A Single-Cell Platform for Longitudinal Monitoring of Protoplast Physiology and Transcriptomics

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INTRODUCTION

Protoplasts are a versatile system for studying cellular differentiation, stress responses, and genetic manipulation. However, conventional bulk assays obscure individual cell behaviors, making it difficult to distinguish whether increased cell numbers reflect enhanced proliferation or improved survival.

To address this, we applied the Cellanome R3200 platform, which integrates imaging, AI-powered cell segmentation, and automated micro-3D printing to capture thousands of individual protoplasts in bio-compatible hydrogel compartments called CellCage™ enclosures (CCEs) within an 8-lane flow cell. CCEs are tunable in shape, size, and permeability, allowing optimization for different cell types. Cells remain alive within CCEs as nutrients and reagents diffuse through the hydrogel, enabling longitudinal live-cell experiments over days. Multiple cellular phenotypes, including proliferation, viability, and morphology, can be quantified via bright-field and fluorescence imaging (up to 4 channels). Cells are then lysed within their compartments for mRNA capture and sequencing, with data matched back to individual CCEs.

We profiled protoplasts from Marchantia thallus, Arabidopsis root, and Arabidopsis aerial seedling tissues encapsulated in 120-micron CCEs. Across these tissue types, we recovered thousands of cages per sample, detecting up to 2,000 genes and 5,000 molecular barcodes (MBCs) per cage. Notably, intact protoplasts yielded very high MBC counts, exceeding 60,000 to 110,000 per cell in some instances. UMAP analysis revealed distinct transcriptional clusters within each tissue type, and differential expression analysis identified tissue-specific marker genes.

This workflow also accommodates perturbations such as hormone treatments or CRISPR-mediated editing, with the ability to sequence detect single-guide RNAs in addition to mRNAs from each CCE. By integrating longitudinal imaging with single-cell transcriptomics, this platform enables direct linkage of phenotypic readouts to gene expression without inference across separate experiments, supporting applications in plant cell biology, health, and resilience.

