

Novel interface to biological systems for retinal prosthetics

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ABSTRACT

The development of retinal prostheses requires a method for interconnecting an imaging system to the retina. Such a system must be able to individually address and stimulate retinal neurons, a significant advance from current technology. As a step toward this goal, we present a novel electronic-to-biologic interface using microfabricated apertures in a silicon substrate. Apertures are created in a thin silicon nitride membrane, after which the surface is appropriately modified to support cell growth. Excitable cells are seeded on the device and imaged using Ca²⁺-sensitive fluorescent dyes in either an inverted or confocal microscope. Using rat pheochromocytoma (PC12) cells, we show the ability to stimulate locally through the apertures. The device allows for the stimulation of cells at precise locations, a necessary requirement for future high-resolution retinal prostheses.

INTRODUCTION

Finding the appropriate interface between electronic devices and the nervous system is a critical step in developing successful neural prosthetics. With this work, we present a first step towards a chemical-based neural prosthetic. We set out to build a biomimetic, microfabricated device that mimics the localized chemical release of a synapse. While our interest is in building an artificial prosthetic retina, this device has applications for all neural systems.

When devising a system to stimulate the nervous system with an external device, electrical stimulation is the most commonly used. Electrical stimulation provides a strong advantage of simplicity; the signals generated by an external source, such as a photodiode array, are electrical. Furthermore, the microelectronics industry provides a great deal of experience in handling electrical signals with precision at the submicron scale. And there has already been a great deal of work towards electrical retinal prostheses [1, 2].

Nevertheless, electrical stimulation has serious neurobiological challenges that chemical stimulation can resolve. The retina consists of as many as 55 cell types [3], including 10 to 15 retinal ganglion cell types, all in very close proximity. Specificity in the retina is provided by different neurotransmitter/receptor systems, including both inhibitory and excitatory transmitters. It is difficult for electrical stimulation to provide specificity for the variety of retinal cell types. Whereas neurons release specific neurotransmitters necessary to excite specific cells, electrical stimulation nonspecifically activates all cells. Additionally, some neurotransmitters inhibit cell activation; inhibition by electrically hyperpolarizing a cell using extracellular electrodes difficult.

An additional challenge with electrical stimulation is localization. Cells are electrically transparent. Electric fields easily pass through tissue, stimulating cells in three dimensions around the electrode. All cells in a volume are equally well stimulated by an electrical pulse.

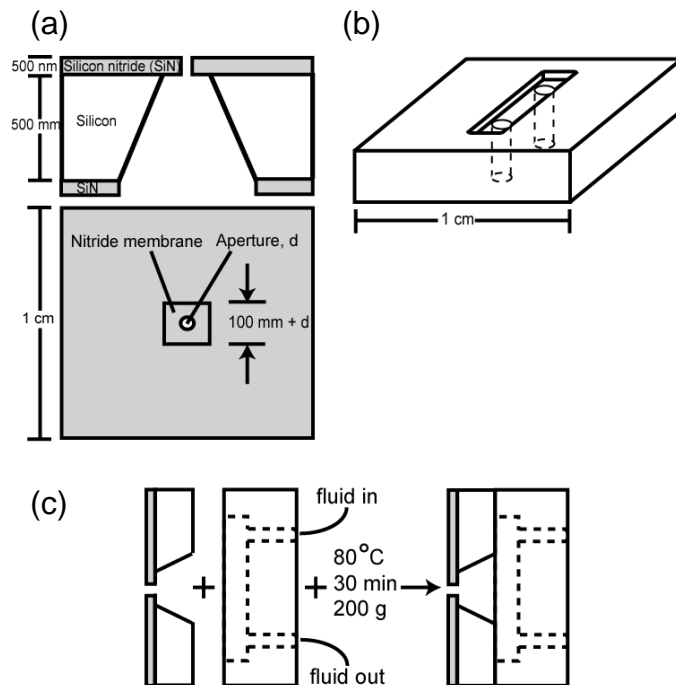


Figure 1. (a) Cartoon depicting the silicon aperture part of the device for localized chemical delivery. (b) Cartoon depicting the fluidic channel part of the device. (c) Description of the bonding process to combine the two pieces.

For example, bipolar cells, which are the first layer of cells stimulated by the photoreceptors, are intermixed throughout the retina, all in very close proximity. Both ON and OFF bipolar cells will be simultaneously excited by the same electrical stimulus, whereas normally one type would be depolarized and the other hyperpolarized by a photoreceptor glutamate pulse [3]. An electrical stimulus loses this information, limiting its effectiveness.

A device using chemical stimulation addresses these issues by providing the specific neurotransmitters released by the neural system. Cells are chemically opaque; thus, localization is possible. In addition, with an appropriate design, a device can provide multiple transmitters (inhibitory and excitatory) to mimic more closely the native synaptic circuit. In photoreceptor-degenerative diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP)[4], where the photoreceptors die, yet the retinal circuitry remains intact, this type of approach provides a clean, biomimetic solution to vision loss.

