Enhancing the Development of hPSC-neurons by Engineering the Extracellular Matrix: an MEA and Microscopy Study

Summary

In a recent study[1], Smith and colleagues used nanotopographically-patterned transparent multiwell microelectrode arrays (MEAs) to study neuronal structure, network connectivity, and sensitivity to synaptic blockers in cultured hPSC-neuron monolayers. The authors found that the nanotopographical cues in the culture environment – which were designed to mimic the *in vivo* extracellular matrix — served to direct the structural organization of axonal growth cones and increase neuronal activity when compared to traditional flat culture surfaces. Further, the authors demonstrated that calculated IC_{50} values for a known synaptic inhibitor measured using nanopatterned cells are closer to known *in vivo* values. Smith *et al.*'s data demonstrate the potential for nanotopographically-patterned substrates to enhance the development of neuronal cultures and potentially improve pre-clinical predictions of CNS drug effects.

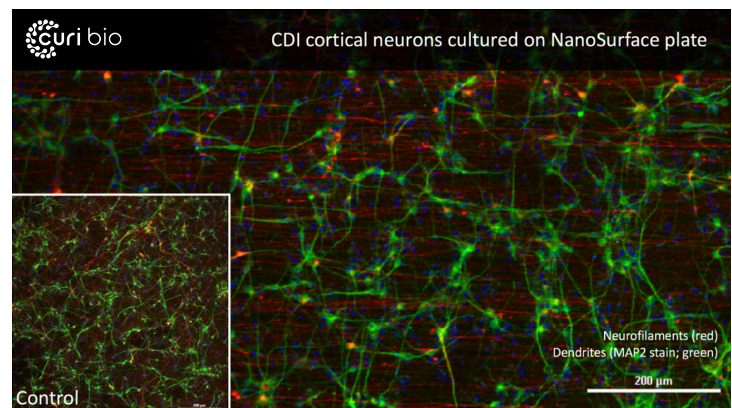

Figure 1: On NanoSurface Plates, *neurofilaments (red) align along the direction of the nanotopography. On conventional cultureware, orientation is random.*

Introduction

Pre-clinical toxicity assays hold great promise to reduce cost and harm while speeding potentially life-saving drugs to market. Typically, these assays are reliant on results from *in vitro* cell based assays, with the hypothesis that they can sufficiently recapitulate the essential characteristics of *in vivo* systems to predict adverse effects in the human body. Despite the significant amount of effort put into developing cell-based assays, significant numbers of new chemical entities (NCEs) that pass pre-clinical screens eventually fail during clinical trials, are marked with black box warnings, or in some cases are even removed from the market after release. Consequently, there is great interest in enhancing the predicative power of pre-clinical cell-based assays.

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Due to their ability to recapitulate specific phenotypes of various cells and tissues without the need for expensive or ethically-fraught sourcing, human pluripotent stem cells (hPSCs) have potential to significantly improve preclinical screening applications. However, many hPSC cells are analyzed at early stages of development and can oftentimes lack mature structural and functional *in vivo* phenotypes required for predictive results. Years of research have produced dozens of strategies that can be implemented to enhance the maturation of hPSCs *in vitro* , and it has been demonstrated that these approaches can increase the predictive capacity of these cells.

Many of these techniques utilize increasing levels of complexity to drive the functional maturation of hPSCs. Unfortunately, this complexity is frequently at odds with productivity as they are incompatible with the highthroughput screening techniques required for the development of NCEs.

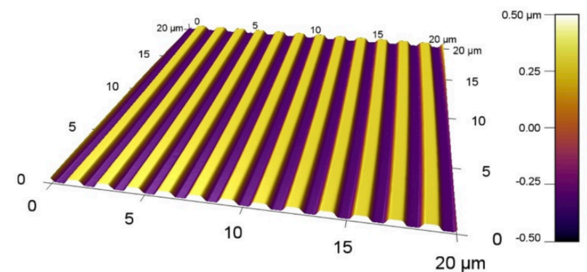

Figure 2: NanoSurface cultureware surface. *Atomic force microscopy image of the culture surface shows the highlyprecise and accurate nanoscale features that are used to mimic the native extracellular matrix.*

The extracellular matrix (ECM) plays an important role in the differentiation, development, and function of various cell types. Cells use structural and chemical cues from their immediate environment to drive a number of processes. In addition to playing a vital role in determining the 'health' of a cell or tissue, the ECM plays a critical role in directing hPSC differentiation. Various methods are employed to recapitulate *in vitro* the aspects of the ECM that are important in these processes. These methods share in the tradeoffs between complexity and throughput that are inherent in most maturation strategies. In the study described here, Smith *et al.* sought to develop a method to drive the maturation of hPSCs for pre-clinical *in vitro* screening by engineering the cellular microenvironment in a manner that was compatible with highthroughput techniques.