LEVERAGING CELLANOME R3200 PLATFORM FOR MULTI-MODAL PROFILING OF CELLS

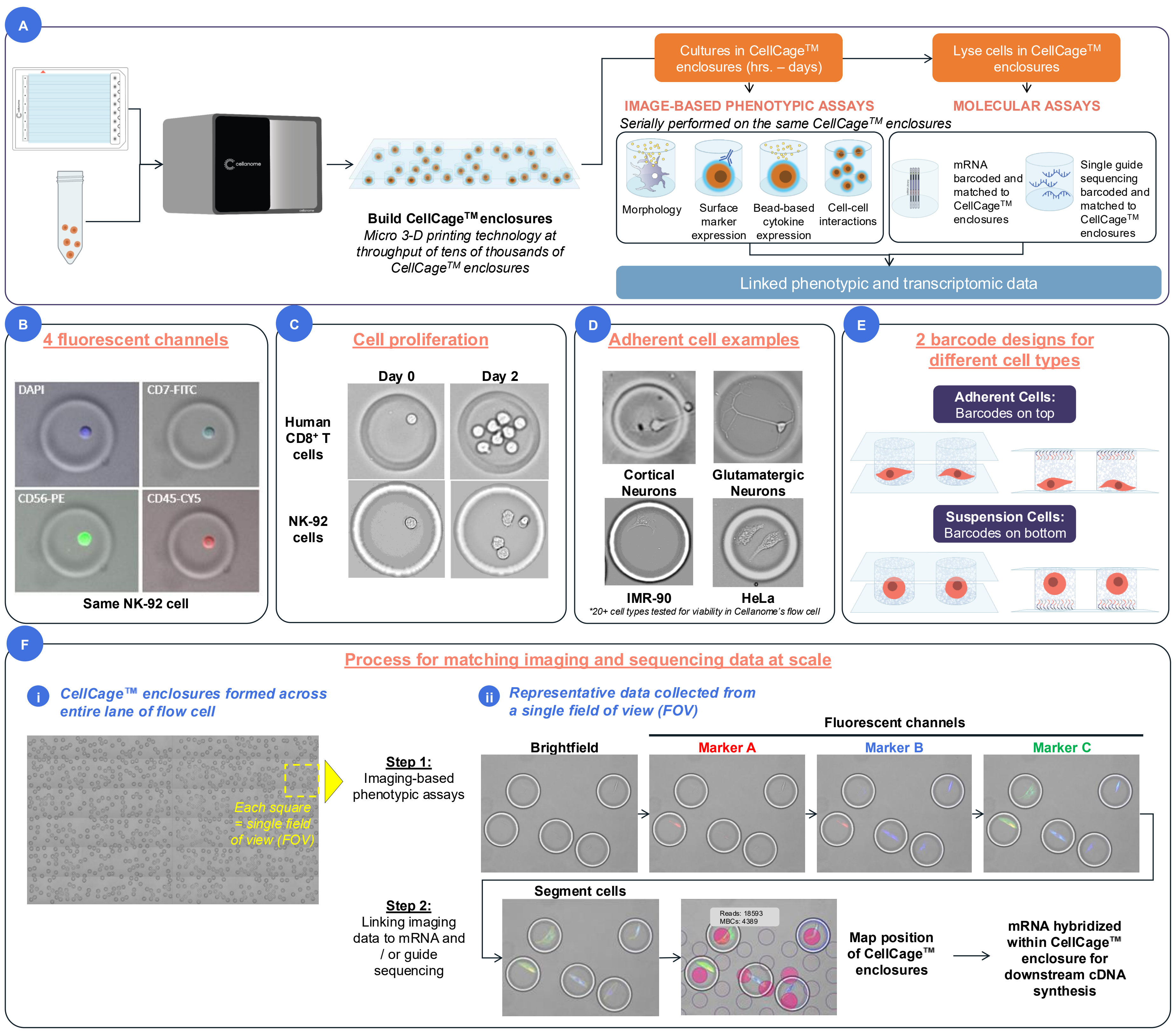


Fig 1. Cellanome's Technology enables the measurement of multiple phenotypic and functional assays from the same cells in CellCage™ enclosures (CCEs).

(A) Tens of thousands of cells are mixed with hydrogel precursor and loaded on an 8-lane flow cell. Cell positions are identified and CellCage™ enclosures (CCEs) automatically generated around cells with light-guided polymerization. Bio-compatible CCEs can be formed around single cells, multiple cells, or cells with objects (e.g., cytokine beads). CCEs are permeable to reagents enabling long-term culturing and a variety of imaging-based, longitudinal phenotypic and functional assays to be performed on the same cells (e.g., small molecules, immunofluorescent antibodies). Cells are lysed within CCEs to release cellular mRNA that is used for downstream library prep and sequencing off the instrument. **(B)** Fluorescent imaging of the same NK-92 cell in CCEs following staining with α-CD7-FITC, α-CD56-PE, α-CD45-CY5 and DAPI. **(C)** Brightfield imaging of activated human T cells and NK-92 cells on days 0, and 2 of culture in CCEs. **(D)** Cellanome technology is compatible with diverse cell types, including adherent cells. Images represent examples of adherent cell types tested that were viable on Cellanome's flow cells. **(E)** Flow cells for sequencing experiments are designed to be compatible with suspension and adherent cells by barcoding either the bottom or top surfaces. **(F-i)** CCEs are formed across entire flow cell lanes at the scale of ten of thousands. **(F-ii)** In a single representative field of view (FOV), overview of how imaging data is linked to mRNA. Encapsulated cells are serially imaged across brightfield and fluorescence channels. In these representative images, three fibroblast markers are detected on the same cells. After imaging data is collected, Cellanome's computer vision capabilities segment cells, identify barcode positions on the flow cell surface, and match them to constructed CCEs. Cells are then lysed within CCEs and mRNA captured to the barcodes on the flow cell's surface prior to cDNA synthesis.