RESULTS AND DISCUSSION

Device fabrication for localized fluid delivery

A device for localized fluid delivery consists of two components, one for localization and one for fluid manipulation. For localization, our devices use small apertures (5 or 10 μm) in a thin silicon nitride membrane (Figure 1(a)). By providing an aperture of small enough size, fluid delivery is limited in both volume and location. The devices are 1 cm^2 chips, with a thickness of roughly 0.5 mm. The silicon nitride is patterned using plasma etching to create the aperture and a square hole in the backside of the wafer. The silicon is etched anisotropically along the (111) plane at an angle of 54.7° to the wafer surface, using the silicon nitride as an etch mask. The square hole in the backside of the wafer is chosen to yield a region 100 μm larger than the aperture. This leaves a thin silicon nitride membrane freely spanning the region without any silicon support. Silicon nitride is transparent in the visible, so cells are easily imaged through this membrane. Because of the high tensile strength of silicon nitride, this nitride membrane is quite strong and stable; it readily withstands the forces generated during processing.

After localization, the other necessary component is fluid delivery to the aperture. To accomplish this, a channel made from poly(dimethylsiloxane) (PDMS) is attached beneath of the aperture. A master mold is made from 300 μm thick SU-8 photoresist on a silicon wafer using conventional photolithography and a mask made on a transparency using an office printer [5, 6]. The channels are 900 μm wide, 150 μm deep, and 8 mm long, while the PDMS is poured approximately 5 mm deep. A cartoon depicting this design is shown in Figure 1(b).

Once the PDMS has cured, the channels are attached to the silicon chip. The PDMS is diced into 1 cm^2 pieces, with one channel per device. Both the silicon and the PDMS are cleaned in a dilute hydrochloric acid solution (1:4), followed by air plasma at 100 W for 60 seconds. The PDMS channel is aligned and centered on top of silicon aperture, and bonded by squeezing the pieces together (~ 0.2 N) and heating on a hot plate (see Figure 1(c)). Once complete, this bond is irreversible; the PDMS will tear before separating from the silicon nitride.

Since rat pheochromocytoma cells (PC12) will not readily adhere to most substrates, including silicon/silicon nitride, it is therefore necessary to treat the chips before seeding with cells [7]. The devices are first immersed in poly(D-lysine) at 50 $\mu\text{g}/\text{ml}$ for 30 minutes at room temperature. The poly(D-lysine) provides a sticking layer for an application of mouse laminin, to which the PC12 cells adhere and spread. After rinsing the devices in phosphate-buffered saline (PBS), the laminin is applied at 5 $\mu\text{g}/\text{ml}$ in PBS for 8 hours in an incubator (37°C , 6.5% CO_2). The chips are rinsed in PBS and are ready for use.

Fluorescent dye loading and measurement

Measurement of bradykinin stimulation is accomplished by observing changes in intracellular Ca^{2+} levels using fluo-4 (Molecular Probes, Eugene, OR). The loading solution is made from fluo-4 reconstituted in DMSO at 1 mM mixed in Ringer's solution (135 mM NaCl, 5 mM KCl, 10 mM D-glucose, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, pH 7.2) to a final fluo-4 concentration of 1 μM .

The stimulating solution is a mixture of bradykinin (Sigma, St. Louis, MO), Ringer's, and sulforhodamine 101 (Sigma). Bradykinin is reconstituted in Ringer's at 1 mg/ml (1 mM), and then diluted to the desired testing concentration. Sulforhodamine (Texas Red) is reconstituted in DMSO at 8 mM, and added to the stimulating solution to yield a final concentration of 4–8 μM . The Texas Red dye provides a means to visualize simultaneously the fluid flow and stimulation.

Changes in fluorescent levels were observed with either an inverted fluorescence microscope or an upright confocal microscope. The inverted microscope, used for the single-cell stimulation data, is a Nikon TE300 (10x, 0.30 NA) with a Hamamatsu Orca ER CCD camera. The data was collected with Metamorph (Universal Imaging Corporation, Downingtown PA). The confocal microscope, used for the multi-cell and two-color experiments, is a Nikon E800 (10x dipping objective, 0.30 NA) with a Nikon PCM 2000 confocal unit. Two lasers were used simultaneously to excite the fluo-4 (Argon ion, 488 nm) and Texas Red (HeNe, 543 nm). Images were sampled with two PMTs simultaneously (515/30 bandpass and 605/32 bandpass filters), and analyzed using SimplePCI (Compix Inc., Cranberry Township, PA).

Microfluidic delivery of cell stimulants

The microfluidic system supplies a small amount of stimulant through the aperture. The design is to flow bradykinin through the channel and allow the chemical diffusion through the

aperture. While there are multiple methods for moving fluids in microchannels, such as electroosmotic flow, we chose a pressure-driven flow using a syringe. The flow through the aperture is a combination of the pressure gradient created by the syringe and diffusion.

