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Single cell and neural process experimentation using laterally applied electrical fields between pairs of closely apposed microelectrodes with vertical sidewalls

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Abstract

As biomedical research has moved increasingly towards experimentation on single cells and subcellular structures, there has been a need for microscale devices that can perform manipulation and stimulation at a correspondingly small scale. We propose a microelectrode array (MEA) featuring thickened microelectrodes with vertical sidewalls (VSW) to focus electrical fields horizontally on targets positioned in between paired electrodes. These microelectrodes were fabricated using gold electroplating that was molded by photolithographically patterned SU-8 photoresist. Finite element modeling showed that paired VSW electrodes produce more uniform electrical fields compared to conventional planar microelectrodes. Using paired microelectrodes, 3μm thick and spaced 10μm apart, we were able to perform local electroporation of individual axonal processes, as demonstrated by entry of EGTA to locally chelate intra-axonal calcium, quenching the fluorescence of a pre-loaded calcium indicator dye. The same electrode configuration was used to electroporate individual cells, resulting in the targeted transfection of a transgene expressing a cytoplasmically soluble green fluorescent protein (GFP). In addition to electroporation, our electrode configuration was also capable of precisely targeted field stimulation on individual neurons, resulting in action potentials that could be tracked by optical means. With its ability to deliver well-characterized electrical fields and its versatility, our configuration of paired VSW electrodes may provide the basis for a new tool for high-throughput and high-content experimentation in broad areas of neuroscience and biomedical research.

Keywords

Microelectrode array; Neurons; Axon; Electroporation; Electrodes with vertical sidewalls; Field stimulation

1. Introduction

In recent years, there has been growing interest in developing experimental methods to study single cells in order to probe deeper into intracellular events and to understand variations among individual cells and not be limited by the averaging effects from studying whole populations of cells (Andersson and van den Berg, 2004). Single cell analyses benefit from

methods in which different cells on a single platform can be individually subjected to widely varying conditions and observed. At smaller scales, there is even need for localized stimulation or delivery of material into specialized, sub-cellular structures. The new experimental techniques at the microscale have come to rely in part on the various functions performed by microelectrodes. Historically, the use of microelectrodes to deliver localized electrical fields to individual cells has yielded many proven applications in basic biomedical research and in drug discovery (Albrecht et al., 2004; Huang et al., 2007; Jain and Muthuswamy, 2007; Lin et al., 2004; Pearce and Williams, 2007; Pine, 2006; Rajaraman et al., 2007; Ravula et al., 2006; Stett et al., 2003; Voldman et al., 2002). Microelectrodes have typically been used to stimulate and record signals from electrically active cells, such as neurons and muscle cells (Ravula et al., 2006; Stett et al., 2003; Taketani and Baudry, 2006). However, microelectrodes have also been used to transiently open cell membranes via electroporation to deliver normally impermeant materials, such as macromolecules and genetic material, into cells (Chang et al., 1992; Fox et al., 2006; Huang et al., 2007; Jain and Muthuswamy, 2007; Lee et al., 2006; Lin et al., 2004; Olofsson et al., 2003; Yuan, 2007). More recently, AC electrical fields have been used to spatially manipulate or trap cells based on the principle of dielectrophoresis (Albrecht et al., 2004; Gascoyne and Vykoukal, 2002; Voldman et al., 2002; Wang et al., 2007).

Microelectrodes are now routinely produced in array formats using techniques from microelectronic fabrication (Pearce and Williams, 2007; Stett et al., 2003; Taketani and Baudry, 2006). The basic construction of such microelectrodes consists of planar metal films lithographically patterned and etched on a planar glass substrate. However, a drawback of these planar electrodes is the high non-uniformity of electric fields induced along the edges of these electrodes (Wang et al., 2007). To circumvent this fundamental disadvantage, alternative electrode configurations have been devised to reduce field non-uniformities. In particular, the fabrication of thickened, three-dimensional electrodes eliminates sharp edges along the substrate while also reducing the impedances between electrode and sample (Barbier et al., 2006; Gross et al., 2002; Heuschkel et al., 2006; Rajaraman et al., 2007; Thiebaud et al., 1997; Voldman et al., 2002; Wang et al., 2007). These raised structures can range from simple “hillocks” (Thiebaud et al., 1997) or etched conical structures (Heuschkel et al., 2006) to specifically molded electrodes shaped like posts, pillars, or blocks rising vertically from a substrate, which could then be integrated with various microfluidic devices (Rajaraman et al., 2007; Voldman et al., 2002; Wang et al., 2007). Designs involving these specifically shaped electrodes have taken advantage of photolithographically patterned photoresist to serve as molds to precisely shape the deposition of metal by electroplating (Rajaraman et al., 2007; Song and Ajmera, 2003; Voldman et al., 2002; Wang et al., 2007). Electrodes with smooth, vertical sidewalls have been produced using this method, and some investigators have used these electrodes to apply electrical fields horizontally, with biological samples positioned in the specific target region between electrode sidewalls. This use of laterally applied electrical fields from vertical sidewalls provides a greater spatial uniformity in field strength and also confers an important advantage for imaging, since the cells or subcellular structures of interest are positioned in between and not obscured by the electrodes. So far, this concept for microelectrode design has only been used on cells freely suspended in fluid media, and the spacing between electrodes have been much larger than the individual cells (Voldman et al., 2002; Wang et al., 2007).

