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# A simplified method for long-term maintenance of human induced pluripotent stem-cell derived neural networks

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***Abstract*—In-vitro models of neuronal networks have become a powerful tool for modeling network activity in the human brain. The exploration of network properties has largely been made possible via microelectrode arrays (MEAs). However, addressing certain tissue engineering challenges remains imperative for their long-term utilization. Maintaining human neural assemblies on glass MEAs is difficult as cells often clump, peel, and spontaneously detach from the surface. Such difficulties in long-term human neuronal network culture has prompted utilization of rat astrocytes, conditioned media, and genetically modified neurons. These methods may not accurately capture the micro-environment of the brain and require significant expertise, making them less universally applicable across diverse studies. Our method produced four successful human induced pluripotent stem cell-derived neuron-astrocyte co-culture with >90 days viability without xeno sources or genetic modification. The presence of mature neurons was confirmed with live staining. Recordings taken from one culture exhibited neuronal spiking activity at an amplitude of ∼200 $\mu$V and evidence of network-wide activity. Synchronized bursting was also observed at a rate of 0.34 - 1.08 Hz in recording data. This demonstrates the feasibility of an easily implementable solution for long-term culturing of active neural networks.**

***Index Terms*—stem cells, microelectrode arrays, neural networks**

## I. Introduction

Stem-cell based therapies are a promising restorative approach to major neurological injuries such as stroke. However, prior clinical trials which involved injection of human allogenic neural stem-cells directly into the brain injury site failed to demonstrate significant improvement in neurological function [1] [2]. This failure is hypothesized to be due to a lack of functional integration of the neural stem-cells into the local environment. As such, engineering techniques that can recreate the structure of the lost neural tissue and reprise the behavior previously encoded by the brain areas being replaced are likely necessary to achieve effective recovery with stem cell-based therapies. To this end, robust in-vitro human stem cell-derived neural tissue models that exhibit prolonged viability and neuronal activity are needed to facilitate comprehensive study of tissue engineering strategies and network programming principles. Unfortunately, such models are not readily available due to the difficulty in maintaining stem-cell derived neuronal networks for extended periods of time. This in turn precludes studies in network activity as human neurons have not yet reached full maturity.

Neural networks are often cultured on glass microelectrode arrays (MEAs) to enable investigations into network activity. However, maintaining human stem cell-derived neural networks on these arrays for extended time periods (>90 days) is challenging. In particular, since neurons are negatively charged, they adhere poorly to the glass surface of MEAs (which are also negatively charged), and tend to detach after short periods of time. Modifications such as plasma treatment followed by poly-D-lysine (PDL) or polyethylenimine (PEI) coatings and repeated additions of extracellular matrix (ECM), e.g., laminin, are commonly used to address this issue. However, even with such modifications, detachment may still occur due to degradation of the PDL/PEI coating. The use of overlying microfluidic devices with microtunnels and decellularized primary human brain matrix to tether cells to the glass substrate has been reported as alternative approaches to studying neuronal cultures long-term [3] [4]. However, these methods may lack future scalability and may not be readily accessible.

To address the problem of long term viability in neural networks, we propose a simplified method for culturing human induced pluripotent stem-cell (hIPSC) derived neuron-astrocyte co-cultures on glass MEAs. This method does not require genetic modification or animal cell harvesting to establish spiking neural networks. Instead, by co-culturing neurons with hIPSC-derived astrocytes that produce endogenous ECM, we demonstrate that long-term neural network survival is possible on glass MEAs with more readily available techniques.

## II. Methods

### A. Overview

hIPSCs were differentiated into neural progenitor cells and astrocytes. These cell populations were cultured on four glass

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substrate MEAs. The glass MEAs were coated using poly-D-lysine (PDL) with regular vitronectin supplementation.

NPCs and astrocytes were placed on the four modified MEAs and were maintained and differentiated into mature neural networks. Live cell staining was performed to confirm the presence of mature neurons. Frequent eletrophysiological recordings were performed and analyzed to monitor evolution of neuronal activity.

