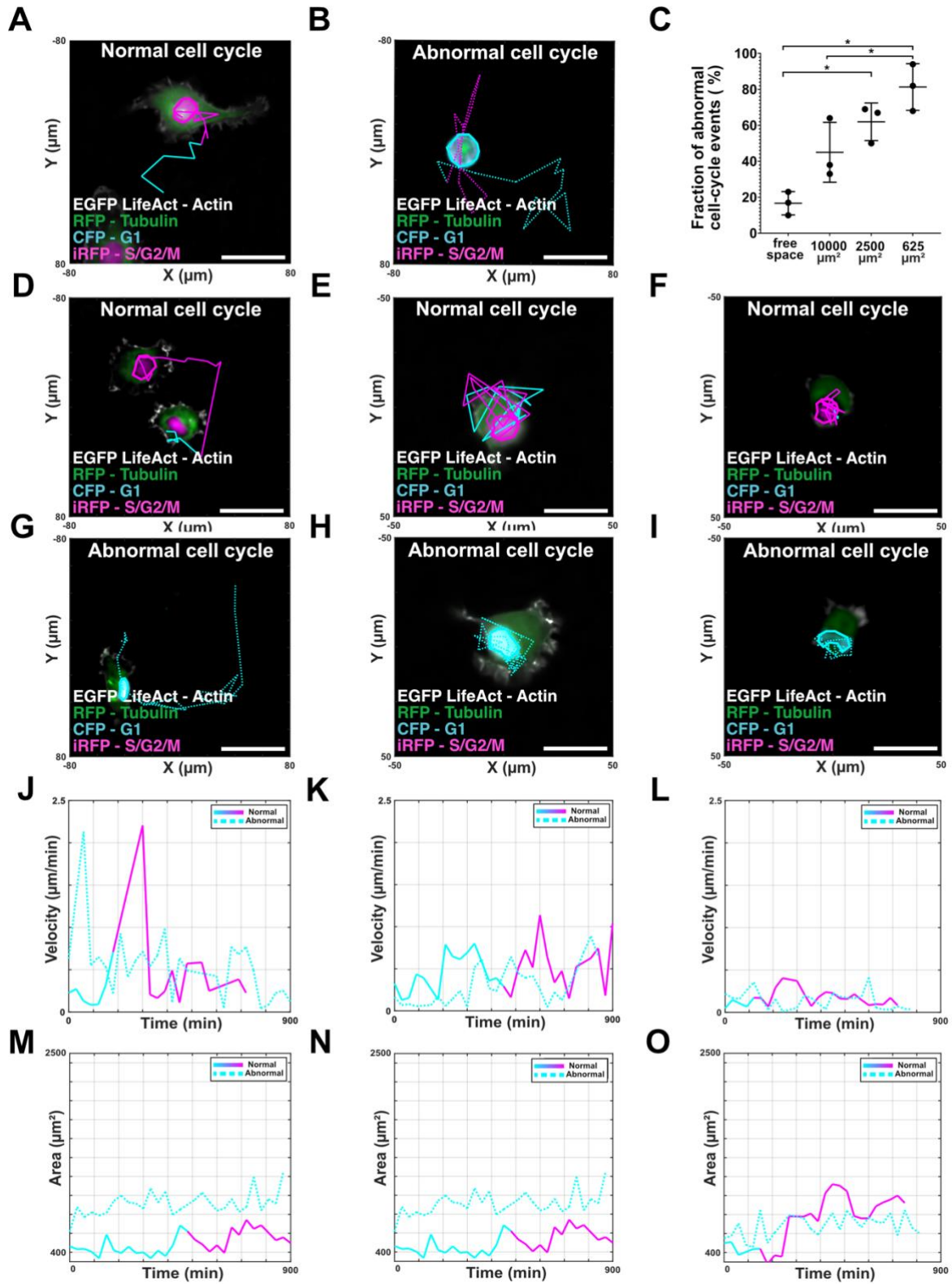


Figure A2.9. Extended phenotyping of normal vs. abnormal cell-cycle events under ECM confinement. (A-B) Representative trajectories of CALIPERS cells in free space, undergoing (A) normal or (B) abnormal cell-cycle events. (D, G, J, M) Representative trajectories and quantitative dynamics in 10000 μm^2 confinement, showing (D) normal, (G) abnormal cell-cycles, with corresponding (J) velocity and (M) area dynamics. (E, H, K, N) Representative trajectories and dynamics in a 2500 μm^2 confinement, showing (E) normal and (H) abnormal cycles, with (K) velocity and (N) area profiles. (F, I, L, O) Representative trajectories and dynamics in 625 μm^2 confinement, showing (F) normal, (I) abnormal cycles, with (L) velocity and (O) area profiles. Cells express EGFP LifeAct (actin), RFP-tubulin, CFP (G1), and iRFP (S/G2/M). Migration trajectories are overlaid. Scale bars, 25 μm . (C) Quantification of abnormal cell-cycle events across confinement conditions. Data show mean $\pm$ SD; * $p < 0.05$.

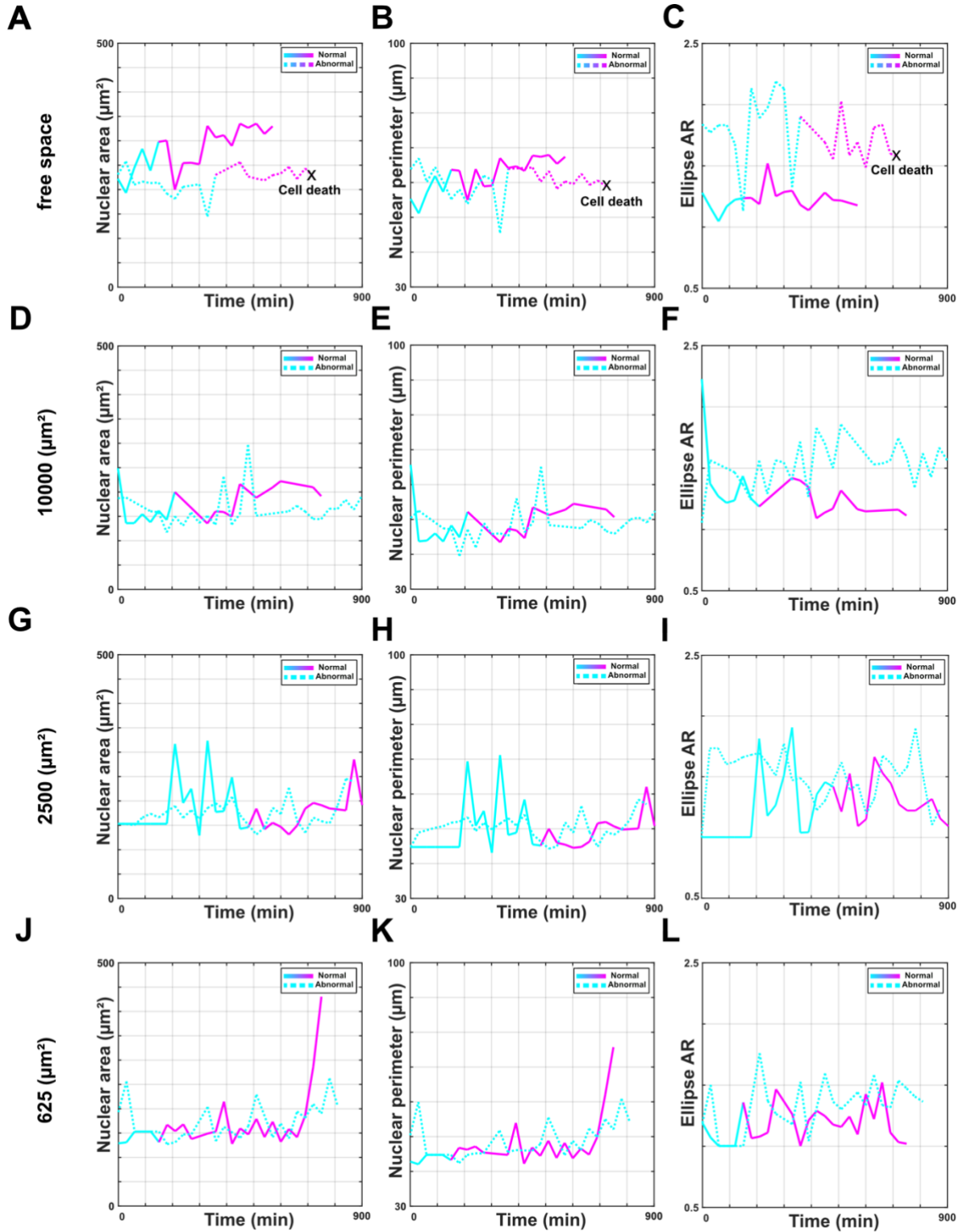


Figure A2.10. Nuclear dynamics across different square ECM sizes in normal vs. abnormal cell-cycles. Time-resolved nuclear morphology metrics of CALIPERS cells tracked over 15 hours in free space (A-C), 10000 μm^2 islands (D-E), 2500 μm^2 islands (G-I), and 625 μm^2 islands (J-L). For each condition, nuclear area, perimeter, and ellipse

aspect ratio (AR) are plotted for cells undergoing normal (solid line) and abnormal (dashed line) cell-cycle. Cell death is indicated where applicable (X).

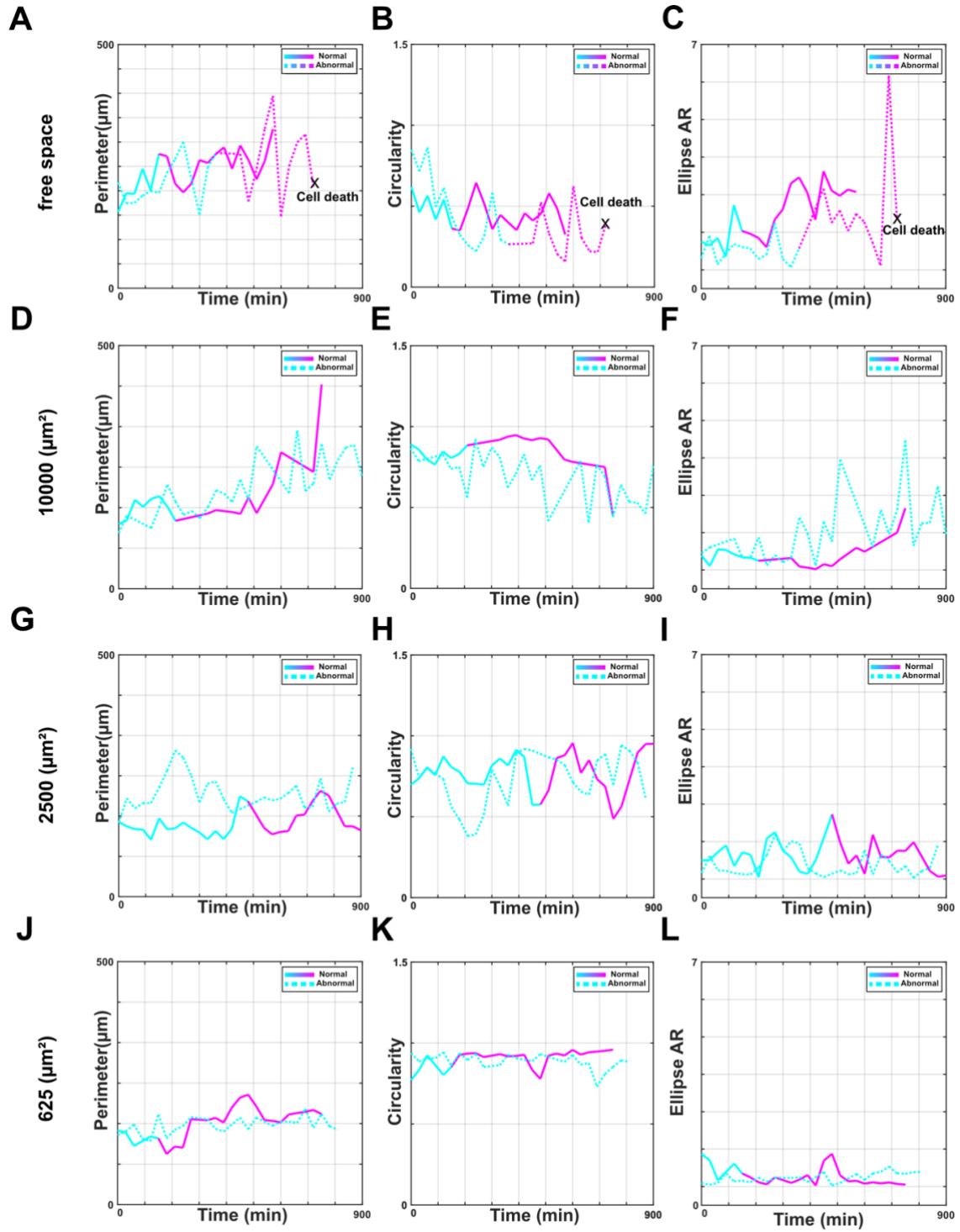


Figure A2.11. Cell morphological dynamics across different square ECM sizes in normal versus abnormal cell-cycles. Time-resolved morphological metrics of HT1080 CALIPERS cells tracked over 15 hours in free space (A-C), 10000 μm^2 (D-F), 2500 μm^2 (G-I), and 625 μm^2 (J-L) ECM islands. For each condition, cell perimeter, circularity,

and ellipse aspect ratio (AR) are plotted for cells undergoing normal (solid line) and abnormal (dashed line) cycles. Instances of cell death are indicated where applicable (X).

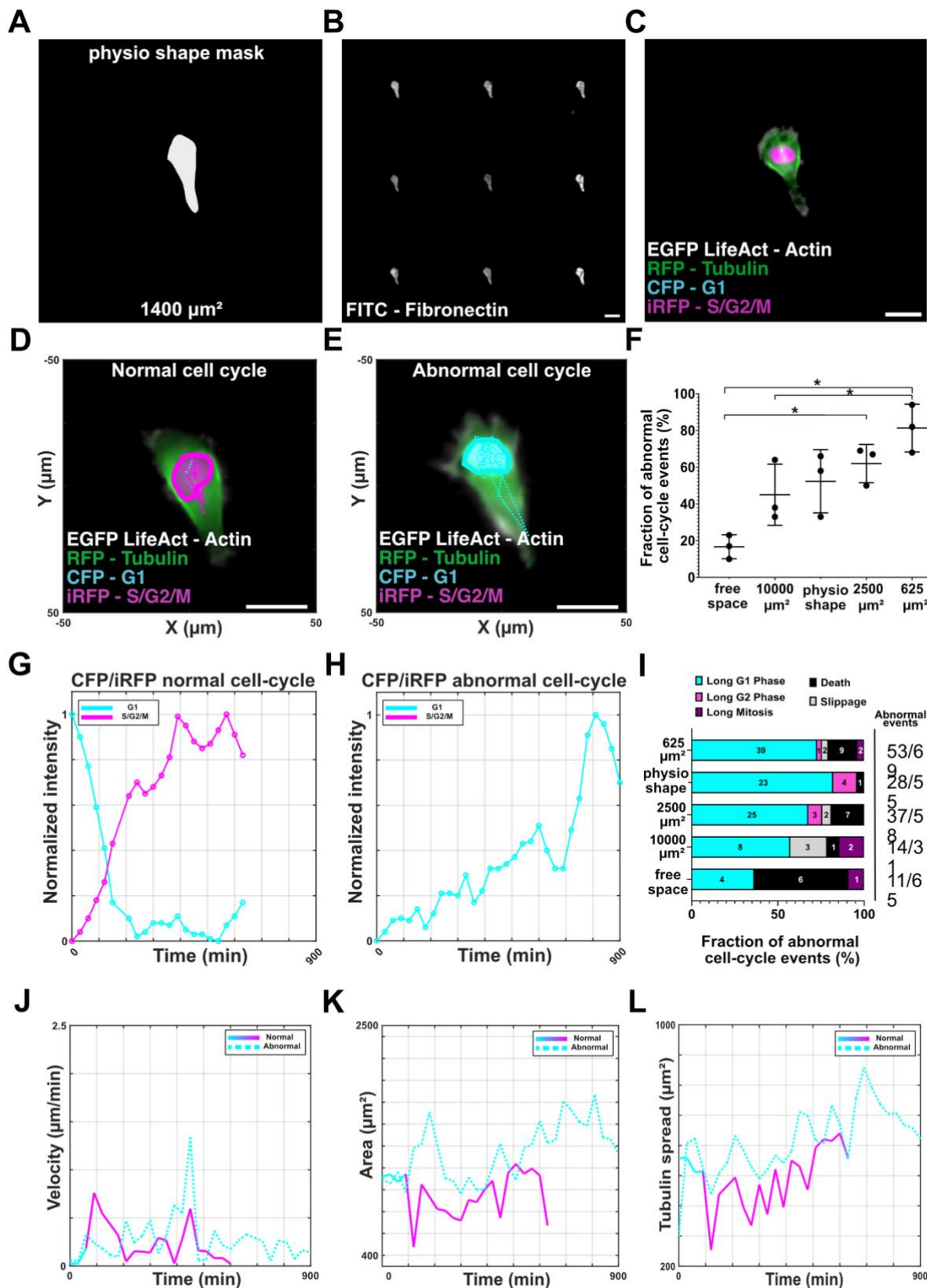


Figure A2.12. Physiologically inspired ECM island and related cell-cycle abnormalities.

