A NEURONAL NETWORK DISPLAY FOR NEUROXICITY SCREENING

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ABSTRACT

We present a rapid, reproducible and sensitive neurotoxicity testing platform that combines the benefits of neurite outgrowth analysis with cell patterning. This approach involves patterning neuronal cells within a hexagonal array to standardize the distance between neighbouring cellular nodes, and thereby standardize the length of the neurite interconnections. This feature coupled with defined assay coordinates provides a streamlined display for rapid and sensitive analysis. We have termed this the network formation assay (NFA).

KEYWORDS: Microarray, Neurotoxicity Screening, Neuronal Network, Dose Response Analysis

INTRODUCTION

There is an urgent need for the widespread screening and identification of chemicals that pose a health risk to the human nervous system. Standard protocols for neurotoxic risk assessment involve the use of *in vivo* rodent models. This approach is lengthy, resource intensive and requires many animals, making it impractical for screening large numbers of chemicals. As a result there is currently a strong emphasis on substituting many animal experiments with simplified *in vitro* cell culture experiments for a high-throughput screening approach to chemical assessment [1].

In vitro nervous system models must accurately mimic the critical cellular events of neurodevelopment and plasticity. Axonal and dendritic outgrowths (collectively termed neurites) are defining morphological characteristics of the differentiated neuronal phenotype that are essential for neuronal connectivity and network function. As such the outgrowths are hallmark neurodevelopmental end-point indicators and are popularly used as the subject of measurement in the neurite outgrowth assay. However, the assay is manually intensive or requires fixation and staining for use with automated high content screening platforms. Despite progress in this area, the neurotoxicology endeavour would greatly benefit from a standardized *in vitro* analysis platform that provides a simple end-point read-out for rapid and accurate high content screening. To address this challenge we have developed a neuronal microarray platform for use as a simple neurite interconnection display [2].

CONCEPT

Neurites serve as hallmark neurodevelopmental end-point indicators which can be correlated with *in vivo* observations and thereby provide predictive value for screening the neurotoxicity effects of test substances where the molecular targets and modes of action are unknown. Traditionally, neurite outgrowth assays involve the use of sparsely seeded cell cultures (for illustration see Fig. 1(A)), with the resource intensive task of identifying single neurons and measuring the lengths of their neurite outgrowths. The presented NFA combines the benefits of the neurite outgrowth assay with cell micropatterning to provide a spatially standardized analysis platform. A uniformly spaced hexagonal array of adhesion nodes is used to pattern neurons. Neurite outgrowths interconnect the cellular nodes and result in the development of a neuronal network (for illustration see Fig. 1(B)). Critically, the array system standardizes the neurite outgrowth length, thereby eliminating length measurements. The distance is also designed to satisfy standard neurite classification criteria which require a length equal to or greater than one or more cell body diameters. The use of analysis coordinates and a common outgrowth length both significantly streamline the identification and measurement effort for high throughput neurotoxicology screening.

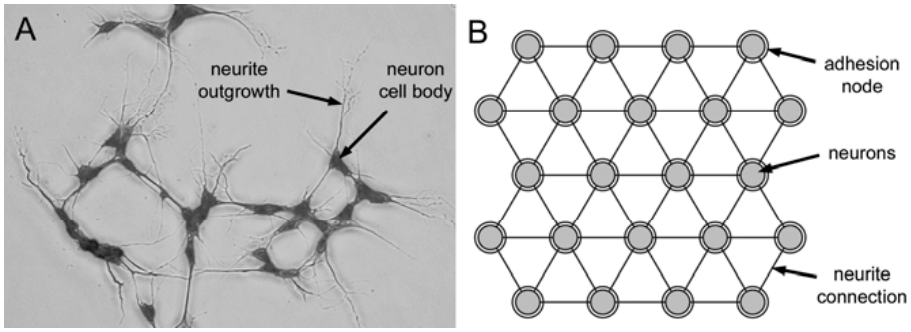

Figure 1: Differentiated SH-SY5Y neurons cultivated on standard tissue culture substrates have an irregular distribution and elaborate complex and 'chaotic' neurite outgrowths (A). Cells were fixed and stained with Giemsa to aid visualization. Illustration of the NFA end-point where hexagonally patterned neurons are interconnected by neurite outgrowths (B).