HEAT-SHOCK PROTOPLAST REPORTER LINE

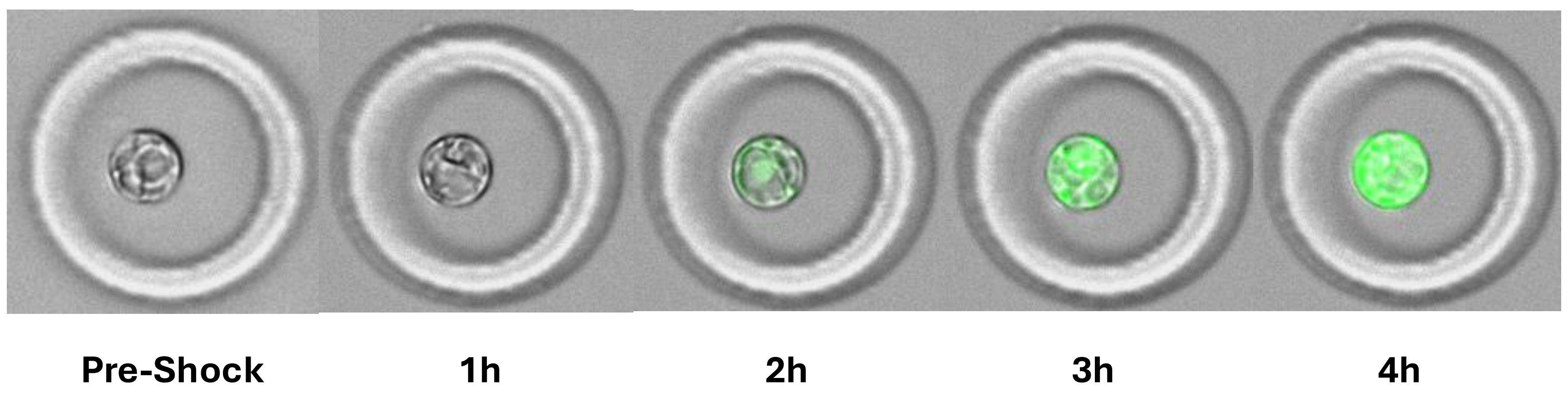
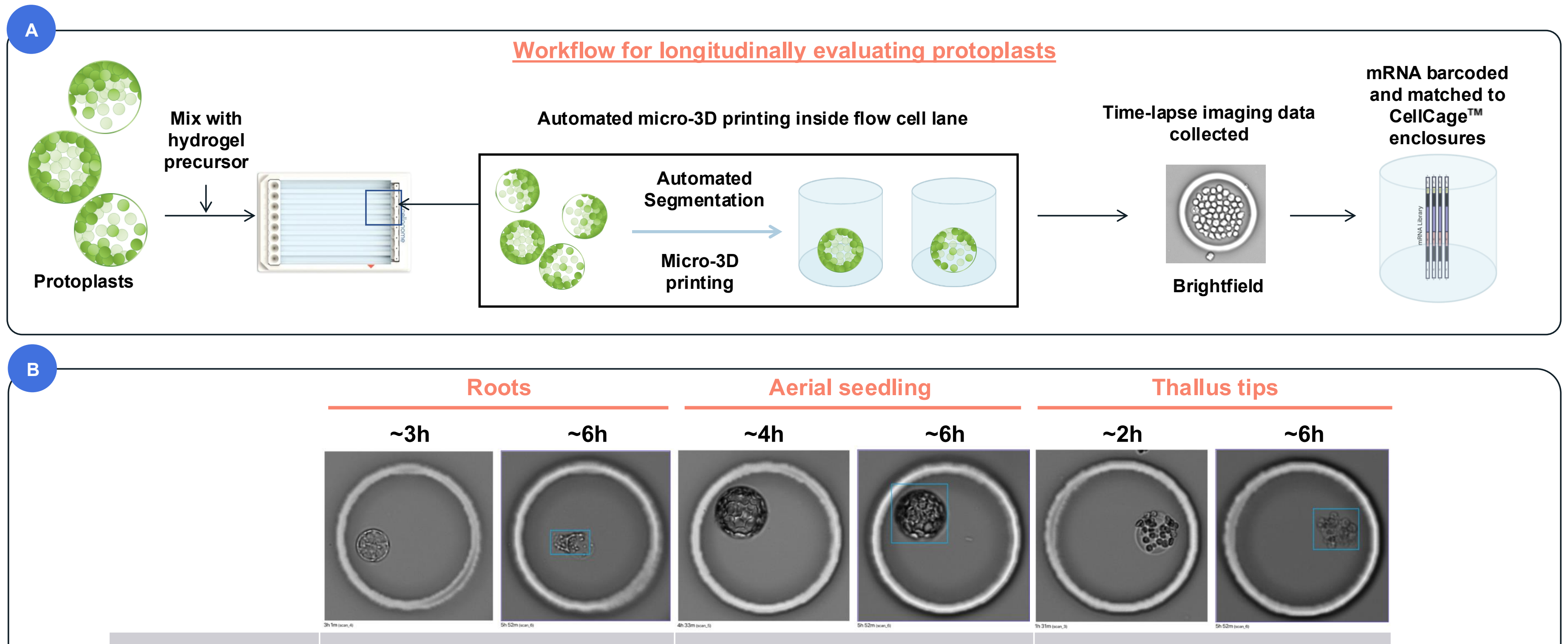


