Localized Neurotransmitter Release for Use in a Prototype Retinal Interface

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Purpose. Current neural prostheses use electricity as the mode of stimulation, yet information transfer in neural circuitry is primarily through chemical transmitters. To address this disparity, this study was conducted to devise a prototype interface for a retinal prosthetic based on localized chemical delivery. The goal was to determine whether fluidic delivery through microfabricated apertures could be used to stimulate at single-cell dimensions.

Methods. A drug delivery system was microfabricated based on a 5- or 10-μm aperture in a 500-nm thick silicon nitride membrane to localize and limit transmitter release. The aperture overlies a microfluidic delivery channel in a silicone elastomer. To demonstrate the effectiveness of this transmitter-based prosthesis, rat pheochromocytoma cells (PC12 cell line) were grown on the surface of the device to test the precision of stimulation, using bradykinin as a stimulant and measuring fluorescence from the calcium indicator, fluo-4.

Results. The extent of stimulation could be controlled accurately by varying the concentration of stimulant, from a single cell adjacent to the aperture to a broad area of cells. The stimulation radius was as small as 10 μm, corresponding to stimulation volumes as small as 2 pL. The relationship between the extent of stimulation and concentration was linear.

Conclusions. The demonstration of localized chemical stimulation of excitable cells illustrates the potential of this technology for retinal prostheses. Although this is only a proof of concept of neurotransmitter stimulation for a retinal prosthesis, it is a significant first step toward mimicking neurotransmitter release during synaptic transmission. (*Invest Ophthalmol Vis Sci.* 2003;44:3144–3149) DOI:10.1167/iovs.02-1097

Making a stable physiologic interface between an electronic device and neurons is one of the major goals in the quest to develop a functional retinal prosthesis (Iezzi R, McCallister P, Abrams G, et al. ARVO Abstract 4545, 2000).[1–6] Neural prosthetics have shown great promise for reinnervating damaged regions of nervous system organs. Already, neural interfaces to the spinal cord,[1] ear,[7] and retina[8] have been implanted in humans with partial restoration of lost function in some cases. However, all the prosthetic devices developed so far are based on an electrical interface, typically an electrode array or electrode cuff, to generate field potentials that depolarize nerve cells. Electrical stimulation has the advantage of simplicity, but, in clinical disease, it is neurotransmitter stimulation from damaged presynaptic neurons, rather than electric field stimulation, that is actually lost. For example, in macular degeneration, a leading cause of blindness in the United States, the atrophy of rod and cone cells in the retina means a loss of glutamate-releasing synapses to bipolar cells.[9] Furthermore, chemical stimulation is neuronally selective, whereas electrical stimulation is inherently nonselective. Electrically based devices have yet to demonstrate the ability to stimulate in a physiologic way complex sensory organs such as the retina, which have both depolarizing and hyperpolarizing signal pathways. The ideal neural stimulator of a prosthetic retina would use neurotransmitters to mimic these natural functions.

Although several groups have investigated the problems involved in localized chemical delivery, none of these devices has demonstrated the resolution to stimulate at the level of single cells.[10] In the development of a prosthetic interface for the retina, the stimulation of single or a few cells is important if high spatial resolution (i.e., good vision) is to be achieved. Furthermore, to minimize possible toxic effects from overstimulation, a chemical prosthetic device should have precise control over the location and quantity of neurotransmitters. We report the development of a microfabricated device that uses microfluidics to deliver transmitters through apertures on the order of single-cell dimensions, and we demonstrate its effectiveness for the controlled stimulation of one or many cells. This technology mimics localized transmitter release by a synapse and opens a new approach to prosthetic neural stimulation in the retina.

Methods and Materials

Silicon Microfabrication

Fabrication was performed at the Stanford Nanofabrication Facility (SNF) with 4-inch, <100> crystal plane orientation, boron-doped, double-polished silicon wafers, nominally 500 μm thick.[11] Using low-pressure chemical vapor deposition (LPCVD), a thin layer (500 nm) of silicon nitride was deposited on the surface of the wafers. Standard contact photolithography and plasma etching of the silicon nitride defined the small features (e.g., the apertures). The larger features on the backside of the wafer were similarly defined by using backside alignment, contact photolithography, and plasma etching. With the features defined in the silicon nitride, the wafers were placed in 20% tetramethylammonium hydroxide (TMAH) at 95°C for approximately 6 hours. The silicon nitride acted as a mask, allowing the TMAH to etch anisotropically through the wafer along the (111) crystal plane.

