ANALYSIS OF AXON GUIDANCE IN SINGLE NEURONS USING A LARGE ARRAY OF MICROFLUIDIC GRADIENT GENERATORS

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ABSTRACT

In this paper, we report a large-scale study of netrin-1 induced axon guidance in primary mammalian neurons using an integrated platform that combines a large array of microfluidic gradient generators. We adapt a previously reported microfluidic method of creating gradients on open-surfaces termed “microjets”, combine it with live-cell, auto-focus, time-lapse imaging to create an integrated platform. We not only show that netrin-1 acts as a bi-functional molecule, having both attractive and repulsive effects on the growth-cone; but also discover a significant dependence of the absolute concentration on the turning response dynamics of the growth cone. Our platform highlights the potential of large-scale, cell-benign, microfluidic devices in unraveling new biological insights into the dynamic behavior of cells navigating its way through a chemotropic gradient field.

KEYWORDS: Axon Guidance, Netrin, Neuron, Gradient

INTRODUCTION

Gradients of biochemical molecules have been widely implicated in axonal guidance, cell migration, stem cell differentiation, and immune response [1]. The challenges associated with investigating the complex processes of axon guidance in vivo without interference from organism-wide responses motivates the development of platforms that can present precisely controlled gradients of diffusible biochemical cues to individual cells and track their response in real time. Over the last decade, microfluidics has emerged as a very compelling technology for interrogating cells in culture with precisely controlled, deterministic, stable, steady-state or dynamic concentration gradients of biochemical cues.

We have previously described a method for generating steady-state gradients on open surfaces [3], in which it is possible to expose axons to a gradient of a diffusible biomolecule, with negligible shear imparted on the cultured cells [2]. In our earlier version of the microjets device [2], where dissociated neurons were all cultured in the same gradient chamber, we observed that even in the presence of a netrin-1 gradient, many axons seemed to grow towards adjacent cell-bodies, thereby hinting at a role played by factors secreted by neighboring cells in influencing the direction of axon growth. In order to decouple the possible effect of secreted factors from neighboring cells, it becomes imperative that we subject individually isolated neurons to an externally applied gradient of netrin-1, and track their axonal migration over time. Therefore, to achieve this, we have implemented a parallel array of single cell microfluidic gradient generators, each of them having the same exact gradient. Using live-cell, time-lapse microscopy, we track the chambers that contain a single neuron, and gather data from approximately 150-250 neurons per experiment, which is at least an order of magnitude higher than what has so far been reported.

EXPERIMENTAL

The “microjets” gradient generator array is fabricated by contact-transferring a 250 µm poly(dimethylsiloxane) (PDMS) micro-structure, “exclusion-molded” off a master template created using multi-layer standard SU-8 photolithography on silicon wafers, as described earlier [3]. Each unit of the array, henceforth called the gradient chamber, is 250 µm high, 200 µm wide and 300 µm long, and is fed on each side by a row of 12 microjets, 2 µm high and 10 µm wide (Fig 1). The gradient chambers are 1.2 mm apart, resulting in a 16x63 rectangular array. The cured PDMS membrane after being picked up from the silicon master, with a fluoro-polymer backed mylar sheet (3M Scotchpak Release Liner 9744), is plasma bonded to a glass cover-slide [3].

After the assembly, characterization and sterilization of the devices, dissociated neurons from E18 mouse hippocampus (12,000 cells/mL) are allowed to settle onto the device, the surface of which is pre-coated with polylysine (50 µg/mL, 1 hour) and laminin (5 µg/mL, 8 hours). The cells are cultured in the chambers for 24 hours (in case of axon guidance studies), or 2 hours (in case of axon specification studies). For the axon guidance studies we interrogated the neurons with a gradient of netrin (200 ng/mL on one end and media on the other), which resulted in a linear gradient across the 200 µm width of the chamber, for 6-12 hours. We used trace amounts of fluorescent-BSA to visualize the gradient. We obtained time-lapse images every 15 minutes, using a TE-2000 Nikon inverted epifluorescence microscope.