In this study, we present a refined method of making paired vertical sidewall (VSW) electrodes that permits sidewalls to be closely apposed to direct uniform electrical fields in a confined volume in order to exclusively target individual cells and even subcellular processes. We focused on adherent cells and their cellular processes since many cell types require adhesion to a substrate in order to differentiate, polarize, function, and survive. We paid particular attention to neurons, because they are electrically active cells and are highly

polarized, projecting long axons, which are highly specialized sub-cellular structures essential for neuronal function. In this study, we demonstrate the capability of paired VSW electrodes for experimentation on isolated individual axons within a dense neuronal field and to direct electrical fields specifically to these subcellular structures to perform focused field stimulation of individual axons and localized, axonal electroporation for delivery of reagents. On the scale of whole cells, we also demonstrate that paired VSW electrodes can likewise focus electroporation to an individual cell from amongst a large population to perform targeted gene transfection.

2. Methods

2.1. Finite element modeling

To illustrate the benefits of using electrical fields applied laterally between two closely spaced vertical sidewall electrodes, finite element modeling of the steady-state electrical field in the region between electrodes on glass and immersed in low-conductivity media was performed using COMSOL Multiphysics. We compared the electrical fields generated by a pair of planar electrodes with the fields generated by a pair of raised, three-dimensional VSW electrodes (Fig. 1, S1). For this demonstration, the voltages along the surface of opposing electrodes were held to explicit potentials of 1 and 0V, respectively. The modeling was performed in a two-dimensional environment and depicted the vertical cross-sections of the opposing electrodes. For each type of electrode (planar vs. VSW), the induction of transmembrane potentials in axonal processes of neurons was represented in two separate situations by: (1) positioning a circular cross-section (representing an axon) midway in between electrodes; or (2) in a separate simulation, by positioning the axon immediately adjacent to one of the electrodes (Fig. S1 in Supplementary material).

2.2. Electrode fabrication

The fabrication of VSW electrodes was based on previous methods for creating raised gold microelectrodes (Song and Ajmera, 2003; Voldman et al., 2002; Wang et al., 2007) but also included important augmentations to the fabrication process to produce paired microelectrodes that were not only shaped with vertical sidewalls but also positioned in close apposition, separated by only 10 μ m. Briefly, a foundation of titanium and gold was deposited via ion beam evaporation on clean pyrex wafers. The first layer of photolithography patterned the gold film to serve as the outline for both the raised electrodes and planar electrical traces. In the second lithographic step, SU-8 25 (MicroChem Corp.) was spin-coated onto the wafer, and the footprint for the raised electrodes was then exposed and developed onto the SU-8 negative photoresist, forming the mold for electroplating the gold VSW electrodes (Fig. 2, Step 2). To perform the electroplating, the wafer, connected to a current source as the cathode, was immersed into a stirred, heated electroplating solution (TSG-250, Transene), along with an opposing platinum mesh anode. A steady current of 0.01mA/mm² of open area was applied to the wafer, with a resulting gold deposition rate of about 0.1 μ m/min. For paired electrodes spaced 10 μ m apart, a gold thickness of over 10 μ m could be achieved, though 3 μ m was typically desired (Fig. 2, Step 3). After completing the deposition of the raised electrodes via electroplating (Fig. 2, Step 4), the SU-8 photoresist was stripped, leaving precisely molded gold electrodes (Fig. 3, S2). Bare titanium was then stripped. The pyrex wafer, with completed electrodes was then diced and packaged for use in a biocompatible cell culture dish with electrical interconnects (Fig. S2 in Supplementary material). Each culture dish consisted of over 90 electrode pairs, in three separately addressable sets of 30.

2.2.1. Challenges for closely apposed electrodes—While electroplated, three-dimensional electrodes have previously been demonstrated using SU-8 molding, a particular