Fluidic Channel Fabrication

The chemical delivery channels were composed in poly(dimethylsiloxane) (PDMS). A mold for the elastomer was fabricated in an epoxy-

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based negative resist (SU-8 2100; MicroChem, Inc., Newton, MA) on a silicon wafer using conventional photolithography. The SU-8 was spun on the wafer in two layers of 150 μm and exposed with a contact aligner with a mask made on a transparency by using an office printer.[12,13] The PDMS (Sylgard 184; Dow Corning, Midland, MI), mixed at a 10:1 ratio of elastomer to curing agent, was poured over the mold to a depth of approximately 5 mm. Glass capillaries were placed vertically in the PDMS at the ends of the channels to provide external access to the channels. After curing, the glass capillaries were removed and the PDMS was diced into 1-cm^2 pieces. A PDMS piece and a silicon chip were cleaned for 8 minutes in 1:4 HCl-H_2O, followed by a plasma cleaning at 100 W for 60 seconds. The two pieces were manually aligned and placed on a hot plate at ~80°C under a 200-g weight for 30 minutes.

Chip Treatment

The PC12 cells used in the study do not readily adhere to most substrates, including silicon-silicon nitride. It was therefore necessary to treat the chips before seeding with cells.[14] The devices were first immersed in poly(D-lysine) at 50 μg/mL for 30 minutes at room temperature. The lysine provides a sticking layer for mouse laminin, to which the PC12 cells adhere and spread. After the devices were rinsed in phosphate-buffered saline (PBS), the laminin was applied at 5 μg/mL in PBS for 8 hours in an incubator (37°C, 6.5% CO_2). The chips were rinsed in PBS and were ready for use. Mass-prepared devices were frozen in PBS at −18°C and used within approximately 2 weeks.

Fluorescent Dye Loading and Measurement

Measurement of bradykinin stimulation was accomplished by observing changes in intracellular Ca^{2+} levels using fluo-4 (Molecular Probes, Eugene, OR). The loading solution was made from fluo-4 reconstituted in dimethyl sulfoxide (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 devices on which cells had been seeded were rinsed in Ringer's and immersed in loading solution for 20 minutes at room temperature. They were rinsed again and allowed to sit for 30 to 40 minutes longer at room temperature in Ringer's solution.

The stimulating solution was a mixture of bradykinin (Sigma-Aldrich, St. Louis, MO), Ringer's solution, and sulforhodamine (Sulforhodamine 101; Sigma-Aldrich). Bradykinin was reconstituted in Ringer's at 1 mg/mL (1 mM), and then diluted to the desired testing concentration. Sulforhodamine was reconstituted in DMSO at 8 mM, and added to the stimulating solution to yield a final concentration of 4 to 8 μM. The solution was warmed to 37°C during the stimulation experiments. The testing dish consisted of a 35-mm Petri dish, through the bottom of which two holes were drilled. Polyethylene tubing was inserted through each hole, and sealed with silicone rubber. During testing, these pieces of tubing were inserted into the access holes of the device. Fluid was delivered through this tubing to the channel using pressure-driven flow from a syringe. The syringe (1 mL) was driven by hand at an average flow rate of 16 μL/s (500 μL in 30 seconds).

