Novel system for culturing and analyzing single neurospheres for high-scale study of physiological-relevant neuronal functions and transcriptomes

Nicholas Elder[2], Olaia F. Vila[1], Sina Farahvashi[1], Mostafa Ronaghi[1], Gary Schroth[1], Faranak Fattahi[2]

[1]Cellanome, Foster City, CA; [2]Univ. of California San Francisco, San Francisco, CA.

ABSTRACT

We present an innovative technology that encapsulates and maintains human neurospheres for extended periods to investigate the dynamics of axon growth, network formation, synapse development, and neurodegeneration. This system enables precise manipulation, imaging, and profiling of individual neurospheres, thus opening new research avenues to study neuron-target interactions, axogenesis, neurodegeneration, and neuronal physiology at an unprecedented resolution.

Traditional molecular and functional assays often involve disruptive processes such as cell dissociation, droplet encapsulation, or flow-based microfluidics, which perturb neurons and alter their physiological state, reducing the reliability of biological findings. Furthermore, these methods do not permit the study of live neurons within functional networks, limiting the potential for multi-modal assays that correlate dynamic physiological properties with molecular profiles.

Our platform addresses these challenges by enabling the comprehensive study of individual neurospheres, either in isolation or in networks. To achieve this goal, we generated neurospheres from 17-day old V2a interneurons derived from human pluripotent stem cells[1,2]. Groups of 100 or 200 neurons were aggregated in AggreWells for 2 days before being transferred to our flow cells. Our novel technology then captures individual neurospheres in, customizable, biocompatible compartments enabling longitudinal monitoring over days to weeks. Throughout this period, multi-modal analysis can be conducted on the same neurosphere by integrating morphology, function, and transcriptomic data through live imaging and barcoded library generation for RNA-sequencing.

By preserving the integrity and function of neurospheres, this technology enables large scale and in-depth assessment of neuronal functions such as synapse formation and axon growth in response to various perturbations. Our goal is to leverage this system to perform high throughput multimodal screens to advance our understanding of neural degeneration and repair.

A NOVEL MICRO-3D PRINTING TECHNOLOGY ENABLES HIGH-THROUGHPUT AND MULTIMODAL EVALUATION OF NEUROSPHERES

GENERATE NEUROSPHERES

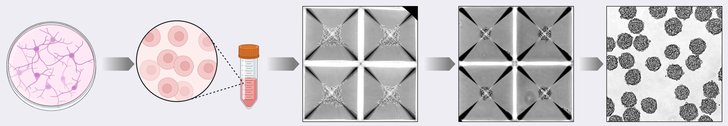

Figure 1A. Generation of Neurospheres. *V2a* neurons are dissociated to single cells and seeded into AggreWells at a density of 100-200 cells per well. After 48 hours, neurons have formed compact neurospheres.

BUILD CELLCAGE™ ENCLOSURES

Figure 1B. Creation of CellCage™ enclosures. After passing through a 100 um filter, hundreds of neurospheres are mixed with a hydrogel precursor and loaded into a flow cell. CellCage™ enclosures are formed around neurospheres by light-guided polymerization.

PHENOTYPIC ASSAYS

MORPHOLOGY & CELL GROWTH

CALCIUM IMAGING

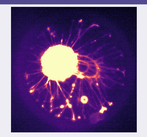

Figure 1C. Long term maintenance and live phenotypic assays. CellCage™ enclosures are permeable to reagents enabling long-term culturing and a variety of imaging-based, longitudinal phenotypic and functional assays to be performed on the same cells (e.g., small molecules, immunofluorescent antibodies)

MOLECULAR ASSAYS

CELLCAGE™ ENCLOSURE RNA SEQUENCING

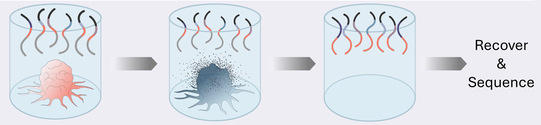

Figure 1D. Positionally matched molecular assays. Cells can be lysed within CellCage™ enclosures to generate positionally barcoded cDNA for downstream library preparation and sequencing off the Cellanome instrument.

MATCHED PHENOTYPIC AND TRANSCRIPTOMIC DATA

CUSTOM ENCAPSULATION OF NEUROSPHERES IN CELLCAGE™ ENCLOSURES

A. AUTOMATED CELLCAGE™ ENCLOSURE FORMATION

B. TUNABLE CELLCAGE™ ENCLOSURE SIZE

C. TRACK NEURITE GROWTH OVER TIME

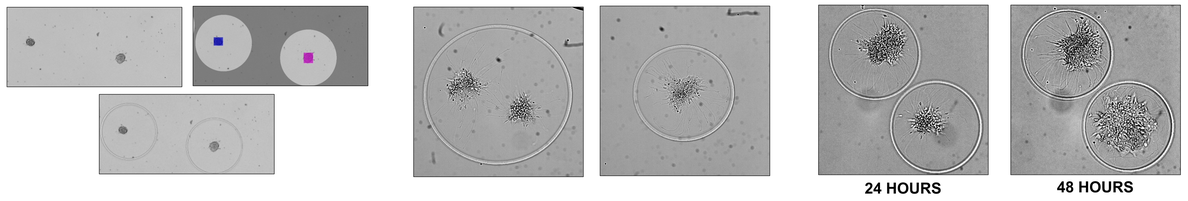

Figure 2. Plating and culture of neurospheres for imaging analysis within CellCage™ enclosures (A) Neurospheres are mixed with a hydrogel precursor and loaded in the flow cell lane. Neurospheres are automatically detected via Cellanome's segmentation algorithm and CellCage™ enclosures are generated around them. Neurospheres are then cultured in the CellCage™ enclosures off instrument for days to weeks. **(B)** The size of the CellCage™ enclosures can be defined as necessary to accommodate room for neurite growth or to include several neurospheres. Shown respectively, are 375 and 250 um diameter CellCage™ enclosures. **(C)** Individual neurospheres are imaged over time to assess morphological changes and cell proliferation.