The team developed a method to modify the surface of a multiwell-MEA with a conductive polymer that is amenable to high-precision nanoscale patterning (Fig. 2). With this approach, the authors successfully recapitulated some of the structural aspects of the extracellular matrix and saw improvements in both structural and functional phenotypes of hPSC-derived neurons and cardiomyocytes. This study demonstrates that controlling the architecture of the extracellular matrix can directly improve the functional phenotypes of stem-cell derived neurons, which may greatly enhance the predictive capability of cell-based assays.

Findings

The authors used commercially-available hPSC-derived glutamatergic neurons and cultured them on patterned MEA surfaces for 21 days. After the culture period, the cells were analyzed with the MEA and with fluorescence microscopy. Across both techniques, they found that neurons cultured on nanopatterned surfaces exhibited more mature phenotypes.

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Structural Studies

Structurally, hPSC-neurons responded to nanoscale extracellular matrix cues by extending neuritic processes along the length of the underlying nanotopography. Fluorescent stains against neurofilament protein (NF) on patterned versus unpatterned cells (Fig. 3A & B, red channel) show that axons grow along the direction of the topography, which is indicated by the yellow arrow. Interestingly, dendrites immunostained using antibodies against microtubule-associated protein 2 (MAP-2) do not show such structural organization on either flat or nanopatterned substrates (Fig. 3A & B, green channel). These data may indicate that while axonal growth cones respond to extracellular matrix cues, dendritic trees are likely influenced by other factors. The authors hypothesized that this may mimic the layered model of the cortex, where axons run uniaxially between cortical layers and support denser synaptic connects, which were examined in more detail in this study.