Changes in fluorescence levels were observed with either an inverted fluorescence microscope or an upright confocal microscope. An inverted microscope was used for some single-cell stimulation data (10×; 0.30 numeric aperture [NA]; model TE300; Nikon, Melville, NY) with a charge-coupled device (CCD) camera (Orca ER; Hamamatsu, Hamamatsu City, Japan). The data were collected with an integrated imaging system (Metamorph; Universal Imaging Corp., Downingtown, PA). A confocal microscope was used for the multicell and two-color experiments (model E800, 10× dipping objective, 0.30 NA; Nikon, with a Nikon PCM 2000 confocal unit). Two lasers were used simultaneously to excite the fluo-4 (argon ion, 488 nm) and sulforhodamine (HeNe, 543 nm). Images were sampled with two photomultiplier tubes simultaneously (515/30 bandpass and 605/32 bandpass filters) at a rate of 0.33 Hz, and analyzed using image-analysis software (SimplePCI; Compix Inc., Cranberry Township, PA).

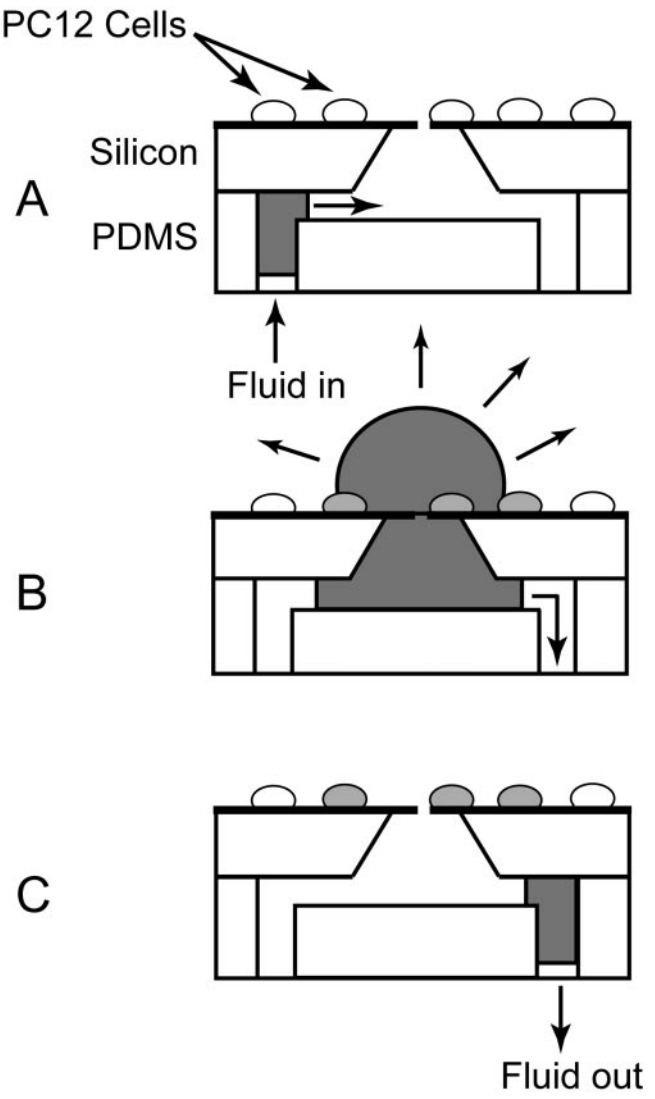

FIGURE 1. Schematics and fluorescence micrographs depicting focal neurotransmitter delivery to cells. (**A**) Fluid is injected through the fluid inlet. (**B**) As the fluid moves underneath the aperture, a small amount of fluid passes through the aperture. Cells near the aperture, cultured on top of the silicon nitride membrane, are stimulated. (**C**) The remainder of the fluid moves through the channel, and leaves the device through the fluid outlet.

RESULTS

Microfabrication to Facilitate Localized Fluid Delivery

To accomplish the precision necessary for localized release, we microfabricated the device shown in Figure 1. The neurotransmitter pulse is delivered to the channel inlet (Fig. 1A), moves under the aperture where some fluid passes through to the cells (Fig. 1B), and finally exits through the channel outlet. The core of our device is a microfabricated circular aperture with a diameter of 5 to 10 μm, smaller than most cell somas and approaching the size of a synaptic ending. Our apertures were created in a 500-nm silicon nitride membrane (Figs. 2A, 2B). After treatment with extracellular matrix proteins, the