### *B. Glass surface coating with PDL/Vitronectin*

60-channel glass MEAs (60MEA200/30iR-ITO-gr, Multichannel Systems, Reutlingen, Germany) with titanium nitride electrodes insulated with silicon nitride underwent $O_2$ plasma treatment for 1.5 minutes at 100 Watts and 0.2 mbar pressure. Subsequently, PDL solution (100 $\mu$g/mL, Sigma, Burlington, MA) was applied to the glass surface in a sterile fashion. MEAs were placed in an airtight container overnight. The PDL solution was aspirated and the MEA surface was gently washed with sterile 18 MΩ deionized (DI) water and allowed to completely air dry. A layer of human recombinant vitronectin (Gibco, Carlsbad, CA) was applied at a concentration of 0.5 $\mu$g/cm$^2$ for 1 hour before cells were deposited.

### *C. hIPSC and Neural Network Culturing and Maintenance*

hIPSCs (BJ1C33 line, UCI Stem Cell Core) were differentiated into NPCs using the dual SMAD inhibition technique mediated by a commercial neural induction medium (StemCell Technologies, Vancouver, Canada) [5]. IPSC-derived astrocytes were generated following the protocol described in [6], which involves sequential exposure to LIF, FGF2 and EGF, and finally CNTF.

Both cell populations above were maintained on vitronectin-coated microplates using commercial medium (Stem Cell Technology, Vancouver, Canada) until ready to transfer to MEAs. All cultures were maintained in a humidified incubator at 5% $CO_2$ and 37°C. More specifically, NPCs were maintained using Neural Progenitor Medium (Stem Cell Technology, Vancouver, Canada), while astrocytes were maintained using commercial BrainPhys medium supplemented with SM1 (B27 equivalent), N2, and ascorbic acid (200 nM) (Stem Cell Technology, Vancouver, Canada).

hIPSC-derived NPCs and astrocytes were mixed in a 1:1 ratio and plated at a density of $5.5 \times 10^4$ cells/cm$^2$ onto the modified MEAs. The resulting co-cultures were maintained and matured into neural networks using the commercial BrainPhys medium described above, further supplemented with human recombinant brain-derived neurotrophic factor (BDNF, 20 ng/mL) (Stem Cell Technologies, Vancouver, Canada). Co-cultures underwent ∼50% media changes every 2-3 days. NPCs were expected to fully differentiate into neurons by 21 days days in-vitro (DIV).

### *D. Imaging*

NeuO neuron live stain (Stem Cell Technologies, Vancouver, Canada) was added to co-culture conditions at a final concentration of 0.2 $\mu$M [7]. Images were taken with a fluorescence microscope (EVOS FLoid, ThermoFisher Scientific, Waltham, MA) after at least 21 DIV. The resulting images were inspected to determine the presence of mature neurons.

