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Towards a Neurotransmitter-Based Retinal Prosthesis Using an Inkjet Print-head

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Abstract. **Electronic chips that provide a patterned stimulus to cells in the retina may provide a viable treatment for age-related macular degeneration. A surrogate MEMS device, in the form of a print-head from a desktop printer, has been used to eject a pattern of neurotransmitters (bradykinin) onto living rat pheochromocytoma (PC12) cells. Fluorescent calcium imaging was used to measure the patterned stimulation of individual cells. The chemical stimulation of cells by directed microfluidic delivery may have applications in retinal prosthetic devices, and in other prosthetic implants in the nervous system.**

Key Words. **retinal prosthesis, neurotransmitters, inkjet printing, age-related macular degeneration**

Introduction

While neural prosthetics have shown great promise for repairing damaged regions of nervous system organs, all prosthetic devices developed so far are based on an electrical interface, typically an electrode array, to generate field potentials that depolarize nerve cells (Zrenner, 2002). Electrical stimulation has the advantage of simplicity, but it is neurotransmitter stimulation from the damaged pre-synaptic neuron, rather than electric-field stimulation, that is actually lost in clinical disease. Whereas electrical stimulation is inherently non-selective, chemical stimulation may have definite advantages in stimulating complex sensory organs such as the retina by selective stimulation of ''on'' and ''off'' signal pathways. In our research group, we are interested in developing a vastly more physiologic interface for a prosthetic retina based on neurotransmitter stimulation (Peterman et al., 2002, 2003). The objective is to deliver a neurotransmitter solution in very precise quantities and at well-defined locations, mimicking a synapse. Figure 1 shows a schematic of a full-scale, neurotransmitter-based retinal prosthesis.

To reach this eventual goal of a full retinal prosthesis based on neurotransmitter stimulation, many technical problems must be overcome. A full prosthesis should have a large array of stimulation sites, the possibility of multiple transmitters, on-demand fluidic delivery, and a renewable transmitter supply. Some potential issues, such as aperture clogging, have been solved by work in the microfluidics field, particularly with respect to inkjet printing. As microfluidics continues to evolve, chemical based prosthetic chips will likely become a powerful method for stimulating the nervous system (Vastag, 2002). Accordingly, the use of microfabricated devices approaching the dimensions of synaptic vesicles (50–100 nm) will open enormous possibilities for high-resolution chemical stimulation.

Direct inkjet printing has already been used to successfully fabricate organic thin film transistors for all-polymer transistor circuits (Kawase et al., 2001; Sirringhaus et al., 2000) and to synthesize DNA probes on glass substrates (Hughes et al., 2001). For printing on fragile substrates, such as biological cells and porous materials based on polymer matrices, the piezoelectric dispensing of fluid by a MEMS device is suitable because it is non-destructive (unlike a bubble jet ejector, which may cause chemical degradation as a result of temperature changes) and can deliver minute (of the order of picoliters) and consistent volumes to precise locations within microns (Schober et al., 1993). In our experiment the total amount of fluid delivered is orders of magnitude less than for standard printing applications, so that the transfer of excess fluid is not an issue.

Electrical stimulation of cells has the advantage that it allows integration with standard devices, such as photodiode arrays, which are readily available from the microelectronics industry. There has already been a great deal of work on electrical retinal prosthesis (Humayun, 2001; Rizzo et al., 2001), including clinical trials.

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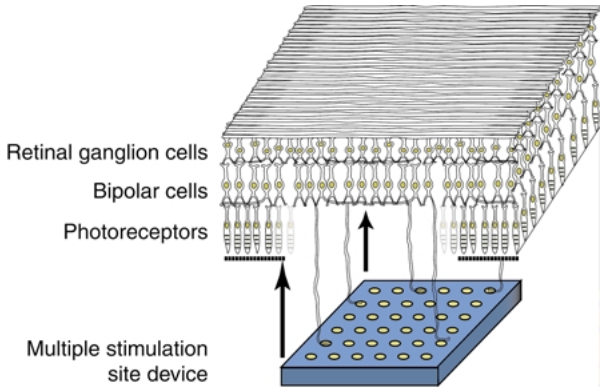

Fig. 1. Schematic of a full-scale, neurotransmitter-based retinal prosthesis.

However, electrical stimulation is non-specific, and can lead to cell damage (Liu et al., 1999). In contrast, chemical stimulation is specific to the neurotransmitters being used to excite chosen cells.

