A NOVEL MULTI-MODAL PLATFORM TO MORE ACCURATELY IDENTIFY AND ISOLATE SENESCENT CELLS

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INTRODUCTION

Senescent cells are a promising, but potentially controversial biomarker candidate for aging because no single marker is specific and expressed over cells' lifecycles. Methods bypassing this problem with orthogonal validation across multi-modal measurements (e.g., proteins, genes, morphology) are sub-optimal, in part, because no single technology longitudinally collects these metrics from the same, single cell. Thus, populations identified as senescent for quantification or enrichment are likely impure, making it challenging to draw confident conclusions around cells caveated as "senescent-like."

To address these challenges, we developed a method for identifying and quantifying senescent cells by leveraging a novel cell biology platform. This platform compartmentalizes tens of thousands of single cells within permeable hydrogel compartments, integrating imaging and nucleic acid extraction for linked, multi-modal analysis of the same cell across functional, phenotypic and genomic data. We validated this method detects multi-modal hallmarks of senescence (e.g., morphology, SASPs, p16/p21, and / or transcriptome) from the same, etoposide-induced IMR90 fibroblasts. Because this method can multi-modally identify cells and unambiguously link converged markers to the transcriptome, we believe this method can play a meaningful role in driving consensus on the identity and utility of senescent cells as an aging biomarker.

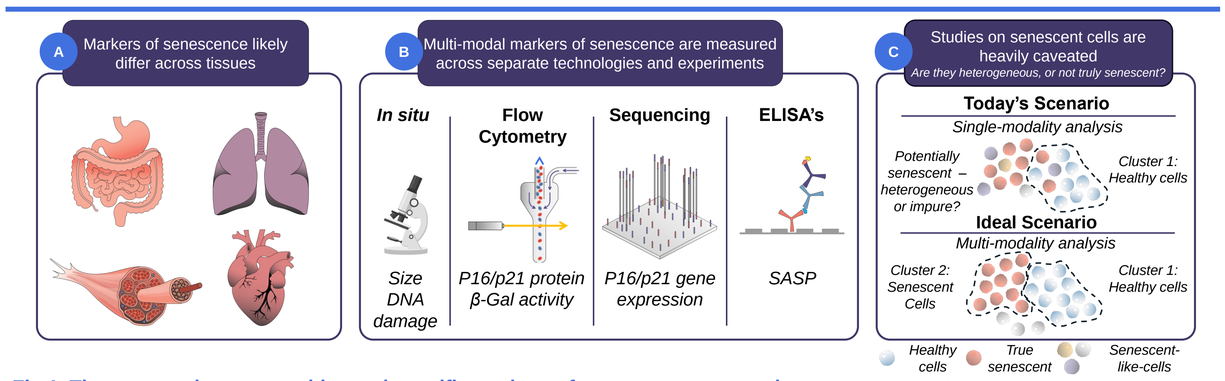


Fig 1. There are no known sensitive and specific markers of senescence across tissue. **(A)** Senescent cells accumulate in tissues with age, but markers of senescence likely vary across cell types and tissues. **(B)** To increase confidence in the identity of senescent cells, significant orthogonal validation is done with different markers across different technologies. **(C)** Because no single technology can collect multi-modal markers from the same, individual cells, researchers leverage single-modal markers to quantify cells, resulting in likely impure or incomplete quantification. This project aims to tackle this challenge by developing a method that measures multi-modal markers of senescence from the same, single cells.

