Automated Targeting of Cells to Electrochemical Electrodes Using a Surface Chemistry Approach for the Measurement of Quantal Exocytosis

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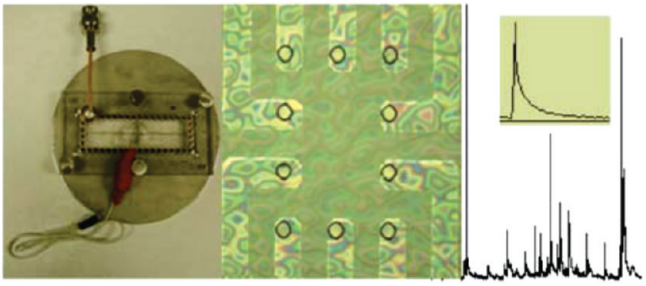
Abstract



Here, we describe a method to fabricate a multichannel high-throughput microchip device for the measurement of quantal transmitter release from individual cells. Instead of bringing carbon-fiber electrodes to cells, the device uses a surface chemistry approach to bring cells to an array of electrochemical microelectrodes. The microelectrodes are small and cytophilic in order to promote adhesion of a single cell, whereas all other areas of the chip are covered with a thin cytophobic film to block cell attachement and facilitate the movement of cells to electrodes. This cytophobic film also insulates unused areas of the conductive film; thus, the alignment of cell docking sites to working electrodes is automatic. Amperometric spikes resulting from single-granule fusion events were recorded on the device and had amplitudes and kinetics similar to those measured using carbon-fiber microelectrodes. Use of this device will increase the pace of basic neuroscience research and may also find applications in drug discovery or validation.

Keywords: Automatic cell positioning, quantal exocytosis, amperometric detection, lab-on-a-chip, microfabrication, electrochemical microelectrodes

Transmitters are signaling molecules that are packaged inside small vesicles within cells. Transmitters are released from cells as vesicles fuse with the plasma membrane and release their contents to the outside of the cell in the process of exocytosis. In excitable cells, the principle stimulus for exocytosis of the transmitter is the elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that results from Ca^{2+} influx through voltage-gated Ca^{2+} channels. Release of the contents of individual vesicles (quantal exocytosis) can be measured using electrochemical microelectrodes placed immediately adjacent to the cell surface (*1*). In particular, carbon-fiber amperometry has been used to detect the spike of current that results from oxidation of biogenic amines released from an individual vesicle that fuses near the surface of the electrode (*2*, *3*). Amperometric spikes are analyzed to determine the amount of transmitter released from each vesicle, the kinetics of the release, and the flux of transmitter through a nanometer-sized fusion pore that often precedes the full fusion reaction (*4*, *5*).

Use of carbon-fiber amperometry together with pharmacological agents or genetic perturbations has greatly contributed to our understanding of the molecular mechanisms of exocytosis. For example, amperometric measurements have shown that the drug L-DOPA provides symptomatic relief for Parkinson's disease by increasing the amount of dopamine packaged into individual vesicles (*27*). In contrast, botulinum toxin A, which is used for the treatment of cervical dystonia in addition to its use as a cosmetic, inhibits exocytosis in part by disturbing the structure of the fusion pore and delaying fusion pore expansion (*28*). However, routine use of the technique for probing the mechanisms whereby drugs and toxins modulate exocytosis has been hampered by the labor-intensive nature of performing experiments using carbon-fiber electrodes.

Electrochemical electrodes on microchips have recently been used as an alternative to carbon-fiber microelectrodes to record quantal exocytosis (*6*−*15*). Microfabricated devices offer the potential for higher throughput and massively parallel recordings and can be batch fabricated to reduce costs. A new challenge with these devices is that, rather than bringing the microelectrode to

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the cell with a micropositioner, one must bring the cell to the microchip electrode. In addition, it is essential that the electrode be very small (cell sized) in order to have an acceptably small level of current noise and to reduce the occurrence of overlapping spikes so that individual spike parameters can be measured. Random seeding of cells on microchip electrode arrays is not ideal because some electrodes do not have an adjacent cell and others have many cells so that there is a low probability of recording release events from a single cell.

Three recent reports describe microfluidic approaches to automatically target cells to electrochemical electrodes (*11*, *15*, *16*). Spegel et al. used solution flow through a small aperture on a chip to bring cells to the working electrode and report a nearly 90% success rate of targeting cells (*11*). Dittami and Rabbitt trapped cells over an electrode using a constriction in a microfluidic channel (*16*). Our group has used multiple microfluidic cell traps etched in silicon to move cells to electrodes (*15*). A drawback of microfluidic approaches is that a balance must be achieved between using a pressure gradient sufficiently large to bring the cell to the electrode yet be gentle enough to avoid cell damage or induce unintended effects on exocytosis, which is sensitive to membrane tension (*17*).

There are many reports of patterning individual cells using cytophilic islands containing extracellular matrix proteins surrounded by cytophobic regions containing materials which resist protein adsorption (*18*) (most commonly poly(ethylene glycol)). Directly patterning cytophilic and cytophobic materials using photolithography is challenging because the solvents used to process photoresists can damage sensitive biomolecule films (although see, e.g., ref *19*). Therefore, the most common approach to pattern adherent cells is to use microcontact printing whereby cytophilic materials are stamped onto a substrate (*18*). The stamp is usually fabricated from the flexible polymer poly(dimethysiloxane), which is molded onto a thick photoresist that is patterned using photolithography. For our application, the use of microcontact printing would be challenging because it would require aligning the flexible stamp with the microchip substrate with micrometer resolution in order to stamp the cytophilic material precisely in register with the electrochemical electrode (although see ref *20*).