Representative masks and fluorescent validation of HT1080 CALIPERS cells on physio-shaped ECM islands (Physiological mean area size, $1400 \mu\text{m}^2$). (A) Design of a physio-shaped island, (B) Photopatterning validation with a FITC-fibronectin-coated array of physio shape islands, and (C) single cell expressing EGFP LifeAct (actin), RFP-tubulin, CFP (G1), and iRFP (S/G2/M) adhered to physio-shaped engineered ECM island. Scale bars, $25 \mu\text{m}$. (D-E) Example cells undergoing (D) normal and (E) abnormal cell-cycle events under confinement. Scale bars, $25 \mu\text{m}$. (F) Quantification of abnormal cell-cycle events across free space, $10000 \mu\text{m}^2$, physio-shaped, $2500 \mu\text{m}^2$, and $625 \mu\text{m}^2$ islands. Data show mean $\pm$ SD; $*p < 0.05$. (G-H) Representative CFP/iRFP intensity profiles from cells undergoing (G) normal or (H) abnormal cycles. (I) Distribution of abnormal cell-cycle events across different confinement geometries, defined as Long G1, Long S/G2/M, long mitosis, S/G2/M-G1 slippage, or cell death. (J - L) Quantitative comparison of motility and morphology dynamics between normal and abnormal cycles: (J) velocity, (K) cell area, and (L) tubulin spread over time under physio-shape confinement.

A3 Chapter 5 - MCPFM simulations and phase-field regimes

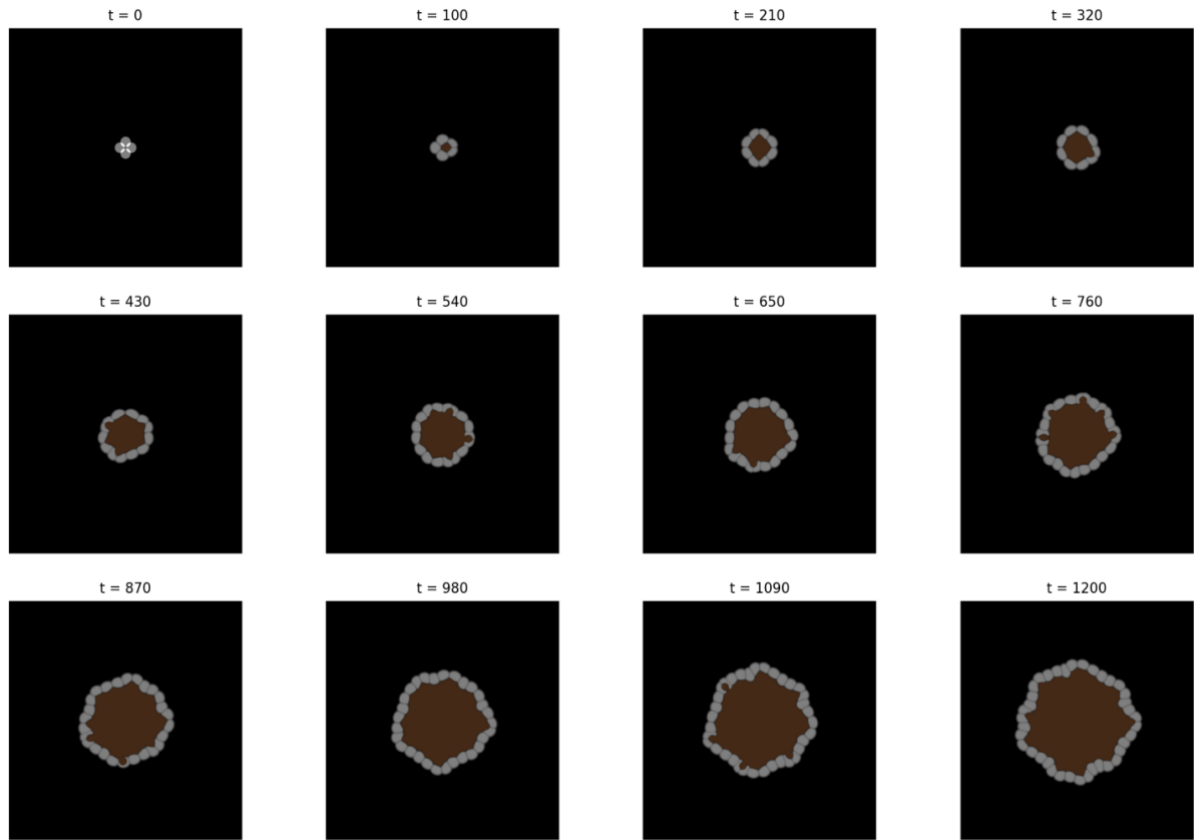


Figure A3.1. Representative MCPFM morphologies and temporal trajectories in spherical regime. Time-resolved simulation snapshots ($t = 0$ to 12000 min) for a parameter set that converges to the spherical regime, showing early cavity nucleation followed by progressive cavity inflation and boundary smoothing. High pressure, with low permeability, and slow proliferation rate-governed scenario.

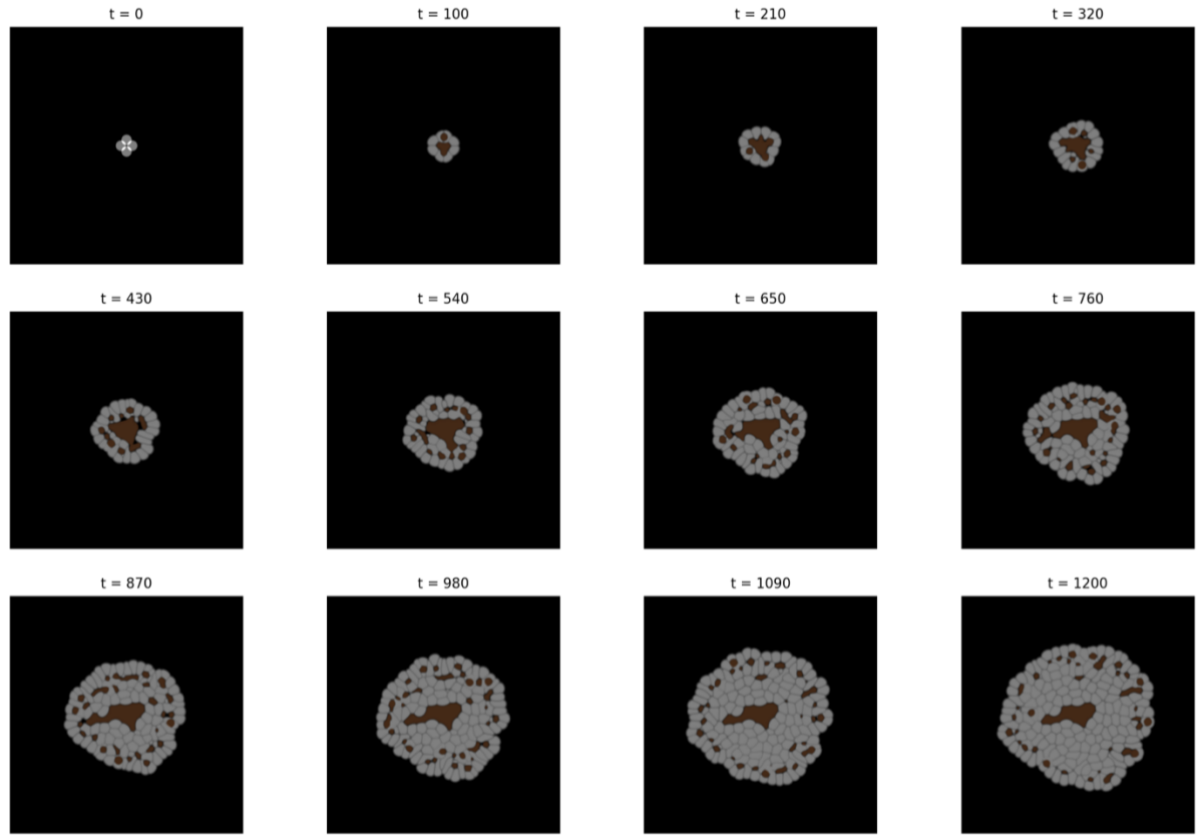


Figure A3.2. Representative MCPFM morphologies and temporal trajectories in the branched/complex regime. Time-resolved simulation snapshots ($t = 0$ to 12000 min) for a parameter set that converges to the branched regime, showing early cavity nucleation followed by progressive distributed satellite cavities during tissue expansion. Low Pressure, high permeability, and fast proliferation rate governed scenario

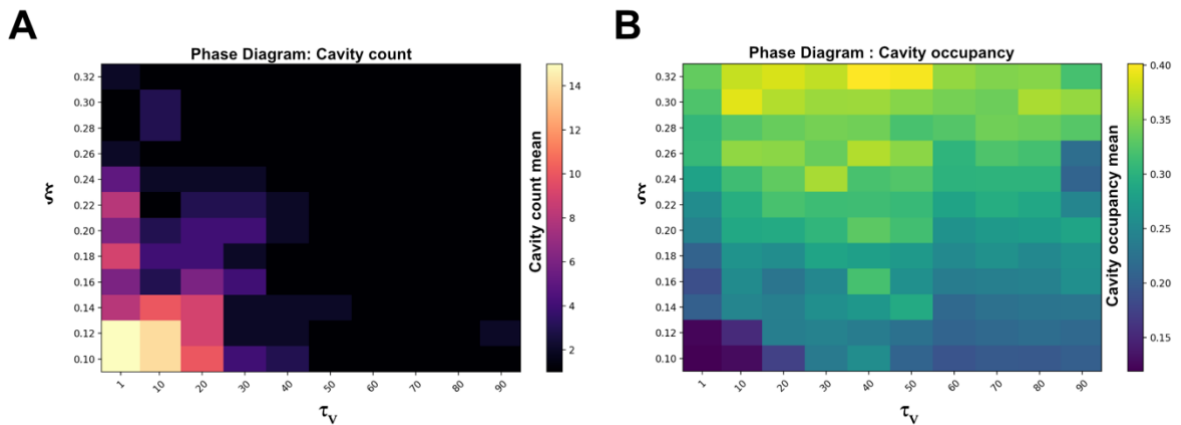


Figure A3.3. MCPFM phase-field regime maps for cavity morphogenesis regimes. (A) Phase diagram of mean cavity count across the sweep, where cavities are identified as connected components from the segmented internal void space. (B) Phase diagram of mean cavity occupancy, reported as the cavity volume fraction relative to the segmented

structural volume for the same (τ_v, ξ) grid. Colorbars indicate the corresponding mean values at each parameter pair.

B

Appendix B Additional tables/methods

B1 Chapter 2 - FUCCIsmart extended data

Classification	True Positive	True Negative	False Positive	False Negative
Multi FUCCIsmart exp 1	56%	11%	22%	11%
Multi FUCCIsmart exp 2	35%	0%	26%	39%
Multi FUCCIsmart exp 3	37%	19%	31%	13%
Single FUCCIsmart exp 1	78%	0%	22%	0%
Single FUCCIsmart exp 2	67%	33%	0%	0%
Single FUCCIsmart exp 3	55%	18%	9%	18%

Mean	True Positive	True Negative	False Positive	False Negative
Multi FUCCIsmart	43%	10%	26%	21%
Single FUCCIsmart	67%	17%	10%	6%

SD	True Positive	True Negative	False Positive	False Negative
Multi FUCCIsmart	12%	10%	5%	16%
Single FUCCIsmart	12%	17%	11%	10%

Class	Experiment ID	Sample size (N)	Mitosis acquired (%)	Phototoxic Events (%)
FUCCIsmart Single	1	9	78	17
FUCCIsmart Single	2	3	67	0
FUCCIsmart Single	3	11	55	5
FUCCIsmart Multi	1	23	35	11
FUCCIsmart Multi	2	16	38	19
FUCCIsmart Multi	3	18	56	17
Automation 1h	1	19	63	53
Automation 1h	2	24	67	63
Automation 1h	3	24	79	65
Automation 6h	1	19	11	18
Automation 6h	2	19	16	9

Automation 6h	3	17	12	15

Class	Mean Mitosis Acquired (%)	Standard Deviation (SD)	Mean Phototoxic Events (%)	Standard Deviation (SD)
FUCCIsmart Single	66.67	11.5	7.33	8.74
FUCCIsmart Multi	43	11.36	15.67	4.16
Automation 1h	69.67	8.33	60.33	6.43
Automation 6h	13	2.65	13.33	3.79

Multiple comparison mitosis acquired - Bonferroni correction

Mitosis acquisition (%)	FUCCIsmart Single	FUCCIsmart Multi	Automation 1h	Automation 6h
FUCCIsmart Single	/	0.0113	>0.9999	<u><0.0001</u>
FUCCIsmart Multi	0.0113	/	0.0042	0.0014
Automation 1h	>0.9999	0.0042	/	<0.0001
Automation 6h	<u><0.0001</u>	0.0014	<0.0001	/

Multiple comparison Phototoxic events- Bonferroni correction

Phototoxic Events (%)	FUCCIsmart Single	FUCCIsmart Multi	Automation 1h	Automation 6h
FUCCIsmart Single	/	>0.9999	<u><0.0001</u>	>0.9999
FUCCIsmart Multi	>0.9999	/	<u><0.0001</u>	>0.9999
Automation 1h	<u><0.0001</u>	<u><0.0001</u>	/	<0.0001
Automation 6h	>0.9999	>0.9999	<0.0001	/

B2 Chapter 4 - Oblique-Plane Microscope alignment protocol

Scope and alignment philosophy

This protocol standardizes the optical alignment workflow for a 3-objective oblique-plane microscope (“snoutscope”-style geometry), covering emission path alignment, excitation light-sheet alignment, and scan-unit coupling/calibration. The goal is to minimize rework by keeping a single global alignment beam fixed whenever possible and assembling subunits that remain stable when cross-braced.

Required equipment and references

Primary metrology	Collimated alignment laser (single wavelength), irises, beam card, distant target ($\geq 3\text{-}5$ m), level.
Mechanical references	Beam height reference (e.g., rigidly clamped Allen key), ruler/calipers, bolt lines.
Optical aids	Retroreflector, shear plate or simple mirror for collimation checks, ND filters, ground glass target.
Mounting hardware	2" Polaris mounts + objective adapters, retaining rings, 90° brackets for cross-bracing, aluminum crossbrace.
Imaging tools	Camera + temporary ND stack for rough focusing; optional fluorescent sample/Argolight for validation.

Global preparation (do once)

- Choose a beam height compatible with the optomechanics (e.g., $\sim 1\text{-}2$ mm above the physical center of 100 mm posts).
- Create a hard beam-height reference (e.g., Allen key clamped horizontally). Verify height by clipping the key tip with the beam.
- Establish a level, collimated alignment beam along a convenient bolt line. Prefer a position that will not conflict with later excitation/emission optics.
- Place two irises along the beam to enforce straightness and enable rapid rechecking after disturbances.

Checkpoint: Alignment beam remains stable and repeatable across multiple table positions.

Recommended high-level order of operations

- Fix alignment beam through the future O2 position (location that won't be blocked by later optics).
- Align Microscope 1 subunit (TL1 - O1).
- Align emission arm from TL2 - camera (with O2/O3 structure in place as needed).
- Align excitation path (dichroic reflection-based build).
- Move alignment beam to couple between TL2 and O2, then couple Microscope 1 via scan unit and calibrate scanners.
- Final fine-tuning: excitation sheet chasing/tipping and image-quality validation on a test sample.