CELLANOME TECHNOLOGY

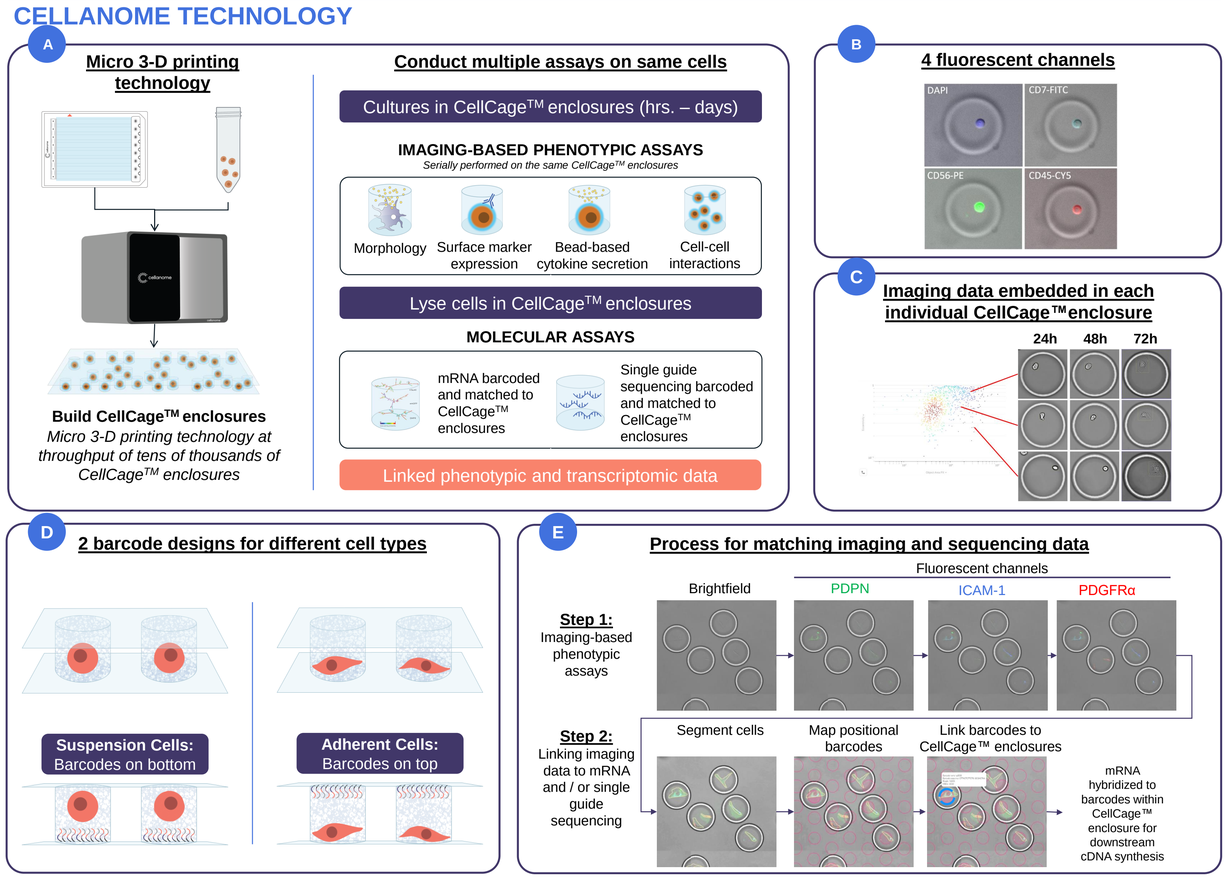


Fig 2. Cellanome's Technology enables the measurement of multiple phenotypic and functional assays from the same cells in CellCage™ enclosures. **(A)** Tens of thousands of cells are mixed with hydrogel precursor and loaded on an 8-lane flow cell. Cell positions are identified and CellCage™ enclosures automatically generated around cells with light-guided polymerization. Bio-compatible CellCage™ enclosures can be formed around single cells, multiple cells, or cells with objects (e.g., cytokine beads). CellCage™ enclosures 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 CellCage™ enclosures to generate positionally barcoded cDNA for downstream library prep and sequencing off the instrument. **(B)** Fluorescent imaging of the same NK-92 cell in CellCage™ enclosures following staining with α-CD7-FITC, α-CD56-PE, α-CD45-CY5 and DAPI. **(C)** Quantification of CellCage™ enclosures embedded with longitudinal imaging data. Each dot represents the aggregate relevant signals within each CellCage™ enclosure. Representative images of adherent fibroblasts. **(D)** Flow cells are designed to be compatible with suspension and adherent cells by barcoding either the bottom (suspension) or top (adherent) surfaces. **(E)** Step-by-step overview of how imaging data is linked to mRNA. Encapsulated cells are serially imaged across brightfield and fluorescence channels. In these representative images, 3 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 CellCage™ enclosures. Cells are then lysed within CellCage™ enclosures and mRNA captured to the barcodes on the flow cell's surface prior to cDNA synthesis.