challenge for our application was the fabrication of paired VSW electrodes that were spaced only 10 μ m apart, which required a small, vulnerable strip of SU-8 to remain firmly adhered to the substrate between closely apposed electrodes during the electroplating process but then be cleanly removable afterwards (Fig. 2, Step 3, asterisk). During electroplating, delamination of this strip from the substrate resulted in misshapened electrodes, footing along the base of the electrodes, or overt electroplating of gold under the SU-8 and bridging of the electrode pairs. By contrast, the SU-8-based molding of gold demonstrated by Voldman et al. (2002) and Wang et al. (2007), had been used to fabricate microelectrodes with wider spacing and thus did not require such narrow strips of SU-8. Further, Voldman's electrodes benefited from their cylindrical shape, which minimized delamination of surrounding SU-8 (Voldman, 2001), while for Wang et al., the inter-electrode spacing was sufficiently large that imperfections along the base were relatively unimportant. However, Song and Ajmera (2003) did produce closely spaced, electroplated structures and addressed the associated difficulties by inserting a thin foundation of a standard, hard-baked photoresist underneath the SU-8 to serve as a release layer that would allow the SU-8 to be easily removed after the electroplating process. This additional layer required an additional photolithographic step. For our process, we used SU-8 exclusively and placed priority on maintaining the integrity and adhesion of the SU-8 through thorough cleaning of the substrate, omission of the usual underlying Omnicoat release foundation under the SU-8, and proper baking. To obtain smooth and straight sidewalls for the SU-8 mold, we permitted only wavelengths above 350 nm to expose the SU-8 during photolithography and limited the total exposure energy to 210mJ/cm². After the electroplating process, however, the adhesion and stability of the SU-8 prevented its easy removal using standard stripping solution (SU-8 Stripper, Microchem Corp.) or even plasma ashing. Much of the hardened and adherent SU-8 within the 10- μ m wide gap region between VSW electrode pairs remained. However, these remnants could be stripped by brief treatment of heated piranha solution (3:1, H₂SO₄:H₂O₂), which did not attack the gold. The refinements that we added to the SU-8-based molding of electroplating electrodes yielded a process that did not employ a separate photoresist layer underlying the SU-8 but could nevertheless reliably produce closely apposed, thickened electrodes with smooth, vertical sidewalls and separated by a clean 10 μ m gap.

2.3. Cell culture

For cell culture, the gold surfaces of the VSW electrodes were rendered cell repellant by deposition of a self-assembled monolayer of methyl-poly-ethylene-glycol (mPEG-SH, Nektar), which only assembled on the gold surface and not on the glass substrate. The glass surface was then coated with properly oriented L1-Fc adhesion molecules on the glass surface as performed by Suh et al. (2004) (details in Supplementary material). The use of L1 (Kamiguchi and Lemmon, 1997) adhesion molecule was intended to enhance the affinity for axons to the glass substrate and to minimize the fasciculation of axons with each other, thus increasing the incidence of isolated, individual axons extending into the target regions between closely apposed VSW electrode pairs.

Neurons were obtained using established protocols. Briefly, hippocampi were surgically removed from dissected brains of embryonic day 15–16 mice, and cells were isolated via trituration and enzymatic digestion (Brewer et al., 1993; Kaech and Banker, 2006). Neurons were plated directly onto the coated substrates containing electrodes and maintained in Neurobasal media (Gibco Invitrogen) supplemented with B27 (Gibco Invitrogen) and Gluta-MAX (Gibco Invitrogen). Cultures were maintained for 3–7 days to allow neurons to project axons from the cell bodies. Electrode demonstrations were performed on axons that extended into the confined gap region in between closely apposed VSW electrodes. In our unguided culture, a plating density of 400 cells/mm², 11 $\pm$ 7% of the paired electrodes had

axons extending through, while at a plating density of 800 cells/mm², 26±7% had axons in between.

For culture of 3T3 fibroblasts, a substrate of adsorbed poly-lysine was sufficient for attachment and growth. Cells were deposited onto the VSW electrode arrays and maintained in minimal essential media supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin. Cultures were maintained for several days to permit the cells to proliferate. Electroporation demonstrations were performed on cells situated in the target area between paired VSW electrodes.

2.4. Electrical signal

Electrical signals were delivered to the VSW electrodes using a custom-built circuit similar to the one developed by Lee et al. (2006), which was modeled after a potentiostat to stabilize the voltage delivered to the electrodes, regardless of the load impedance between them. A function generator (Agilent 22330A) provided the desired waveforms, and a power supply (Agilent E3631A) supplied the necessary current to the circuit, enabling it to track the waveform provided by the function generator. The voltage delivery circuit monitored various types of waveforms faithfully and had a bandwidth of over 100 kHz.

2.5. Electroporation

Axon electroporation was detected using the influx of extracellular EGTA (MW 380, Sigma–Aldrich), a calcium chelator, into the intracellular domain of neurons and axons followed by the optical monitoring of free intracellular calcium using fluorescent calcium indicators that were pre-loaded into the neurons. Due to the high affinity of EGTA for calcium, even a small quantity of EGTA introduced into the cytoplasm can significantly quench the fluorescence of the calcium indicator dye. Prior to electroporation, neurons cultured with the arrays of paired VSW electrodes were pre-loaded with the calcium indicator Fluo-4AM (Invitrogen) using established protocols. Briefly, 10μM of Fluo-4 AM was introduced into the culture media and incubated with the cells for 20min at 37°C. Next, the culture was washed twice with PBS, and an electroporation media with 20mM of EGTA was introduced. This media, which was of much lower conductivity (~320 mS/m) than normal culture media, was buffered with MOPS and adjusted to a pH of 7.2 under atmospheric conditions. The VSW electrode array, with the live neurons and their axons, was then mounted on a heated microscope stage, and electrically connected to the signal delivery circuits. To perform the actual electroporation, five trains each with five 400μs biphasic pulses were delivered over a period of 100ms to axons located specifically in between VSW electrodes. The voltage levels were adjusted at 0.5V increments to determine the threshold at which visible changes occurred (i.e. fluorescence quenching). Before and after the delivery of electrical signals, time-lapse images were recorded by a Retiga Q-Imaging EXi, cooled CCD camera mounted on a standard inverted microscope (Nikon TE 2000) under 20× objective magnification using a FITC filter with illumination by a 150WHg lamp (Optiquip). The sequences of images were recorded on a desktop PC operating Simple PCI Imaging software (Hamamatsu Corporation). This time-lapse sequence provided the means to accurately measure the magnitude of the quenching of fluorescence as well as the time scales associated with the spread of EGTA along the axon and ultimately to the cell body.