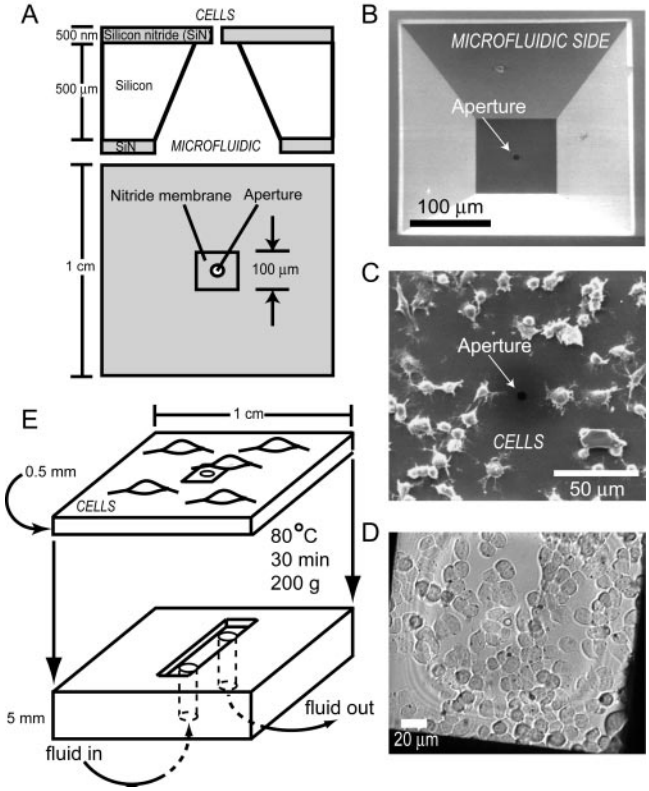

FIGURE 2. (**A**) Schematic depicting the silicon aperture part of the device for localized chemical delivery. (**B**) Scanning-electron micrographs (SEMs) of the bottom of the silicon chip and aperture. The fluid was ejected through the 10-μm aperture from the bottom of the chip onto the top where the cells reside. (**C**) SEM of PC12 cells adherent and growing on top of the device. (**D**) Light microscope image though the silicon nitride window of PC12 cells growing on the device. Note the cell spanning the aperture at the center of the frame. (**E**) Schematic showing design and assembly of the fluidic channel.

silicon nitride readily supported growth and adhesion of PC12 cells. Figure 2C shows a scanning electron micrograph of PC12 cells growing near an aperture, and Figure 2D is a bright-field image of cells spreading across the aperture.

To deliver fluid to the underside of the aperture, we fabricated a channel 820 μm wide and 300 μm deep in poly(dimethylsiloxane; PDMS). The channel was irreversibly bonded to the silicon beneath the aperture (Fig. 2E). Using pressure-driven flow, we moved the fluid through the channel and ejected it from the aperture. The fluid flow and ejection were visualized with fluorescent dyes. The ejected fluid diffused quickly as it moved away from the aperture. When a high concentration of fluorescent dye was ejected, the fluid moved beyond the edges of the window and was seen to move radially outward from the aperture. At low concentrations, discerning the fluid flow through the aperture was difficult because of the background fluorescence through the transparent silicon nitride window.

Controlled Stimulation of Cells

To show that our model aperture can function as a prosthetic interface, we cultured PC12 cells on the surface (as in Fig. 2E) and studied the effect of a continuous flow of a transmitter stimulant, bradykinin, through the microfluidic channel underneath the aperture. The goal was to determine whether we could achieve stimulation through the aperture and to explore the extent of cellular stimulation during a steady flow of transmitter under the aperture. After culturing the PC12 cells on the chip surface, we loaded the cells with a calcium-sensitive fluorescent dye (fluo-4) to monitor transmitter stimulation. When only Ringer's solution with fluorescent dye was delivered through the channel, no cell stimulation was observed. When a low concentration of bradykinin (5 μM) was used as the stimulant underneath the aperture (Figs. 3A, 3C) a very limited area of excitation was observed which, in this example, involved only a single cell that was close to the aperture (Fig. 3A, arrow). Between the time of first recognizing cell stimulation (t_0) and 6 seconds later, only the one cell remained stimulated, and cells located farther from the aperture were not excited. In four separate experiments, we found that the radius of cells stimulated remained less than 25 μm, on the order of individual cell dimensions, when concentrations lower than 5 μM bradykinin were used.