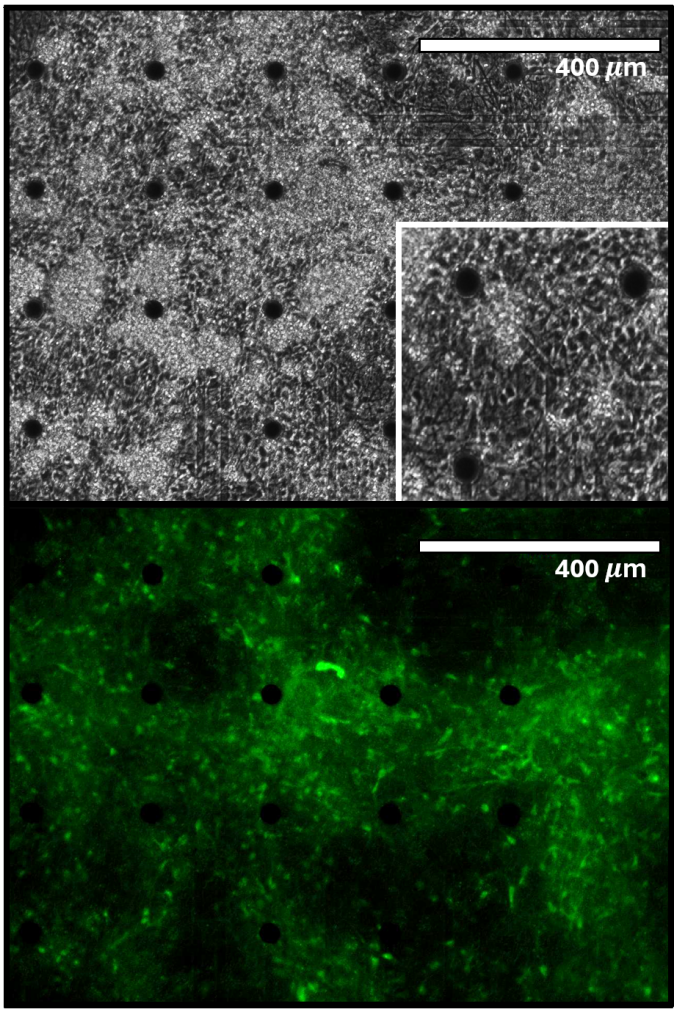


Fig. 1. *Top:* Representative phase contrast image of cultured neural network on glass microelectrode array taken at DIV 80 (10x magnification). *Inset:* Further magnified to display cell morphology. *Bottom:* Fluorescence image of same field of view with NeuO live stain labeling mature neurons. Unstained regions are likely populated with astrocytes.

### *E. Recording and Analysis of MEA Activity*

A custom-built circuit board was designed and fabricated to interface with the MEAs via spring-loaded pins. Amplifier array integrated circuits (Intan RHS2116, Intan Technologies, Santa Monica, CA) included on the custom circuit board to enable up to 64 channels of recording. A controller (Intan RHS Controller, Intan Technologies, Santa Monica, CA) was connected to the amplifier arrays and used to acquire neural signals from the MEA at 30 kHz/channel and 16-bit resolution. Recordings were performed for up to 60-s long intervals at each session within a 37°C incubator.

Data was analyzed in MATLAB as follows. For all recordings, channels with impedance above 3.5 MΩ were discarded. The neural activity time series was subjected to a 60 Hz notch filter and a 200 Hz high pass filter. The filtered signal

was common-average referenced. A spike detection algorithm [8] was applied to the resulting signal to identify all action potential events. The presence of spiking activity was used to determine the presence of neural activity within the co-culture. To assess the evolution of neural activity, the average spiking rate (spikes/s) was calculated across all channels and compared across recordings to determine the evolution over time. Neural networks have a tendency to exhibit "bursting" activity as they mature. Burst activity was determined by convolving the aggregate number of spikes across all channels with a sliding boxcar kernel. The resulting time-series was subjected to time-discrete fast Fourier transform to obtain the power spectrum. The center frequency of the resulting power spectrum was used to estimate the bursting rate.

## III. Results

Four MEAs were plasma treated followed by PDL and vitronectin surface coating. NPCs and astrocytes were successfully differentiated from IPSCs and deposited onto the MEAs. These co-cultures were maintained and matured until failure. Imaging with NeuO live stain was performed and showed the presence of mature neurons, and a representative image is shown in Fig. 1. All co-cultures survived past 90 DIV. By 120 DIV, a single co-culture remained. The survival curve is shown in Fig. 2. Two co-cultures failed due to detachment from the glass substrate, and one failed due to fungal infection.

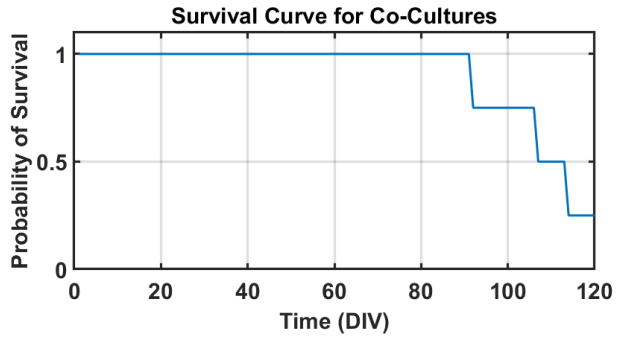


Fig. 2. Survival curve of four co-cultures over time. Two co-cultures were lost due to detachment, and one due to fungal infection.