2.6. DNA plasmid transfection

3T3 fibroblasts cultured on the VSW electrode arrays were used for gene transfection studies. Electroporation was performed on near confluent cultures using 300μg/mL of an EGFP plasmid in a low conductivity media (~60 mS/m). The electroporation wave-form (described in the previous section) was applied at 5V, and the cells were incubated an

additional 5 min in the poration media, with the plasmids. The cells were then washed with PBS and returned to the normal culture conditions in MEM media with 5% FBS and at 37°C. After 6 h of incubation, the cells were observed under the microscope. Successfully transfected cells were fluorescent as seen under a FITC filter due to the synthesis of freely soluble cytoplasmic GFP protein arising from EGFP plasmid.

2.7. Field stimulation

The demonstration of field stimulation used high-speed optical imaging of the changes in transmembrane potentials of neurons and axons. The neurons were pre-loaded with the voltage-sensitive di-8-ANEPPS dye (Invitrogen) using established protocols. The culture was then washed, and a non-HEPES buffered, CO₂-independent media (Hibernate E Media with Low-Fluorescence, BrainBits, LLC) was introduced to the culture. To demonstrate field stimulation, we identified neurons whose axonal hillocks were positioned roughly in the target area between two opposing VSW electrodes. Real time changes in membrane voltage were monitored using a NeuroCCD System (Redshirt Imaging LLC) with a SciMeasure 80×80 CCD camera mounted on the microscope. The supporting Neuroplex software captured images at 0.5ms intervals and was coordinated with the stimulation delivered to the electrodes. The recorded images were then processed within the Neuroplex software to track the progression of axon depolarization from the point of stimulation.

3. Results

3.1. Finite element modeling of electrical fields

To illustrate the benefits of VSW electrodes, we compared finite element modeling of the electrical fields resulting from a 1-volt difference between a pair typical planar electrodes vs. a pair of our VSW electrodes in a low conductivity media (Fig. S1 in Supplementary material). For example, a pair of planar electrodes, spaced 10μm apart, will have an electrical field profile that varies in strength by a factor of four, with very high field strengths at the electrode edge and much lower strength mid-way in between (Fig. S1A and C in Supplementary material). In contrast, for a pair of VSW electrodes 3μm tall and likewise spaced 10μm apart, the field strength varied only by approximately 30% along the substrate in between the sidewalls (Fig. S1B and C in Supplementary material).

3.2. Rational design of VSW electrodes for axons

Since we intended our VSW electrodes to focus electrical fields on single cells and subcellular components, we set the gap between electrode pairs at 10μm. While the electrical fields applied by VSW electrodes are more uniform than those induced by planar electrodes, the parameter most important for use on individual cells is actually the variation of transmembrane voltages induced at various locations between the electrode pairs. For a linear axonal process in between an electrode pair and running perpendicular to the applied electrical field lines (Fig. 1A), the variation in transmembrane potential can be explicitly calculated by applying our FEM modeling to the two limiting cases with low conductivity media and glass substrate: (1) the axon (represented as a 1-μm diameter, circular cross-section) positioned immediately adjacent to an electrode; and (2) the axon positioned midway. The difference in the induced axon membrane potential between these two cases (Fig. 1B) was greatest when using the thinnest electrodes (Fig. 1B, S1D–G): the axon adjacent to an electrode experienced approximately double the membrane potential of the axon positioned midway. However, this disparity diminishes as the thickness of the electrode is increased, finally reaching zero with electrodes approximately 3μm thick (Fig. 1B, F–G). Thus, for axons with approximately 1μm diameter, the optimal thickness for VSW electrode pairs spaced 10μm apart is an electrode thickness of about 3μm. With VSW electrodes at this thickness, the induced axon membrane potential is only very slightly

affected by its position within the 10 μ m gap. Based on this information, we fabricated arrays of VSW microelectrodes using these specifications (Figs. 2 and 3).

These simple computational models highlighted the benefits of using raised VSW electrode pairs with laterally applied electrical fields. The greater accuracy in controlling applied electrical fields and transmembrane potentials is essential for more refined experimentation on individual cells and subcellular processes.