CALCIUM IMAGING OF NEUROSPHERES IN CELLCAGE™ ENCLOSURES

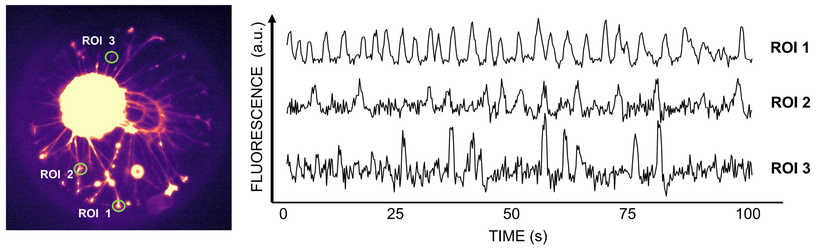

Figure 3. Assessment of spontaneous neurosphere activity with calcium imaging on Cellanome's Instrument. (A) Neurospheres were incubated with Fluo-4AM dye for 1 hour at 37'C. The dye was then washed out and replaced with fresh media. Images were acquired at 2 Hz. **(B)** Representative traces of calcium imaging in three distinct regions of interest (ROI).

CUSTOM CELLCAGE™ ENCLOSURES FOR DIRECTING NEURITE OUTGROWTH

SOLID CELLCAGE™ ENCLOSURE

WINDOWED CELLCAGE™ ENCLOSURE

MAZE CELLCAGE™ ENCLOSURE

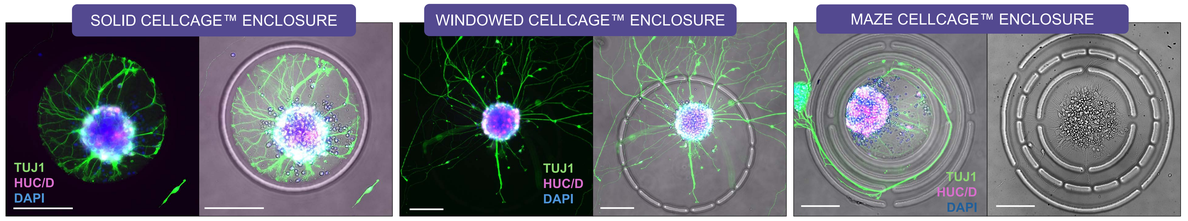

Figure 4. CellCage™ enclosures can be configured to study different neurite outgrowth behaviors. CellCage™ enclosures can be modified to have openings of configurable sizes and / or layered in different patterns to restrict or permit neurite outgrowth. This can allow spatially restricted neurospheres to contact one another or to test neurite pathfinding in response to external cues, such as added morphogens. Scale bar is 130 um.

TRANSCIPTOME ANALYSIS OF INDIVIDUAL NEUROSPHERES

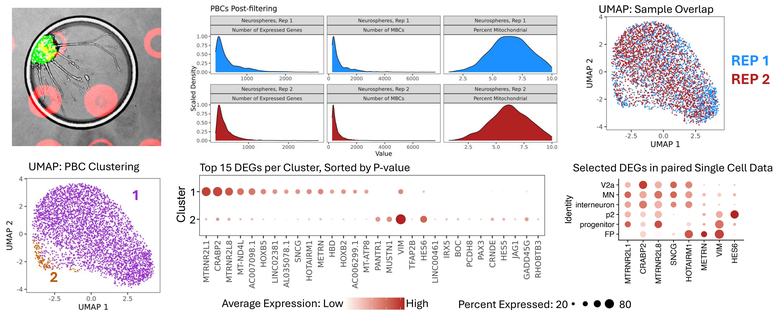

Fig 5. mRNAseq of single V2a neurospheres. CellCage™ enclosures were formed around single neurospheres located under a barcoded surface. Neurospheres were then lysed, and their barcoded mRNA was sequenced off Cellanome's instrument. Replicate libraries overlapped well with one another and positional barcodes could be clustered to identify differentially expressed genes.

DISCUSSION

Here were report a novel workflow for studying neurospheres in isolation. Encapsulation of single neurospheres enables the collection of definitively mapped data from multiple modalities across hundreds of individual neurospheres. We demonstrate this technology's utility for studying neurite formation and activity and envision advancing these methods further for studying a variety of neuron functions, including neurite outgrowth, degeneration, and / or interaction between neurospheres made of different neural populations.

The ability to link transcriptional profiles to dynamic neural functions can empower the discovery of novel genes regulating healthy neural activity from physiologically relevant aggregates. We believe these novel multi-modal datasets will advance studies aiming to decipher the underlying mechanisms of neurodegenerative disease.

REFERENCES

1. Butts, J. C. *et al.* Differentiation of V2a interneurons from human pluripotent stem cells. *Proc Natl Acad Sci USA* **114**, 4969–4974 (2017).
2. Butts, J. C. *et al.* V2a interneuron differentiation from mouse and human pluripotent stem cells. *Nat. Protoc.* **14**, 3033–3058 (2019).