When a high concentration of stimulant (100 μM bradykinin) was used initially to fill the prosthetic channel and was maintained beneath the aperture, a larger circle of cellular stimulation occurred than with the 5-μM concentration of bradykinin (Figs. 3B, 3D). As the drug passed through the aperture, we saw initially only a small radius of stimulation with one or a few cells (t_0). After 6 seconds, the radius of excitation was much larger.

It is known that PC12 cells can respond variably to chemical stimulation.[15] As a demonstration that this highly localized stimulation was a result of localized transmitter delivery and not a reflection of variable cell responsiveness, we performed two-stage experiments. A high concentration of transmitter (100 μM bradykinin) was run underneath the aperture immediately after 1 minute of observation at low concentration (5 μM bradykinin). The higher concentration always stimulated additional cells farther from the aperture than those stimulated at the lower concentration. In addition, the flow of Ringer's solution without a stimulant did not result in cell stimulation.

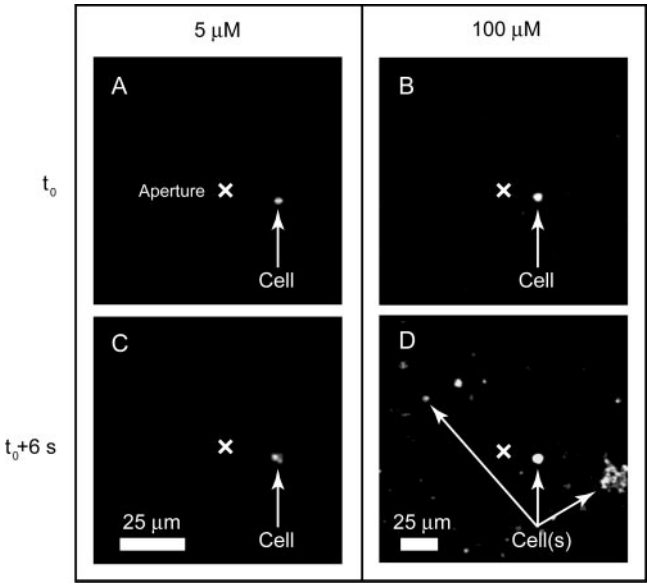

FIGURE 3. Time-lapse confocal micrographs demonstrating single and multiple cell stimulation. The aperture (*arrow* indicates location) is 10 μm in diameter. A background image taken before bradykinin application is subtracted from the images. The panels show the effects of two different concentrations of bradykinin. (**A**, **B**) Initially, at both concentrations, only one cell was stimulated. (**C**) Six seconds after the first frame. No additional cells had been stimulated with the 5 μM solution, but (**D**) additional cells had been stimulated with the 100 μM solution.

