

Studies on Competitive Responses in Neurons to Extracellular Cues Using Microfabricated Systems

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Abstract

The successful creation of biomimetic interfaces requires the thorough understanding of how cells perceive and integrate extracellular cues. Nerve cells respond strongly to environmental signals and although there are a variety of stimuli that influence neuron behavior, there are no systematic studies on competitive responses, i.e. when cells are presented with simultaneous but independent stimuli. We have designed microfabricated structures in poly(dimethyl siloxane) (PDMS) with the purpose of allowing individual neurons to be presented with different stimuli in competition. We report the creation of cross-shaped wells where single cells are positioned in the center of the patterns, while contact guidance and chemical guidance cues are localized precisely on the arms of the structures. The chemical stimulus is provided by a Nerve Growth Factor (NGF) gradient immobilized using a photosensitive moiety; the physical stimulus is provided by microchannels. With the designed system we can investigate competition among growing neurites and therefore, better understand cell responses to the extracellular environment. Ultimately, this knowledge will allow us to create materials capable of controlling neuron polarization and neurite outgrowth.

Introduction

Interfaces between cells and engineered devices are critical for the field of tissue engineering and biomaterials. For these applications, it is essential to be able to manipulate cell-material interactions to promote very specific responses such as survival, differentiation, proliferation or migration. An important aspect that contributes to the successful design of these biomimetic interfaces is to comprehend how cells integrate continuously the extracellular stimuli to produce a favorable response.

Neurons are cells that are particularly influenced by extracellular cues during several phases of their development, for instance in cell migration processes [1], polarization [2] and axon steering [3] and regeneration [4]. Numerous environmental stimuli, including substrate topography [5], growth factors [6], extracellular matrix components [7], conducting materials [8] and support cells [9], have been investigated with the purpose of inducing precise responses in nerve cells. However, the majority of these stimuli have been investigated individually, with only a few studies on competitive responses to simultaneous and independent stimuli. This project contributes to this unexplored but relevant area, as cells in natural processes are not presented only with a single and unique stimulus but with combinations of multiple stimuli instead.

Here we describe the design of a microfabricated system to analyze the responses of neurons to extracellular stimuli in competition, in particular physical and chemical guidance cues. This system consists of a microfabricated cross-shaped well capable of containing an individual neuron in the center, while presenting simultaneously different guidance cues. Physical stimulus is presented as microchannels in one of the arms of the cross (1-3 μm deep

and 0.5-2 μm width). The chemical guidance is obtained by immobilizing a gradient of NGF using an azido compound ($-\text{N}_3$).

Experimental Details

Microfabrication

The Cr mask was fabricated using a clean glass slide coated with a chromium layer and poly(methyl methacrylate) resist (4% in Chlorobenzene, Microchem, Newton, MA). Patterns were written with electron beam (E-beam) lithography, followed by development (isopropyl alcohol: methyl isobutyl ketone, 3:1; Sigma, St. Louis, MO) and Cr etching (nitric acid and ceric sulfate, Transene, Danvers, MA). Masters were fabricated with SU-8 photoresist (SU-2002, Microchem, Newton, MA) on silicon using conventional photolithographic methods. Finally, PDMS structures were created by replica molding (Sylgard 184, Dow Corning). Microchannels were fabricated using E-beam lithography, aluminum lift-off and reactive ion etching (RIE) of silicon wafers.

Nerve Growth Factor (NGF) immobilization

An intermediate compound containing the azido group was obtained according to previously published methods [10]. This intermediate was further conjugated to poly(allylamine) (Sigma, St. Louis, MO). NGF (2.5S-NGF, Invitrogen, Carlsbad, CA) was immobilized by casting subsequent layers of the poly(allylamine) conjugate and a superimposed layer of the protein as it is described somewhere else [11,12]. A UV lamp or Nd:YAG laser (355 nm) was used to expose and fix the protein selectively, followed by extensive washing. Immunochemistry studies were performed by incubating a primary anti-NGF antibody (Abcam, Cambridge, MA) followed by incubation with a secondary antibody fluorescently labeled with TRITC (Sigma, St. Louis, MO).