One co-culture displayed robust spiking and bursting activity for which the results are reported here. For this co-culture, 4 recordings were performed from DIV 71 - 105. Using the data analysis approach described in Methods, the number of active channels and their average firing rate was determined across all recordings and summarized in Table I. A representative raster plot and the calculated aggregate firing rate at 71 DIV from the active co-culture is shown in Fig. 3. Using the aggregate firing rate, the fundamental frequency (see Fig. 4 for representative power spectrum) of bursting activity was calculated across all sessions and summarized in Fig. 5. We observed that as the co-culture matured, the fundamental frequency changed from ∼0.34Hz to ∼1.08 Hz over a period of 34 days.

## IV. Discussion

This study demonstrates the feasibility of a simplified method to co-culture hIPSC-derived neurons and astrocytes for a time duration adequately long enough to observe robust

TABLE I
AVERAGE NUMBER OF ACTIVE CHANNELS AND THEIR FIRING RATES FOR A SINGLE CO-CULTURE OVER TIME.

| Time (DIV) | # of Active Channels (%) | Firing Rate (spikes/s) |
|---|---|---|
| 71 | 6 (11.76) | 14.91 |
| 85 | 14 (27.45) | 13.40 |
| 100 | 15.83 (31.04) | 12.55 |
| 105 | 14.2 (27.84) | 12.20 |

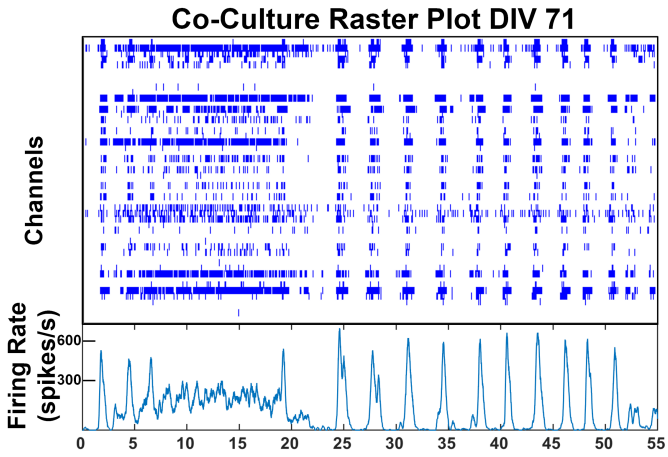


Fig. 3. *Top:* Representative raster plot of a single co-culture. *Bottom:* Corresponding aggregate firing rate exhibiting a bursting pattern.

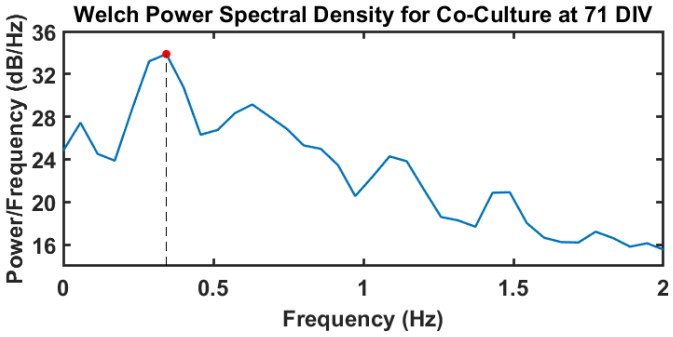


Fig. 4. Representative power spectrum calculated from aggregate firing rate plot seen in Fig.3. Fundamental bursting frequency (∼0.34 Hz) is labeled.

