An Innovative Single-Cell System for Monitoring Protoplast Physiology Provides Insights into Plant Cellular Responses

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INTRODUCTION

Protoplasts—plant cells that have been separated from one another through enzymatic digestion of the cell wall—provide a versatile cell-based system for both fundamental biology and agricultural applications. Using delivery methods such as polyethylene glycol-mediated transformation or viral vector transduction, DNA, RNA, and proteins can be introduced into protoplasts to manipulate processes including cell wall synthesis, cell division, and differentiation. Thus, protoplasts are an effective in vitro system for studying questions that range from understanding cellular differentiation to how plants respond to stimuli like stress and hormones. However, traditional approaches rely on bulk measurements, which can be challenging to interpret because they mask individual cellular behaviors. Thus, nuances, like survivability of individual cells in response to perturbations cannot be differentiated from greater numbers of live cells resulting from enhanced proliferation. To overcome these limitations, we leveraged a novel technology that converges multi-modal evaluation of live, cellular behavior with transcriptomics at single-cell resolution.

EXPERIMENTAL APPROACHES FOR PROTOPLAST ANALYSIS

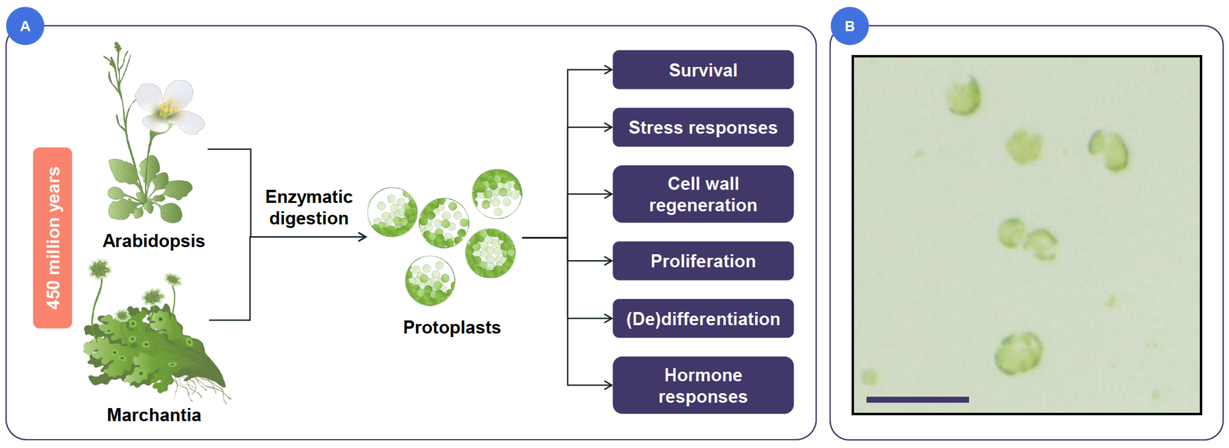


Fig 1. Plant cells can be isolated through cell wall digestion and phenotypes can be studied in bulk.

(A) *Arabidopsis* and *Marchantia* are plant model systems that represent a divergence of 450 million years. Cell walls from these plant tissues are digested with digestive enzymes (e.g., driselase, cellulase, macerozyme) to release individual plant cells in the form of protoplasts. Various processes can then be studied including cell survival, stress responses, and proliferation. **(B)** Example protoplasts following digestion released from *Arabidopsis* seven-day-old seedlings. Scale bar, 80 micrometers. **(C)** Schematic depicting new insights from cell types that can be revealed by multi-modal single-cell profiling of protoplasts in comparison to traditional bulk measurements.