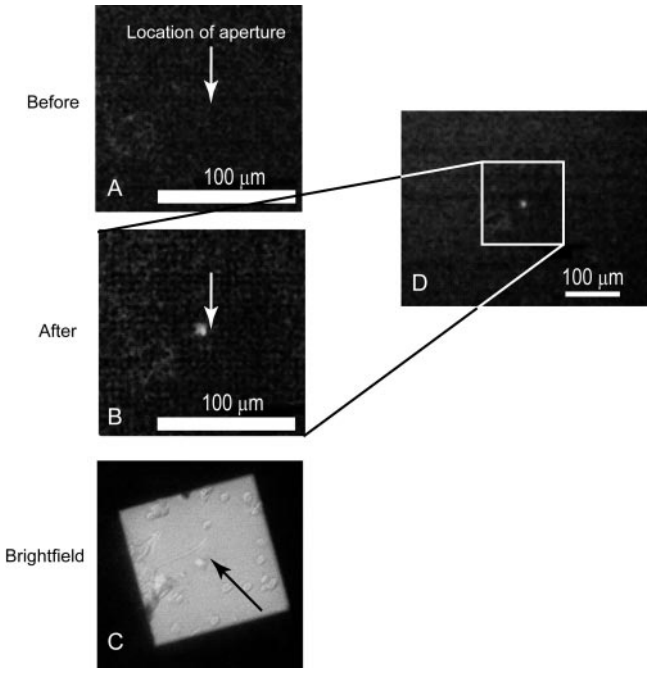

FIGURE 4. Single-cell stimulation with short pulses. *Arrows*: aperture location. (**A**) Before application of bradykinin. (**B**) After application of bradykinin (50 μM). Only the cell nearest the aperture was stimulated. This cell was sitting at the aperture's edge (not visible), as shown in the bright-field image (**C**). Many other cells were visible through the nitride window, none of which glowed before or after the bradykinin stimulus. The surrounding cells were viable, yet were not exposed to a concentration of bradykinin high enough to stimulate them. The square visible in the bright-field image is the transparent silicon nitride membrane shown in Figure 2. (**D**) Large-scale image after excitation, demonstrating that no other cells were stimulated.

Stimulation was a consequence of the localized bradykinin delivery and not of fluid flow near the cells.

We also performed experiments using a short pulse (<1 second) of transmitters in the microfluidic channel. Cells were cultured to cover much of the chip, but when 50 μM bradykinin was pulsed under the aperture, only a single cell near the aperture was stimulated, whereas cells farther away were not stimulated (Fig. 4). The small aperture, in conjunction with the brief period of pulsed exposure, controlled the maximum distance from the aperture at which cells became stimulated—in this case, within the dimensions of a single cell.

Quantifying the Spread of Cell Stimulation

We measured the radius of cell stimulation as a function of the time that the transmitter flowed underneath the aperture for two transmitter concentrations. As bradykinin passed through the aperture, a wave of cell stimulation was observed progressing radially outward. An example of a stimulation wave as bradykinin passed over a layer of cells cultured around the aperture is diagrammed in Figure 5A, with circles representing the maximum distance at which cells were stimulated at successive 3-second intervals. The fluorescence image shows the status after all the cells in the region had been stimulated (12 seconds) with 100 μM bradykinin. Because transmitter fills the volume overlying the aperture, we plotted the volume as overlying half spheres, corresponding to the radii of stimulation that we measured. Figure 5B shows data from two experiments comparing concentrations of 10 and 100 μM. For each concentration, the data points follow a straight line. When, alternatively, we compared the calculated volume of stimulation at a fixed measurement time (9 seconds), as a function of transmitter concentration (Fig. 5C), we also found a linear relationship. For the experiments in Figure 4 in which only a single cell was stimulated, this corresponded to a radii of 10 μm and therefore to excitation volumes, calculated as above, as low as 2 pL.

DISCUSSION

Our microfabricated device mimics in principal a synaptic junction from which a very small amount of neurotransmitter is ejected from a point source (the vesicle), providing a locally high concentration of neurotransmitter that decays to undetectable levels farther from the source. By choosing a small enough concentration with small enough apertures, we can limit stimulation to a region on the order of single-cell dimensions. This level of accuracy is a necessary requirement for a retinal prosthesis.

We chose to use silicon microfabrication to produce our devices. Microfabrication is a powerful technology for producing devices with structures on the scale of biological systems. In fact, it is possible with microfabrication to make apertures on the order of 50 to 100 nm, the size of a synaptic vesicle,