The fluid is supplied by inserting a 24-gauge Teflon hoses into each access hole. One-milliliter tuberculin syringes are used to drive the fluid through the hoses, at a rate of 10 to 30 $\mu\text{l/s}$. Injected volumes range from 250 μl through 1000 μl , taking 15 to 60 seconds to deliver. The average flow rate is 16 $\mu\text{l/s}$; when combined with the channel geometry, this yields a Reynolds number of approximately 3100, above the limit for laminar flow. Being above the laminar limit is an advantage in this system. There is a 500 μm gap between the channel and the aperture due to the wafer thickness. Nonlaminar flow allows mixing to occur by methods other than diffusion, speeding the rate at which bradykinin will reach the aperture.

With the fluidic delivery system in place, cell stimulation is accomplished by delivering an appropriate amount of stimulant to the cells. Rat pheochromocytoma cells (PC12) were chosen because of their usefulness as a neurobiological model [8, 9], and because of their ease of care and ready availability. This cell line changes intracellular Ca^{2+} levels upon a bradykinin stimulus, reaching a maximum change at an external bradykinin concentration of 1 μM [9]. The cells are seeded on the chips at least four hours before testing to allow them to adhere.

The two parameters of control over stimulation radius are concentration and volume. By adjusting either the concentration or the volume of bradykinin supplied, the distance from the aperture at which cells are stimulated is controlled. When a large total quantity of bradykinin is delivered to the aperture (high concentration or large volume), many cells are stimulated. This is shown in Figure 2, where a wave of stimulated cells is visible. Bradykinin was driven through the channel at 100 μM for approximately 21 seconds. At the starting time, no cells are stimulated. After 3 seconds, cells within 40 μm are stimulated as the bright cell to the left and below the aperture in the middle frame shows. Two frames later, after another 6 seconds, cells further away (100 μm) are stimulated. The bottom intensity cross-section shows further from the aperture two cells that have been stimulated.

CONCLUSION

The focus of research on retinal prostheses has been on electrical stimulation. While electrical stimulation offers many advantages in terms of simplicity, it lacks the ability to mimic the nervous system on a fundamental level. By approaching the problem from a chemical stimulation standpoint, a more biomimetic solution to this problem is possible. As a first stepping-stone towards a chemical-based retinal prosthetic, we have shown the ability to stimulate cells locally using a neurobiological model system. By varying the amount and concentration of neurotransmitter supplied through a microaperture, the stimulation distance can be controlled. While much work is needed, this is the framework for a new approach to stimulating the nervous system.

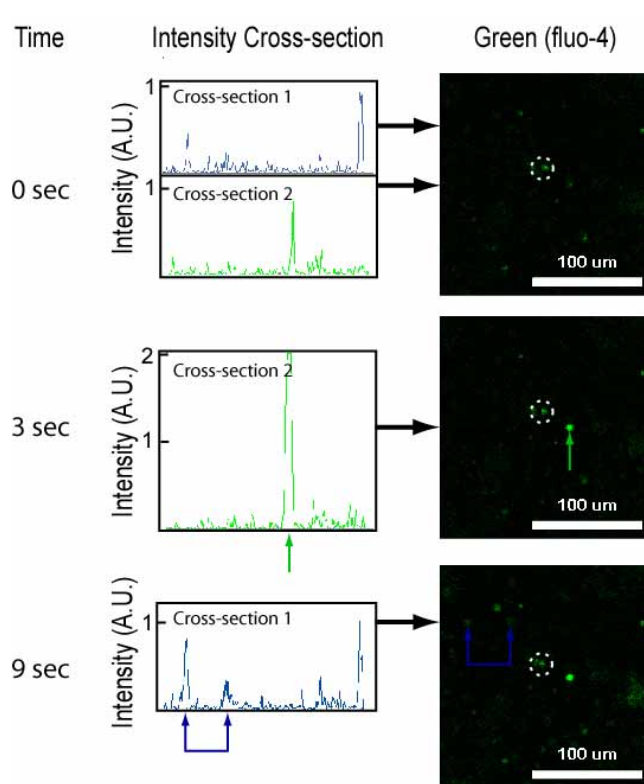


Figure 2. Time-lapse confocal micrographs of multi-cell stimulation. The aperture is 10 μm in diameter (half the size of a cell body) and is located at the center of the dotted circle. A Ringer's solution containing 100 μM bradykinin is applied just after the first displayed frame. Intensity cross-sections (arbitrary units, constant scale) indicate which cells are stimulated. The top intensity plot shows two cross-sections at time zero. As the fluid radiates outward from the aperture, cells are stimulated. Within 3 seconds, a cell 25 μm from the aperture is stimulated (middle plots, green arrow). After 9 seconds, cells 100 μm away are stimulated (bottom plots, blue arrows). Other cells in the region are stimulated; the arrows indicate only representative events.

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