LEVERAGING CELLANOME TECHNOLOGY FOR MULTI-MODAL PROFILING OF CELLS

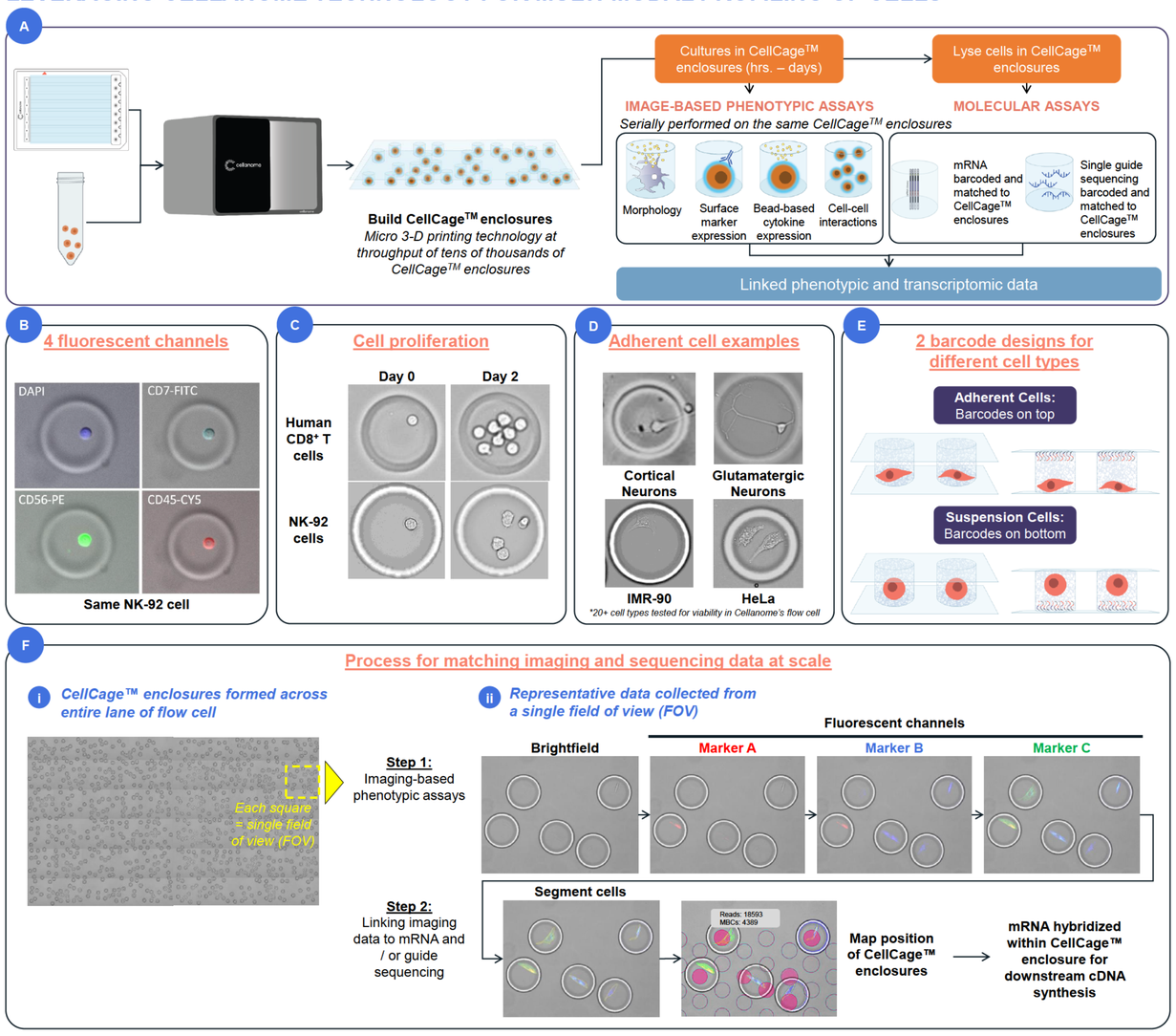
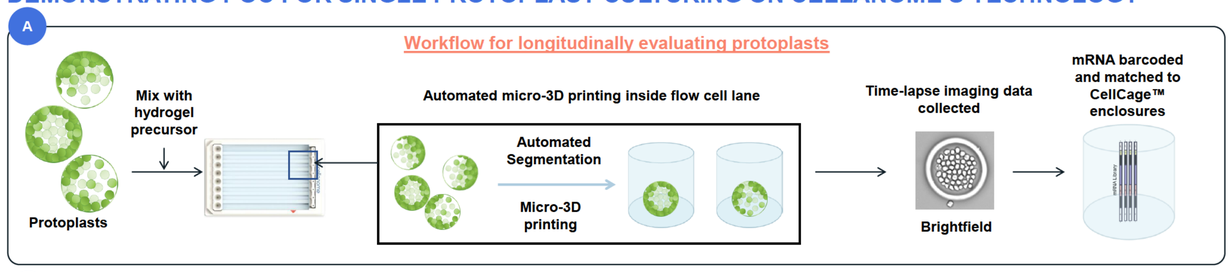