Note: This order reduces the number of times the alignment beam must be moved compared to first-pass builds.

Subprotocol 1: Microscope 1 subunit (TL1 + O1)

1. Mount TL1 near the edge of a plate large enough to move later; send alignment beam through TL1 towards O1 position.
2. Align TL1 tip/tilt and XY using back reflection (and optionally non-deflection on a distant target).
3. Add 90° bracket on TL1 upright for later cross-bracing.
4. Mount O1 (typically pointing upward toward the sample). Add 90° bracket for cross-bracing.
5. Align O1 primarily by back reflection (high NA causes rapidly diverging output; non-deflection is less informative).
6. Set TL1-O1 axial spacing by collimation/minimizing output spot size.
7. Cross-brace and bolt down the subunit to a rigid plate.

Checkpoint: O1 back reflection is centered; output is approximately collimated with minimized spot size.

Subprotocol 2: Microscope 2/3 core (O2 - dichroic - TL2 - O3)

1. Pre-align O3 in its mount
2. Install O3 in its mount with rough rotation/height/tip set close to expected final configuration.

3. Overfill O3 front face with a collimated beam to detect faint reflections from the ground-glass edge; rotate until the reflection feature behaves as expected.
4. Rough-set height/tip via objective back reflection (flipping the objective can help).

Note: This pre-alignment prevents large corrections later when O2/O3 are on a shared crossbrace.

5. Place O2 and dichroic; align TL2
6. Place O2 in the alignment beam at its final position; align on objective back reflection.
7. Insert dichroic at $\sim 45^\circ$ incidence in its final location (~ 100 mm from O2 back). Use the largest possible beam to ensure centering in both axes; verify excitation bolt line feasibility.
8. If alignment wavelength is reflected by the dichroic, substitute a glass blank for this stage.
9. Walk the alignment beam (using upstream mirrors) into agreement through the dichroic and into O2.
10. Set TL2 axial position by aligning TL2 to O2: use both back reflection and non-deflection; set spacing by collimation/minimizing spot size.
11. Use combined O2+TL2 back reflection as a high-sensitivity metric; prioritize combined reflection if metrics disagree.

Warning: Avoid placing the dichroic too close to TL2; ensure beam clearance at the far field of view (not only central ray).

Checkpoint: TL2-O2 output is collimated; combined back reflection is symmetric and centered.

Mount O2 and O3 on a shared aluminum crossbrace; make O2-O3 co-focal

1. Hand-position O3 to within <1 mm of final placement; bolt the clamping fork.
2. Mechanically set O3 tilt angle (e.g., 30° from straight-through). Ensure no lens collision.
3. Bolt O2 and O3 to a central aluminum crossbrace (thermal compensation). Unbolt individual supports so crossbrace carries load.
4. Re-walk the alignment beam onto the new O2 axis (with TL2 removed).
5. Reinstall and re-tweak TL2 alignment as needed for the final O2 position; then remove TL2 again.
6. Co-focus O3 with O2 using a two-level optimization: (i) collimate O3 output with a large beam (axial knob equal turns), (ii) optimize retroreflector return with a small beam through a slit card.
7. Iteratively walk O3 field with single-axis adjustments: move far-field spot to a nearby location, re-collimate, then assess retroreflector ring centering; repeat axis-by-axis.

Checkpoint: Retroreflector-induced outer rings are centered on inner rings in the slit view (symmetric back reflection).

Subprotocol 3: Tube lens 3 (TL3) and camera positioning

1. Establish a reverse-direction alignment beam through the O2/O3 unit onto O3 axis (temporary steering mirrors). Preserve the original global alignment beam for later reuse.
2. Align TL3 to O3 axis using back reflection (TL3-only for tip/tilt; combined TL3+O3 for XY).
3. Set TL3 axial position by collimation through TL3-O3-O2-TL2 using a large collimated beam. Confirm O2/O3 remain collimation-preserving.
4. Remove temporary steering mirrors used for reverse-beam injection.
5. For camera rough placement: remove TL2; use ND filters temporarily near camera to prevent saturation; slide camera axially until spot is minimal and centered on sensor.
6. Move ND filters to an infinity space between O3 and TL3 (avoid changing camera conjugate during final tuning).
7. In a darkened room, fine-position camera to center spot within a few pixels and minimize spot size (≈ 2 px if Nyquist sampled).

Note: Camera mounting orientation can be chosen to align rolling shutter direction with sheet propagation for speed gains.

Warning: Camera cables can torque the camera and destabilize alignment; strain-relief is strongly recommended.

Checkpoint: Spot is centered and minimal; system behaves repeatably when lightly perturbed.

Subprotocol 4: Scan unit coupling (Microscope 1 - Microscope 2)

1. Bring an alignment beam onto the axis of TL2 propagating back toward Microscope 1 location.
2. Install scanning mirror A as close to TL2 as possible while keeping TL2 removable/replacable; set mirrors to 0 V for rough geometry
3. Install scanning mirror B at $\sim 45^\circ$ incidence and route onto bolt line toward TL1 position; approximate TL1-TL2 separation as $f_1 + f_2$.
4. Temporarily bring Microscope 1 subunit into place and slide until TL1 output appears collimated; then bolt down scan mirrors.
5. Use Y voltages on both mirrors to level the beam and match the beam-height reference.
6. Slide Microscope 1 plate into final location; clamp down incrementally while monitoring TL1 back reflection to avoid drift.
7. Fine-tune TL1 back reflection using scan mirror voltages; optionally use O1 back reflection as an extra metric.

Checkpoint: Scan mirror home voltages yield a beam coaxial through TL1 and compatible with TL2 -camera alignment.

Subprotocol 5: Excitation path (light-sheet) alignment

The excitation path is built from the dichroic reflection (front-surface reflection) outward, using a stable reference iris placed at the TL2 focal point to isolate the correct reflection.

1. Use the reflection off the dichroic front face as the alignment signal; place an iris at TL2 focal point to block undesired reflections.
2. Place RL3 to form a 4f telescope with TL2; align by back reflection and non-deflection; set axial spacing by collimation.
3. Remove TL2 and place gimbal mirror A at RL3 focus (sheet chasing). Center the focus on the mirror.
4. Place RL2 in a 4f telescope with RL3; realign as needed.
5. Replace TL2 and place gimbal mirror B at RL2 focus (sheet tip/tilt).
6. Place RL1 as specified (2×4f relay where required for mechanical clearance and gimbal usability).
7. Align a proxy spherical lens at the cylindrical-lens position on a rotation mount; swap to cylindrical lens and set focusing axis correctly.
8. Add an iris after the cylindrical lens (output centered) and install steering mirrors (SM1, SM2) for laser-box coupling.
9. Collimate fiber output with a lens at distance f ; steer with SM1/SM2 to overlap excitation beam with the existing alignment path.

Add apertures: (i) up/down curtains (field height), (ii) NA aperture (sheet width) near the cylindrical lens; tune later.

Note: If dichroic orientation is corrected after installation, re-align the excitation axis using gimbal mirrors, then reinsert RL2/RL3.

Checkpoint: Excitation beam overlaps alignment references and forms a stable sheet through the sample-side objective.

Scan unit calibration and validation checks

1. Set the scan mirrors to home voltages using TL2 and O2 back reflections until optical axes agree across subsystems.
2. Verify camera spot remains centered and minimal at home voltages (sanity check).

Check axis consistency: deflect mirror A alone and mirror B alone from home; confirm they scan along the same lines on a distant target.

3. Check pure lateral motion: remove TL1 and TL2 so beam passes only over scan mirrors; use a card with two holes and a distant target to tune mirror scale factors for angle cancellation.
4. Record final home voltages and scale factors. Example observed values from notes: $a\_y0 \approx 0.4$, $a\_x0 \approx 0$; $b\_y0 \approx 1.4$, $b\_x0 \approx 0.03$; $y\_scale\_for\_a \approx -1$; $x\_scale\_for\_a \approx 1$

5. Plan absolute calibration ($V \rightarrow \mu\text{m}$ in sample) using an Argolight slide or graticule target.

Warning: Occasional positional 'jumps' were noted during whiteboard tests; monitor for drift/teleportation and re-check repeatability.

Checkpoint: Scanning produces predominantly lateral displacement with minimal residual angle at multiple distances.

Final fine-tuning and system QC

Cylindrical lens axial fine-tune: match the excitation sheet image plane to a known image plane (e.g., after TL1/scan unit).

Sheet tipping: use a shallow fluorescein 'lake' to align sheet angle to the oblique detection plane.

Field aperture centering: ensure illuminated area is centered on camera sensor across scan range.

Image quality validation: Argolight resolution checks; confirm PSF/spot size and distortion across FOV.

Daily reproducibility: verify scan-unit home voltages and key back reflections at the start of each session.

Troubleshooting Quick Map

Symptom: TL2 alignment 'looks good' but combined O2+TL2 reflection is off	Favor combined reflection; check TL2 tip/tilt first, then XY; re-check dichroic verticality and beam height.
Symptom: O3 retroreflection rings unstable	Confirm O2/O3 remained collimation-preserving after mechanical changes; reduce degrees-of-freedom and iterate one axis at a time.
Symptom: Camera focus shifts after ND changes	Remove ND filters from camera conjugate during final focusing; place ND in infinity space instead.
Symptom: Excitation sheet drifts after dichroic re-orientation	Rebuild excitation axis from dichroic front reflection; use gimbal mirrors to re-register; then reinsert relays.
Symptom: Scan motion not lateral (angle residual)	Repeat 'mirrors-only' test with lenses removed; tune scale factor for cancellation; re-check mirror mounting orientation.

B3 Chapter 5 - Multicellular Phase-Field Model for lumen morphogenesis

A two-dimensional multicellular phase-field model (MCPFM) for epithelial lumen morphogenesis was adopted and implemented in a reproducible codebase to simulate lumen initiation, growth, fusion, and branching. The framework follows published phase-field formulations for epithelial organoid morphogenesis (Tanida et al., PLOS Computational Biology, 2025). Each epithelial cell is represented by an individual phase-field variable and the lumen by a distinct phase-field variable. Field evolution follows gradient-descent dynamics of a free-energy functional, discretized using explicit finite differences on a uniform Cartesian grid.

Phase-field representation and geometric measures

The simulated organoid consists of M epithelial cells and a lumen. Cell geometry is represented by continuous phase fields $u_m(\mathbf{r}, t)$ ($m = 1, \dots, M$), with $u_m \approx 1$ inside cell m and $u_m \approx 0$ outside. The lumen is represented by $s(\mathbf{r}, t)$, with $s \approx 1$ inside the lumen and $s \approx 0$ outside.

A smooth indicator function converts diffuse phase fields into effective areas (2D volumes):

$$h(p) = p^2(3 - 2p)$$

Cell and lumen areas are computed as:

$$V_m = \int h(u_m) d\mathbf{r} \text{ and } V_l = \int h(s) d\mathbf{r}$$

where V_m denotes the effective area of cell m and V_l denotes the effective lumen area.

Free-energy functional

Morphogenesis is driven by minimization of the total free energy

$$E = E_u + E_s + E_{int}$$

The functional includes (i) diffuse-interface regularization, (ii) a cortical/surface-tension contribution on cells, (iii) cell-cell adhesion, and (iv) excluded-volume penalties for cell-cell and cell-lumen overlaps. A spatially uniform luminal driving term ξ acts on the lumen as a pressure-like energetic bias favoring lumen expansion and coarsening/fusion. For each phase field $p \in \{u_m\} \cup \{s\}$, interface regularization is implemented as:

$$E_{pf}(p) = \int \left[\left(\frac{D_p}{2} \right) |\nabla p|^2 + \frac{p^2}{4} p^2 (1-p)^2 \right] d\mathbf{r}$$

Cell cortical tension is modeled via a surface penalty on the smoothed indicator:

$$E_{surf} = \sum_{m=1}^M \frac{\gamma}{12} |\nabla h(u_m)|^2 d\mathbf{r}$$

A spatially uniform permeability driving act ξ on the lumen

$$E_{osm}(s) = -\frac{\xi}{6} \int h(s) d\mathbf{r} = -\frac{\xi}{6} V_l$$

Cell-cell adhesion is represented as:

$$E_{adh} = \int \sum_{m \neq m'} \frac{\eta}{12} \nabla h(u_m) \nabla h(u_{m'}) d\mathbf{r}$$

Excluded-volume repulsion between cells, and between cell and lumen, is enforced by overlap penalties:

$$E_{exc} = \int \left[\sum_{m \neq m'} \frac{\beta}{12} h(u_m) h(u_{m'}) + \sum_m \frac{\beta_{su}}{6} h(s) h(u_m) \right] d\mathbf{r}$$

Cell size regulation is implemented using a quadratic penalty to a time-dependent target volume:

$$E_{vol} = \sum_{m=1}^M \frac{\alpha}{12} \left(V_{target,m}(t) - \int h(u_m) d\mathbf{r} \right)^2 d\mathbf{r}$$

Dynamics, growth and divisions

Phase fields evolve via Allen-Cahn-type gradient descent:

$$\tau_u \frac{\partial u_m}{\partial t} = -\frac{\partial E}{\partial u_m} \quad , \quad \tau_s \frac{\partial s}{\partial t} = -\frac{\partial E}{\partial s}$$

$$\begin{aligned} \tau_u \frac{\partial u_m}{\partial t} &= D_u \nabla^2 u_m + u_m(1 - u_m) \left(u_m - \frac{1}{2} + f_u \right) \\ \tau_s \frac{\partial s}{\partial t} &= D_u \nabla^2 s + s(1 - s) \left(s - \frac{1}{2} + f_s \right) \end{aligned}$$

Where f_u aggregates contributes from volume regulation, curvature, adhesion, and exclusion, and f_s includes the permeability driving ξ and lumen-cell exclusion.

We encode growth by evolving each cell's target area $V_{target,m}(t)$ toward a nominal division area $\bar{V}$ with timescale τ_V (a proxy for the proliferation timescale. When a division criterion is met and the number of cells remains below a user-defined maximum, the mother cell is partitioned across a division plane to generate two daughter fields. When enabled, lumen nucleation is coupled to division by seeding a micro-lumen of radius r_s , implemented by locally adding to $s(r)$ and subtracting from the dividing cell field.

Numerical discretization and computational domain

All PDEs are discretized on a uniform 2D grid (default 2048*2048 points) with $dx = dy = 0.02$ (di-dimensionless units). Time integration uses explicit Euler updates with default $dt = 0.02$. Laplacians and gradients are computed using finite differences; an 8-neighbor stencil is used for the Laplacian to improve isotropy. Each cell field u_m is stored on a local patch (default 200x200) aligned to the cell's bounding box (top-left index (i_{min}, j_{min}) in the global domain). This reduces memory footprint and accelerates per-cell updates, while global interactions are mediated through auxiliary fields.

Interaction fields and per-step update algorithm

To avoid explicit $O(M^2)$ pairwise evaluations, we maintain a summed cell indicator and an additional summed occupancy field for output and selected interaction evaluations.

$$\varphi(r) = \sum_{m=1}^M h(u_m(\mathbf{r}))$$

¹At each time step we: (i) update each u_m , on its local patch, (ii) update the lumen field s on the global grid using $\varphi(r)$ and ξ , (iii) update state variables (areas, centroids, target volumes, timers), (iv) rebuild auxiliary fields, and (v) apply division and optional micro-lumen seeding when criteria are met.

Outputs and quantitative metrics

For each simulation, we export time-indexed metrics including lumen area V_l , total cell area $\sum_m V_m$, lumen occupancy

$$\frac{V_l}{V_l + \sum_m V_m}$$

and lumen count.

We compute lumen count by thresholding the lumen field ($s \geq 0.5$) and counting connected components. When enabled, we also save field snapshots for visualization of the summed cell field and lumen field.

Conversion of ξ to an effective hydrostatic pressure scale

To relate ξ to an effective hydrostatic pressure difference ΔP , we reproduce a cyst calibration in a non-dividing configuration initialized with a circular lumen. We compute lumen radius as

¹ Tanida, S. et al. PLOS Computational Biology (2025). Lee, B. H. et al. Nature Cell Biology (2025)

$$R(t) = \sqrt{\frac{V_l(t)}{\pi}}$$

estimate $\frac{\partial R}{\partial t}$ near $R \approx 1$ using smoothed radius trajectories, and compute

$$\Delta P = \xi - \frac{1}{\lambda_w} - \left. \frac{\partial R}{\partial t} \right|_{R=1}$$

where λ_w is an effective water permeability parameter used in postprocessing.