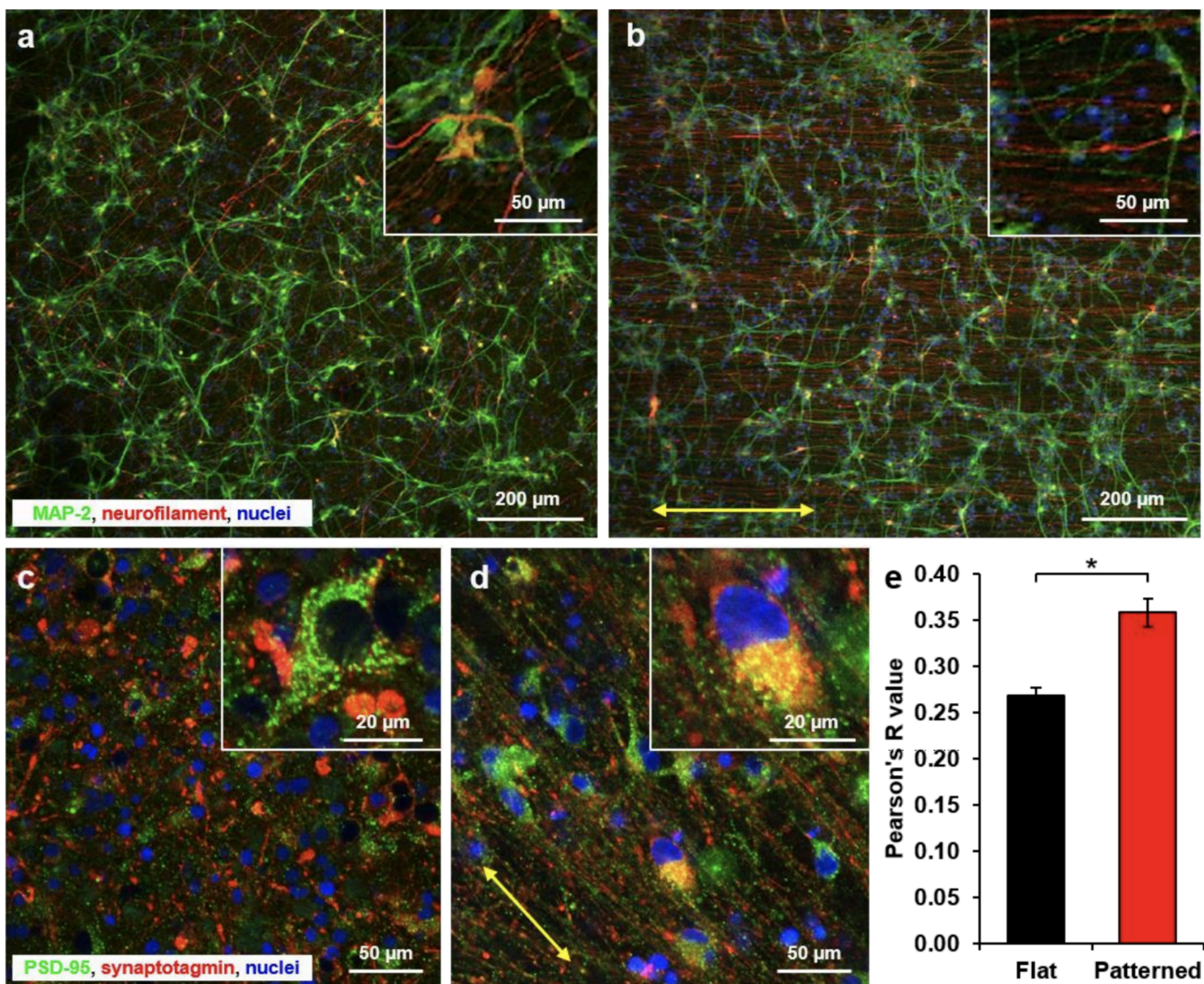

*__Figure 3: Structural impact of nanotopographically patterned Nafion substrates on hPSC neurons.__ Human PSC-Ns on flat __(A)__ versus patterned __(B)__ substrates stained for cytoskeletal proteins. Inset shows detail of neurite orientation. Human PSC-Ns on flat __(C)__ versus patterned __(D)__ substrates immunostained for synaptic markers. Inset shows detail of the staining associated with a single cell. For __(B)__ and __(D)__, the yellow arrow indicates the orientation of the underlying nanotopography. __(E)__ Pearson correlation coefficients are an indicator of the relative levels of pre- and post-synaptic marker co-localization. In presented data, *p < 0.002.*

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In addition to cytoskeletal structural proteins, the investigators stained for markers of synaptic formation. Postsynaptic density protein 95 (PSD-95) is a scaffolding protein that is typically located in the post-synaptic density of neurons, while synaptotagmin is a membrane protein that is localized to the pre-synaptic region. Increased co-localization of these proteins suggests a possible increase in synapse formation. When compared to flat cultures, hPSC-neurons cultured on nanopatterned surfaces show greater protein co-localization compared to flat cultures (Fig. 3C & D). Quantitative analysis using the mean Pearson correlation coefficient also indicates that colocalization is increased in patterned cultures (Fig. 3E). Consequently, the authors hypothesized that nanopatterned cultures enable increased synapse formation.

Function

To assess functional performance of hPSC-neurons, the authors cultured cells on flat and nanopatterned MEA surfaces (Fig. 4A & B). The MEA allowed for characterization of electrical parameters under various conditions. When assayed for network burst behavior, significant differences between flat and patterned cultures were seen. Spikes per network burst and spikes per burst per channel were increased on nanopatterned cells compared to cells on flat surfaces (Fig. 4C & D), while the number of electrodes participating in the reading was unchanged. Network burst duration (Fig. 4E) was also increased on nanopatterned cells, suggesting that the shaping of the extracellular environment increases synaptic crosstalk.