In this paper we give a proof of concept that a simple, surrogate MEMS printing device, such as a print-head from a commercial ink jet printer, can be used to dispense a pattern of drops of neurotransmitter on demand to stimulate living rat pheochromocytoma cells (PC12 cell line), as shown in Figure 2(a). We note that that a commercial ink-jet printer ejects drops at the rate of 50 kHz, whereas retinal cells are activated only at about 25 Hz. Our experiment suggests the possibility of designing a customized MEMS device for chemical stimulation of cells. An extensive discussion and evaluation of important parameters (such as stimulant concentration, temporal dynamics, and materials issues) related to the design of an artificial synapse chip is given elsewhere (Peterman et al., 2002, 2003).

Methods

Inkjet printer

We used an Epson 740 Stylus color printer for dispensing neurotransmitter onto the PC12 cells. This commercial printer has a particularly simple and robust drop ejection technology (called micro piezo technology) in which electrical pulses cause the ink reservoir wall to deflect inward, projecting ink through the nozzle. Figure 2(b) shows a scanning electron micrograph of a gold-plated inkjet print-head. The ink jet nozzles are separated by

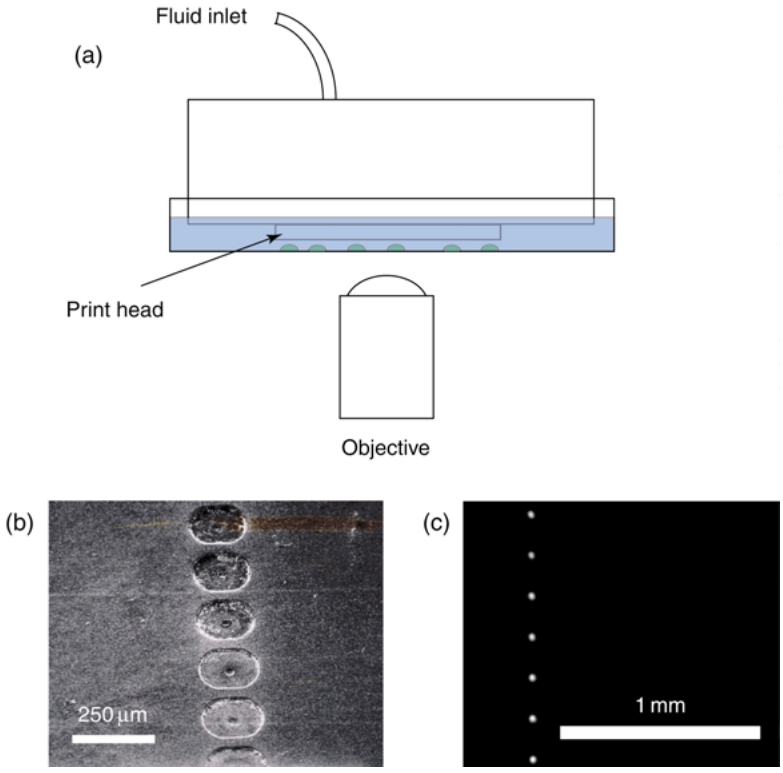

Fig. 2. (a) Schematic of experimental setup. The print-head was lowered into the center of the Petri dish, into a Ringer's solution, and over a glass coverslip upon which PC12 cells were cultured; (b) scanning electron micrograph of an inkjet print-head; and (c) fluorescence micrograph of fluorescein droplets used in alignment of the print-head.

about 200 microns, and the nozzle openings are about 20 microns in diameter.

Drop volumes are in the 6 picoliter range, corresponding to drop diameters of about 20 microns. The color print-head has three rows of 48 nozzles (Figure 2(b)). Having many rows of multiple nozzles is helpful in aligning the nozzles directly over cells. Another advantage of using an Epson 740 inkjet printer is that the print-head can be removed from the carriage rail of the printer and replaced by a dummy print-head, allowing the printer to operate normally while the active print-head (with the connecting electrical cable) can be positioned above a dish with a solution containing PC12 cells. The inkjet cartridges were removed from the print-head and replaced with either a syringe containing fluorescein dye (alignment) or a syringe containing neurotransmitter (stimulation). The entire system was easily set up with the print-head positioned above the objective of an inverted microscope, as shown in Figure 2(a). To determine the precise location of the nozzles on the print-head when it was aligned over the glass coverslip, a syringe containing fluorescein dye was connected to the print-head and squeezed manually. The resulting pattern is shown in Figure 2(c). No activation of cells was observed in this case since no stimulant was present. The horizontal positions of the microscope stage and print-head were fixed after this positioning. Once the horizontal position was fixed, the glass coverslip with PC12 cells was placed in the center of the 100 mm Petri dish, the dish was filled with solution, and the print-head was lowered in place on a custom-made *z*-axis positioner.