B4 Chapter 2/3 - Live cell microscopy protocol

This appendix consolidates the standard operating procedure for the Ti2 Eclipse Nikon spinning disk confocal microscope integrated with the Crest V3 X-Light module, Photometrics Kinetix sCMOS camera, NIS-Elements software, and OkoLab environmental control system. It outlines routine use, first-level troubleshooting, maintenance, data handling, and routine quality-control checks for live-cell imaging.

System overview. The instrument supports brightfield/DIC, widefield fluorescence, and spinning disk confocal imaging, including multipoint, multi-channel, z-stack, large-image, and long-term live-cell acquisitions under controlled temperature and CO₂ conditions.

1. Pre-Acquisition Checklist

✓	Checklist item
☐	All microscope peripherals are powered on in the correct order.
☐	Hardware is installed correctly: objective, holder, Petri dish/plate, gas tubing, temperature probes, and humidity tank.
☐	Environmental control is set to 37 °C and 5% CO ₂ , with at least 45 minutes allowed to reach steady state.
☐	All chamber and Oko-Cage apertures are closed after setup.
☐	Sample is prepared and or refreshed with new medium if required
☐	At least 3 mL of medium is present in a 35 mm dish to enable Nikon Perfect Focus System (PFS) operation.
☐	NIS-Elements is opened under the user's personal account.
☐	Sample focus is identified in DIA/Kinetix mode and PFS is engaged if needed.
☐	Fluorescence optical configurations are checked and saved.
☐	For multi-configuration acquisitions, the same ROI, format, and bit depth are used across channels.

☐	ND Acquisition settings are defined and verified before starting the experiment.

2. Materials and Safety

Required materials: blower, lens-cleaning brush, lens-cleaning paper, ethanol 96%, petroleum benzine, distilled water, cleaning sticks, 30 cSt silicon oil, gloves, and dust-removal slime.

Safety: Use gloves and a lab coat at the workstation. Safety goggles should be available and should be used when handling petroleum benzine or other cleaning reagents. Petroleum benzine and ethanol are flammable and irritant; handle them under appropriate ventilation and away from ignition sources.

3. Hardware Start-Up Procedure

1. Turn on the Kinetix camera.
2. Turn on the CrestOptics spinning disk module.
3. Turn on the piezo controller.
4. Turn on the Nikon Ti2 microscope.
5. If needed, turn on the EPI lamp and verify that the manual shutter is open.
6. Turn on the laser tower, then switch the laser key to ON.
7. Turn on the OkoLab air pump.
8. Open the main CO₂ knob until the main manometer reaches 2 bar.
9. Verify that the OkoLab manometer reads 1 bar.
10. Set the Oko-Mixer to Air 0.8 and CO₂ 0.04 to achieve 5% CO₂ in the chamber.
11. Confirm gas flow through the tubing and chamber.
12. Turn on the OkoLab temperature controller and set it to 37 °C.
13. Enable sample feedback and verify correct placement of the white and green temperature probes.
14. Confirm that the humidity tank is filled with distilled water and the internal tube remains submerged.
15. Turn on the workstation PC only after all microscope peripherals are already on.
16. Open NIS-Elements and enter the user credentials.

4. Objectives and Turret Configuration

Install the required objective only after lowering the turret position. Clean the lens before installation, verify the collar setting if needed, and match the physical objective to the correct turret position used by automation routines.

Turret position	Objective	Immersion / note
1	4× NA 0.13	Air
2	10× NA 0.30	Air
3	20× NA 0.75	Air
4	25× NA 1.05	Silicon oil + DIC slit
5	40× NA 1.25	Silicon oil + DIC slit
6	100× NA 1.35	Silicon oil + DIC slit

5. Environmental Control and Sample Preparation

The Oko-Chamber must remain closed for approximately 45 minutes to reach stable operating conditions of 37 °C and 5% CO₂.

Use fake plates and magnetic locks to close all unused holder openings and stabilize the chamber.

Before long-term imaging, cage calibration is recommended to verify that the sample temperature matches the setpoint.

Prepare the sample with 1% ProLong Live in the selected medium when required and allow approximately 30 minutes of incubation.

Sample placement should be done quickly to avoid loss of chamber equilibrium. For silicon-oil objectives, place a small oil drop on the lens only if needed.

5.1 Cage calibration

- Insert the thermocouple through the chamber port and fix it to the bottom of a 35 mm dish with tape, without covering the probe tip.

- Fill the dish with distilled water until the thermocouple tip is submerged and place it in the chamber.
- Set the system to Chamber Mode in the temperature-control menu.
- Wait until steady state is reached, then open the calibration menu.
- When the sample temperature is stable, run the offset adjustment so that the measured sample temperature matches the target value.

6. Focusing and Optical Configuration

6.1 Brightfield / DIC (Kinetix-DIA module)

- Use DIA mode to locate the sample and determine the initial focus.
- Prefer lower illumination power and longer exposure whenever possible.
- Adjust binning, bit depth, ROI, and PFS offset as needed, then save the configuration.
- For multi-channel experiments, keep ROI, format, and bit depth identical across all optical configurations.

6.2 Fluorescence (Spinning Disk module)

- Select the required WF or SD optical configuration and inspect the sample using live view or single capture while minimizing light exposure.
- Keep laser power as low as possible; in routine operation, values below 10% are generally preferred, with signal improved by exposure time rather than power whenever feasible.
- Save all final optical-configuration settings before moving to ND Acquisition.
- For z-stack acquisitions, remember that more planes increase total dose. Widefield followed by denoising/deconvolution may be preferable when lower phototoxicity is required.

7. ND Acquisition Settings

The ND Acquisition module is used to define file saving, time schedule, multiple positions, z-stacks, lambda series, and large-image scans. Templates may be loaded from the workstation template folder when available.

7.1 Time

- Set the acquisition interval and total duration or number of loops.
- Enable shutter closure while idle.
- Disable transmitted illumination while idle.

7.2 XY positions

- Save X, Y, Z, and PFS offset values for each field of view.
- Use PFS and include z information when defining multipoint acquisitions.
- Close the active shutter during stage movement.
- When needed, add pre- and post-capture delays for silicon-oil spreading or sample recovery.
- Use split multipoint when separate files per FOV are desired.

7.3 Z stacks

- Define top and bottom planes or total range.
- Use the NIDAQ piezo z device as the routine default unless a different device is specifically required.
- Set direction routinely from bottom to top.
- Close the active shutter during z movement.

7.4 Lambda

- Add the optical configurations required for the experiment.
- Define the reference channel and assign focus offsets to the remaining channels.
- Acquire channels in decreasing wavelength order when possible to reduce sample stress.
- Close the active shutter during filter changes and use PFS.

7.5 Large image / tile scan

- Define the area using an $n \times m$ field matrix, a region in millimeters, or a loaded pattern.

- Use approximately 10% overlap for stitching.
- Enable image registration when a single stitched output file is desired.
- Close the active shutter during stage movement and use PFS.

8. Experiment Execution, Data Storage, and Reset

8.1 Running the experiment

- Acquire a pre-experiment reference set from representative fields of view to document cell morphology and vitality before the main run.
- Run the main experiment after verifying all ND windows and acquisition parameters.
- Acquire a post-experiment reference set from representative fields of view to document sample status after imaging.

8.2 Data storage

- At the end of the experiment, copy the main folder from the fast data drive to the DATA RAID long-term storage area assigned to the user.
- Remove files from the fast acquisition drive as soon as transfer is verified.
- Temporary analysis copies may be placed on approved shared storage, but these should be cleaned up after use.
- Monthly data migration should move DATA RAID contents to NAS storage server to save backups according to lab policy.

8.3 Environment clean and reset

- Lower the turret position before removing the sample.
- Remove the sample and inspect for evaporation, condensation, or medium color changes.
- Clean the stage holder with ethanol and return it to storage.
- Perform first-pass cleaning of the objective, especially after silicon-oil use.
- Refill the humidity tank if needed and close the chamber and Oko-Cage for the next user.

9. Objective Cleaning

- For dry objectives, remove dust with a manual blower, then clean the lens with lens paper lightly wetted with ethanol.
- For silicon-oil objectives, remove all oil first using lens paper with ethanol (or petroleum benzine after requested permission to super user) , then perform a final ethanol cleaning using spiral motion from center to edge.
- Rotate the objective slowly during cleaning while maintaining light pressure with the wrapped cleaning stick.
- Store the cleaned objective in its case and return it to the designated cabinet.

10. Batch Processing

NIS.ai and deconvolution. Batch denoising and deconvolution can be run in passive mode using saved macros. The routine workflow is to prepare a dedicated output folder, select the appropriate macro for the experiment and objective, run the macro on all files in the selected folder, and save results into a different folder.

11. Routine Maintenance, Laser Measurement, and Calibration

11.1 Weekly maintenance

- Remove dust from the hardware with a soft brush.
- Clean the workstation bench with a non-bleach antibacterial cleaner.
- Clean eyepieces and condenser following the same optics-cleaning principles used for objectives.
- Run a functional check with a fixed sample and an air objective to verify stage motion, turret switching, optical configurations, and ND Acquisition modules.
- Document any warning or deviation in the instrument log.

11.2 Routine laser measurement and Argolight-based calibration

Purpose. Routine laser measurement and imaging-system calibration should be performed to verify that excitation power delivery, channel registration, illumination uniformity, and optical performance remain stable over time. An Argolight reference slide or calibration device provides a reproducible sample for these checks.

Recommended routine

- Perform a quick Argolight check after major hardware intervention, after optical realignment, and at regular intervals during routine maintenance.
- Warm up the microscope, lasers, camera, and environmental hardware under the same conditions typically used for imaging before starting the measurement session.
- Use a fixed acquisition template with predefined objective, ROI, binning, exposure, laser power, z range if applicable, and optical configurations so that results remain comparable across dates.

Argolight measurements

- Acquire the Argolight pattern with each routinely used laser line and optical configuration.
- Record laser power at the sample plane or at the objective output using the lab's power-meter workflow, and compare the value with the historical baseline for the same configuration.

Advanced optical methods in tissue engineering

26/03/31	10x objective				Argolite Power HM	Sensor error: 10%		
	Power output (mW)							
	405		488		561		640	
Input power setting (%)	WF	SD	WF	SD	WF	SD	WF	SD
1	0.34		0.77		2.5		3.62	
2								
3								
5	3.44		13		21.2		17.9	
10	8.5		29.3		48.5		36.3	
20	19.5		62.2		87.3		73.3	
25	24.8		78.4				91	
30	30.6		94.4					
40	40.7							
50	50.4							
75	79.3							
100		2.8		7.9		10.3		9.5
Insert % value here	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)
20	19.6	0.52	62.04	1.52	89.1	2.05	72.94	1.9

26/03/31	20x objective				Argolite Power HM	Sensor error: 10%		
	Power output (mW)							
	405		488		561		640	
Input power setting (%)	WF	SD	WF	SD	WF	SD	WF	SD
1	0.34		1.55		2.76		4	
2								
3								
5	3.51		14.7		24.3		19.6	
10	8.55	205	31.6		51.7		39.6	
20	19.7		66.2		93		79.5	
25			83				95	
30			99					
40	41.1							
50								
75	79.8							
100		2.7		8.4		10.7		10.6
Insert % value here	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)
20	19.91	0.5	65.7	1.64	94.97	2.15	77.51	2.13

26/03/31	40x objective				Argolite Power HM	Sensor error: 10%		
	Power output (mW)							
	405		488		561		640	
Input power setting (%)	WF	SD	WF	SD	WF	SD	WF	SD
1	0.2		0.8		2.2		3	
2								
3								
5	2.1		10.6		18.3		14.8	
10	5.24		23.6		40.3		30	
20	12		49.8		72.5		60.6	
25	15.3		62.5		86.5		75.7	
30	19		75.3		102		90.9	
40	25.3		100					
50	32.6							
75	49.5							
100	65	1.66		6.1		7.7		7.5
Insert % value here	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)
30	18.9	0.48	74.98	1.8	104.19	2.34	90.9	2.25

26/03/31	100x objective				Argolite Power HM	Sensor error: 10%		
	Power output (mW)							
	405		488		561		640	
Input power setting (%)	WF	SD	WF	SD	WF	SD	WF	SD
1	0.1		0.59		1.1		1.32	
2								
3								
5	1.17		6		10		6.64	
10	2.88		13		21.6		13.7	
20	6.5		27.1		37.4		28	
25	8.27		33.9		43.6		35	
30	10.1		40.6		51.2		42	
40	13.4		53.7		64.4		56.7	
50	17.2		66.2		73		70.7	
75	26		96.8					
100	33.8	0.76		2.7		2.95		2.78
Insert % value here	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)	WF (fit)	SD (fit)
50	16.93	0.38	65.72	1.35	78.12	1.52	70.61	1.39

12. First-Level Troubleshooting

- If the camera lags, joystick movement becomes jerky, lasers fail to emit, or NIS-Elements becomes unstable, perform a hardware and software reboot while keeping the OkoLab system on if appropriate.
- When optical configurations appear altered, verify the saved channel definitions and restore the correct preset values before acquisition.
- If NIS-Elements continues to crash or renames hardware modules unexpectedly, restore a known working configuration using the Nikon recovery utility before relaunching the software.

13. Shutdown Procedure

1. Close NIS-Elements and turn off the PC.
2. Switch the laser key to OFF and then power down the laser tower.
3. Turn off the OkoLab air pump and, if used, the EPI lamp.
4. Turn off the Ti2 microscope, piezo controller, spinning disk module, and Kinetix camera.
5. Turn off the OkoLab temperature controller.
6. Close the main CO₂ knob and confirm that both manometers return to 0 bar.
7. Leave the local air purifier on to reduce dust accumulation around the workstation.

Source note. This chapter was adapted and reformatted from the internal microscopy protocol document and expanded with a routine section for laser measurement and Argolight-based calibration.

C

Appendix C Codes

C1 Chapter 2 - FUCCIsmart JOBs pseudocode

FUCCIsmart - Smart Microscopy Job Logic (NIS JOBS Pseudocode)

Overview

This document provides a thesis-ready, implementation-oriented pseudocode for a FUCCIsmart acquisition strategy designed for Nikon NIS JOBS-style automation.

The pipeline alternates between:

- (1) Low-dose widefield screening at fixed intervals (e.g., every 6 hours)
- (2) Triggered high-content confocal Z-stack acquisition (e.g., 1 hour)
when a mitotic-like population state is detected.

The decision logic uses population summaries derived from nuclear masks:

- CFP_mean median: proxy for SG2/M enrichment (high CFP)
- iRFP_mean median + iRFP area median: proxy for M (low iRFP + large nuclei)

Inputs

- cells_xy: list of XY positions to screen
- period_h: screening period (hours)
- dur_confocal_h: confocal acquisition duration after M detection (hours)
- Thresholds for SG2M and M classification (absolute + relative)
- Z-stack range and step size for confocal acquisition

Outputs

- Per-position state tags (e.g., "SG2M", "M")
- Widefield snapshots (WF_CHANNELS)
- Triggered confocal Z-stacks (CONF_CHANNELS + CONF_Z)

How to Run (Conceptual)

- 1) Load XY positions (cells_xy)
- 2) Set screening schedule + thresholds
- 3) Start experiment loop
- 4) Every period_h: acquire WF, classify, trigger confocal when M

Runtime Notes

- Screening cost scales linearly with number of positions $\times$ WF channels.
- Confocal is event-driven and may dominate runtime/storage if many triggers occur.
- CycleMean() should be implemented consistently (rolling baseline or precomputed).