EXPERIMENTAL

The neuronal arrays were prepared by thin film poly(dimethylsiloxane) (PDMS) microcontact printing as described by Frimat and co-workers [2]. The hexagonal array contained 70-μm-diameter cell adhesion nodes, separated by a distance of 100 μm. Each chip contained 25 arrays, each with 367 adhesion nodes. Human SH-SY5Y neuroblastoma cells (DSMZ, Germany) were first differentiated using 10 μM *trans* retinoic acid (Sigma) for 3 days and then patterned for the neurotoxicity screening experiments. To demonstrate the NFA the arrayed cells were exposed to acrylamide, a reference neurotoxic compound. The SH-SY5Y neuron arrays were then cultured in 7 mL of serum-containing media along with acrylamide at final concentrations ranging from 0–5 mM. Array occupancy and neurite interconnection values were recorded (n = 3). Array occupancy was defined as the percentage of the 367 cell adhesion nodes that contain one or more cells. The NFA involved a streamlined analysis approach, measuring the number of neurites connecting immediately neighbouring neuronal nodes and not connections to more distant nodes. Difficulties discerning multiple connections between the same neuronal node neighbours were also obviated by classifying these together as a single connection. To reduce the impact of sample–sample pattern occupancy variations values are converted to neurite interconnections per occupied neuronal node. Neuronal networks were imaged using an inverted microscope (IX71, Olympus). Cytotoxicity was measured using the CellTiter-Blue® Cell Viability Assay (Promega).

RESULTS AND DISCUSSION

Randomly seeded SH-SY5Y cells become clustered on the array, with multiple cells occupying the adhesion nodes and with other adhesion nodes remaining vacant. Nevertheless, the across chip, chip-to-chip and batch-to-batch pattern occupancy was reproducibly high (>70%). To demonstrate the advantages of the neuronal array a dose response experiment using the neurotoxin acrylamide was undertaken with differentiated SH-SY5Y neurons at passage 12. Dose dependency results following 24 hours of exposure to acrylamide are compared with data from the CellTiter-Blue® Cell Viability Assay in Fig. 2. The NFA produced a sigmoidal dose response curve over two orders of magnitude and was 7.55-fold more sensitive than the cytotoxicity test, revealing specific neurotoxicity effects whereas the viability assay only captures gross cytotoxic effects. The viability assay gave a 50% inhibition concentration (IC_{50}) of 5.1 mM (95% CI 4.2–6.1). In contrast, the concentration that caused a 20% reduction in network formation (20% network inhibition, NI_{20}) relative to controls was 0.26 mM (95% CI 0.12–0.46). These values are equivalent to the 20% SH-SY5Y neurite degeneration (ND_{20}) acrylamide concentration reported by Nordin-Andersson and co-workers [3]. An additional NFA experiment involving SH-SY5Y cells at passage 10 gave a NI_{20} value of 0.28 mM (95% CI 0.17–0.38). This reproducibility and the narrow 95% confidence intervals demonstrate the robust character of the assay.

The simplicity of the NFA analytical read-out enables rapid data acquisition, requiring only 3 hours to manually undertake the dose response analysis (367 nodes, with 7 concentrations in triplicate: 7707 nodes). In contrast, the manual assessment of neurite length is a labour intensive task requiring approximately 10 hours for the analysis of just 200 cells [1]. The positioning of neuronal cells and their neurite interconnections at pre-defined coordinates is also highly desirable for high throughout analysis using automated image capture systems.