Fig 2. Cellanome's Technology enables the measurement of multiple phenotypic and functional assays from the same cells in CellCage™ enclosures (CCEs).

(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 generating cDNA 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.

DEMONSTRATING POC FOR SINGLE PROTOPLAST CULTURING ON CELLANOME'S TECHNOLOGY

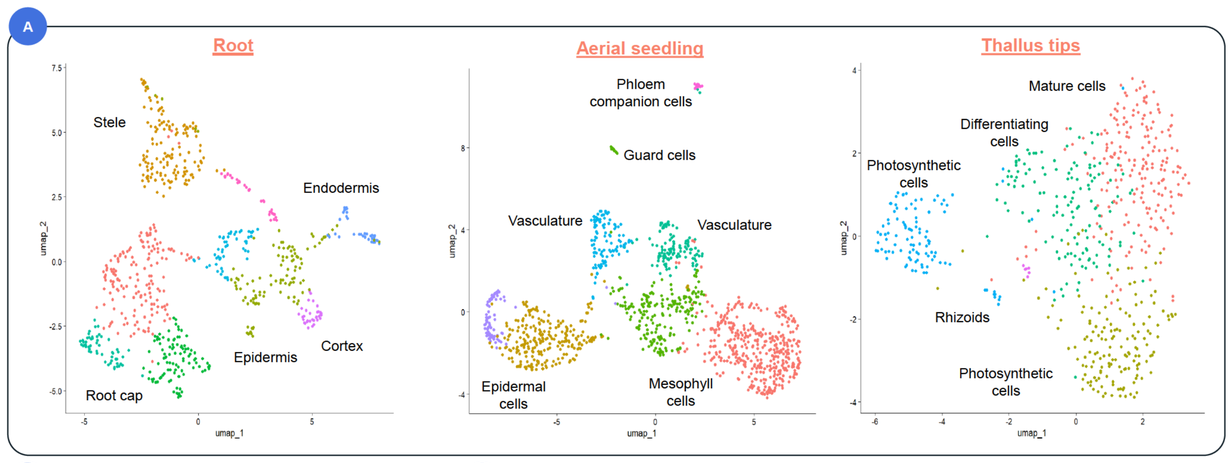


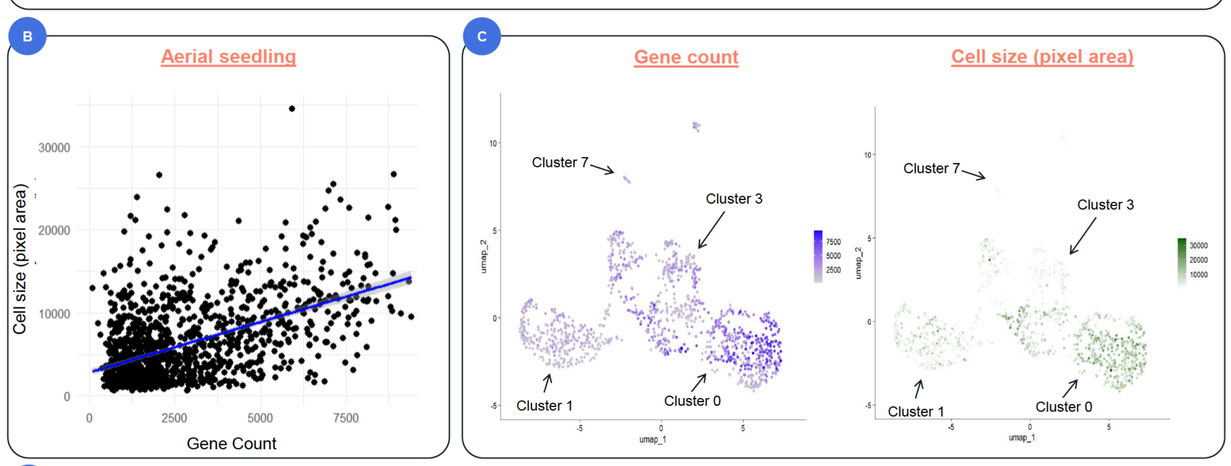
	Roots		Aerial seedling		Thallus tips	
# of cells detected	8,626		4,795		13,468	
# of CCEs	3,368		2,212		4,624	
# of cells enclosed in CCEs	3,368		2,212		4,624	
% of cells enclosed in CCEs	39%		46%		34%	