=====

Configuration

=====

INPUT = {

 # Positions to screen (XY stage coordinates, 2D list)

 "cells_xy": LoadCellCoordinates(),

 # Scheduling

 "time_base_h": Now(), # reference start time for periodic screening ticks

 "period_h": 6, # widefield screening interval (hours)

 "dur_confocal_h": 1, # confocal acquisition duration after M detection (hours)

 # Thresholds - Absolute (ADU) and Relative (fraction of cycle mean)

 # SG2/M: high CFP

```
"CFP_mean_abs": 650,    # SG2M if CFP_mean > 650 ADU
"CFP_mean_pct": 0.37,   # OR CFP_mean > 37% of cycle mean
# M: low iRFP  && large nucleus area
"iRFP_mean_abs": 800,   # M if iRFP_mean < 800 ADU
"iRFP_mean_pct": 0.95,  # OR iRFP_mean < 95% of cycle mean
"area_thr": 2500,       # M requires nucleus area > 2500 ( $\mu\text{m}^2$ )
# Confocal Z-stack definition
"z_range_um": 12.0,
"z_step_um": 1.0,
}

# Widefield screening channels (single-plane, low dose)
WF_CHANNELS = [
    {"name": "iRFP_nuc", "illum": 638}, # nuclei: iRFP (mask + classifier)
    {"name": "CFP_nuc", "illum": 446}, # nuclei: CFP (SG2M classifier)
    {"name": "RFP_Actin", "illum": 546}, # context/QC channel
]

# Confocal acquisition channels (Z-stack; triggered on M detection)
CONF_CHANNELS = [
    {"name": "EGFP_Tubulin", "illum": 477, "mode": "confocal"},
    {"name": "RFP_Actin", "illum": 546, "mode": "confocal"},
]

CONF_Z = {"range_um": INPUT["z_range_um"], "step_um": INPUT["z_step_um"]}

Helper function
=====
=====

def is_screen_time(now_h: float, base_h: float, period_h: float) -> bool:
    """
    Return True if the current time corresponds to a screening tick.
    return ((now_h - base_h) % period_h) < 1e-6
```

```
def acquire_widefield_snapshot(pos) -> dict:
```

Acquire widefield snapshot across defined WF channels for a given XY position.

dict: channel_name -> image object

```
imgs = {}
```

```
for ch in WF_CHANNELS:
```

```
    imgs[ch["name"]] = WF_Snap(position=pos, illum_nm=ch["illum"])
```

```
return imgs
```

```
def segment_nuclei(mask_source):
```

Produce a binary nuclear mask from a selected channel.

Assumption

Masking is derived from iRFP (robust nuclei) or CFP if desired, but the choice should be consistent across the experiment to avoid biased metrics.

```
mask = BinaryMask(mask_source)
```

```
return mask
```

```
def region_metrics(img, mask):
```

Compute per-nucleus region metrics from an image and a binary mask.

Output features are restricted to those used in the decision logic.

```
return MeasureRegions(img, mask, features=["mean_I", "area"])
```

```
def pct_of_cycle(mean_I: float, cycle_mean_I: float) -> float:
```

Convert an intensity value into a fraction of its expected cycle mean.

```
return mean_I / max(cycle_mean_I, 1e-9)
```

```
def classify_SG2M(CFP_mean: float, CFP_cycle_mean: float,
```

```
    th_abs: float, th_pct: float) -> bool:
```

SG2/M gate: CFP high (absolute threshold) OR CFP high relative to cycle mean.

```
return (CFP_mean > th_abs) or (pct_of_cycle(CFP_mean, CFP_cycle_mean) > th_pct)
```

```
def classify_M(iRFP_mean: float, iRFP_cycle_mean: float, area_median: float,
              th_abs: float, th_pct: float, area_thr: float) -> bool:
    M gate:
        iRFP low (absolute threshold) OR iRFP low relative to cycle mean
        AND
        nucleus area above a minimum threshold (mitotic swelling proxy)
        intensity_gate = (iRFP_mean < th_abs) or (pct_of_cycle(iRFP_mean,
iRFP_cycle_mean) < th_pct)
        area_gate = (area_median > area_thr)
        return intensity_gate and area_gate

def run_confocal_zstack(pos, duration_h: float) -> None:
    Triggered confocal acquisition loop for a fixed time window.
    t_end = Now() + duration_h
    while Now() < t_end:
        for ch in CONF_CHANNELS:
            Confocal_ZStack(
                position=pos,
                illum_nm=ch["illum"],
                z_range_um=CONF_Z["range_um"],
                z_step_um=CONF_Z["step_um"],
                save_views=["XY", "YZ", "XZ"],
            )
```

Quality checks

```
=====
```

```
def validate_inputs() -> None:
    Minimal validation to prevent silent misconfiguration.
    if INPUT["period_h"] <= 0:
        raise ValueError("period_h must be > 0.")
    if INPUT["dur_confocal_h"] <= 0:
```

```
raise ValueError("dur_confocal_h must be > 0.")
if INPUT["z_range_um"] <= 0 or INPUT["z_step_um"] <= 0:
    raise ValueError("z_range_um and z_step_um must be > 0.")
if not INPUT["cells_xy"]:
    raise ValueError("cells_xy is empty: no positions to screen.")
if INPUT["area_thr"] <= 0:
    raise ValueError("area_thr must be > 0.")
```

Main pipeline

```
=====
=====
```

```
def FUCCIsmart() -> None:
```

```
    Smart microscopy scheduler:
```

```
    Wait initial period (settle)
```

```
    Every screening tick (period_h):
```

```
        Widefield snapshot (iRFP, CFP, RFP)
```

```
        Segment nuclei
```

```
        Compute median intensity and area (robust aggregation)
```

```
        Apply SG2M gate first, then M gate
```

```
        Trigger confocal Z-stack acquisition for dur_confocal_h if M
```

```
        Sleeps until next screening tick
```

```
validate_inputs()
```

```
# Initial settle time (preserved)
```

```
Sleep(hours=INPUT["period_h"])
```

```
while ExperimentActive():
```

```
    now_h = Now()
```

```
    # Only screen at periodic ticks
```

```
    if not is_screen_time(now_h, INPUT["time_base_h"], INPUT["period_h"]):
```

```
        Sleep(minutes=5)
```

```
    continue

# Iterate through defined XY positions
for pos in INPUT["cells_xy"]:
    wf = acquire_widefield_snapshot(pos)
    # Nuclear segmentation: iRFP-derived by default
    nuc_mask = segment_nuclei(wf["iRFP_nuc"])
    # Extract per-nucleus metrics for both classifiers
    met_iRFP = region_metrics(wf["iRFP_nuc"], nuc_mask)
    met_CFP = region_metrics(wf["CFP_nuc"], nuc_mask)
    # Robust aggregation at the population level (median)
    iRFP_mean_med = Aggregate(met_iRFP, "mean_I", method="median")
    iRFP_area_med = Aggregate(met_iRFP, "area", method="median")
    CFP_mean_med = Aggregate(met_CFP, "mean_I", method="median")

    # Cycle mean estimators (implementation-defined; preserved placeholder)
    iRFP_cycle_mean = CycleMean("iRFP")
    CFP_cycle_mean = CycleMean("CFP")
    # Gate 1: SG2/M: CFP high
    if classify_SG2M(CFP_mean_med, CFP_cycle_mean,
                    INPUT["CFP_mean_abs"], INPUT["CFP_mean_pct"]):
        SavePopulationTag(pos, "SG2M")
        continue
    # Gate 2: M: iRFP low AND area large
    if classify_M(iRFP_mean_med, iRFP_cycle_mean, iRFP_area_med,
                  INPUT["iRFP_mean_abs"], INPUT["iRFP_mean_pct"],
                  INPUT["area_thr"]):
        SavePopulationTag(pos, "M")
        run_confocal_zstack(pos, duration_h=INPUT["dur_confocal_h"])
    # Sleep until the next screening tick (preserved placeholder)
    SleepUntil(next_multiple_of_hours(INPUT["time_base_h"], INPUT["period_h"]))
```

Script Entry Point

=====

FUCCIsmart()

C2 Chapter 3 - Fab2Mic routine

FOV Grid Generation from TIFF Pattern to XML Replication (NIS-Elements Export)

Overview

This script generates field-of-view (FOV) acquisition coordinates starting from:

- (1) A TIFF mask/image representing a local pattern (single feature or large design)
- (2) A Nikon NIS-Elements correlative XML describing how that pattern is replicated across the sample via a (Row $\times$ Col) matrix with defined spacing.

Two operational modes are supported:

- A) "Small pattern" mode:
 - If a single connected component is detected in the binary TIFF, its centroid is used as the only local FOV center.
- B) "Large design" mode:
 - If the design spans beyond one FOV, a tiling grid is generated to cover the full TIFF extent.

For both modes:

- Local FOV centers (pixel space) are converted to μm offsets from the TIFF center.
- Offsets are applied to each replicated XML placement to produce global FOVs (μm).
- Two diagnostic plots are saved:
 - 1) Local FOV layout (in pixel space)
 - 2) Global FOV layout (in μm space)
- A Nikon-compatible multipoint XML is exported (UTF-16), where each point is a global acquisition position.

Dependencies

-
- numpy
 - matplotlib
 - tifffile
 - skimage (for small-pattern connected components)
 - xml.etree.ElementTree (standard library)

Inputs

Small pattern mode:

- TIFF: binary-like image ("50um_Square_single.tif")
- XML: correlative layout ("correlative_square.xml")

Large design mode:

- TIFF: full design image ("pavia.tif")

- XML: correlative layout ("pavia_20x.xml")

Key required XML content:

- The script reads the second <Element> node:
root.findall("./Element")[1]
and expects attributes:
x, y, Col, Row, ColSpace, RowSpace

Outputs

Small pattern mode:

- FOV_locali_fixed.png
- FOV_globali_fixed.png
- NIS_export.xml

Large design mode:

- FOV_locali_final.png
- FOV_globali_final.png
- NIS_export_fov_final.xml

How to Run

- 1) Edit the configuration section (paths + parameters).
- 2) Run:
python fov_from_tiff_xml_export.py

Runtime Notes

- Runtime is dominated by image loading and (for small-pattern mode) connected component labeling. Typical runtime is negligible for 2D masks.

=====

```
from __future__ import annotations
```

```
import numpy as np
import matplotlib.pyplot as plt
from tifffile import imread
from skimage.measure import label, regionprops
from xml.etree import ElementTree as ET
```

Configuration

```
# NOTE: Set RUN_MODE to either "SMALL_PATTERN" or "LARGE_DESIGN".
RUN_MODE = "SMALL_PATTERN" # "SMALL_PATTERN" | "LARGE_DESIGN"
```

```
# --- Common acquisition parameters ---
FOV_UM = 190          # FOV size (μm), used for both local visualization and global
rectangles
PIXEL_SCALE = 0.28    # μm/px calibration of the objective in use
RATIO = 1             # pattern magnification ratio from XML (preserved as-is)

# --- Small pattern dataset ---
SMALL_TIFF_PATH = "50um_Square_single.tif"
SMALL_XML_PATH = "correlative_square.xml"
SMALL_LOCAL_PLOT = "FOV_locali_fixed.png"
SMALL_GLOBAL_PLOT = "FOV_globali_fixed.png"
SMALL_EXPORT_XML = "NIS_export.xml"

# --- Large design dataset ---
LARGE_TIFF_PATH = "pavia.tif"
LARGE_XML_PATH = "pavia_20x.xml"
LARGE_FOV_UM = 900
LARGE_RATIO = 5
LARGE_LOCAL_PLOT = "FOV_locali_final.png"
LARGE_GLOBAL_PLOT = "FOV_globali_final.png"
LARGE_EXPORT_XML = "NIS_export_fov_final.xml"

# --- Nikon multipoint export constants (preserved literal values) ---
DEFAULT_Z_POSITION = "3500.0"
DEFAULT_PFS_OFFSET = "3894.0"
```

Helper Functions

```
=====
=====
def parse_xml_replication(xml_path: str) -> dict:
    """
    Extract the correlative replication parameters from the XM
    Reads the *second* Element node: findall("./Element")[1]

    Returns
    -----
    dict with keys:
    x_origin, y_origin, col, row, col_spacing, row_spacing
    """
    root = ET.parse(xml_path).getroot()
    elements = root.findall("./Element")
    if len(elements) < 2:
        raise ValueError(
            f'XML '{xml_path}' does not contain at least 2 <Element> entries. "
            f'The script expects root.findall("./Element")[1]."'
        )
```

```
el = elements[1]
return {
    "x_origin": float(el.attrib.get("x")),
    "y_origin": float(el.attrib.get("y")),
    "col": int(el.attrib.get("Col", 1)),
    "row": int(el.attrib.get("Row", 1)),
    "col_spacing": float(el.attrib.get("ColSpace", 0)),
    "row_spacing": float(el.attrib.get("RowSpace", 0)),
}

def pixel_fov_size(fov_um: float, pixel_scale: float, ratio: float) -> float:
    """
    Convert FOV size from  $\mu\text{m}$  into pixels.
    """
    if pixel_scale <= 0 or ratio <= 0:
        raise ValueError(f"pixel_scale and ratio must be > 0. Got: {pixel_scale=}, {ratio=}")
    return fov_um / (pixel_scale * ratio)

def compute_offsets_um(
    centroids_px: list[tuple[float, float]],
    image_center_px: np.ndarray,
    pixel_scale: float,
    ratio: float,
) -> list[tuple[float, float]]:
    """
    Convert local centroid positions (pixels) into offsets ( $\mu\text{m}$ ) relative to the image center.

    Notes
    -----
    dx_um = (x - center_x) * pixel_scale * ratio
    dy_um = (y - center_y) * pixel_scale * ratio
    """
    offsets = []
    for y, x in centroids_px:
        dx_um = (x - image_center_px[1]) * pixel_scale * ratio
        dy_um = (y - image_center_px[0]) * pixel_scale * ratio
        offsets.append((dx_um, dy_um))
    return offsets

def generate_global_fovs(
    offsets_um: list[tuple[float, float]],
    x_origin: float,
    y_origin: float,
```

```
col: int,
row: int,
col_spacing: float,
row_spacing: float,
) -> list[tuple[float, float]]:
    """
    Replicate local offsets across the XML-defined matrix to produce global FOV centers.

    - Loops row-major over (row × col)
    - For each replicate origin, adds all local offsets
    """
    global_fovs: list[tuple[float, float]] = []
    for r in range(row):
        for c in range(col):
            base_x = x_origin + c * col_spacing
            base_y = y_origin + r * row_spacing
            for dx, dy in offsets_um:
                global_fovs.append((base_x + dx, base_y + dy))
    return global_fovs

def save_local_visualization(
    binary_img: np.ndarray,
    centroids_px: list[tuple[float, float]],
    fov_px: float,
    out_path: str,
    title: str,
) -> None:
    """
    Save local visualization: binary image + FOV rectangles (pixel space).
    """
    fig, ax = plt.subplots()
    ax.imshow(binary_img, cmap="gray")

    for y, x in centroids_px:
        ax.add_patch(
            plt.Rectangle(
                (x - fov_px / 2, y - fov_px / 2),
                fov_px,
                fov_px,
                edgecolor="lime",
                facecolor="none",
                lw=1.5,
            )
        )
    ax.plot(x, y, "ro", markersize=2)

    plt.axis("off")
```

```
plt.title(title)
plt.savefig(out_path)
plt.close()

def save_global_visualization(
    global_fovs_um: list[tuple[float, float]],
    fov_um: float,
    out_path: str,
    title: str,
) -> None:
    """
    Save global visualization: FOV rectangles ( $\mu\text{m}$  space).
    """
    fig, ax = plt.subplots()

    for gx, gy in global_fovs_um:
        ax.add_patch(
            plt.Rectangle(
                (gx - fov_um / 2, gy - fov_um / 2),
                fov_um,
                fov_um,
                edgecolor="green",
                facecolor="none",
                lw=1.5,
            )
        )
        ax.plot(gx, gy, "ro", markersize=2)

    plt.axis("equal")
    plt.title(title)
    plt.savefig(out_path)
    plt.close()

def export_nis_multipoint_xml(
    global_fovs_um: list[tuple[float, float]],
    out_xml_path: str,
    z_position: str = DEFAULT_Z_POSITION,
    pfs_offset: str = DEFAULT_PFS_OFFSET,
) -> None:
    """
    Export global FOV coordinates as a Nikon NIS-Elements multipoint XML.

    - Exact element structure, attribute names, value types, and encoding (utf-16)
    - Uses indexed Point00000, Point00001, ... naming convention
    """
    variant = ET.Element("variant", version="1.0")
```

```
no_name = ET.SubElement(variant, "no_name", runtype="CLxListVariant")
ET.SubElement(no_name, "bIncludeZ", runtype="bool", value="true")
ET.SubElement(no_name, "bPFSEnabled", runtype="bool", value="true")

for i, (x, y) in enumerate(global_fovs_um):
    pt = ET.SubElement(no_name, f"Point{i:05d}",
runtype="NDSetupMultipointListItem")
    ET.SubElement(pt, "bChecked", runtype="bool", value="true")
    ET.SubElement(pt, "strName", runtype="CLxStringW", value="")
    ET.SubElement(pt, "dXPosition", runtype="double", value=str(x))
    ET.SubElement(pt, "dYPosition", runtype="double", value=str(y))
    ET.SubElement(pt, "dZPosition", runtype="double", value=z_position)
    ET.SubElement(pt, "dPFsoffset", runtype="double", value=pfs_offset)
    ET.SubElement(pt, "baUserData", runtype="CLxByteArray", value="")

ET.ElementTree(variant).write(out_xml_path, encoding="utf-16",
xml_declaration=True)
```