RESULTS AND DISCUSSION

The operational principle of the device is conceptually schematized in Fig 1. Very low volumes of fluid (~100pL/min), ejected from the microjets on being driven by a syringe pump at a controlled flow-rate, create a gradient in the open chamber, where a single neuron is cultured (Fig 1). Each row of microjets from all the gradient chambers is connected to a common fluidic inlet port through 40 µm width of microchannels that are routed through a binary distribution network so as to ensure equal hydrodynamic resistance from the port to each chamber. The gradient is established within 5 minutes of initiating flow through the microjets in the device. The shape and profile of the gradient can be altered by modulating the flow rate driving the flow in the two sets of microjets. Unlike in most microfluidic
gradient generators, in our device the gradient is primarily created by convection (i.e. the effect of diffusional transport is negligible). Therefore, the resulting concentration profile is fairly independent of the molecular weight of the species, which allows us to use tracer molecules to visualize the gradient.

Using food coloring dyes we were able to visualize the formation of the gradients (Fig 2a). We also used a previously described method based on Beer-Lambert law [4] to measure the surface gradient in the chambers (Fig 2b), which is a more accurate indication of what the cells sense. In order to visualize the surface gradient, 1 mM fluorescein mixed with 45 mM Orange G was flowed through one set of microjets, while the bath, chambers and the other set of microjets was filled with 45 mM Orange G. The protocol utilizes the light absorption spectrum of Orange-G, a non-fluorescent dye that absorbs energy strongly at the excitation wavelength (490 nm) of the dye (fluorescein), but weakly at its emission wavelength (540 nm). Orange-G competes with fluorescein in solution for excitation energy; at 45 mM (with a 0.6 NA objective), it results in an effective penetration length of ~4.9μm for fluorescein [4]. Since the excitation decays exponentially from the surface, 95% (0.95 ≈ 1-1/e) of the fluorescence intensity that we detect comes from the volume that is within ~15 μm of the surface. The slope of the surface gradient formed in our 1008 chamber device (Fig. 2b) is spatially and temporally consistent and repeatable, that is, the slope is invariant across chambers (Fig. 2c) and over time (Fig 2e). When plotted across chambers, the mean slope was -12.72° with a standard deviation of 2.07° (Fig 2d).

![Fig 1: Schematic of a unit of the microjet array device](image1)

![Fig 2: Characterization of the microjet array gradient: (a) Micrograph of the device filled with food-coloring dyes (scale bar = 2 mm). (b) Surface gradients in a 16x16 section of the device (scale bar = 200 μm), seen with Orange-G and fluorescein. (c) Plots of the fluorescent intensity across the chamber for all the 1008 units in the device. (d) Mean and standard deviation of the slopes. (e) Plot of the fluorescence intensity in a chamber (arbitrarily selected) over 21 hours.](image2)
We analyzed 6-hour long time-lapse images from 3 separate experiments with 152, 162 and 279 chambers having single neurons. For each time-point, the difference in angle between the axon-growth vector and the gradient direction is plotted as a normalized cumulative histogram. About 61% of the axons get attracted towards the gradient, within 30 minutes of getting exposed to a gradient, whereas there is no bias in turning in the absence of a gradient (Fig. 3a). In the first 30 minutes, the axons on the higher concentration side were attracted towards the gradient, whereas the ones on the lower concentration side were repelled (Fig. 3b). Further analysis revealed that the axons on the higher concentration side of the chamber, alternated between being attracted and repelled by the gradient in a bistable fashion (Fig. 3c), whereas the ones on the lower concentration side were progressively attracted towards the gradient (Fig. 3d).

**CONCLUSION**

We have shown the proof-of-principle demonstration of a 16x63 parallel array of open-access, microfluidic chambers capable of studying gradient sensing in single neurons. This platform (a) produces a stable gradient with negligible shear forces on the culture surface, (b) is amenable to long-term culture of neurons, and (c) enables us to study in parallel the response of a large number of single cells to biochemical gradients, thereby accelerating the discovery of complex mechanisms governing cell migration and axon guidance.

**REFERENCES**


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