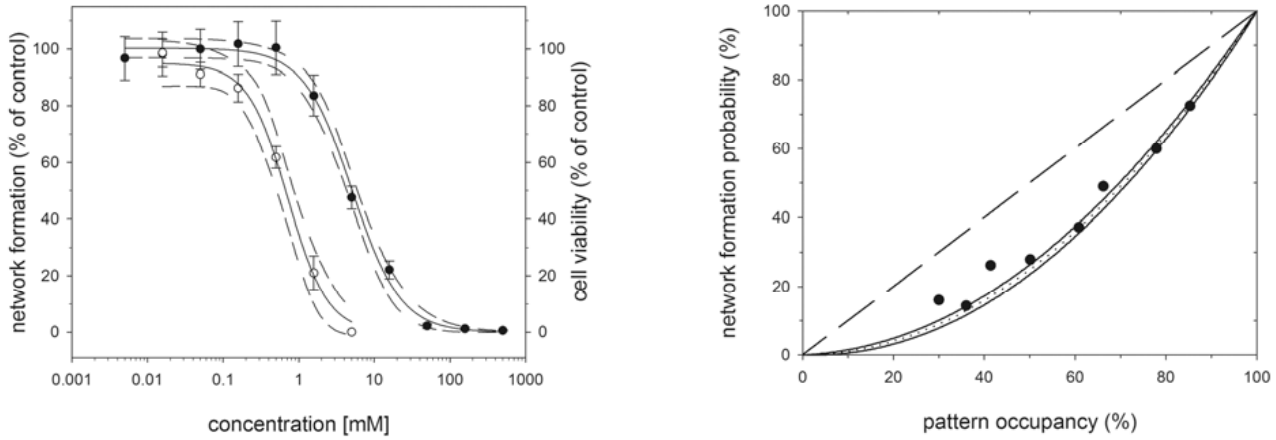

Figure 2: Dose response curves from the NFA (open circles) and the CellTiter-Blue® Cell Viability Assay (black circles). Data was obtained following 24 hours of exposure to acrylamide using cells at passage 12. Data points with standard deviation are plotted along with non-linear regression curves for the mean (continuous line) and the 95% confidence intervals (dashed lines). Figure 3: Simulation of occupancy versus network formation probability. The 95% confidence intervals (continuous lines) are plotted with the linear connection per occupied node prediction (dashed line) and experimental data points (black circles).

The presence of unoccupied adhesion nodes reduces the probability of neurite outgrowths to connect with neighbouring neuronal nodes. A simulation was developed to obtain a statistical appreciation of this phenomenon and aid data normalisation in circumstances where there is a high variation in chip-to-chip pattern occupancy. Results from a 10,000 cycle simulation are presented in Fig. 3. To validate the simulation, serial cell dilutions were patterned on the microarrays. Following a 24 hour period for neurite outgrowth the number of connections per node was measured and supported the

prediction generated by the simulation. Critically, network formation probability was especially sensitive to variations at high levels of pattern occupancy.

To further validate the NFA we have used mouse embryonic neurons (the gold standard cell type) which retain many *in vivo* morphological, biochemical and electrophysiological characteristics. These *ex vivo* cells require a poly-*L*-lysine coating and the provision of narrow (~3 µm) tracks connecting the adhesion nodes to support neurite outgrowths. Shown in Fig. 4(A), the thin film PDMS printing technique was successfully used to establish a primary neuronal network. However, issues of pattern reproducibility and stability limit the useful scope of these polymer patterns. In place of these we are currently developing patterning techniques based on the use of poly(ethylene glycol) (PEG) coatings. PEG-ylated surfaces resist protein and cell adhesion, but following UV- or plasma-based oxidative patterning support the assembly of proteins. Patterns of fluorescently labelled proteins are documented in Fig. 4(B) and the resultant neuronal networks in Fig. 4(C). Importantly, this protein patterning approach is suitable for a tremendous range of proteins, including those required for the adhesion and maintained differentiation of neural stem cells. These non-tranformed and self-renewing cells will further aid standardization of the NFA.

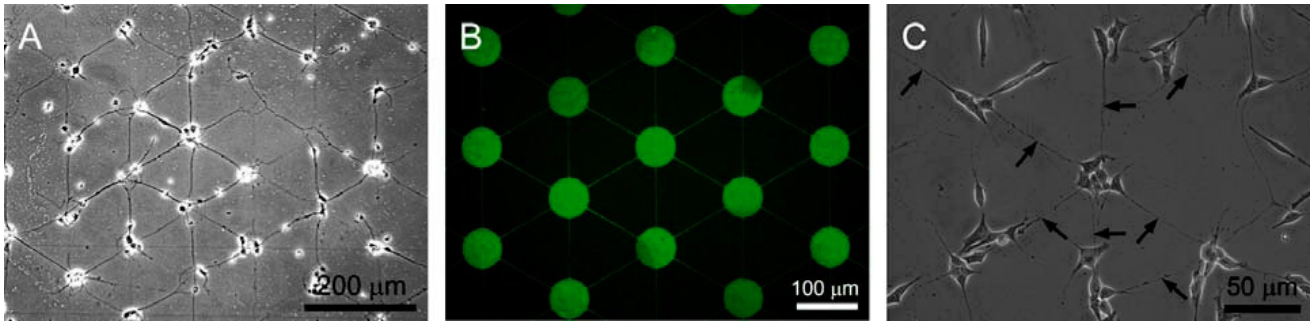

Figure 4: A mouse embryonic neuronal network developed on a PDMS micropattern (A). Plasma-based oxidative patterning of PEGylated surfaces for the directed assembly of proteins (B) and the resultant neuronal network (C).

CONCLUSION

The NFA standardizes the neurite outgrowth length and assigns assay coordinates to greatly streamline the analysis process. The simple assay enabled the reproducible, rapid and sensitive assessment of neurite outgrowth inhibition by the neurotoxic compound acrylamide. Straightforward developments are required for the widespread adoption of the assay to progress the neurotoxic hazard assessment effort as well as impact the field of neurodevelopmental biology.

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