Fig 2. Longitudinal monitoring of heat-shock-induced GFP expression in plant protoplasts on the R3200.
Arabidopsis root protoplasts carrying a heat-shock-responsive promoter driving GFP were loaded and encaged in CellCages on the R3200 flowcell. Cells were subjected to a 1-hour heat treatment at 37 °C followed by recovery at ambient temperature. Brightfield and fluorescence images of the same individual cell were captured at the indicated time points. GFP signal first becomes detectable at approximately 2 hours post-treatment, with progressive accumulation and redistribution of fluorescence from a punctate, perinuclear pattern to a more diffuse cytoplasmic distribution by 4 hours. This experiment demonstrates that the R3200 platform supports dynamic perturbation and longitudinal single-cell imaging of plant protoplasts, enabling real-time tracking of transcriptional responses within individually caged cells.

DEMONSTRATING MULTI-MODAL DATA FOR SINGLE PROTOPLAST CULTURING ON R3200

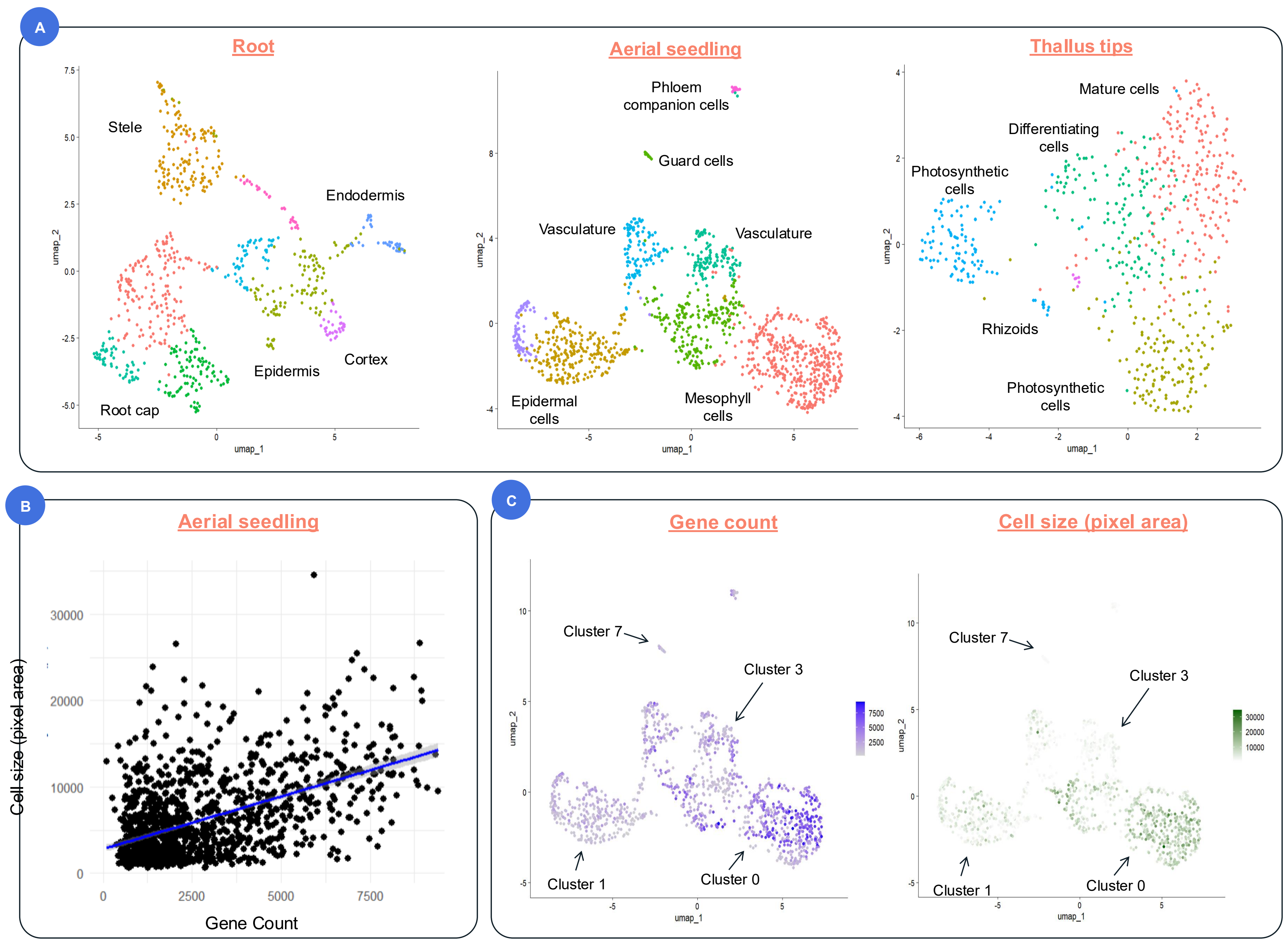


	Roots	Aerial seedling	Thallus tips
# of cells detected	8,626	4,795	13,468
# of CCEs	3,368	2,212	4,624
# of cells enclosed in CCEs	3,368	2,212	4,624
% of cells enclosed in CCEs	39%	46%	34%

Fig 3. Diverse protoplasts can be enclosed and longitudinally imaged at single-cell resolution.

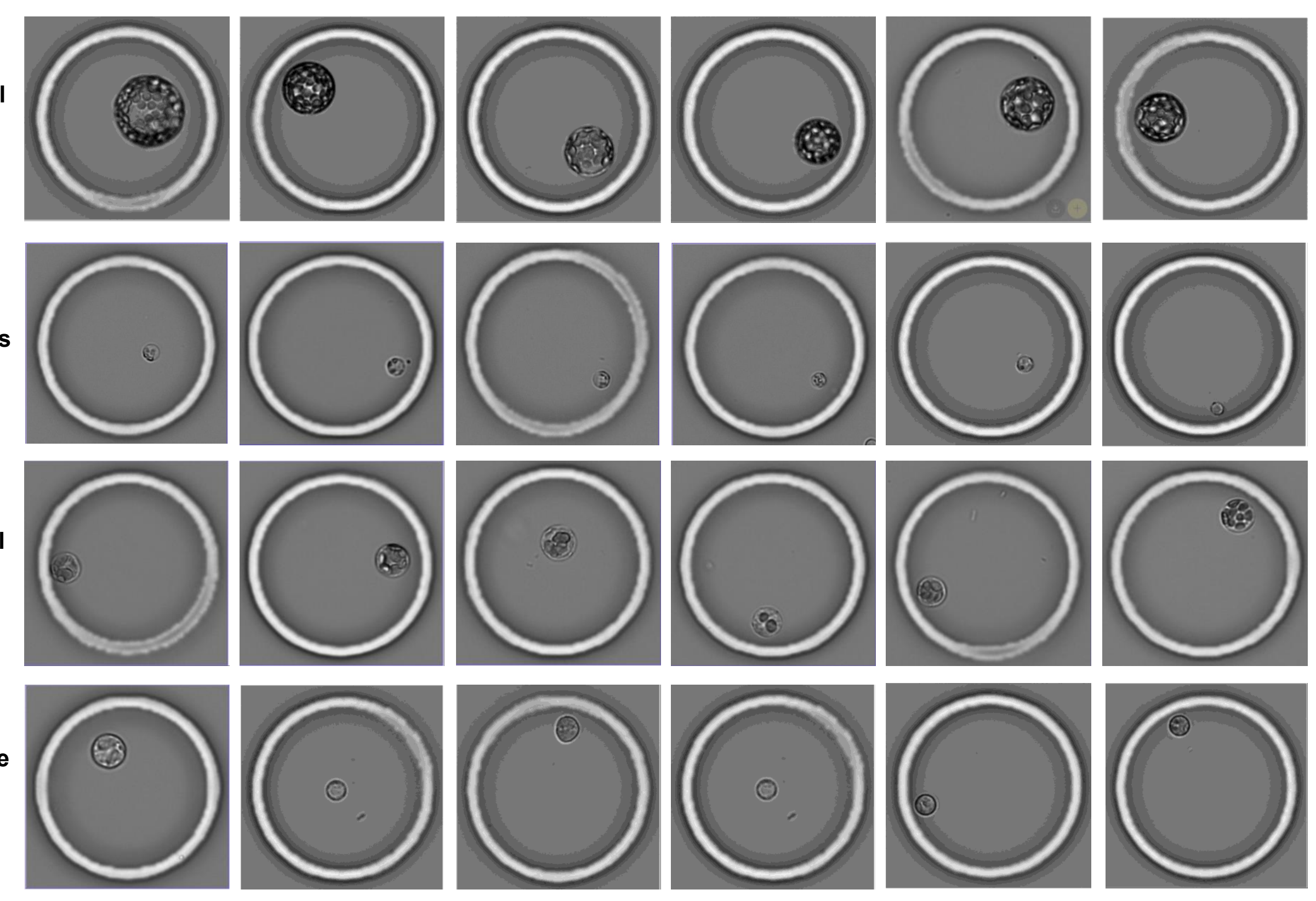
(A) Following a similar process as Fig 2, protoplasts are mixed with a hydrogel precursor and loaded onto flow cells. Thousands of protoplasts are successfully detected and enclosed in CCEs for *Arabidopsis* 7-day-old roots and aerial seedlings and *Marchantia* 11-day-old thallus tips. **(B)** Example images of protoplasts encapsulated in CCEs and associated capture and detection metrics. Blue boxes in CCEs indicate detection of cells through Cellanome segmentation.