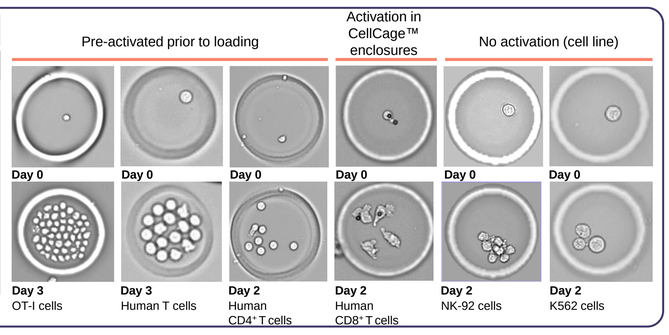
TESTING BROAD CELL TYPES TO SUPPORT ATLAS INITIATIVES

A

Primary cells

Source	Cells	Cell type	Tissue
Human	PBMCs – CD8⁺ T cells	Lymphoid	Blood
	PBMCs – Monocytes	Myeloid	Blood
	K562 cells	Lymphoid	Blood
	NK-92	Lymphoid	Blood
Mouse	OT-I (CD8⁺ T cells)	Lymphoid	Spleen isolation
	P14 (CD8⁺ T cells)	Lymphoid	Spleen isolation

**Note: CellCage™ enclosures' sizes vary depending on experiment needs and cell type*



B

Primary cells

Source	Cells	Cell type	Tissue origin
Human	Hs 255.T	Fibroblast	Colon
	Hs 675.T	Fibroblast	Colon
	Cortical neurons	Neuronal / glial	Brain
	Glutamatergic neurons		
	Astrocytes (Glial)		
	V2a	Interneuron	Spinal cord
	UNA + Fibs	Fibroblast	Skin
	UNA+ ESCs	Embryonic	Blastocyst
	UNA+ iPSCs	Fibroblasts	Skin
	IMR90	Fibroblasts	Lung
	U87	Glia	Brain glioma
	HeLa	Epithelial	Cervix
	HEK293T	Epithelial	Kidney
Mouse / Other	CHO	Epithelial	Ovary
	Dendritic Cells (e.g., mutu-DC or primary)	Myeloid	Bone marrow
	MC38	Epithelial	Colon
	AT-3	Epithelial	Mammary

**Note: UNA = vector used to promote neuronal differentiation*

Fig 3. Cell types tested for viability on Cellanome's Technology. **(A)** Circulating suspension blood cells. **(B)** Adherent tissue cells