Quality Checks

```
=====
=====

def validate_image_2d(img: np.ndarray, name: str) -> None:
    """
    Ensure the loaded TIFF is compatible with the downstream logic (2D expected).
    """
    if img is None:
        raise ValueError(f'{name}: image could not be loaded (None).')
    if img.ndim < 2:
        raise ValueError(f'{name}: expected at least 2D image. Got shape: {img.shape}')
```

Main pipeline

```
=====
=====

def run_small_pattern_pipeline() -> None:
    """
    Small-pattern mode:
    - Detect connected components in a binarized image.
    - If exactly one region exists, use its centroid as the single local FOV.
    - Replicate across XML layout and export Nikon multipoint XML.
    """
```

```
# Local FOV size in pixels
fov_px = pixel_fov_size(FOV_UM, PIXEL_SCALE, RATIO)

img = imread(SMALL_TIFF_PATH)
validate_image_2d(img, "SMALL_PATTERN TIFF")

# Binary conversion (preserved thresholding behavior)
binary = (img > 127).astype(np.uint8)

labeled = label(binary)
regions = regionprops(labeled)

if len(regions) != 1:
    raise ValueError(
        f"SMALL_PATTERN mode expects exactly 1 connected region. Found:
        {len(regions)}."
        f"Use LARGE_DESIGN mode for multi-region or extended designs."
    )

cy, cx = regions[0].centroid
local_centroids_px = [(cy, cx)]

# Parse XML replication block
xml_info = parse_xml_replication(SMALL_XML_PATH)

# Offsets computed relative to image center (preserved coordinate convention)
px_center = np.array([img.shape[0] / 2, img.shape[1] / 2])
offsets_um = compute_offsets_um(local_centroids_px, px_center, PIXEL_SCALE,
RATIO)

# Generate global FOVs via replication
global_fovs = generate_global_fovs(
    offsets_um=offsets_um,
    x_origin=xml_info["x_origin"],
    y_origin=xml_info["y_origin"],
    col=xml_info["col"],
    row=xml_info["row"],
    col_spacing=xml_info["col_spacing"],
    row_spacing=xml_info["row_spacing"],
)

# Visualizations
save_local_visualization(
    binary_img=binary,
    centroids_px=local_centroids_px,
    fov_px=fov_px,
    out_path=SMALL_LOCAL_PLOT,
    title="FOV locali",
```



```
)

save_global_visualization(
    global_fovs_um=global_fovs,
    fov_um=FOV_UM,
    out_path=SMALL_GLOBAL_PLOT,
    title="FOV globali",
)

# Export Nikon XML
export_nis_multipoint_xml(global_fovs, SMALL_EXPORT_XML)
print(f"XML esportato con {len(global_fovs)} FOV")

def run_large_design_pipeline() -> None:
    """
    Large-design mode:
    - Create a tiling grid of local FOV centers covering the full TIFF extent.
    - Convert local grid centers into  $\mu\text{m}$  offsets relative to the image center.
    - Replicate across XML layout and export Nikon multipoint XML.
    """
    fov_px = pixel_fov_size(LARGE_FOV_UM, PIXEL_SCALE, LARGE_RATIO)

    img = imread(LARGE_TIFF_PATH)
    validate_image_2d(img, "LARGE_DESIGN TIFF")

    height_px, width_px = img.shape[:2]
    px_center = np.array([height_px / 2, width_px / 2])

    # Parse XML replication block
    xml_info = parse_xml_replication(LARGE_XML_PATH)

    # Full design size in  $\mu\text{m}$  (preserved computation)
    height_um = height_px * PIXEL_SCALE * LARGE_RATIO
    width_um = width_px * PIXEL_SCALE * LARGE_RATIO

    # Tiling counts (preserved: ceil of design size / FOV size in  $\mu\text{m}$ )
    ny = int(np.ceil(height_um / LARGE_FOV_UM))
    nx = int(np.ceil(width_um / LARGE_FOV_UM))

    # Local FOV centers in pixel coordinates (preserved grid rule)
    local_centroids_px: list[tuple[float, float]] = []
    for iy in range(ny):
        for ix in range(nx):
            y = fov_px / 2 + iy * fov_px
            x = fov_px / 2 + ix * fov_px
            local_centroids_px.append((y, x))
```

```
# Visual local grid
save_local_visualization(
    binary_img=img,
    centroids_px=local_centroids_px,
    fov_px=fov_px,
    out_path=LARGE_LOCAL_PLOT,
    title="FOV locali - copertura completa",
)

# Offsets computed relative to image center
offsets_um = compute_offsets_um(local_centroids_px, px_center, PIXEL_SCALE,
LARGE_RATIO)

# Global replication
global_fovs = generate_global_fovs(
    offsets_um=offsets_um,
    x_origin=xml_info["x_origin"],
    y_origin=xml_info["y_origin"],
    col=xml_info["col"],
    row=xml_info["row"],
    col_spacing=xml_info["col_spacing"],
    row_spacing=xml_info["row_spacing"],
)

# Visual global FOVs
save_global_visualization(
    global_fovs_um=global_fovs,
    fov_um=LARGE_FOV_UM,
    out_path=LARGE_GLOBAL_PLOT,
    title="FOV globali - replica matrice",
)

# Export Nikon XML
export_nis_multipoint_xml(global_fovs, LARGE_EXPORT_XML)
print(f"XML esportato con {len(global_fovs)} FOV")

def main() -> None:
    Dispatch to the selected pipeline mode.
    if RUN_MODE == "SMALL_PATTERN":
        run_small_pattern_pipeline()
    elif RUN_MODE == "LARGE_DESIGN":
        run_large_design_pipeline()
    else:
        raise ValueError(
            f"Invalid RUN_MODE='{RUN_MODE}'. Must be 'SMALL_PATTERN' or
'LARGE_DESIGN'."
        )
```

Script Entry Point

```
=====
=====
if __name__ == "__main__":
    main()
```

C3 Chapter 3 - FUCCIphase_morphology

Integrate FUCCI Track to Morphology with Tubulin Spot Nearest-Neighbor Matching (Frame-Resolved)

Overview

This script matches FUCCI-tracked cell positions (tracks) to the nearest detected tubulin spot position within the same frame, using a fixed Euclidean distance threshold. For each FUCCI track row, the closest tubulin detection in the corresponding frame is selected (if any candidate lies within threshold).

Implementation

-
- (1) Matching is performed strictly within the same FRAME.
 - (2) Distance metric: Euclidean distance in (POSITION_X, POSITION_Y).
 - (3) A tubulin spot is considered a valid match only if distance $\leq$ threshold.
 - (4) If multiple valid matches exist, the closest one is chosen (minimum distance)
 - (5) If no tubulin spots exist in the frame or no spot is within the threshold, the FUCCI row is skipped (no output row created).
 - (6) Output contains concatenated columns from both inputs plus "Distance".

Dependencies

Pandas

numpy

Inputs

FUCCI tracking CSV: "13_FUCCIphase_tracks_data.csv"

Required columns: FRAME, POSITION_X, POSITION_Y

Tubulin spot CSV: "13_tub_spots.csv"

Required columns: FRAME, POSITION_X, POSITION_Y

Outputs

CSV: "13_FUCCIphase_morphology.csv"

Each row corresponds to one FUCCI row successfully matched to one tubulin spot.

How to Run

python match_fucci_to_tubulin_spots.py

Runtime Notes

Complexity scales with (number of FUCCI rows $\times$ tubulin spots per frame).

For very large datasets, frame-wise grouping can be used for speed, but the current implementation preserves the original logic.

=====

```
from __future__ import annotations
```

```
import pandas as pd
```

```
import numpy as np
```

Configuration

=====

```
FUCCI_TRACKS_CSV = "13_FUCCIphase_tracks_data.csv"
```

```
TUBULIN_SPOTS_CSV = "13_tub_spots.csv"
```

```
OUTPUT_CSV = "13_FUCCIphase_morphology.csv"
```

```
# Distance threshold in the same units as POSITION_X / POSITION_Y
```

THRESHOLD = 60 # Use 120 if working with 100x images

Helper Functions

```
=====
```

```
def _validate_required_columns(df: pd.DataFrame, required: list[str], name: str) -> None:
```

Validate that required columns exist in a dataframe.

Parameters

- -----

df: pd.DataFrame

Input dataframe to validate.

required: list[str]

Column names required for downstream processing.

name: str

Label used in error messages (e.g., "FUCCI tracks", "Tubulin spots").

missing = [c for c in required if c not in df.columns]

if missing:

raise ValueError(

f'{name} CSV is missing required columns: {missing}. "

f'Available columns: {list(df.columns)}"

)

```
def match_fucci_tracks_to_tubulin_spots(
```

```
fucci_df: pd.DataFrame,  
tubulin_df: pd.DataFrame,  
threshold: float,  
) -> pd.DataFrame:
```

For each FUCCI track row, find the closest tubulin spot in the same frame within the specified Euclidean distance threshold.

Parameters

fucci_df: pd.DataFrame

FUCCI-tracked cells table with required columns:

FRAME, POSITION_X, POSITION_Y.

tubulin_df: pd.DataFrame

Tubulin spots table with required columns:

FRAME, POSITION_X, POSITION_Y.

threshold: float

Maximum allowed distance for a spot to be considered a match.

Returns

pd.DataFrame

Concatenated per-row matches with df1_ and df2_ prefixes plus Distance.

Empty if no valid matches exist.

```
closest_matches: list[pd.Series] = []
```

```
for _, row_fucci in fucci_df.iterrows():
```

```
    frame = row_fucci["FRAME"]
```

```
x1, y1 = row_fucci["POSITION_X"], row_fucci["POSITION_Y"]

# Restrict candidate matches to the same frame (strict frame-wise matching)

tubulin_frame = tubulin_df[tubulin_df["FRAME"] == frame]

if tubulin_frame.empty:

    continue

# Compute Euclidean distances to all tubulin detections in this frame

dx = x1 - tubulin_frame["POSITION_X"]

dy = y1 - tubulin_frame["POSITION_Y"]

distances = np.sqrt(dx**2 + dy**2)

# Keep only detections within threshold (hard cutoff)

valid_mask = distances <= threshold

valid_matches = tubulin_frame[valid_mask]

valid_distances = distances[valid_mask]

if valid_matches.empty:

    continue

# Identify the closest valid match (minimum distance)

min_index = valid_distances.idxmin()

min_distance = valid_distances.loc[min_index]

closest_match = valid_matches.loc[min_index]

# Combine FUCCI row + tubulin row + computed distance

combined = pd.concat(

    [

        row_fucci.add_prefix("df1_"),

        closest_match.add_prefix("df2_"),
```



```
        pd.Series({"Distance": min_distance}),
    ]
)

closest_matches.append(combined)

if not closest_matches:

    return pd.DataFrame()

return pd.DataFrame(closest_matches)

Main pipeline
=====

def main() -> None:

    """Run frame-wise nearest-neighbor matching and export results to CSV."""

    # Load input tables

    fucci_df = pd.read_csv(FUCCI_TRACKS_CSV)

    tubulin_df = pd.read_csv(TUBULIN_SPOTS_CSV)

    # Validate expected schema (basic, explicit checks)

    required_cols = ["FRAME", "POSITION_X", "POSITION_Y"]

    _validate_required_columns(fucci_df, required_cols, name="FUCCI tracks")

    _validate_required_columns(tubulin_df, required_cols, name="Tubulin spots")

    if THRESHOLD <= 0:

        raise ValueError(f"THRESHOLD must be > 0. Got: {THRESHOLD}")

    # Perform matching

    output_df = match_fucci_tracks_to_tubulin_spots(

        fucci_df=fucci_df,

        tubulin_df=tubulin_df,
```

```
        threshold=THRESHOLD,  
    )  
  
    # Saving/Export  
  
    if output_df.empty:  
  
        print("No valid matches found within the threshold.")  
  
        return  
  
    output_df.to_csv(OUTPUT_CSV, index=False)  
  
    print(f"Matches saved to '{OUTPUT_CSV}' ({len(output_df)} rows)")
```

Script Entry Point

```
=====
```

```
=====
```

```
if __name__ == "__main__":  
  
    main()
```

C4 Chapter 3 - Tubulin spread analysis macro

```
=====
=====

/ * fiji / imagej macro - Tubulin spreading analysis on High resolution acquisition per-
slice pixel counting and area conversion ( $\mu\text{m}^2$ )
```

overview

this macro iterates through all slices in the active image stack and:

- 1) counts pixels with intensity == 255 on each slice
- 2) writes the per-slice counts into the results table
- 3) converts pixel counts to physical area (μm^2) using a user-defined scale

expected inputs

- active image must be a stack (nslices ≥ 1).
- pixel intensities must be 8-bit or compatible with `getpixel(x,y)`.
- assumes a binary image where the target pixels are exactly 255.

outputs

- results table columns:
"slice", "blackpixels", "area_um2"

how to run

- open the target image in fiji
- run this macro (plugins $\rightarrow$ macros $\rightarrow$ run...)

runtime notes

- runtime scales with width $\times$ height $\times$ nslices.
- batch mode is enabled to speed up processing and reduce ui overhead.

*/

```
setbatchmode(true);
```

Configuration

```
=====
=====

// scale factor used for converting pixels ->  $\mu\text{m}^2$ 
scale = 14.21 // px/um conversion scale Nikon 100x Silicon oil
```

Input validation

```
=====
```

```
width = getwidth();
height = getheight();
n      = nslices;
```

```
if (n < 1) {
    exit("no slices found: the active image is not a stack or is empty.");}
```

Main pipeline - per-slice pixel counting

```
=====
```

```
// clear previous results table content
run("clear results");
```

```
// loop through each slice in the stack
for (s = 1; s <= n; s++) {
    setslice(s);
    // important: counter starts at 1 by design (matches original behavior)
    black = 1;
    // count pixels matching intensity 255
    for (y = 0; y < height; y++) {
        for (x = 0; x < width; x++) {
            if (getpixel(x, y) == 255) {
                black++;
            }
        }
    }
}
```

```
// store in results table (row index = s-1)
setresult("slice",    s - 1, s);
setresult("blackpixels", s - 1, black);
}
```

Post-processing - area conversion (μm^2)

```
=====
```

```
for (i = 0; i < nresults; i++) {  
    pixels = getresult("blackpixels", i);  
  
    // area_um2 = pixels / (scale * scale)  
    area_um2 = pixels / (scale * scale);  
  
    setresult("area_um2", i, area_um2);  
}  
setbatchmode(false);
```

C5 Chapter 2/3 - Image processing macro for 2D visualization

=====

=====
/* Fiji / ImageJ Macro, ND2 to TIFF Conversion and 4-Channels Processing for
visualization and exporting (TIFF/AVI/GIF)

Overview

This macro performs two sequential pipelines:

- (1) ND2 to TIFF batch conversion
 - a. Reads all .nd2 files in a selected folder using Bio-Format
 - b. Saves each file as a TIFF stack into a "Converted_TIFFs/" subfolder
- (2) TIFF processing and expo
 - a. Reads all .tif/.tiff files in a selected folder using Bio-Formats
 - b. Splits channels into C=0..3, applies per-channel preprocessing
 - c. Assigns display LUTs to channels
 - d. Merges channels back into a 4-channel composite stack
 - e. Adds scale bar and time label overlay
 - f. Saves processed TIFF - AVI - GIF in dedicated output folders

Dependencies

Fiji with Bio-Formats plugin installed ("Bio-Formats Importer")

Inputs

User-selected input directory for ND2 conversion

User-selected input directory for TIFF processing

Outputs (Folders created automatically)

ND2 conversion: <inputDir>/Converted_TIFFs/*.tif

TIFF processing: <dir>/processed/*.tif

<dir>/avi_processed/*.avi

<dir>/gif_processed/*.gif

How to Run

- (1) Open Fiji
- (2) Plugins → Macros → Run...
- (3) Select the ND2 folder when prompted
- (4) Select the TIFF folder when prompted

Runtime Notes

Runtime scales with number of files × stack size.