3.3. Single axon electroporation

To demonstrate the ability of closely spaced VSW electrode configurations to target subcellular elements, we directed electrical fields to individual axons running in between VSW electrode pairs and performed highly localized electroporation on these neuronal processes. For this demonstration, neurons were pre-loaded with a fluorescent calcium indicator, and maintained in a low conductivity (~320 mS/m) media with 20mM of EGTA (Fig. 4A). Biphasic pulses were applied across the VSW electrode pairs. When poration was achieved, the otherwise impermeable EGTA locally entered the axon, chelating the intra-axonal calcium and locally quenching the Fluor-4 fluorescence. The electrical threshold for successful fluorescence quenching was achieved at 2.0–4.5V ($n = 12$, mean $\pm$ SD = 3.7 $\pm$ 0.8 V). By contrast, in our control tests using calcium free media with only 1mM EGTA, this quenching was never observed in response to electrical signals. Based on our FEM modeling, this applied voltage across electrode pairs corresponded to a transmembrane voltage of 240–540mV for a 1 μ m diameter axon, which is consistent with the generally accepted range of voltage thresholds for membrane electroporation (Chang et al., 1992).

Upon electroporation, the intra-axonal fluorescence was immediately quenched locally, while the fluorescence of the cell body and surrounding neurons remained unchanged (Fig. 4B and C). This dimming was clearly visible using time-lapse fluorescence imaging. In many cases, the quenching of fluorescence subsequently spread along the axon over the course of 10–20 s, eventually reaching the neuronal cell body (Fig. 4D). This progression of quenching most likely resulted from the diffusion of EGTA from the region of initial entry towards the more distal and more proximal segments of the axon and eventually the cell body, where it continued to chelate intracellular calcium. The quenching of fluorescence appeared to progress faster along the distal reaches of the axon compared to the proximal segment likely as a result of diffusion of free calcium out of the cell body and transiently replenishing calcium being sequestered at the proximal axonal segment by the advancing EGTA.

Following the electroporation treatment, the axons remained morphologically intact, and the parent neuron appeared to remain viable. In preliminary studies employing the same paired VSW electrodes to study the effects of various electrical stimulation protocols on axon cell biology, we found that the application of these brief electrical pulses not only allowed membrane integrity to be maintained, but likewise did not have any apparent effect on critical aspects of axonal biology such as the normal transport of organelles within axons (unpublished work; manuscript in preparation).

3.4. Whole cell electroporation and transfection

In addition to the demonstration of local axonal electroporation, paired VSW electrodes were also used to electroporate whole cells. Using 3T3 fibroblasts cultured on the electrode array in the same manner as neurons, we performed gene transfection experiments on cells positioned in the gap between electrode pairs. Electroporation was performed in the presence of a low conductivity media containing 400 μ g/mL of EGFP plasmids and with a train of 10 biphasic pulses, 400 μ s wide and at 5V. This voltage was deliberately chosen to

be somewhat higher than the threshold for electroporation, since immediate feedback regarding membrane poration was not possible in this demonstration. Following electrical pulses, the electroporation medium was exchanged with a normal maintenance medium. After 6 h of incubation following electroporation, we observed cells positioned in between the electrode pairs that were fluorescent and thus successfully transfected (Fig. 5, S3). More extensive studies are required to identify the voltage thresholds required for whole cell transfection via electroporation using paired VSW electrodes and to quantitatively characterize its rate of success.

3.5. Field stimulation of neurons

VSM electrode configurations were also used to perform the more conventional function of directed field stimulation of individual axons. Stimulation signals were applied through a VSW electrode pair to trigger local axonal depolarization of sufficient magnitude that was then propagated as an action potential along the length of the axon. Because of the compatibility of VSW electrode configurations with optical imaging, the depolarization and the lateral spread of the resulting action potential was tracked with optical means with the neurons pre-loaded with voltage-sensitive dyes. Successive images captured at 0.5ms intervals revealed the progression of membrane depolarizations representing the conduction of an action potential along the axon (Fig. 6).

4. Discussion

We demonstrated the fabrication and different uses of pairs of closely apposed vertical sidewalls electrodes in delivering focused and well-characterized electrical fields to a highly localized volume suitable for single cell and even single axon experimentation. VSW electrodes spaced only 10 μ m apart were fabricated by precise molding of gold deposited via electroplating on a planar glass substrate. This configuration of microelectrodes applied more uniform electrical fields compared to conventional planar electrode configurations constructed from thin conductive films. Using VSW electrodes, we delivered small molecules exclusively into specific segments of individual axons from primary neuronal cultures and monitored the effects in real time. We also demonstrated that electroporation can be applied at the whole cell level using VSW electrodes by successfully transfecting cultured 3T3 cells positioned in between electrodes with a gene encoding soluble green fluorescent protein (GFP). Finally, we demonstrated that VSW electrodes were effective for extracellular stimulation of individual axons. Due to the optical compatibility of our electrode configuration, the depolarization and spread of action potentials along the axon could be monitored optically in real time, allowing the experimentalist to both precisely trigger and monitor action potential generation and conduction in the same axon.