Glass slide treatment for cell growth

Cells from the PC12 cell line were cultured on glass coverslips. The coverslips were first cleaned in ethanol, then treated in poly(D-lysine) at 50 μg/ml for at least 30 minutes at room temperature. This treatment was enough to provide an adhesive layer for the cells. The cell cultures were prepared according to a standard protocol (Greene and Tischler, 1976). Experiments are done within 24 hours of seeding, as the cell adhesion begins to deteriorate after this point. The cell density during stimulation experiments was approximately 1×10^4 cells/cm^2, or roughly 50 cells in a visible region.

Fluorescent dye loading of cells and measurement of cell stimulation

Measurement of bradykinin stimulation was accomplished by observing changes in intracellular Ca^{2+} levels (Appell and Barefoot, 1989), using fluo-4 (Molecular Probes, Eugene, OR). The loading solution was 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 cover slips upon which cells had been seeded were rinsed in Ringer's solution, and immersed in loading solution for 20–30 minutes at room temperature. They were rinsed again and allowed to sit for 30–40 minutes longer in Ringer's solution at room temperature.

The stimulating solution was made of bradykinin (Sigma, St. Louis, MO), reconstituted in Ringer's at 100 μg/ml ($\sim$ 100 μM). Changes in fluorescent levels were observed with an inverted fluorescence microscope. The inverted microscope was a Nikon TE300 (10×, 0.30 NA) with a Hamamatsu Orca ER CCD camera. The data was collected and analyzed with Metamorph (Universal Imaging Corporation, Downingtown, PA).

Results and Discussion

We demonstrated that ink-jet printing of neurotransmitters can stimulate living cells. Figure 3 shows time-lapse fluorescence micrographs of cell stimulation when the inkjet print-head was activated with a specific pattern, in this case, the letter ''A''. Since the print-head was fixed,

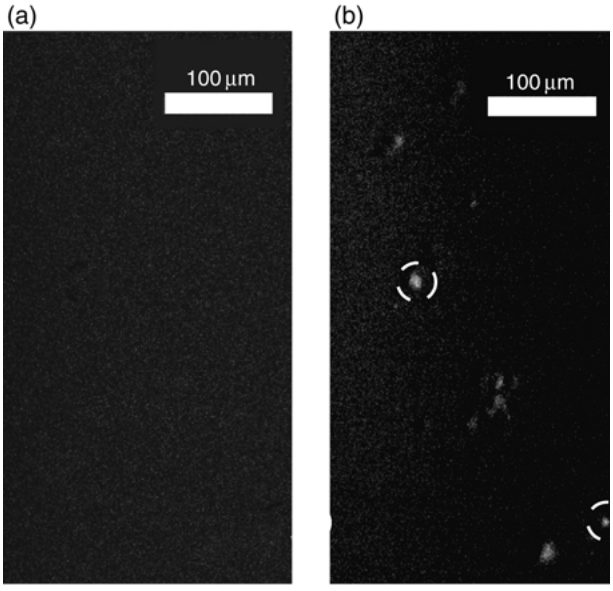

Fig. 3. *Panels showing before (a) and after (b) fluorescence micrographs of stimulated PC12 cells. A background image has been subtracted from both frames. A Ringer's solution containing 100 μM bradykinin was applied just after the first frame by activating the printer. The fluorescence intensity from several stimulated cells as a function of time is shown in Figure 4. The print-head nozzles run approximately vertically down the frame. The central cell shown in Figure 3(b) is directly below a nozzle on the print-head.*