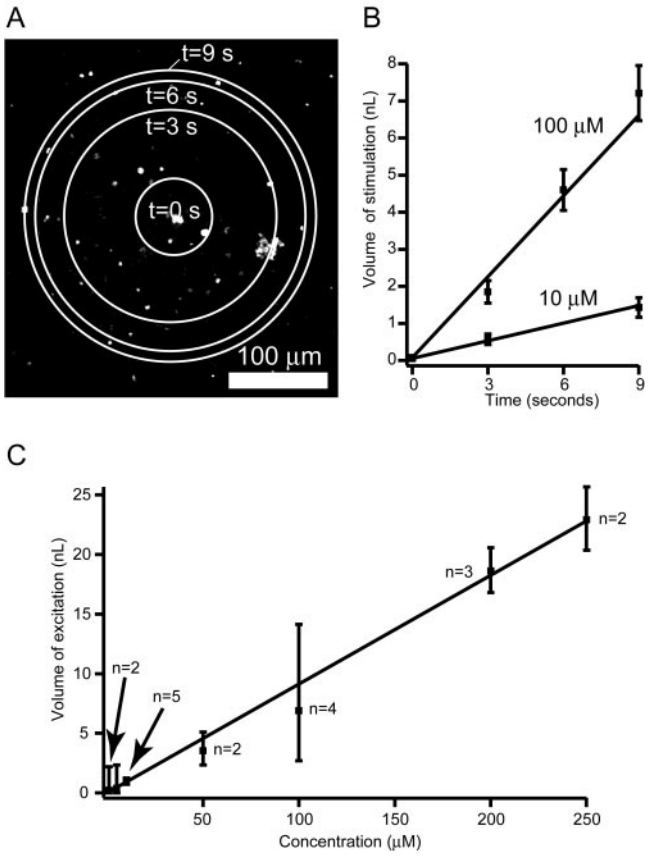

FIGURE 5. (**A**) A confocal image 12 seconds after the application of 100 μM bradykinin. The smallest *circle* is the radius at which cells were stimulated 3 seconds after the beginning of fluid flow. Moving outward, each *circle* represents the stimulation distance after an additional 3 seconds. The *circles* are centered on the aperture. (**B**) The volume of a hemisphere corresponding to each measured radius has been plotted as a function of time for two concentrations of supplied bradykinin, showing a linear fit to the data points. The volume increases linearly with time. (**D**) The volume of excitation at 9 seconds for four different bradykinin concentrations increases linearly with concentration. *Solid line*: linear fit to the data.

should that turn out to be useful. A small aperture is crucial for limiting egress of transmitter and thus providing a point source for stimulation.

The apertures were fabricated in silicon nitride, which has several advantages as a substrate: First, it is transparent in the visible spectrum, and cells are therefore easily imaged through the membrane; second, the high tensile strength of silicon nitride allows the membrane to withstand readily the manipulations generated during processing, surface treatment, and cell culture; third, because of its strength, the silicon nitride membrane can be made very thin so that the diffusion time through the aperture is very short. This short diffusion time is important because, much like a synaptic cleft, where the distance between synaptic endings is very small for rapid transport, the thin silicon nitride membrane in this device allows for rapid refreshing and delivery of fluids. Finally, silicon nitride is inert to cells and readily supports cell growth.

The microfluidic system used pressure to flow the transmitter under the aperture. The fluid moved through the channel, parallel to the silicon nitride membrane and aperture. As the fluid passed the aperture, a small amount of the transmitter ejected through the aperture, while the unused portion of the transmitter flowed out of the system. Fluid moved through the aperture only when pressure was applied to the channel, suggesting a pressure-driven component of the flow. Other methods for moving fluids in microchannels exist, such as electro-osmotic flow, but our goal with this first model prosthesis was simply to demonstrate an effective interface rather than to compare delivery systems. In this model demonstration, the channels were large compared with the scale of the aperture. For a retinal prosthesis, in which many channels and apertures would be necessary, the channel size must be much smaller. Channels for fluidic delivery have been demonstrated as small as 10 nm,[16] but in this prototype device, the channel size was chosen for ease of fabrication. The photolithography was performed with a transparency mask produced on an office printer to minimize costs and simplify assembly, with the drawback of low lithographic resolution. Nevertheless, the small size of the aperture, coupled with the large transaperture volume, allowed the aperture to act as a point source of stimulation.

For testing localized stimulation, we chose PC12 cells as a neurobiological model. PC12 cells are similar to neurons in function and respond to bradykinin by changing their intracellular calcium levels, reaching a maximum change at roughly a 1 μM concentration of bradykinin.[17] The response time of PC12 cells to bradykinin is slower than the response time of retinal neurons to neurotransmitters[18,19]; consequently, the temporal aspects of this device are probably better than demonstrated in this study. PC12 cell growth on the silicon nitride was excellent after treatment with extracellular matrix proteins.