MULTI-MODAL WORKFLOWS ON CELLS ENCAPSULATED IN CELLCAGE™ ENCLOSURES

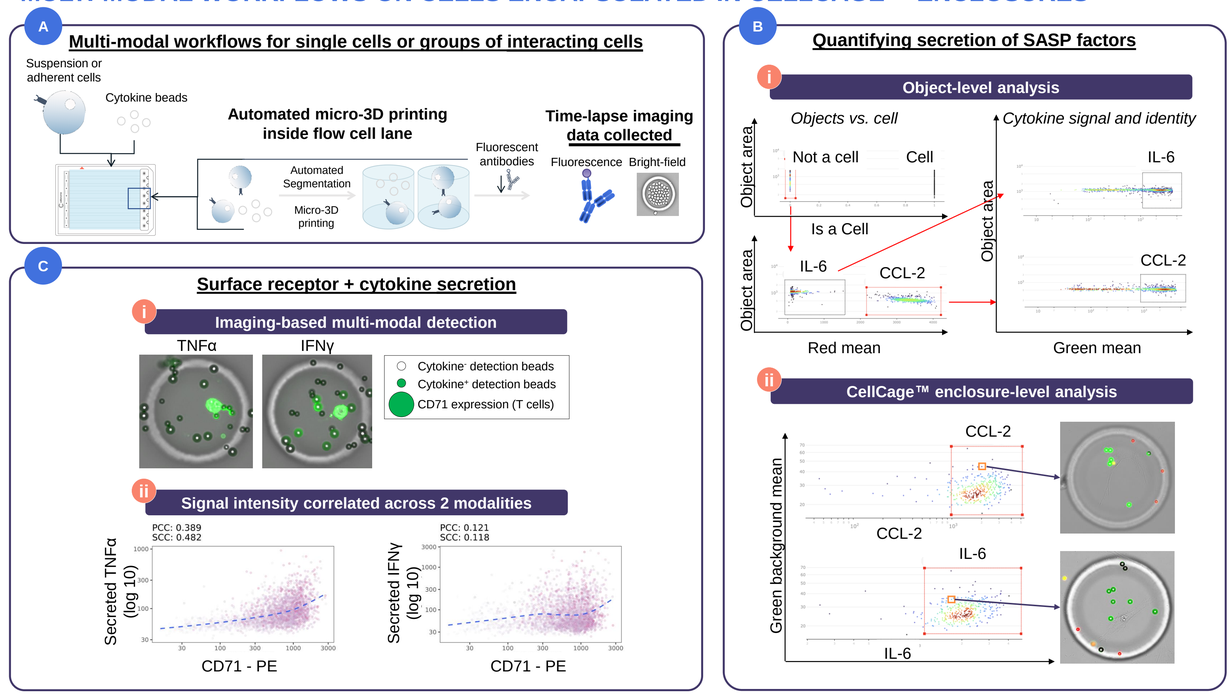


Fig 4. Secretion of SASP factors can be converged to morphology and cell-surface expression with Cellanome's Technology. **(A)** Schematic of workflow for multi-modal assays on Cellanome's Technology. POC was established for assays converging cytokine secretion to surface markers. For cytokine secretion assays, PBMCs or fibroblasts were activated with α-CD3 / α-CD28 in flask, mixed with cytokine detection beads and hydrogel precursor, and loaded onto a Cellanome flow cell. Cells were detected and CellCage™ enclosures formed around cells alone or with proximal cytokine beads. Media containing reagents that label cells or beads, including α-CD71 (lymphocyte activation), α-IFNγ / α-TNFα / α-IL-6 / α-CCL-2 (cytokine beads) were perfused onto the flow cell. Subsequently, time-lapse images of cells within CellCage™ enclosures were collected for multi-modal evaluation. **(B)** Analysis strategy for isolating fluorescence signal to cytokine detection beads. **i)** Representative gating strategy distinguishing fibroblast cells from non-cell objects detected within CellCage™ enclosures. Identity of cytokine secreted can be determined by signal from distinct fluorescence channels. **ii)** Representative gating strategy for analyzing aggregate signal of all objects within a single CellCage™ enclosure, with each dot representing an individual CellCage™ enclosure. This enables quantifying the number of cytokine positive CellCage™ enclosures and therefore, single cytokine-secreting fibroblast cells. **(C)** Converging cytokine secretion to surface receptors on individual cells. **i)** Representative images generated to collect multi-modal data (secreted cytokines, surface receptors). **ii)** Signal intensity of cytokine beads and surface receptor expression can be quantified and linked to perform pairwise analysis across modalities, with each dot representing an individual CellCage™ enclosure.