Our development and specific uses of closely spaced VSW electrode pairs was motivated by the increasing need for microscale probing of individual cells and subcellular components for functional analysis. Electroporation has emerged as a versatile and widely used mechanism for intracellular delivery of membrane-impermeable molecules and gene transfections. For single cell electroporation and delivery, numerous methods have been developed for both *in vivo* and *in vitro* settings but usually require the positioning of user-operated electrodes, capillaries, or micropipettes to address one cell or a small group of cells at a time (Fox et al., 2006; Olofsson et al., 2003; Yuan, 2007). More recently, microelectrode arrays (Huang et al., 2007; Jain and Muthuswamy, 2007; Lin et al., 2004; Olofsson et al., 2003) and microfluidic devices (Fox et al., 2006; Olofsson et al., 2003) have also been developed for such uses. However, attempts on subcellular electroporation have been rare despite the potential uses for such techniques. In one demonstration, a pair of manually positioned, carbon-fiber microprobe electrodes was used to porate a nascent process projecting from individual hippocampal progenitor cells in culture (Lundqvist et al.,

1998). The electroporation permitted the influx of extracellular calcium into the process and eventually the whole cell. Another study identified the discrete locations of electroporation on a cell's membrane under a given electrical field but did not explicitly control or restrict the poration to any particular subcellular region (Teruel et al., 1999; Teruel and Meyer, 1997). In contrast to these demonstrations, our configuration of paired VSW combined precise spatial control as well as real-time observation of electroporation. In addition to performing electroporation, paired VSW electrodes also served the more conventional function of field stimulation of cultured neurons. The benefit of our focused electrical fields is the ability to target and stimulate a specific neuron without effecting neighboring cells. Meanwhile, the uniformity of the electrical field permits the identification of specific voltage ranges that are sufficient to trigger stimulation of the target while avoiding disruption of the cell membrane. With laterally applied electrical fields, this configuration is also well-suited for the increasingly popular use of voltage-sensitive fluorescent dyes and real-time optical imaging of membrane depolarization and propagation of action potentials (Djurisic et al., 2003; Loew et al., 1992).

Our particular interest in the axonal processes of neurons is due to their importance in intracellular transport and the conduction of action potentials. Given the axon's essential role, substantial research has been focused on axon development as well as mechanisms governing axon biology in health and disease (Coleman and Perry, 2002). Here we have developed a versatile electrode array that can apply well-defined and targeted electrical fields to specific neurons and axons while simultaneously permitting the observation of intracellular and intra-axonal activities, such as axonal transport as well as high-speed events like the propagation of action potentials. The ability to locally introduce experimental drugs, molecules, and reagents exclusively into a specific axonal segment via electroporation represents a new experimental technique that will likely be useful in studies of axonal function and architecture. For example, axon electroporation may allow researchers to specifically intervene in axonal processes in studies of axonal responses to injury or regeneration. Moreover, even our specific assay of delivering EGTA to chelate intracellular calcium can itself be used to switch off the ability of specific neurons to propagate action potentials, which depends on calcium. Such a technique could, for example, be used to functionally silence specific neurons in a neuronal circuit. By combining these functions with the capability to perform field stimulation on specific neurons, our VSW electrode system can greatly enhance the variety of experimentation on single neurons and single axons while increasing the content that can be obtained. The broad variety of experimental topics that can be addressed by our device ranges from studies of neural circuit behavior, synaptic plasticity, learning and memory, to applications within neural machine interfaces. Furthermore, our VSW electrode system can be augmented with the integration of cell micropatterning techniques (Chang and Wheeler, 2006; Chang and Sretavan, 2008) that are compatible with the fabrication of the VSW arrays. Thus, while our current configuration of VSW arrays consists of only three independently addressable sets of electrodes, precise micropatterning and guidance of axonal outgrowth along with careful redesign of array configuration can increase the number of individually addressable channels. This will permit explicit positioning and alignment of neurons and axons relative to specific VSW electrodes to possibly form high-density arrays for high content and highly parallel experimentation.

5. Conclusion

We have proposed, fabricated, and demonstrated the use of paired vertical sidewall electrodes to apply focused and well-defined electrical fields to individual cells and cellular processes. This configuration was motivated by the need for microelectrodes that generate more uniform (and thus more easily characterized) electrical fields. As demonstrated by finite element modeling, there is much less uncertainty regarding the field strength