SINGLE-CELL mRNA SEQUENCING LINKED TO SINGLE-CELL PROTOPLAST SIZE



D

Example cells in selected clusters



Mesophyll cells Cluster 0

Gene count average	3,965
Cell size (pixel area) average	9,193
Gene count / cell size ratio	0.43

Guard cells Cluster 7

Gene count average	895
Cell size (pixel area) average	1,633
Gene count / cell size ratio	0.55

Epidermal cells Cluster 1

Gene count average	1,189
Cell size (pixel area) average	3,321
Gene count / cell size ratio	0.36

Vasculature cells Cluster 3

Gene count average	1,865
Cell size (pixel area) average	1,835
Gene count / cell size ratio	1.01

Fig 4. Heterogeneity in protoplast size can be further investigated by generating linked scRNA-sequencing data.

(A) Individual cell mRNA sequencing of protoplasts within different CCEs allows successful umap clustering and identification of distinct cell-type populations for *Arabidopsis* and *Marchantia*. **(B)** A positive correlation is observed between cell size (pixel area) and gene counts per cell for *Arabidopsis* aerial seedling protoplasts. **(C)** Cell size and gene count can be more specifically examined across clusters, revealing distinct relationships for each cell type including mesophyll (cluster 0) guard cells (cluster 7), epidermal cells (cluster 1), and vasculature cells (cluster 3). **(D)** Examples of encapsulated protoplasts for the cell types described in Fig 4C showing cell size and morphology alongside gene count average, cell size average, and corresponding ratio statistics.

FUTURE DIRECTIONS

We are leveraging the R3200 platform to explore plant cellular responses under diverse perturbations, such as hormonal treatments, stress conditions, and CRISPR-mediated gene editing, enabling precise dissection of regulatory pathways at single-cell resolution. By integrating longitudinal imaging with transcriptomic profiling, we will uncover connections between protoplast behavior, such as cell wall regeneration and survival, linked to underlying gene expression programs. This system will be adapted to study various plant species, including crops, to reveal species-specific cellular responses and advance agricultural applications. Furthermore, we will optimize the encapsulation and transformation workflows to enhance protoplast viability and throughput, ultimately enabling predictive insights into plant health and resilience.