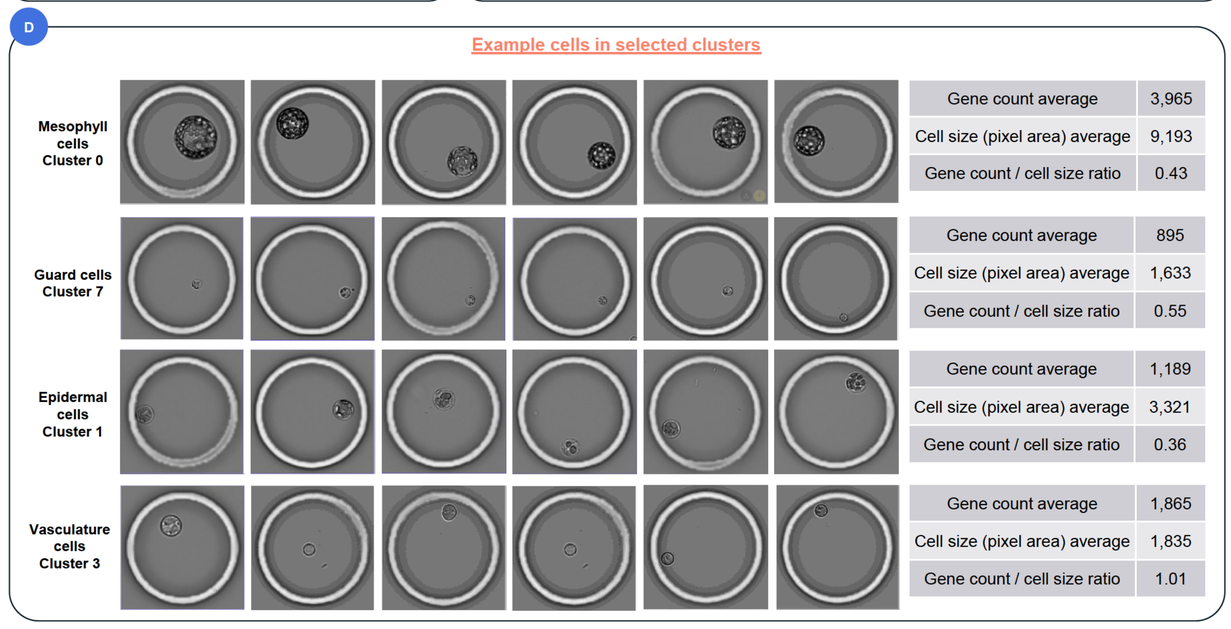
Fig 3. Diverse protoplasts can be enclosed and longitudinally imaged at single-cell resolution.

(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







	Mesophyll cells Cluster 0	Guard cells Cluster 7	Epidermal cells Cluster 1	Vasculature cells Cluster 3
Gene count average	3,965	895	1,189	1,865
Cell size (pixel area) average	9,193	1,633	3,321	1,835
Gene count / cell size ratio	0.43	0.55	0.36	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 aim to leverage the Cellanome 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.