SORTING OF DIFFERENTIATED NEURONS USING PHOTOTRANSISTOR-BASED OPTOELECTRONIC TWEEZERS FOR CELL REPLACEMENT THERAPY OF NEURODEGENERATIVE DISEASES

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ABSTRACT

A major challenge in cell replacement therapy for neurological diseases is the sorting of differentiated neurons because conventional cell sorting techniques require cells in suspension. Recently, spherical glass beads have been demonstrated as a movable growth substrate, allowing transport of differentiated neurons without dissociation. Phototransistor-based optoelectronic tweezers provides a gentle mean to optically manipulate cells. By combining these two approaches, we demonstrated fluorescent-activated automatic sorting of differentiated neurons. We envision employing this cell sorting system for cell replacement therapy to treat neurodegenerative diseases and other nerve injuries.

KEYWORDS

Neuron, Cell sorting, Dielectrophoresis

INTRODUCTION

Neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, arise from damages to the nervous system. The nervous system, unlike many other tissues, has a limited capacity for self-repair; mature nerve cells lack the ability to regenerate. For this reason, cell replacement therapy [1], where new cells are transplanted to replace those lost through diseases and injuries, has drawn great interests. In cell replacement therapy, different types of cells will be required for each particular condition. For example, dopaminergic neurons are required to treat Parkinson's disease (Fig. 1). One of the challenges in this field is to find a way to isolate specific nerve cells from a mixed population. Conventional cell sorting methods only work with cells in suspension: those disassociated from their supporting substrate. However, only immature neurons can be dissociated. Furthermore, neurons in suspension lost their delicate processes and do not allow long-term treatments. A sorting system for differentiated neuron is lacking.

Recently, colloids, such as glass beads, have been demonstrated as moveable supports for neuronal growth[2]. This cell culturing technique enables the relocation of individual differentiated neurons without dissociation from the beads. In addition, Optoelectronic Tweezers[3] (OET) is an optical manipulation techniques utilizes light-induced dielectrophoresis (DEP). OET provides noninvasive optical manipulation of cells with single cell resolution. Cell sorting utilizes OET devices have been demonstrated. Cells have been sorted based on viability[3] and types[4]. The development of Phototransistor-based OET (Ph-OET) [5] further provides the capability of actuation of cells in cell culture media and physiological buffers, an important feature for handling more sensitive cells. Here, we demonstrate fluorescent-activated sorting of differentiated neurons by combining a novel cell culture technique[2] and optoelectronic tweezers [3] (OET) opto-fluidics.

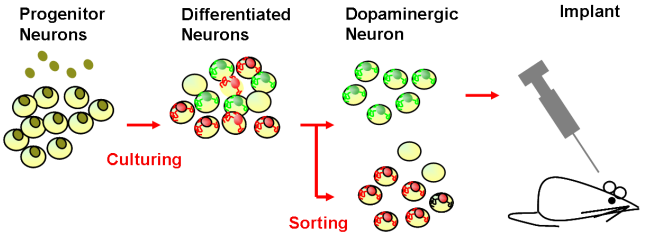


Fig. 1 Process for cell replacement therapy for Parkinson's disease. The steps includes culturing neurons, sorting of neurons, and implant dopaminergic cells to the damaged tissue.

DEVICE PRINCIPLE

Spherical Glass Beads as Movable Growth Substrate

Neurons grow and attach to its growth substrate through the binding of adhesion proteins. The binding force is in the range of a few nano-Newton, far exceeding the force available for conventional cell manipulation techniques, such as DEP, optical tweezers and hydrodynamic forces. Therefore, traditionally, cells can only be handled as suspension particles. One way to overcome this limitation is to use micro-spherical glass beads as both the growth substrate and the carrier for cell manipulation. Growing on micro-spherical beads, these neurons (Fig.2b, c) can be transported without dissociation from its growth substrate. With their large curvature, these beads mimic the conventional planar cover slips in typical cultures. It has been shown that neurons cultured on these beads can reach maturation, and can be subjected to genetic manipulation and treatments[2].