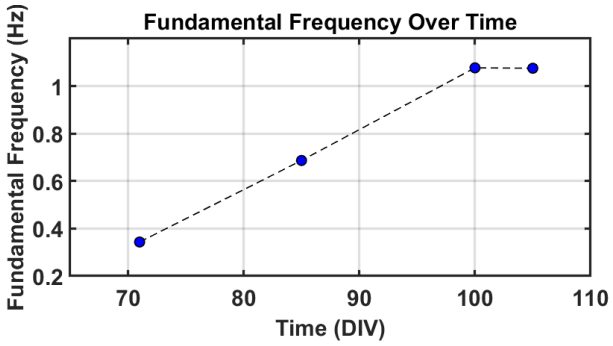


Fig. 5. Bursting fundamental frequency seen in a single co-culture over time.

spiking and network bursting activity. Although this approach requires further refinement to improve on the robustness of its outcomes, these findings indicate that it is possible to engineer long lasting hIPSC-derived neural networks with minimal complexity. Achieving long-term viability and activity in hIPSC derived neural networks is critical to developing in-vitro models of network activity in the human brain. In particular, our hIPSC-derived neural network exhibited a bursting rate of up to 1.08 Hz (Fig. 5), which is comparable to the low delta band oscillations (0.3-1 Hz) observed in premature infants' EEG [9]. These models can be used to engineer techniques to encode complex behavioral information for the purposes of bio-computing or restoring lost neurological function.

To circumvent the short lifespan of neural networks on MEAs, many resort to lentiviral transfected stem-cells to force accelerated neuronal differentiation [10]. This introduces significant genetic modifications to neurons, increases protocol burden, and has the potential to influence network learning properties and alter cell fate in immature NPCs [11]. Other proposed methods utilizing engineered micro-tunnels or primary brain samples require significant expertise or are difficult to obtain [3], [4]. Our method does not require the use of genetic modification or non-human components, increasing convenience and potential for clinical translation. Furthermore, the inclusion of hIPSC-derived astrocytes likely facilitates longer neural network viability as astrocytes are known to produce extracellular matrix and trophic factors for neurons [12]. Although a time investment is required to generate lines of hIPSC-derived astrocytes, these cells can be placed in cryostorage and readily thawed and expanded as needed. Newer culturing methods like cerebral organoids generate mature assemblies of human neurons and astrocytes but suffer from limited contact area for recording, making studies in network activity difficult [13].

Despite the long survival and imaging evidence of the presence of mature neurons, only 1 out of the 4 co-cultures in this study were found to have robust spiking and bursting activity. Here, it is possible that the overgrowth of astrocytes likely contributed to occluding the MEA electrodes from proper contact with neurons and their processes [14], [15]. This issue can be addressed by optimizing the initial ratio of astrocytes to NPCs, avoidance of factors such as GDNF, or applying arresting agents to prevent glial overproliferation [15]. The co-cultures in this study also ultimately experienced detachment and peeling from the glass substrate indicating that frequently used surface coatings such as PDL or PEI may degrade over time. This leads to a loss of the positively charged coating on the glass substrate, and in turn exposes the neural network to electrostatic repulsive forces between the negatively charged glass and neurons. This can potentially be addressed by permanent surface modification to increase the positive charge of the glass surface, e.g., with amino-silanes. Future work to improve neural network viability will likely need to address electrostatic forces between neurons and the substrate surface. Achieving long-term survival of human neuronal networks on MEAs enables the development of training paradigms to encode specific behavioral information. The consequences of such encoding are far-reaching and include applications in bio-computing and training human neural networks to recapitulate functional behaviors, thereby paving the path for restorative therapies to neurological injury.

## REFERENCES


[1] D. Kalladka, J. Sinden, K. Pollock, C. Haig, J. McLean, W. Smith, A. McConnachie, C. Santosh, P. M. Bath, L. Dunn, *et al.*, "Human neural stem cells in patients with chronic ischaemic stroke (pisces): a phase 1, first-in-man study," *The Lancet*, vol. 388, no. 10046, pp. 787–796, 2016.

[2] K. W. Muir, D. Bulters, M. Willmot, N. Sprigg, A. Dixit, N. Ward, P. Tyrrell, A. Majid, L. Dunn, P. Bath, *et al.*, "Intracerebral implantation of human neural stem cells and motor recovery after stroke: multicentre prospective single-arm study (pisces-2)," *Journal of Neurology, Neurosurgery & Psychiatry*, 2020.