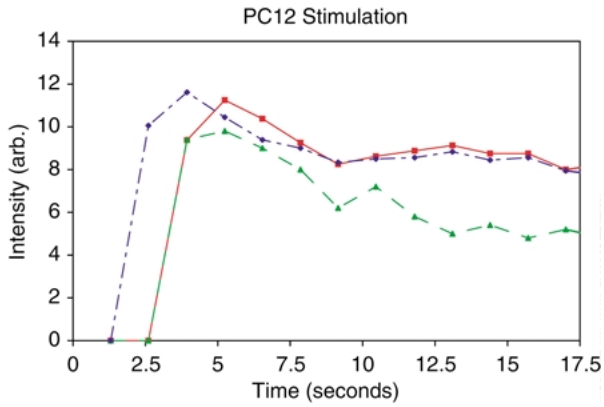

Fig. 4. *Time evolution of fluorescence intensity from stimulated cells.*

the choice of the activation pattern was arbitrary, i.e., the letter ''A'' would not itself be printed onto the cells. The left-hand frame (Figure 3(a)) shows cells before stimulation. The right-hand frame (Figure 3(b)) shows the same cells 1.5 seconds after stimulation by Ringer's solution containing (100 μM) bradykinin. Several representative cells are labeled in Figure 3(b). A comparison of the two frames shows the change in fluo-4 intensity due to changes in intracellular Ca^{2+} levels. The time dependence of the intensity of three representative cells is given in Figure 4 (one of the cells is outside the frames shown in Figure 2). The first excited cell was directly below a nozzle of the print-head. Because the other two cells whose intensity is plotted in Figure 4 are further away from a nozzle on the print-head, the stimulation starts about 1–2 seconds later. The spread of neurotransmitter from the print-head nozzles is pressure driven, and it is difficult to quantify the spread of the neurotransmitter fluid. However, a spread of about 100 microns per second is consistent with the rate we observe when we eject fluorescein dye from the nozzles. The initial 1.5 seconds time delay between initiation of chemical delivery and cell stimulation corresponds well with the temporal aspects of Ca^{2+} signaling (Bui et al., 1999; Fink et al., 2000).

Two pieces of evidence indicate that these stimulation events were specifically due to bradykinin. When the print-head was activated with only Ringer's solution (with and without fluorescein), no cell stimulation was observed. Additionally, PC12 cells do not have a baseline stimulation rate; without a stimulus, these cells do not activate. This property, while very useful for this work, is unlike neurons that may occasionally fire without an external stimulant. We can deduce that the observed stimulation events are not due to background, but are due to the bradykinin ejected by the printer.

PC12 cells can be stimulated by bradykinin concentrations as low as a few nanomolar, reaching maximum stimulation at $\sim 1\,\mu M$. The applied concentration of $100\,\mu M$ is five orders of magnitude larger than necessary for stimulation. However, the dish containing the print-head was filled with tens of milliliters of Ringer's solution, resulting in the ejected droplets being diluted by at least seven orders of magnitude. As a result of this difference, only the cells near the print-head nozzles were immersed in enough bradykinin for stimulation.

A long-term goal for this application in ophthalmology is to develop a high-resolution neural interface that connects the pixelized signal from a digital camera to individual nerve cells in the retina of patients with age-related macular degeneration, thus bypassing damaged photoreceptors in the retina. While prosthetic retinas currently in development stimulate multiple cells with non-specific metal electrodes, we suggest a device that could stimulate individual dendrites with packets of neurotransmitters allowing spatial and neurochemical specificity.

To establish whether such a device would meet the necessary energy requirements, we estimate the power required to operate a MEMS ejector a few microns in diameter, ejecting droplets at the rate of one every millisecond. The power to charge a capacitor with the above dimensions to fractions of a volt is about a picowatt (10^{-12} W). Since avalanche photodiodes generate nanowatts (10^{-9} W) of power in ambient light, a single photodiode would be able to charge hundreds to thousands of MEMS ejectors to deliver neurotransmitter to the nerve cells. A standard photodiode could be operated in the photovoltaic mode to power the diaphragm in such a system. The energy requirements of the chemical stimulation system described here are much lower than the energy requirements of an electrical stimulation system, because the stimulation process is amplified by the opening of an ion channel by the neurotransmitter-nerve cell interaction.

In summary, a chip with an array of neurotransmitter stimulation ejection sites could be implanted into the retina of the eye to bypass damaged photoreceptor layers and stimulate the surviving retinal neurons. While the materials used in producing inkjet print-heads (esp. silicon) are not the most appropriate for the retina, work towards flexible, biocompatible substrates using polyimides and silicone rubbers is being performed by various groups (Majji et al., 1999; Stieglitz et al., 1997, 1999). This neurotransmitter-based prosthesis would have the potential to serve as a key neural interface in a number of artificial retina systems that are being developed worldwide, in addition to neural systems outside the eye.

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