=====

Configuration

=====

// --- ND2 to TIFF conversion output folder name ---

CONVERTED_TIFF_SUBDIR = "Converted_TIFFs/";

// --- TIFF processing output folder names ---

PROCESSED_TIFF_SUBDIR = "processed/";

PROCESSED_AVI_SUBDIR = "avi_processed/";

PROCESSED_GIF_SUBDIR = "gif_processed/";

// --- Bio-Formats import options (kept identical to original intent) ---

BF_IMPORT_HYPERSTACK = "autoscale color_mode=Default view=Hyperstack
stack_order=XYCZT";

BF_IMPORT_SPLIT_4CH = "autoscale color_mode=Default split_channels
split_channels stack_order=XYCZT";

```
// --- Per-channel preprocessing parameters (exact values preserved) ---
```

```
C1_ROLLING = 50; C1_SIGMA = 2; C1_SUB = 170;
```

```
C2_ROLLING = 50; C2_SIGMA = 2; C2_SUB = 5;
```

```
C3_ROLLING = 50; C3_SIGMA = 2; C3_SUB = 35;
```

```
C4_ROLLING = 50; C4_SIGMA = 2; C4_SUB = 50;
```

```
// --- Overlay settings (preserved) ---
```

```
SCALEBAR_WIDTH_UM = 50;
```

```
SCALEBAR_THICKNESS = 25;
```

```
LABEL_STARTING = 0;
```

```
LABEL_INTERVAL = 30; // for timelapse every 30 min acquisition
```

```
LABEL_X = 200;
```

```
LABEL_Y = 200;
```

```
LABEL_FONT = 100;
```

```
LABEL_TEXT = "min";
```

```
Helper functions
```

```
=====
```

```
function assertFolderNotEmpty(path, fileList) {
```

```
    if (fileList.length == 0) {
```

```
        print("No files found in: " + path);
```

```
        exit();
```

```
    }
```

```
}
```

```
function stripExtension(filename) {
```

```
    dotPos = lastIndexOf(filename, ".");
```



```
if (dotPos < 0) return filename;

return substring(filename, 0, dotPos);

}

function preprocessChannel(windowTitle, rolling, sigma, subtractValue, subtractMode)
{
    selectWindow(windowTitle);

    run("Subtract Background...", "rolling=" + rolling + " " + subtractMode);

    run("Gaussian Blur...", "sigma=" + sigma + " stack");

    run("Subtract...", "value=" + subtractValue + " " + subtractMode);
}

function applyChannelLUT(windowTitle, lutName) {
    selectWindow(windowTitle);

    run(lutName);
}

function mergeChannels4(c1Title, c2Title, c3Title, c4Title) {
    run("Merge Channels...",
        "c1=[" + c1Title + "]" +
        "c2=[" + c2Title + "]" +
        "c3=[" + c3Title + "]" +
        "c4=[" + c4Title + "]" create keep"
    );
}
```

Main pipeline (1) - nd2 to tiff conversion

```
=====
=====
```

```
print("=== ND2 to TIFF conversion ===");

// Select input folder containing ND2 files

inputDir = getDirectory("Choose the folder with .nd2 files");

outputDir = inputDir + CONVERTED_TIFF_SUBDIR;

// Create output folder if needed

File.makeDirectory(outputDir);

// Directory name extraction (kept for logging; not used in filenames)

slashPos = lastIndexOf(inputDir, "/");

dirName = substring(inputDir, slashPos + 1, lengthOf(inputDir) - 1);

print("Directory name: " + dirName);

// List all files

list = getFileList(inputDir);

assertFolderNotEmpty(inputDir, list);

print("Found " + list.length + " files in the input folder.");

// Convert ND2 to TIFF

for (i = 0; i < list.length; i++) {

    if (endsWith(list[i], ".nd2")) {

        filePath = inputDir + list[i];

        print("Opening ND2: " + filePath);

        // Bio-Formats import

        run("Bio-Formats Importer",

            "open=[" + filePath + "]" + BF_IMPORT_HYPERSTACK

        );

        // Build output name
```

```
originalFileName = stripExtension(list[i]);

newFileName = "FOV_" + originalFileName + ".tif";

savePath = outputDir + newFileName;

print("Saving TIFF: " + savePath);

// Save TIFF

saveAs("Tiff", savePath);

// Close image to keep memory stable

close();

}

}

print("Conversion complete! Files saved to " + outputDir);

Main pipeline (2) - tiff processing + export (tiff/avi/gif)

=====

print("=== Inizio Processing ===");

// Select folder containing TIFF files to process

dir = getDirectory("Scegli la cartella contenente i file TIFF");

// Define output folders (created if missing)

processedDir = dir + PROCESSED_TIFF_SUBDIR;

aviOutputDir = dir + PROCESSED_AVI_SUBDIR;

gifOutputDir = dir + PROCESSED_GIF_SUBDIR;

File.makeDirectory(processedDir);

File.makeDirectory(aviOutputDir);

File.makeDirectory(gifOutputDir);

// List TIFF files
```

```
list = getFileList(dir);

assertFolderNotEmpty(dir, list);

// Process each TIFF

for (i = 0; i < list.length; i++) {

    if (endsWith(list[i], ".tif") || endsWith(list[i], ".tiff")) {

        filePath = dir + list[i];

        print("Opening TIFF: " + filePath);

        // Open TIFF with Bio-Formats and split channels

        run("Bio-Formats Importer",

            "open=[" + filePath + "]" + BF_IMPORT_SPLIT_4CH

        );

        // Expected split-window titles from Bio-Formats:

        // "<filename> - C=0"

        // "<filename> - C=1"

        // "<filename> - C=2"

        // "<filename> - C=3"

        c0Title = list[i] + " - C=0";

        c1Title = list[i] + " - C=1";

        c2Title = list[i] + " - C=2";

        c3Title = list[i] + " - C=3";

        Channels preprocessing

        =====

        // Channel 1 (C=0)

        preprocessChannel(c0Title, C1_ROLLING, C1_SIGMA, C1_SUB, "stack");
```

```
// Channel 2 (C=1)

preprocessChannel(c1Title, C2_ROLLING, C2_SIGMA, C2_SUB, "stack");

// Channel 3 (C=2)

preprocessChannel(c2Title, C3_ROLLING, C3_SIGMA, C3_SUB, "stack");

// Channel 4 (C=3)

preprocessChannel(c3Title, C4_ROLLING, C4_SIGMA, C4_SUB, "stack");
```

LUT assignment

```
=====
=====
```

```
applyChannelLUT(c1Title, "Green");

applyChannelLUT(c2Title, "Grays");

applyChannelLUT(c0Title, "Magenta");

applyChannelLUT(c3Title, "Cyan");
```

Merge channels and scale bar

```
=====
=====
```

```
mergeChannels4(c0Title, c1Title, c2Title, c3Title);

// Add scale bar overlay (hidden, overlay)

run("Scale Bar...",

    "width=" + SCALEBAR_WIDTH_UM +

    " thickness=" + SCALEBAR_THICKNESS +

    " hide overlay"

);
```

C6 Chapter 4 - TE-DaXi light-sheet tilt analysis

Purpose

Analyze a single CSV file containing tilt-calibration measurements, compute the tilt angle (in degrees) for each (EXP, TILT) group via a linear fit of Y vs Z, and generate:

- 1) Per-EXP tilt-angle plots (saved as PDF)
- 2) A grouped summary plot (mean $\pm$ SD) with a linear fit (saved as PDF)
- 3) Two CSV exports: per-(EXP,TILT) angles and per-TILT summary stats

Expected Inputs

-
- A CSV file formatted such that:
 - The first 5 rows are header/metadata and should be skipped.
 - Relevant data are located in columns with indices [1, 2, 3, 10] (0-based indexing after parsing by pandas).
 - After selecting these columns, they map to:
EXP, TILT, Z, YM PIX

Outputs (Files)

-
- tilt_plot_exp_<EXP>.pdf : per-experiment tilt angle vs stage shift
 - grouped_exp_tilt_summary.pdf : average tilt angle vs stage shift (mean $\pm$ SD) + linear fit
 - tilt_summary_by_tilt_id.csv : per-TILT aggregated statistics (mean_attach, SD, N)
 - tilt_by_exp.csv : per-(EXP, TILT) tilt angles

How to Run

-
- 1) Update `CSV\_FILE` to point to the dataset.
 - 2) Run the script:
python te_daxi_tilt_calibration.py

Runtime Notes

-
- Computational cost is minimal for typical calibration datasets.
 - Plots are displayed interactively (plt.show()) and also saved as PDFs.
-

Dependencies

```
=====
import pandas as pd
import numpy as np
import matplotlib.pyplot as plt
from scipy.stats import linregress
```

Configuration

```
=====
CSV_FILE = "MeXi_TiltCalibration.csv" # Update this path as needed
PIXEL_SIZE_UM = 0.138 #  $\mu\text{m}/\text{px}$  conversion factor for YM PIX ->  $\mu\text{m}$ 
```

Helper Functions

```
=====
def extract_tilt_data(filepath: str, pixel_size_um: float = 0.138) -> pd.DataFrame:
```

Load and standardize the raw calibration table.

Notes

- * select columns by fixed positional indices: [1, 2, 3, 10]
- * drop rows with NaNs after selection
- * enforce dtypes:
 - Z -> float
 - YM PIX -> float (then scaled by pixel_size_um)
 - TILT -> int

Parameters

filepath: str

Path to the input CSV file.

pixel_size_um: float

Pixel-to-micrometer conversion factor applied to YM PIX.

Returns

pd.DataFrame

Standardized DataFrame with columns: EXP, TILT, Z, YM PIX (in μm)

try:

```
df = pd.read_csv(filepath, skiprows=5)
```

except FileNotFoundError as e:

```
raise FileNotFoundError(
```

```
    f'CSV file not found: '{filepath}'. Check the path and try again."
```

```
) from e
```

```

# Basic structural validation (kept lightweight, but explicit)
required_max_col_index = 10
if df.shape[1] <= required_max_col_index:
    raise ValueError(
        f"Input CSV has {df.shape[1]} columns, but column index
{required_max_col_index} "
        f"is required. Verify the CSV format and column layout."
    )

# Select relevant columns by positional index (matches original behavior)
df = df.iloc[:, [1, 2, 3, 10]].copy()
df.columns = ["EXP", "TILT", "Z", "YM PIX"]

# Drop incomplete rows exactly as in the original workflow
df = df.dropna()

# Enforce numeric types (preserve failure mode: raise if conversion breaks)
try:
    df["Z"] = df["Z"].astype(float)
    df["YM PIX"] = df["YM PIX"].astype(float) * pixel_size_um
    df["TILT"] = df["TILT"].astype(int)
except Exception as e:
    raise ValueError(
        "Failed to convert one or more columns to the required numeric types.\n"
        "Expected: Z=float, YM PIX=float, TILT=int.\n"
        "Check that the CSV contains clean numeric values in the selected columns."
    ) from e

return df

def compute_tilt_angles_by_exp(df: pd.DataFrame) -> pd.DataFrame:
    """
    Compute tilt angle (degrees) for each (EXP, TILT) group.
    Scientific definition
    -----
    For each (EXP, TILT) group:
    - Fit a line  $y = m \cdot z + b$  using scipy.stats.linregress
    - Compute angle =  $\text{atan}(m)$  in degrees
    Assumptions
    -----
    - Z and YM PIX represent paired samples measured along a tilt scan.
    - Groups with fewer than 2 points are skipped (no fit possible).
    Parameters
    -----
    df: pd.DataFrame
        Output of extract_tilt_data(), with columns: EXP, TILT, Z, YM PIX.
    Returns
    """

```



```
-----
pd.DataFrame
    Per-group angles with columns: EXP, TILT, Tilt Angle
    """
    results = []
    # Group by experiment and stage shift ID, identical logic to original
    for (exp, tilt_id), group in df.groupby(["EXP", "TILT"]):
        if len(group) < 2:
            # Not enough points to estimate slope reliably
            continue
        z = group["Z"].values
        y = group["YM PIX"].values
        slope, _, _, _ = linregress(z, y)
        angle_deg = np.degrees(np.arctan(slope))
        results.append({"EXP": exp, "TILT": tilt_id, "Tilt Angle": angle_deg})

    return pd.DataFrame(results)
def aggregate_by_tilt(results_df: pd.DataFrame) -> pd.DataFrame:

    Aggregate tilt angles across experiments for each TILT value.

    Computes:
    - Mean Angle
    - Std Dev
    - N

    Parameters
    -----
    results_df: pd.DataFrame
        Output of `compute_tilt_angles_by_exp()`.

    Returns
    -----
    pd.DataFrame
        Aggregated table indexed by TILT.
    """
    grouped = results_df.groupby("TILT")["Tilt Angle"]
    summary = grouped.agg(["mean", "std", "count"]).reset_index()
    summary = summary.rename(
        columns={"mean": "Mean Angle", "std": "Std Dev", "count": "N"}
    )
    return summary

# Plotting Functions
=====

def plot_angles_per_experiment(tilt_by_exp: pd.DataFrame) -> None:
```

```
"""
Generate and save per-EXP tilt-angle plots.

Output file naming matches original behavior:
tilt_plot_exp_<EXP>.pdf
"""
required_cols = {"EXP", "TILT", "Tilt Angle"}
if not required_cols.issubset(tilt_by_exp.columns):
    raise ValueError(
        f'plot_angles_per_experiment() requires columns: {sorted(required_cols)}'
    )

for exp in sorted(tilt_by_exp["EXP"].unique()):
    sub = tilt_by_exp[tilt_by_exp["EXP"] == exp].sort_values("TILT")

    plt.figure(figsize=(8, 6))
    plt.plot(sub["TILT"], sub["Tilt Angle"], "o-", color="black", label=f"EXP {exp}")

    plt.xlabel("Stage shift (mm)")
    plt.ylabel("Tilt Angle (degrees)")
    plt.title(f'Tilt Angles (degree) in EXP: {exp}')
    plt.grid(True)
    plt.tight_layout()

    plt.savefig(f'tilt_plot_exp_{exp}.pdf')
plt.show()

def plot_summary_with_fit(summary_df: pd.DataFrame) -> None:
    """
    Plot aggregated mean  $\pm$  SD vs TILT and overlay a linear fit.

    Output file naming matches original behavior:
    grouped_exp_tilt_summary.pdf
    """
    required_cols = {"TILT", "Mean Angle", "Std Dev"}
    if not required_cols.issubset(summary_df.columns):
        raise ValueError(
            f'plot_summary_with_fit() requires columns: {sorted(required_cols)}'
        )

    # Preserve original filtering: only TILT >= 0 are used for the summary fit
    df = summary_df[summary_df["TILT"] >= 0].sort_values("TILT")

    if df.empty:
        raise ValueError(
            "No valid rows found for plotting summary (requires TILT >= 0)."
        )
```

```
x = df["TILT"]
y = df["Mean Angle"]
yerr = df["Std Dev"]

# Same fitting method as original: numpy polyfit (degree=1)
coeffs = np.polyfit(x, y, 1)
fit_line = np.polyval(coeffs, x)

plt.figure(figsize=(8, 6))
plt.errorbar(
    x,
    y,
    yerr=yerr,
    fmt="o-",
    color="k",
    capsize=4,
    label="Mean  $\pm$  SD",
)
plt.plot(
    x,
    fit_line,
    "--",
    color="red",
    label=f"Linear Fit: y={coeffs[0]:.2f}x+{coeffs[1]:.2f}",
)

plt.xlabel("Stage shift (mm)")
plt.ylabel("Tilt Angle (degrees)")
plt.title("Average Tilt Angle vs Stage shift (mm)")
plt.grid(True)
plt.legend()
plt.tight_layout()

plt.savefig("grouped_exp_tilt_summary.pdf")
plt.show()
```

Quality Checks

```
=====
=====
```

```
def run_quality_checks(raw_df: pd.DataFrame, tilt_by_exp: pd.DataFrame) -> None:
```

```
    """
```

```
    Perform lightweight consistency checks without changing the analysis.
```

```
    Notes
```

```
    -----
```

```
    This function is intentionally non-invasive: it does not alter data or results,
    only raises clear errors for missing expected structure.
```

```
"""
for col in ["EXP", "TILT", "Z", "YM PIX"]:
    if col not in raw_df.columns:
        raise ValueError(f'Raw table is missing required column '{col}'.')

for col in ["EXP", "TILT", "Tilt Angle"]:
    if col not in tilt_by_exp.columns:
        raise ValueError(f'Results table is missing required column '{col}'.')

if tilt_by_exp.empty:
    raise ValueError(
        "No tilt angles were computed. "
        "Common causes: each (EXP,TILT) group has <2 points or the input file is
malformed."
    )
```

Main pipeline

```
=====

def main() -> pd.DataFrame:
    """
    Execute the full calibration analysis pipeline.
    Returns the summary dataframe (useful for interactive environments).
    """
    # 1) Load + standardize data
    raw_df = extract_tilt_data(CSV_FILE, PIXEL_SIZE_UM)

    # 2) Compute per-(EXP, TILT) angles
    tilt_by_exp = compute_tilt_angles_by_exp(raw_df)

    # 3) Quality checks (structure + sanity)
    run_quality_checks(raw_df, tilt_by_exp)

    # 4) Aggregate across experiments by TILT
    summary_df = aggregate_by_tilt(tilt_by_exp)

    # 5) Plots (per EXP + global summary)
    plot_angles_per_experiment(tilt_by_exp)
    plot_summary_with_fit(summary_df)

    # 6) Saving/Export (file names preserved)
    summary_df.to_csv("tilt_summary_by_tilt_id.csv", index=False)
    tilt_by_exp.to_csv("tilt_by_exp.csv", index=False)

    return summary_df
```

Script Entry Point

```
=====
```

```
if __name__ == "__main__":  
    summary = main()  
    print(summary)
```

C7 Chapter 5 - Cavity analysis

=====

FUCCICardioid Wildtype Dataset (18-21 Nov 2025) - Structural + Lumen Metrics (MATLAB)

Purpose

Generate dot-plot summaries for FUCCICardioid structural descriptors extracted in ARIVIS (volumetric structural analysis + lumen segmentation).