In these experiments, two different models of fluid flow were used. In the first model, the fluid flow was continuous over the time scale of the experiment. As would be expected, the stimulation distance increased with higher bradykinin stimulation. When the concentration was low enough, it became possible to stimulate only a single cell closest to the aperture. In essence, the aperture was acting as a point source for stimulation, in that the concentration of bradykinin leaving was diluted into the bulk fluid and never reached a level high enough to stimulate more distant cells. For a chemical-based retinal prosthetic, stimulation in a very small volume is of utmost importance. Not only would a small volume of stimulant provide high resolution, but it also would limit the amount of transmitter released, extending the life of the device and allowing the natural recycling of the retina to remove the neurotransmitter. The second model of fluid flow used fluid pulses, instead of long-duration flow. For a working visual prosthesis, transmitters should be pulsed underneath the aperture to achieve temporal resolution, as well as the spatial resolution achieved by the aperture. As the stimulation distance decreases, a higher concentration (but lower volume) of stimulant can achieve the same results.

These data indicate that the stimulation effectiveness of transmitters circulated beneath these small apertures is quantifiable and in fact can, under some circumstances, be described by a simple linear equation. This allows accurate modulation of neurotransmitter effects in a model prosthetic interface by adjustments of concentration. In addition, these results indicate that this technique complements well other neurotransmitter-based devices, such as those that use "caged" molecules (Iezzi R, Safadi M, Miller J, et al., ARVO Abstract 5050, 2001).[21]

Conclusions

Although electrical prostheses have been well characterized, little work has been undertaken with neurotransmitter-based prosthetics—the major challenges being accurate spatial control, microfluidic delivery, and a renewable supply of neurotransmitters. A transmitter-based prosthetic would be particularly useful for selective stimulation of retinal circuitry that is heavily dependent on subtle patterns of stimulation and inhibition. For example, there are two primary types of retinal bipolar cells (ON and OFF) that are stimulated by the rods and cones. Because the ON and OFF bipolar cells are spatially colocalized, both cells would be depolarized by a nearby electrical stimulus; however, in normal retinas, OFF cells are depolarized and ON cells are hyperpolarized by a glutamate pulse from the photoreceptors.[20] Thus, neural information would be lost by nonspecifically stimulating both ON and OFF cells with an extracellular electrode array. With an appropriate design, later generations of our device may be able to provide several different neurotransmitters to mimic more closely the biological system. Both stimulating and inhibiting transmitters could be released simultaneously onto colocalized cells, as occurs at synapses in the retinal ganglion cell layer in the eye.

The goal of this work was to build a first-stage microfabricated device that would mimic the synaptic release of neurotransmitters and to demonstrate the feasibility of localized chemical stimulation within the spatial range of a single cell. Our results show that, with low enough neurotransmitter concentrations, such highly localized stimulation is possible. We show that chemical stimulation may approach the precision and spatial resolution that has been achieved with electrical stimulation, but with the additional ability to mimic some of the major functions of synaptic transmission. The use of microfabricated apertures approaching the dimensions of synaptic vesicles may open further possibilities for high-resolution chemical stimulation.

We realize that there is much technical development that must occur before the eventual goal of a full retinal prosthesis is reached. For example, a functioning prosthesis should have a large array of stimulation sites, the possibility of multiple transmitters, on-demand fluidic delivery, and a renewable transmitter supply, as well as ways of preventing aperture and microfluidic channel clogging. These aspects are now being investigated. Moreover, device biocompatibility with the retina, long-term stability, and neurotransmitter metabolism must be addressed. Finally, understanding the delicate balance between neural stimulation and neural toxicity due to repeated neurotransmitter release must also be studied.

In conclusion, we have demonstrated construction of a device that mimics an artificial synapse with spatial resolution

comparable to cellular dimensions. Our prototype device opens new prospects for prosthetic neural tissue stimulation with chemical transmitters in the retina and elsewhere in the nervous system.

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