experienced by targets placed between closely apposed VSW electrodes in contrast to targets placed in proximity to more conventional planar, thin film-based electrodes. Using this VSW configuration, we have demonstrated several important capabilities for studying cells and neurons. Specifically, we performed targeted electroporation on individual axons that are only 1–2 μm in diameter. We also showed that individual cells could be genetically transfected via electroporation. Specifically for neurons, the VSW electrodes can also be used to induce transmembrane depolarization in a single neuron leading to the propagation of action potentials. Because electrical fields are applied laterally from the electrode sidewalls, all of these demonstrations could be viewed in real time with targets positioned in between the electrodes, thus providing an important advantage over conventional microelectrodes. Since the paired VSW electrodes may be reconfigurable into higher-density arrays, they may form the basis for a new tool for high-content and high throughput experimentation and screening on individual cells and cellular processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Albrecht DR, Sah RL, Bhatia SN. Biophys. J 2004;87(4):2131–2147. [PubMed: 15454417]
- Andersson H, van den Berg A. Curr. Opin. Biotechnol 2004;15(1):44–49. [PubMed: 15102465]
- Barbier V, Tatoulian M, Li H, Arefi-Khonsari F, Ajdari A, Tabeling P. Langmuir 2006;22(12):5230–5232. [PubMed: 16732644]
- Brewer GJ, Torricelli JR, Evege EK, Price PJ. J. Neurosci. Res 1993;35(5):567–576. [PubMed: 8377226]
- Chang, DC.; Chassy, BM.; Saunders, JA.; Sowers, A. Guide to Electroporation and Electrofusion. Academic Press, Inc.; 1992.
- Chang, JC.; Wheeler, BC. Advances in Network Electrophysiology. US: Springer; 2006. Pattern Technologies for Structuring Neuronal Networks on MEAs; p. 153-192.
- Chang WC, Sretavan DW. Langmuir 2008;24(22):13048–13057. [PubMed: 18925765]
- Coleman MP, Perry VH. Trends Neurosci 2002;25(10):532–537. [PubMed: 12220882]
- Djurisic M, Zochowski M, Wachowiak M, Falk CX, Cohen LB, Zecevic D. Methods Enzymol 2003;361:423–451. [PubMed: 12624923]
- Fox MB, Esveld DC, Valero A, Luttge R, Mastwijk HC, Bartels PV, van den Berg A, Boom RM. Anal. Bioanal. Chem 2006;385(3):474–485. [PubMed: 16534574]
- Gascoyne PR, Vykoukal J. Electrophoresis 2002;23(13):1973–1983. [PubMed: 12210248]
- Gross M, Altpeter D, Steiglitz T, Schuettler M, Meyer J-U. Sens. Actuators A 2002;96:105–110.
- Heuschkel, MO.; Wirth, C.; Steidl, E.; Buisson, B. Advances in Network Electrophysiology. Taketani, M.; Baudry, M., editors. US: Springer; 2006. p. 69-111.
- Huang KS, Lin YC, Su CC, Fang CS. Lab Chip 2007;7(1):86–92. [PubMed: 17180209]
- Jain T, Muthuswamy J. Biosens. Bioelectron 2007;22(6):863–870. [PubMed: 16635569]
- Kaech S, Banker G. Nat. Protoc 2006;1(5):2406–2415. [PubMed: 17406484]
- Kamiguchi H, Lemmon V. J. Neurosci. Res 1997;49(1):1–8. [PubMed: 9211984]
- Lee ES, Robinson D, Rognlien JL, Harnett CK, Simmons BA, Bowe Ellis CR, Davalos RV. Bioelectrochemistry 2006;69(1):117–125. [PubMed: 16483852]

- Lin YC, Li M, Wu CC. Lab Chip 2004;4(2):104–108. [PubMed: 15052348]
- Loew LM, Cohen LB, Dix J, Fluhler EN, Montana V, Salama G, Wu JY. J. Membr. Biol 1992;130(1):1–10. [PubMed: 1469705]
- Lundqvist JA, Sahlin F, Aberg MA, Stromberg A, Eriksson PS, Orwar O. Proc. Natl. Acad. Sci. U.S.A 1998;95(18):10356–10360. [PubMed: 9724707]
- Olofsson J, Nolkranz K, Ryttsen F, Lambie BA, Weber SG, Orwar O. Curr. Opin. Biotechnol 2003;14(1):29–34. [PubMed: 12565999]
- Pearce TM, Williams JC. Lab Chip 2007;7(1):30–40. [PubMed: 17180203]
- Pine, J. Advances in Network Electrophysiology. Taketani, M.; Baudry, M., editors. US: Springer; 2006. p. 3-23.
- Rajaraman S, Choi S-O, Shafer RH, Ross JD, Vukasinovic J, Choi Y, DeWeerth SP, Glezer A, Allen MG. J. Micromech. Microeng 2007;17:163–171.
- Ravula SK, McClain MA, Wang MS, Glass JD, Frazier AB. Lab Chip 2006;6(12):1530–1536. [PubMed: 17203157]
- Song I, Ajmera PK. J. Micromech. Microeng 2003;13:816–821.
- Stett A, Egert U, Guenther E, Hofmann F, Meyer T, Nisch W, Haemmerle H. Anal. Bioanal. Chem 2003;377(3):486–495. [PubMed: 12923608]
- Suh LH, Oster SF, Soehrman SS, Grenningloh G, Sretavan DW. J. Neurosci 2004;24(8):1976–1986. [PubMed: 14985440]
- Taketani, M.; Baudry, M. Advances in Network Electrophysiology. US: Springer; 2006.
- Teruel MN, Blanpied TA, Shen K, Augustine GJ, Meyer T. J. Neurosci. Methods 1999;93(1):37–48. [PubMed: 10598863]
- Teruel MN, Meyer T. Biophys. J 1997;73(4):1785–1796. [PubMed: 9336174]
- Thiebaud P, de Rooij NF, Koudelka-Hep M, Stoppini L. IEEE Trans. Biomed. Eng 1997;44(11):1159–1163. [PubMed: 9353996]
- Voldman, J. A Microfabricated Dielectrophoretic Trapping Array for Cell-based Biological Assays (Ph.D. thesis). Electrical Engineering and Computer Science. Cambridge, MA: Massachusetts Institute of Technology; 2001. p. 86
- Voldman J, Gray ML, Toner M, Schmidt MA. Anal. Chem 2002;74(16):3984–3990. [PubMed: 12199564]
- Wang L, Flanagan LA, Jeon NL, Monuki E, Lee AP. J. Microelectromech. Syst 2007;16(2):454–461.
- Yuan TF. Cytotechnology 2007;54(2):71–76. [PubMed: 19003020]