Phototransistor-Based OET

These glass beads have typical diameter of 45um to 120um. Ph-OET device can effectively manipulate particles with this dimension. Ph-OET has the advantage of being non-invasive, highly parallel, and providing specific single cell resolution. Fig. 2 shows a schematic illustrating the principle of Ph-OET manipulation of neurons on beads. Ph-OET device consists of sandwiched layers of transparent indium-tin-oxide (ITO) electrode, liquid media containing the cells, a phototransistor array, and a bottom electrode. To actuate the device, an AC voltage is applied between the top and bottom electrodes, and optical patterns are projected onto the phototransistors through the ITO electrode. Without optical illumination, the phototransistors are insulating; thus, the majority of applied voltage is dropped across the phototransistor. With optical illumination, the phototransistors become conductive, so the majority of the voltage is dropped across the liquid layer. This creates a non-uniform electric field in the media. The interaction between the electric field gradient and the induced electric dipole of the particles give rise to a DEP force. Cell culture media and saline buffers have typical conductivity at 1.4 S/m, and the cells experiences negative DEP (nDEP) forces in these media. nDEP force repels the cells away from the electric field maximum. In OET, the electric field maximum corresponds to the optical patterns; therefore the optical pattern acts as a virtual wall that pushes against the cells. Complex optical patterns can be generated with spatial light modulator, allowing transporting and trapping of multiple cells in parallel.

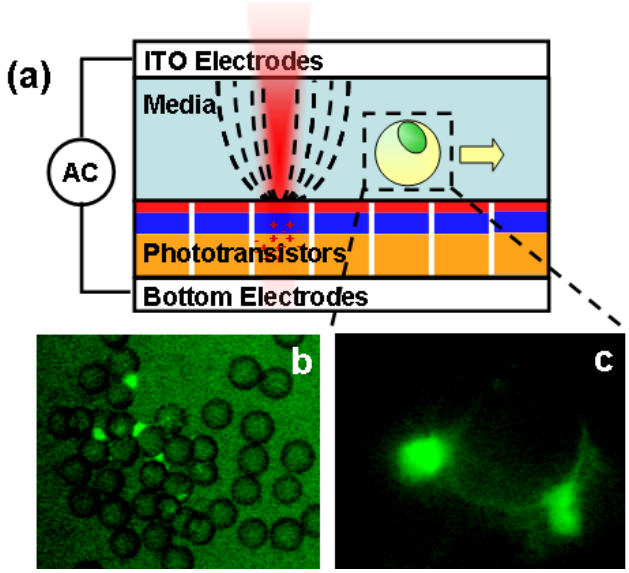


Fig 2. (a) Principle of Ph-OET actuation of differentiated neurons with glass beads as supporting substrate. (b-c) superimposed bright field and fluorescent images of neurons cultured on 45μm glass beads.

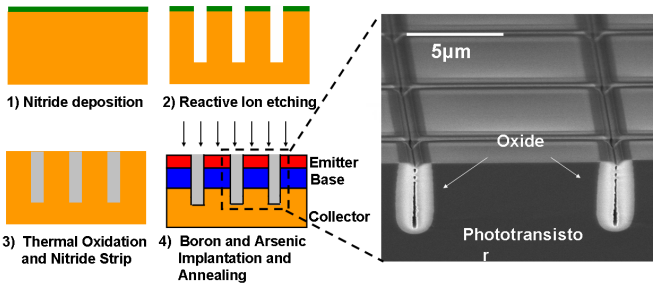
EXPERIMENTAL

Cell Culture

In these experiments, Primary rat hippocampal neurons were cultured on silica beads with 45μm diameter with the following protocol[2]. Borosilicate glass sphere is sterilized in ethanol and dried. The beads are coated with poly-L-lysine (PLL) by immersing the glass beads in 1 mg/mL PLL solution overnight. Hippocampi from embryonic rats were treated with trypsin, and washed. The dissociated cells were plated on PLL-coated glass beads and maintained in neurobasal medium. Cells were cultured for at least 2-4 days, when the processes extend out from the neural cell body.

Device Fabrication

The phototransistor arrays were fabricated on a highly-doped substrate with a 5-μm thick, n-doped epitaxially-grown silicon layer with resistivity of 5-10 Ω-cm. Fig. 3 shows the Ph-OET substrate fabrication process. A 100nm silicon nitride was deposited with LPCVD process at 850°C. Pixels of phototransistor were then defined by lithography in 10um x 10um sizes with 350nm spacing. Reactive ion etching (RIE) etched through both the silicon nitride and the silicon to create electrical isolation between etch pixels. To fill the void created by the etching process, 1μm of silicon dioxide is thermally grown in between the gaps, while silicon nitride film served as an oxidation mask protecting the top surface. The silicon nitride is then removed with phosphoric acid at 160°C. The silicon is then doped with the N+-P-N phototransistor doping profile by two ion implantation followed by annealing steps described below. Boron was implanted first with dosage of 2×10^{12} cm^{-2} and energy of 20 keV, followed by drive-in step at 1000°C for 90 minutes in furnace. Arsenic is then implanted with a dosage of 5×10^{15} cm^{-2} and energy of 10 keV, and annealed at 900°C for 15 minutes. Based on the ion implant and annealing process, the doping concentration of the phototransistor can be estimated with Tsuprem4 device simulation software and the result is listed in the table in Fig. 3. This process provides a planar device surface. Finally, the Ph-OET substrate is coated with PEG to reduce cell or beads adhesion onto the device.