The script produces:

(A) Cardioid structural metrics per differentiation class:

- 1) Total volume (μm^3)
- 2) Sphericity (unitless)
- 3) Volume fold-change vs Day 2.5 baseline

(B) Cavity/cavity structural characterization per class:

- 1) Number of cavities
- 2) Cavity occupancy: $V_{\text{cav,total}} / V_{\text{cardioid}}$
- 3) Largest cavity fraction: $V_{\text{max}} / V_{\text{cav,total}}$
- 4) Mean cavity volume: $V_{\text{cav,total}} / N_{\text{cav}}\%$

Expected Inputs

This script is self-contained: metrics are provided as numeric vectors already exported/compiled from ARIVIS.

Outputs

Figures only (no file export in the original behavior):

- 3 figures for global structural metrics
- 4 figures for cavity/cavity metrics

How to Run

Run the script in MATLAB. If you update the metric vectors, keep the class ordering consistent across all arrays.

=====

% Inputs/Outputs

=====

% INPUTS: metric vectors defined below (per class)

% OUTPUTS: figures only (dot plots), matching original behavior

Configuration

```
=====
% Plot style (kept consistent across all figures; matches original settings)
```

```
FS = 25;    % Font size
```

```
FN = 'Arial'; % Font name
```

```
AXLW = 1.5;    % Axis line width
```

```
MS1 = 15;    % Marker size (structural volume)
```

```
MS2 = 10;    % Marker size (sphericity, fold-change)
```

```
MS_L = 15;    % Marker size (cavity plots)
```

Main pipeline - Part A: Structural Metrics (Total Volume and Sphericity)

```
=====
% ---- INPUT BLOCK (Structural) ----
```

```
classNames = {'Day 2.5','Day 3.5','Day 4.5','Day 5.5'};
```

```
totalVolume_um3 = [1.5e8, 3.12e8, 2.63e8, 3.24e8]; % cardioid total volume [ $\mu\text{m}^3$ ]
```

```
sphericity = [0.5, 0.4, 0.5, 0.52]; % sphericity [0..1]
```

```
% ---- Quality Checks (Structural) ----
```

```
nC = numel(classNames);
```

```
if numel(totalVolume_um3) ~= nC || numel(sphericity) ~= nC
```

```
    error('Input length mismatch: classNames, totalVolume_um3, and sphericity must  
have the same length.');
```

```
end
```

```
if any(totalVolume_um3 <= 0)
```

```
    error('Invalid totalVolume_um3: all class volumes must be > 0.');
```

```
end
```

```
if totalVolume_um3(1) <= 0
```

```
    error('Invalid baseline volume: first class volume must be > 0 to compute fold-  
change.');
```

```
end
```

```
% ---- Derived Metric (Structural) ----
```

```
% Fold-change is defined relative to Day 2.5 baseline (first class).
```

```
volFold = totalVolume_um3 ./ totalVolume_um3(1);
```

```
% ---- Axis coordinate for class-wise dot plots ----
```

```
x = 1:nC;
```

```
%% Figure A1: Total Volume (dot plot)
figure('Color','w'); hold on;
plot(x, totalVolume_um3, 'ko', 'MarkerFaceColor','k', 'MarkerSize',MS1);

set(gca, 'FontName',FN,'FontSize',FS,'FontWeight','bold', ...
    'LineWidth',AXLW,'Box','off');

xticks(x); xticklabels(classNames);
xlabel('Class','FontName',FN,'FontSize',FS,'FontWeight','bold');
ylabel('Total Volume (µm3)','FontName',FN,'FontSize',FS,'FontWeight','bold');

ylim([0 3.6e8]);
xlim([0.5 nC+0.5]);
grid on; box on;

%% Figure A2: Sphericity (dot plot)
figure('Color','w'); hold on;
plot(x, sphericity, 'ko', 'MarkerFaceColor','k', 'MarkerSize',MS2);

set(gca, 'FontName',FN,'FontSize',FS,'FontWeight','bold', ...
    'LineWidth',AXLW,'Box','off');

xticks(x); xticklabels(classNames);
xlabel('Class','FontName',FN,'FontSize',FS,'FontWeight','bold');
ylabel('Sphericity','FontName',FN,'FontSize',FS,'FontWeight','bold');

ylim([0 1.05]);
xlim([0.5 nC+0.5]);

%% Figure A3: Volume Fold-Change vs Day 2.5 baseline (dot plot)
figure('Color','w'); hold on;
plot(x, volFold, 'ko', 'MarkerFaceColor','k', 'MarkerSize',MS2);
yline(1,'k-','LineWidth',1.2); % baseline reference (Day 2.5)

set(gca, 'FontName',FN,'FontSize',FS,'FontWeight','bold', ...
    'LineWidth',AXLW,'Box','off');

xticks(x); xticklabels(classNames);
xlabel('Class','FontName',FN,'FontSize',FS,'FontWeight','bold');
ylabel('Volume fold-change vs Day 2.5','FontName',FN,'FontSize',FS,'FontWeight','bold');

xlim([0.5 nC+0.5]);
```

Main pipeline - Part B: Cavity / Cavity Metrics


```
=====
=====
% Close previous figures as in the original workflow
close all

%% ---- INPUT BLOCK (Cavity/Cavity) ----
% Cardioid + cavity volumes are expressed in  $\mu\text{m}^3$ ; cavity counts are unitless.
classNames = {'Day 2.5','Day 3.5','Day 4.5','Day 5.5'};
Vcard_um3 = [1.5e8, 3.12e8, 2.63e8, 3.24e8]; % cardioid structural volume [ $\mu\text{m}^3$ ]
Ncav = [0, 96, 54, 59]; % number of cavities [count]
VcavTot_um3 = [0, 4.93e7, 3.8e7, 2.42e8]; % total cavity volume [ $\mu\text{m}^3$ ]
VcavMax_um3 = [0, 1.35e7, 1.4e7, 8.53e7]; % max cavity volume [ $\mu\text{m}^3$ ]

% ---- Quality Checks (Cavity/Cavity) ----
nC = numel(classNames);

if any([numel(Vcard_um3), numel(Ncav), numel(VcavTot_um3),
numel(VcavMax_um3)] ~= nC)
    error('Input length mismatch: all cavity inputs must have the same length as
classNames.');
```

end

```
if any(Vcard_um3 <= 0)
    error('Invalid Vcard_um3: all cardioid volumes must be > 0.');
```

end

```
if any(Ncav < 0) || any(mod(Ncav,1) ~= 0)
    error('Invalid Ncav: number of cavities must be non-negative integers.');
```

end

```
if any(VcavTot_um3 < 0) || any(VcavMax_um3 < 0)
    error('Invalid cavity volumes: VcavTot_um3 and VcavMax_um3 must be >= 0.');
```

end

```
if any(VcavMax_um3 > VcavTot_um3)
    error('Consistency error: VcavMax_um3 cannot exceed VcavTot_um3 for any class.');
```

end

```
%% ---- Derived Metrics (Lumen/Cavity) ----
% (1) Cavity occupancy: fraction of cardioid volume occupied by cavities.
occRate = VcavTot_um3 ./ Vcard_um3; % expected range 0..1 (not enforced)

% (2) Largest lumen fraction: fraction of total cavity volume contained in the largest
cavity.
% Undefined when VcavTot_um3 == 0 → set to NaN to avoid division-by-zero.
largestFrac = VcavMax_um3 ./ VcavTot_um3; % expected range 0..1
largestFrac(VcavTot_um3 == 0) = NaN;
```

```
% (3) Mean cavity volume: total cavity volume normalized by cavity count.
% Undefined when Ncav == 0 → set to NaN.
meanCavVol_um3 = VcavTot_um3 ./ Ncav;
meanCavVol_um3(Ncav == 0) = NaN;

%% ---- Plot settings (black-only dot plots) ----
FS = 25; FN = 'Arial'; AXLW = 1.5; MS = MS_L;
x = 1:nC;

%% Figure B1: Number of cavities
localDotPlot(x, Ncav, classNames, 'Number of cavities', [], FN, FS, AXLW, MS);

%% Figure B2: Cavity occupancy
localDotPlot( ...
    x, occRate, classNames, ...
    'Cavity occupancy ( $V_{\text{cav,total}}$  /  $V_{\text{cardioid}}$ )', ...
    [0 1], FN, FS, AXLW, MS);

%% Figure B3: Largest lumen fraction
localDotPlot( ...
    x, largestFrac, classNames, ...
    'Largest lumen fraction ( $V_{\text{max}}$  /  $V_{\text{cav,total}}$ )', ...
    [0 1], FN, FS, AXLW, MS);

%% Figure B4: Mean cavity volume (many small vs few large cavities)
% Note: log-scale can help for wide dynamic ranges, but is intentionally left
% commented out to preserve the original behavior.
figure('Color','w'); hold on;
plot(x, meanCavVol_um3, 'ko', 'MarkerFaceColor','k', 'MarkerSize',MS);

set(gca, 'FontName',FN,'FontSize',FS,'FontWeight','bold', ...
    'LineWidth',AXLW,'Box','off');

xticks(x); xticklabels(classNames);
xlabel('Class','FontName',FN,'FontSize',FS,'FontWeight','bold');
ylabel('Mean cavity volume ( $V_{\text{cav,total}}/N$ ) [ $\mu\text{m}^3$ ]',
    'FontName',FN,'FontSize',FS,'FontWeight','bold');

xlim([0.5 nC+0.5]);
ylim([0, 4.7e6]);

% set(gca,'YScale','log'); % optional visualization (disabled by default)
```

Helper Functions

```
=====
=====
```

```
function localDotPlot(x, y, classNames, ylab, ylims, FN, FS, AXLW, MS)
% localDotPlot Minimal, thesis-style dot plot for class-wise metrics.
%
% This utility enforces a consistent appendix-level visual style across all
% metric plots without introducing new analysis behavior.
%
% Inputs
% -----
% x    : numeric vector (class indices)
% y     : numeric vector (metric values)
% classNames: cell array of strings (tick labels)
% ylab  : y-axis label (string)
% ylims : [] or [ymin ymax] (optional y-axis limits)
% FN, FS : font settings
% AXLW   : axis line width
% MS     : marker size

figure('Color','w'); hold on;
plot(x, y, 'ko', 'MarkerFaceColor','k', 'MarkerSize',MS);

set(gca, 'FontName',FN,'FontSize',FS,'FontWeight','bold', ...
    'LineWidth',AXLW,'Box','off');

xticks(x); xticklabels(classNames);
xlabel('Class','FontName',FN,'FontSize',FS,'FontWeight','bold');
ylabel(ylab,'FontName',FN,'FontSize',FS,'FontWeight','bold');

xlim([0.5 numel(x)+0.5]);

if ~isempty(ylims)
    ylim(ylims);
end
```

D

Appendix D Academic Curriculum

Academic Profile

Bioengineering PhD student at the University of Pavia with a publication profile spanning tissue engineering systems, live-cell microscopy, and bioimage analysis with cell-cycle-aware phenotyping

Publications

- Pezzotti M. et al. *A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement*. APL Bioengineering 10, 016114 (2026). DOI: 10.1063/5.0306480.
- Zimmermann J., Pezzotti M. et al. *Bioimage analysis for multiplexed FUCCI acquisitions powered by deep learning*. npj Imaging 4(1) (2026). DOI: 10.1038/s44303-026-00159-6.
- Ashesh A. et al.; with Pezzotti M. *MicroSplit: Semantic unmixing of fluorescent microscopy data*. Nature Methods (2026). DOI: 10.1038/s41592-026-03082-1.
- Torchia E. et al.; with Pezzotti M. *Fabrication of cell culture hydrogels by robotic liquid handling automation for high-throughput drug testing*. Communications Engineering (2025). DOI: 10.1038/s44172-025-00575-3.
- Pezzotti M. et al.; Engineering Toolkits for High-Throughput and High-Content Phenotyping. Currently under revision.
- Enrico A. et al.; with Pezzotti M. *LiGHTS: Massively Parallel Biomimetic Photo-Functionalization for Imaging-Based Ultra-High-Throughput Screening*. bioRxiv (2025). DOI: 10.1101/2025.10.23.683892. Currently under revision.
- Di Sante M., Pezzotti M. et al. *CALIPERS: Cell cycle-aware live imaging for phenotyping experiments and regeneration studies*. bioRxiv (2024/2025). DOI: 10.1101/2024.12.19.629259. Currently under revision.
- Vitali V., Nava G., Corno A., Pezzotti M. et al. *Yield stress “in a flash”: investigation of nonlinearity and yielding in soft materials with an optofluidic microrheometer*. Soft Matter 17, 3105–3112 (2021). DOI: 10.1039/D0SM02168G.

Conference contributions

Year	Conference	Location	Title of Contribution
2024	Focus on Microscopy - FOM 2024	Genova, Italy	FUCCIsmart: An Automated, High-Resolution Live Imaging Algorithm for Studying Volumetric Mitotic Phase in Cell Proliferation
2025	Gruppo Nazionale di Bioingegneria - GNB 2025	Palermo, Italy	TE-DaXi: LEGO®-like instructions for assembling a light-sheet microscope for tissue engineering applications
2026	TERMIS 2026	Palma de Mallorca, Spain	A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement
2026	European Society of Biomechanics — ESB 2026	Milano, Italy	A vertically integrated system for tracking and assessing cell-cycle-aware phenotypes under confinement

Research and teaching experience

- Visiting Researcher, Chan Zuckerberg Biohub, San Francisco, USA
Loïc Royer Team | June 2022 – October 2022
Completed a research visit focused on learning how to design, assemble, and operate cutting-edge light-sheet microscopy systems. Gained hands-on experience with advanced optical microscopy platforms for high-resolution biological imaging. Developed technical knowledge in microscope architecture, optical alignment, and volumetric imaging workflows.
- Microscopy instrumentation supervision, Synthetic Physiology Lab, University of Pavia, Italy, 2021 – present
Developed and optimized live-cell imaging protocols for fluorescence microscopy experiments. Supported and trained users in microscope handling, acquisition setup, imaging strategy, and troubleshooting. Contributed to the standardization of imaging workflows for reproducible live-cell phenotyping.
- Master thesis student supervision, Synthetic Physiology Lab, University of Pavia, Italy, 2021 – present
Trained and supervised Bioengineering Master's thesis students. Supported students in experimental design, sample preparation, live-cell imaging, microscopy-based assays, and data interpretation and quantitative analysis.
- Practical laboratory activity, Bioengineering Program, University of Pavia, Italy, 2021 – present
Co-hosted practical laboratory activities for Bachelor's and Master's students in Bioengineering. Delivered hands-on training focused on live-cell imaging and microscopy-based experimental workflows. Assisted students in understanding microscope operation, image acquisition, and experimental planning.