Fig. 1.

(A) An axon (dark grey) projecting into a 10 μm gap between a VSW electrode pair (gold). The application of electrical fields (white arrows) in between these electrodes induces a transmembrane potential in the axon segment within the gap. (B) The transmembrane potential induced on the axon in (A) depends on where it is positioned in proximity to an electrode. This plot shows the difference in the induced transmembrane potential for the two limiting cases (shown in the inset) of (1) the axon positioned midway between electrodes and; (2) the axon positioned immediately adjacent to an electrode vs. electrode thickness. This difference is minimized when the VSW electrode is approximately 3 μm thick.

Fig. 2.

Fabrication of paired vertical sidewall (VSW) electrodes. The microfabrication process for these electrodes follows similar methods reported by other groups. (1) A thin foundation of Ti/Au is first deposited on a glass substrate by vapor deposition, and the gold is then patterned and etched via conventional photolithography. (2) Next, the desired footprint of the VSW electrode is patterned in SU-8 photoresist cast over the substrate. The developed SU-8 served as a mold with vertical sidewalls to contain and shape the additional deposition of gold via electroplating. (3) The electroplating step produced gold electrodes up to about 10 μm in vertical thickness. (4) After the deposition, the SU-8 mold was removed, leaving the vertical sidewall electrodes intact. A particular challenge in our fabrication process was to manage the fabrication of VSW electrode pairs spaced just 10 μm apart (note the asterisk in Step 3). During electroplating the SU-8 in this gap had to remain firmly adhered to the substrate. Then, afterwards, the removal of the SU-8 from this gap required improved cleaning techniques to ensure thorough removal of SU-8 remnants while leaving the closely apposed electrode pairs intact.

Fig. 3.

SEM image of an apposed vertical sidewall (VSW) electrode pair, showing the vertical sidewalls and narrow, 10-μm gap in between. This gap region is the target area for individual cells and cellular processes. The gold surfaces are rendered cell repellant by a self-assembled monolayer deposited by mPEG-SH. Our fabrication process was developed to ensure that the sidewalls were vertical and smooth and free of distortions along the base.

Fig. 4.

Targeted electroporation of an individual axon. (A) The axon that projected into the area in between the VSW electrode pair (grey outline) was pre-loaded with Fluo-4 calcium indicator and was visualized under fluorescent imaging. (B) A false-color rendering of the neuron and axon based on brightness levels before the electrical pulse is applied. (C) After an electrical pulse is applied between the electrode pair, the membrane along the axon is porated, permitting the influx on EGTA from the surrounding media into the intra-axonal space. Immediately following poration, the entry of EGTA chelates the intracellular calcium, instantaneously quenching the fluorescence (dimming), apparent within 2 s after the pulse. (D) Over the following few seconds, progressive dimming spreads along the axon

toward the cell body and towards the distal end of the axon. At 12 s after the pulse, the dimming along the axonal hillock (segment near the cell body) became apparent.

Fig. 5. Transfection via electroporation. For 3T3 fibroblasts cultured among the electrode, a cell positioned in between the VSW electrode pair was electroporated with transfected with an E-GFP plasmid. This plasmid was subsequently expressed, resulting in the synthesis of GFP protein that freely dissolved in the cell cytoplasm. (Note therefore that the thicker, central portion of the cell appears brighter than the periphery.) Successfully transfected (and viable) cells were visible under fluorescent imaging. Figure shows an overlay of brightfield and fluorescence illumination.

Fig. 6. Field stimulation. (A) Using the VSW electrodes, a stimulating pulse was applied to the target axon in between the electrode pair. A pulse above the depolarization threshold triggers the propagation of an action potential. (B) Membrane depolarization. Using voltage sensitive dyes and optical measurement, the propagation of action potential between positions 1 and 2 along the axon was monitored in real time. The lag in depolarization between region 1 and 2 was about 1 ms.