Layer	Doping Concentration (cm^{-3})	Thickness (μm)
Emitter (N^+)	1 x 10^{20}	0.15
Base (p)	3 x 10^{16}	0.35
Collector (n)	6 x 10^{14}	4.5

Fig 3. Schematics of the fabrication process of Ph-OET device. The inset is a SEM picture of final device. The bottom table lists the doping concentration and thickness for each N^+-P-N layer.

The Ph-OET substrate was then integrated with glass micro-fluidic channels, and the process is shown in Fig 4. 500nm of amorphous silicon was first deposited onto the borosilicate glass through LPCVD process at 500 ^oC. The thin amorphous silicon served as the etching mask for the subsequence glass etching process. The channel was defined with lithography, followed by a RIE etching of the amorphous silicon. The glass substrate was then immersed in 49% HF solution for 20 minutes to form the fluidic channels 150μm in height. An ITO thin film was then e-beam evaporated onto the glass, and annealed in N_2 at 300^{o}C for 30mins. To bond the channel onto Ph-OET substrate, a thin wet PDMS was stamped onto the ITO side of the glass substrate as adhesive. Pressure was then applied between the Ph-OET and glass channel. Care was taken to prevent PDMS from entering the channels area.

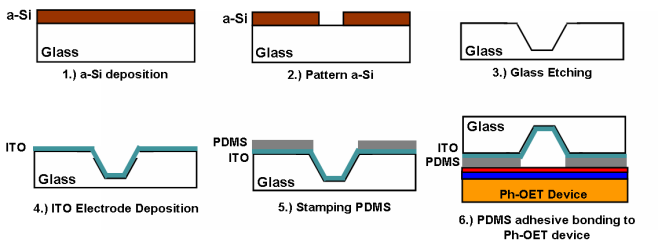


Fig 4. Fabrication process of micro-fluidic channels integration with Ph-OET. The channels were fabricated on Borosilicate glass wafers. The channels were created by HF glass etching. To bond the channels onto Ph-OET device, a thin layer of wet PDMS was applied as the bonding adhesive.

RESULTS

Characterization of Manipulation of Neurons with Ph-OET

In these experiments, optical patterns were generated with a commercial digital projector. The projector light was focused through a 20x objective lens, and has a light intensity of 1.5 W/cm^2 when focused on the sample. Fig.5 shows an example of trapping and moving of single differentiated neurons using Ph-OET device. A square optical pattern was used to trap the glass beads and the neuron to its center. As the optical patterns moves within the field of view, the cell was transported along with the square optical image.

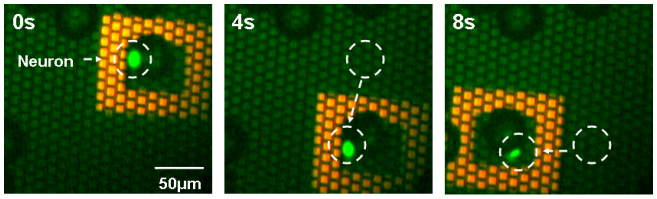


Fig 5. Image sequences of manipulation of single differentiated neuron. Square enclosing optical patterns create a stable trap in the center. Digital projector allows dynamic control of the optical trapping. The neuron on bead moves according to the optical pattern.

The neurons manipulation speed is a function of applied voltage. To measure the actuation speed, a 50μm line pattern was projected onto the device, and a motorized stage was used to move the Ph-OET device at a constant speed in a direction perpendicular to the line pattern. The maximum stage movement speed in which the beads can keep up with the optical line was recorded. A maximum speed of 276μm/s can be achieved with voltage bias of 25Vpp and 1MHz frequency (Fig. 6).

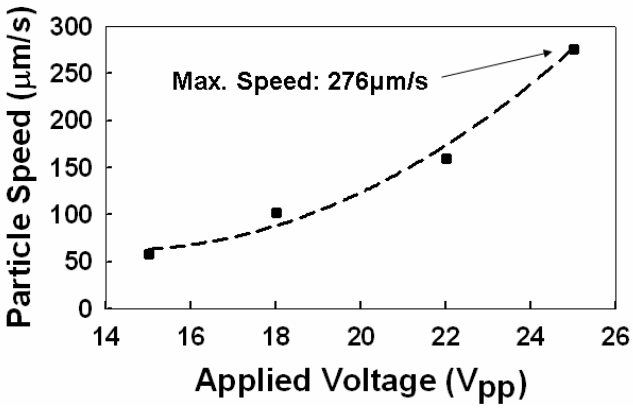


Fig 6. Measured cell manipulation speeds as a function of applied voltage. The voltage bias frequency was 1MHz, and the optical source had a light intensity of 1.5W/cm^2.