DETECTING CLASSIC, MULTI-MODAL HALLMARKS OF SENESCENCE

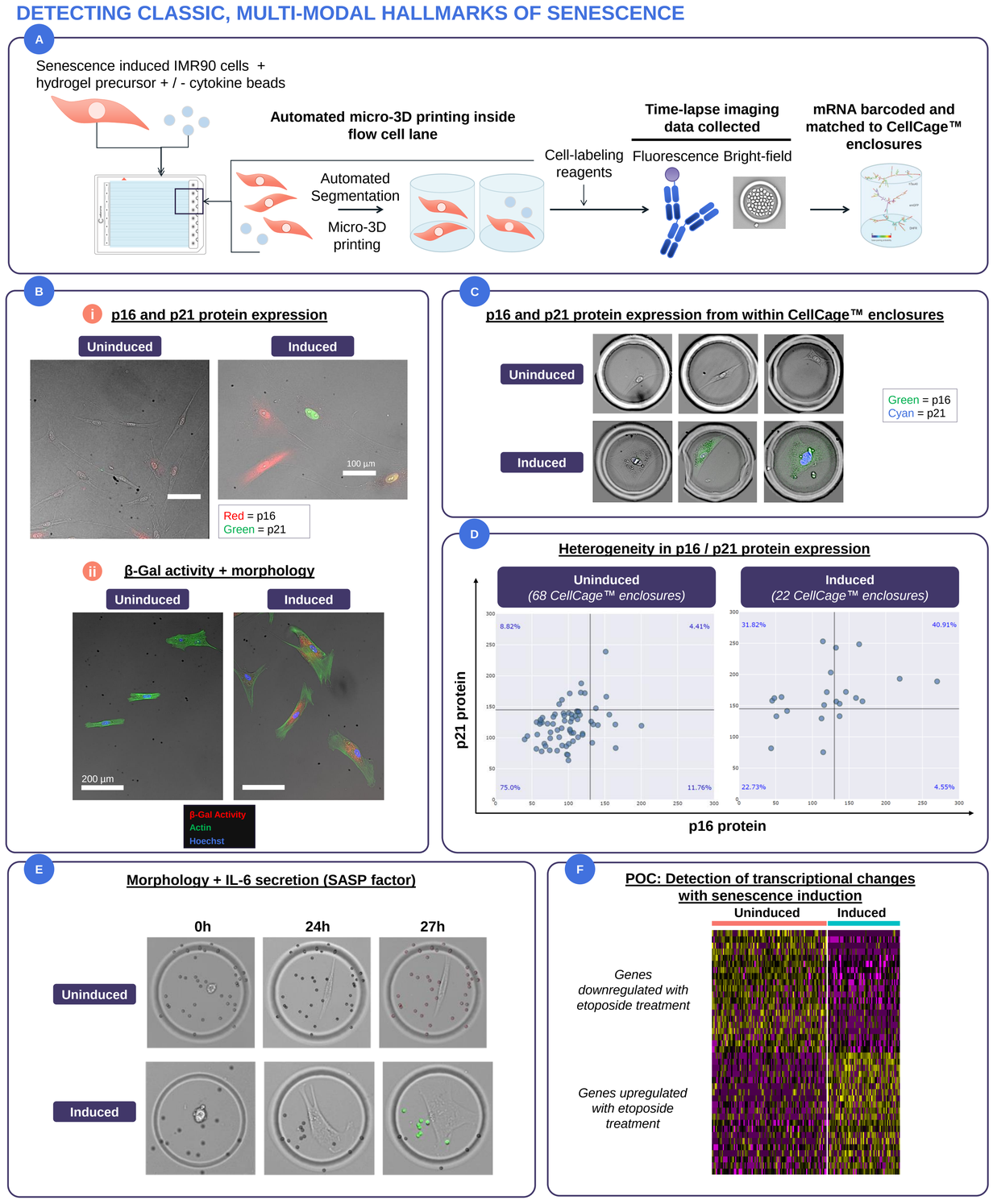


Fig 5. Cellanome's Technology is compatible with etoposide-induced models of senescence. **(A)** Schematic of workflow for evaluating etoposide induced and uninduced IMR90 cells. **(B)** Validating *in vitro* models of senescence are compatible with Cellanome's Technology by evaluating classic hallmarks of senescence. Upon adherence to flow cell surface, fixation / permeabilization and labeling reagents (e.g., fluorescent antibodies) were diffused into flow cell lanes. **i)** Representative images show p16 and p21 protein expression specifically in induced cells. **ii)** Representative images show β-Galactosidase staining (red) and larger morphological size, specifically in induced cells. **(C)** Validating encapsulation within Cellanome's CellCage™ enclosures is compatible with senescence. Detection reagents were diffused as described above, after encapsulation of induced and uninduced cells. Representative images of cells in single CellCage™ enclosures which exhibit larger morphological size and p16 / p21 protein expression in induced cells. **(D)** Preliminary single-cell evaluation of p16 / p21 protein expression of induced and uninduced IMR90 cells reflect expected heterogeneity in intensity and cellular distribution of protein expression. **(E)** Larger morphology and secretion of SASP factor (IL-6) detected from the same induced IMR90 cells co-encapsulated with cytokine detection beads. **(F)** POC that imaging data detecting senescence markers can be further linked to scRNA sequencing data. Each column represents an individual CellCage™ enclosure containing a single IMR90 cell, with genes clustered by upregulation or downregulation in uninduced vs. induced cells.

DISCUSSION AND FUTURE DIRECTIONS

We demonstrate that encapsulation within Cellanome's CellCage™ enclosures is compatible with *in vitro* models of senescence and detecting multi-modal senescence markers from the same individual cells. This includes imaging-based detection of larger morphology with p16 / p21 protein expression, β-Gal activity, and / or secretion of SASP factors, any of which can be linked to single-cell transcriptomics. Our next steps are to 1) continue converging more senescence measurements from the same cells with single experiments, 2) validate this method in more senescence models, and 3) apply multi-modal detection of senescence to experiments co-culturing both senescent and healthy cells. We believe this method will increase confidence in senescence studies, accelerate atlas initiatives seeking to identify tissue-specific senescence markers, and unlock access to novel questions, including kinetics of senescence acquisition and whether senescent cells can transmit this phenotype to healthy cells.

Interested in collaborating? Please reach out!