[3] C. Tamatani, K. Shimba, K. Kotani, and Y. Jimbo, "Activity-and spatial-dependent variations in axonal conduction recorded from microtunnel electrodes," in *2023 15th Biomedical Engineering International Conference (BMEiCON)*, pp. 1–3, IEEE, 2023.

[4] D. Lam, H. A. Enright, J. Cadena, S. K. Peters, A. P. Sales, J. J. Osburn, D. A. Soscia, K. S. Kulp, E. K. Wheeler, and N. O. Fischer, "Tissue-specific extracellular matrix accelerates the formation of neural networks and communities in a neuron-glia co-culture on a multi-electrode array," *Scientific reports*, vol. 9, no. 1, p. 4159, 2019.

[5] S. M. Chambers, C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain, and L. Studer, "Highly efficient neural conversion of human es and ips cells by dual inhibition of smad signaling," *Nature biotechnology*, vol. 27, no. 3, pp. 275–280, 2009.

[6] A. Serio, B. Bilican, S. J. Barmada, D. M. Ando, C. Zhao, R. Siller, K. Burr, G. Haghi, D. Story, A. L. Nishimura, *et al.*, "Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of tdp-43 proteinopathy," *Proceedings of the National Academy of Sciences*, vol. 110, no. 12, pp. 4697–4702, 2013.

[7] J. C. Er, C. Leong, C. L. Teoh, Q. Yuan, P. Merchant, M. Dunn, D. Sulzer, D. Sames, A. Bhinge, D. Kim, *et al.*, "Neuo: a fluorescent chemical probe for live neuron labeling," *Angewandte Chemie International Edition*, vol. 54, no. 8, pp. 2442–2446, 2015.

[8] Z. Nenadic and J. W. Burdick, "Spike detection using the continuous wavelet transform," *IEEE transactions on Biomedical Engineering*, vol. 52, no. 1, pp. 74–87, 2004.

[9] M. André, M.-D. Lamblin, A.-M. d'Allest, L. Curzi-Dascalova, F. Moussalli-Salefranque, S. N. T. Tich, M.-F. Vecchierini-Blineau, F. Wallois, E. Walls-Esquivel, and P. Plouin, "Electroencephalography in premature and full-term infants. developmental features and glossary," *Neurophysiologie clinique/Clinical neurophysiology*, vol. 40, no. 2, pp. 59–124, 2010.

[10] Y. Zhang, C. Pak, Y. Han, H. Ahlenius, Z. Zhang, S. Chanda, S. Marro, C. Patzke, C. Acuna, J. Covy, *et al.*, "Rapid single-step induction of functional neurons from human pluripotent stem cells," *Neuron*, vol. 78, no. 5, pp. 785–798, 2013.

[11] D. Cao, H.-H. Cheung, and W.-Y. Chan, "Doxycycline masks the genuine effect of the doxycycline-inducible transgene by promoting dopaminergic neuron differentiation from human pluripotent stem cells," *Stem Cells and Development*, vol. 28, no. 13, pp. 833–845, 2019.

[12] S. Wiese, M. Karus, and A. Faissner, "Astrocytes as a source for extracellular matrix molecules and cytokines," *Frontiers in pharmacology*, vol. 3, p. 120, 2012.

[13] Q. Huang, B. Tang, J. C. Romero, Y. Yang, S. K. Elsayed, G. Pahapale, T.-J. Lee, I. E. Morales Pantoja, F. Han, C. Berlinicke, *et al.*, "Shell microelectrode arrays (meas) for brain organoids," *Science advances*, vol. 8, no. 33, p. eabq5031, 2022.

[14] Y. Nam and B. C. Wheeler, "In vitro microelectrode array technology and neural recordings," *Critical Reviews™ in Biomedical Engineering*, vol. 39, no. 1, 2011.

[15] A. Blau, "Cell adhesion promotion strategies for signal transduction enhancement in microelectrode array in vitro electrophysiology: An introductory overview and critical discussion," *Current opinion in colloid & interface science*, vol. 18, no. 5, pp. 481–492, 2013.