Cell culture

PC12 cells were cultured with Ham's F12K medium supplemented with 15% horse serum and 2.5 % fetal bovine serum. Cells were primed with NGF (50 ng/ml) for one week.

Results

We have designed microstructures in PDMS with the purpose of containing single neurons. **Figure 1** shows representative images of the microfabrication process. Figure 1c) illustrates an SEM image of a PC12 cell positioned in the center of a pattern.

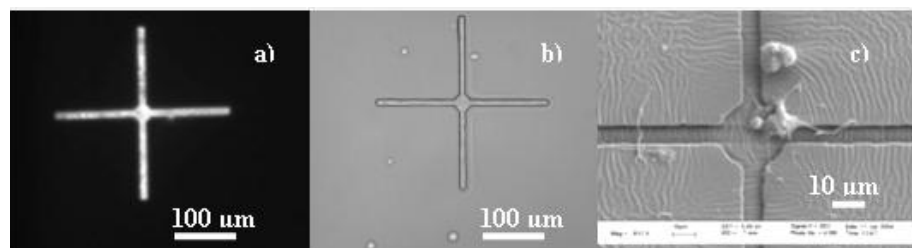


Figure 1. Microfabrication of cross-shaped wells. a) Bright field image of Cr mask; b) Bright field image of PDMS substrate; c) SEM image of PC12 cells on PDMS patterns.

We have begun the process of NGF immobilization using azido compounds. **Figure 2a)** demonstrates the ability to selectively immobilize the protein with UV light exposure, by creating patterns that correspond to a TEM grid used as a mask. Only the areas that were

exposed showed the presence of the growth factor, confirmed by the incubation of a primary antibody and a TRITC-conjugated secondary antibody. Similarly, laser exposure produced selective immobilization of NGF only in the scanned areas (Figure 2b). The activity of the protein was assessed by culturing unprimed PC12 cells and observing successful neurite extension (Figure 2c). Additional experiments are being performed to immobilize gradients in one of the arms of the cross by changing the scanning rate of the laser.

Microchannels of different dimensions have been fabricated both with SU-8 molds (using a designed Cr mask) and Si molds. An example of a structure containing microchannels in one of the arms of the cross is illustrated in Figure 2d).

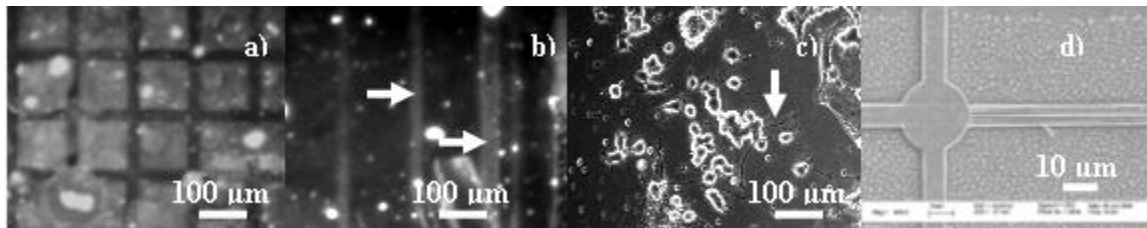


Figure 2. Creation of stimuli. a) Fluorescent image of immobilized NGF (with TEM grid as a mask); b) Fluorescent image of immobilized NGF in lines using a UV laser; c) Phase contrast image of PC12 cells extending neurites on PDMS with immobilized NGF; d) SEM image of a silicon master with microchannels of 1 μm width.

Discussion

We have designed a system for analyzing competitive responses in neurons to simultaneous and independent environmental cues. Cross-shaped patterns have been microfabricated in PDMS for positioning individual cells that can be presented with two different stimuli: physical guidance provided by microchannels, and chemical guidance provided by an immobilized gradient of NGF. Preliminary results demonstrated the feasibility of selectively immobilizing NGF on PDMS substrates, both with a UV lamp and a UV laser. Also, the retained activity of the protein has been corroborated. Additionally, microchannels of different dimensions have been incorporated in the designed micro-wells to provide contact guidance. The implementation of this system will enable us to study decision-making processes in neurons involving engineered stimuli, which is highly relevant to understand cell behavior and create better biomimetic interfaces.

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