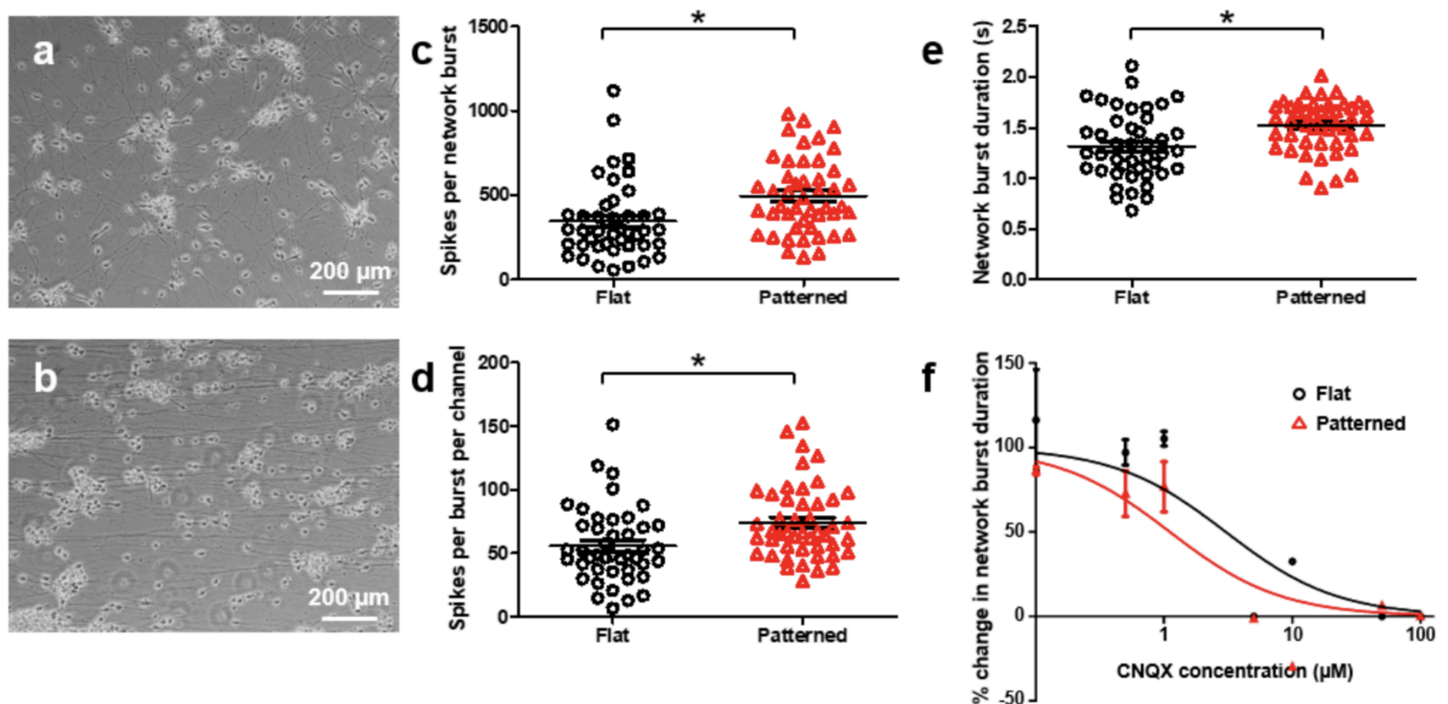

*Figure 4: **Electrophysiological responses of hPSC-neurons to culture on nanoMEAs.** Bright-field images of low-density hPSC-neurons maintained on flat **(A)** versus patterned **(B)** substrates. Number of spikes recorded per network burst from high density hPSC-neuron populations maintained on flat and nanotopographically-patterned MEAs **(C)**. MEA measurement of the mean number of spikes recorded per electrode during individual network bursts from hPSC-neuron populations under both conditions **(D)**. Measurement of network burst duration from hPSC-neuron populations maintained on flat and nanotopographically-patterned MEAs **(E)**. Normalized dose response curve **(F)** illustrating effect of increasing concentrations of the AMPA receptor blocker CNQX on duration of network bursts in unpatterned and patterned hPSCneuron populations. R2 values for the unpatterned and patterned cultures were 0.75 and 0.76, respectively. In all presented data, *p < 0.01.*

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Finally, to test the functional ramifications of the suggested increase in synapse formation in patterned hP-SCneurons, the authors treated cells with cyanquixaline (CNQX), a synapse blocker that acts on the AMPA receptor. Here, the duration of network bursts was analyzed across a range of CNQX concentrations. From these data,dose-response curves were made, and IC_{50} values were calculated. Nanopatterned cultures exhibited a significantly lower IC_{50} value and was much closer to the previously-reported *in vivo* value (Fig. 4F). These results suggest that this approach can be used to enhance the predictive utility of *in vitro* hPSC-neuronal assays without sacrificing high-throughput data collection.

Conclusions

Imparting biomimetic topographical cues to a variety of cells in culture enhances their ability to recapitulate *in vivo* structural and functional phenotypes. Nanotopography, in concert with high-throughput MEA analysis can be an important tool for screening the potential toxic effects of drugs before they pass through a long and expensive development pipeline. This approach presents a multiplexed, scalable, high-throughput platform that provides structural organization for non-invasive, long term electrophysiological and optical assessment of various cellular functional parameters.

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References

[1] A. S. Smith, E. Choi, K. Gray, J. Macadangdang, E. H. Ahn, E. C. Clark, P. Tyler, M. A. Laflamme, L. Tung, J. C. Wu, *et al.*, “Nanomea: a versatile platform for high-throughput analysis of structure-function relationships in human stem cell-derived excitable cells and tissues,” *bioRxiv*, p. 453886, 2018.

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