Cell Sorting

Automatic detection and sorting of these neurons on beads was implemented (Fig. 5a). The neurons were sorted based on their fluorescence signal. The CCD camera and the projector were synchronized by Labview program. In these experiments, the cells were kept in Phosphate Buffered Saline (PBS) solution, in which neurons can be maintained for more than 12 hours. Before sorting, viable cells were stained with Calcein-AM viability dye and

incubated for 15 minutes in room temperature. Viable neurons on the beads showed green fluorescence; where as dead cells or beads without cells would be dark. The cells were transported to the input reservoir with a 1ml pipette. The typical applied voltage in sorting experiment is 20Vpp. These large glass beads have high mass density compared to PBS solution, therefore gravity play an important role in their movement within the device. Taking advantage of their weight, we utilize gravity to bring a constant flow of the cells into the sorting area by tilting the samples. As cells flow through the channel, the CCD camera detects the green fluorescent emitted from the cells. A custom Labview program tracked the cells' locations, and projects an optical pattern close to the cell with a slight position offset. The optical pattern then repelled the cells to the upper channel, while undetected cells and beads passes through unaffected (Fig. 5 (b-d)). In this manner, only viable cells passed through the upper channels and separate from others. The typical beads traveling speed is 30μm/s. This sorting speed indicates an estimated throughput of 3000 cells/hr.

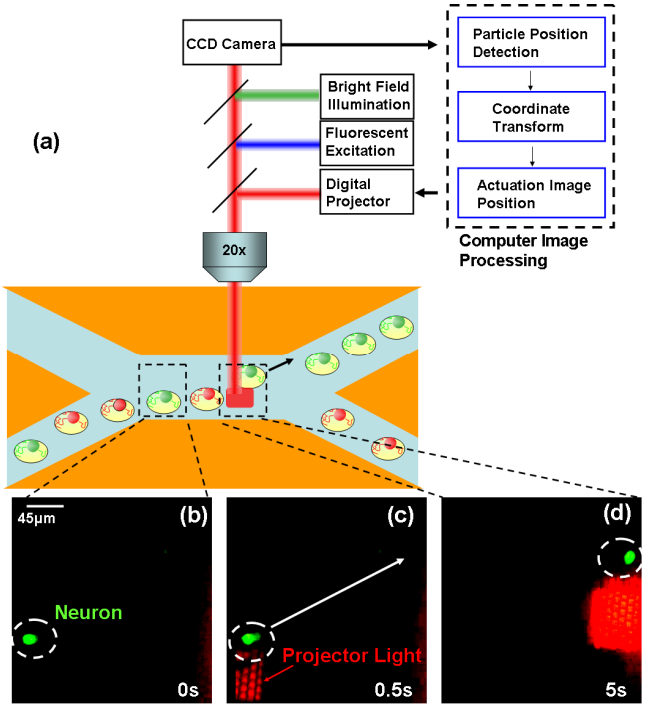


Fig 7. (a) Schematics of Micro-channel integrated Ph-OET device for cell sorting. Cells flow continuously from input to output ports, and cells detected with fluorescence were collected. The process is automated and controlled by Labview program. (b-d) Image sequences of a cell being directed insides the channel upon detection of fluorescent signal. A square optical image with 60μm x 60μm area was projected besides the cell to guild the movement.

CONCLUSION

In summary, we have demonstrated automatic sorting of differentiated neuron. To the best of our knowledge, this is the first time sorting of differentiated neuron cells have been demonstrated. The system utilizes colloid glass carriers as movable growth support, allowing relocation of cells without disrupting cell-substrate adhesion. Ph-OET enables noninvasive manipulation of these neurons with single particle resolution. The sorting device consists of Ph-OET device integrated with micro-fluidic channels. A automatic detection and sorting system is realized. Our computer program synchronizes the CCD camera for detection and the digital projector for cell actuation. The sorting is based on fluorescent signals, and we demonstrate sorting and enrichment of viable neurons. We are aiming for employ this system for cell replacement therapy for nervous diseases. This system may also be applied to other bio-applications where sorting of adherent cells is desired, such as drug screening for neural cells.

ACKNOLOGEMENT

This work was funded by the NIH through the NIH Roadmap for Medical Research, Center for Cell Control, Grant # PN2 EY018228. We also thank Micro-fabrication Laboratory at University of California-Berkeley for assistance in device fabrication.

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