Here, we describe a simple and novel self-aligning approach for targeting individual cells to microelectrodes using differential surface chemistry created with conventional photolithographic methods. We previously have shown that nitrogen-doped diamond-like carbon (DLC:N) is well suited as an electrochemical electrode material for amperometric measurement of quantal exocytosis (*9*) and also promotes adhesion of neuroendocrine cells (*21*, *22*). In contrast, Teflon AF

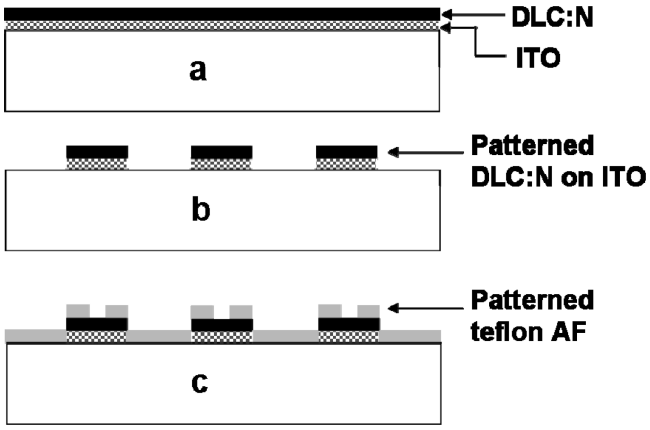

Figure 1. Overview of device fabrication. (a) DLC:N and ITO conductive films are sputter-deposited on a glass slide substrate. (b) The conductive films are patterned into 40 traces using photolithography together with Reactive Ion Etch of DLC:N and wet etch of ITO. (c) The insulating Teflon AF film is spin-coated on the substrate, and openings over the conductive films are made using photolithography with a second photomask and Reactive Ion Etch. Each opening constitutes both a working electrode and a cell docking site.

inhibits cell attachment(*21*) and is also an excellent electrical insulator. Therefore, we use a Teflon AF film both to insulate conductive DLC:N films and to block cell attachment. Cell-sized holes etched through the Teflon AF film define both the working area of the DLC:N electrode and cell docking sites. In addition to presenting the method for fabricating this device, we demonstrate selective targeting of cells to the electrodes and demonstrate functionality and reproducibility with high-resolution sample recordings of quantal exocytosis on the device.

Results and Discussion

Fabrication Overview

Figure 1 presents an overview of the fabrication process flow and is described in more detail in the Methods section. Devices are transparent in order to allow imaging of cells over electrodes using a conventional inverted microscope. The substrate is a standard 25 × 75 mm glass slide. DLC:N is an excellent electrode material because it is transparent, has desirable electrochemical properties (*9*), and forms a cytophilic surface to promote cell attachment (*21*). Since the resistivity of DLC:N is high, however, a transparent, yet highly conductive, indium−tin-oxide (ITO) conductive film (~100 nm) is first sputter-deposited on the glass substrate followed by sputter-deposition of the DLC:N film (40−110 nm thick; Figure 1a). The conductive electrode films are patterned into 40 individual traces using photolithography (Figure 1b).

Insulation of the bulk of the conductive films that come in contact with the cell bath solution is necessary

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to reduce the backround current noise level in order to resolve picoampere-magnitude amperometric spikes. Teflon AF is an excellent electrical insulator and has the additional property of being cytophobic, presumably because it is resistant to the adsorption of extracellular matrix proteins. A 2% Teflon AF solution is spin-coated onto the patterned substrate to give a film thickness of ~250 nm and then cured by baking. A subsequent deposition and patterning of the photoresist using a second photomask is followed by Reactive Ion Etch to open 20 μm-diameter holes in the Teflon to define the working electrodes that also served as cell docking sites (Figure 1c).

Device Layout and Connectivity

Connection pads (3 mm by 3 mm) for each of the 40 electrodes are laid out on the edge of the chip (Figure 2a), whereas the working electrodes/docking sites are laid out in 4 sets of 10 near the center of the chip (Figure 2b). Each set of 10 electrodes is arranged in a square pattern that can fit within the field of view of a 40× microscope objective lens. The device is placed in a custom-built holder that mounts on the microscope stage (Figure 2c). A plastic cover containing numbered holes facilitates connecting a metal pin to the desired electrode. The metal pin, in turn, is connected to the headstage of an EPC-9 potentiostat amplifier. A PDMS gasket serves to hold the drop of solution containing cells over the working electrode. A Ag/AgCl wire is placed in the drop of solution to serve as the ground/reference electrode.

Selective Cell Attachment to Working Electrodes

A drop (~50 μL) of cell bath solution containing ~10^6 cells/mL bovine adrenal chromaffin cells is placed on the device, and ~30 min is allowed for cells to settle on the surface of the chip. A gentle wash with bath solution is then used to remove unattached cells. Figure 3a is a photomicrograph depicting that 7 of the 10 electrode/docking sites are occupied by cells. We quantified cell attachment on 14 electrode arrays and found that 92 of the possible 140 electrode/docking sites (66%) are occupied by cells. We found that 72 of the sites are occupied by single cells, whereas multiple cells reside on 20 of the electrodes. The ratio of single cells to clumps depends on cell culture conditions in that cell clumping is more prevalent when cells are in culture for longer than 24 h.

Since some cells remain on the Teflon AF surface, we quantified the density of cells on the electrodes (DLC:N surface) versus the Teflon AF surface (Figure 3b). The data indicate that cells preferentially attach to the DLC:N electrode surface by a ratio of greater than 15:1 ($p < 0.001$) (*21*).

In order to determine how sensitive cell targeting is to the choice of electrode and insulating material, we fabricated similar devices using ITO as the electrochemical

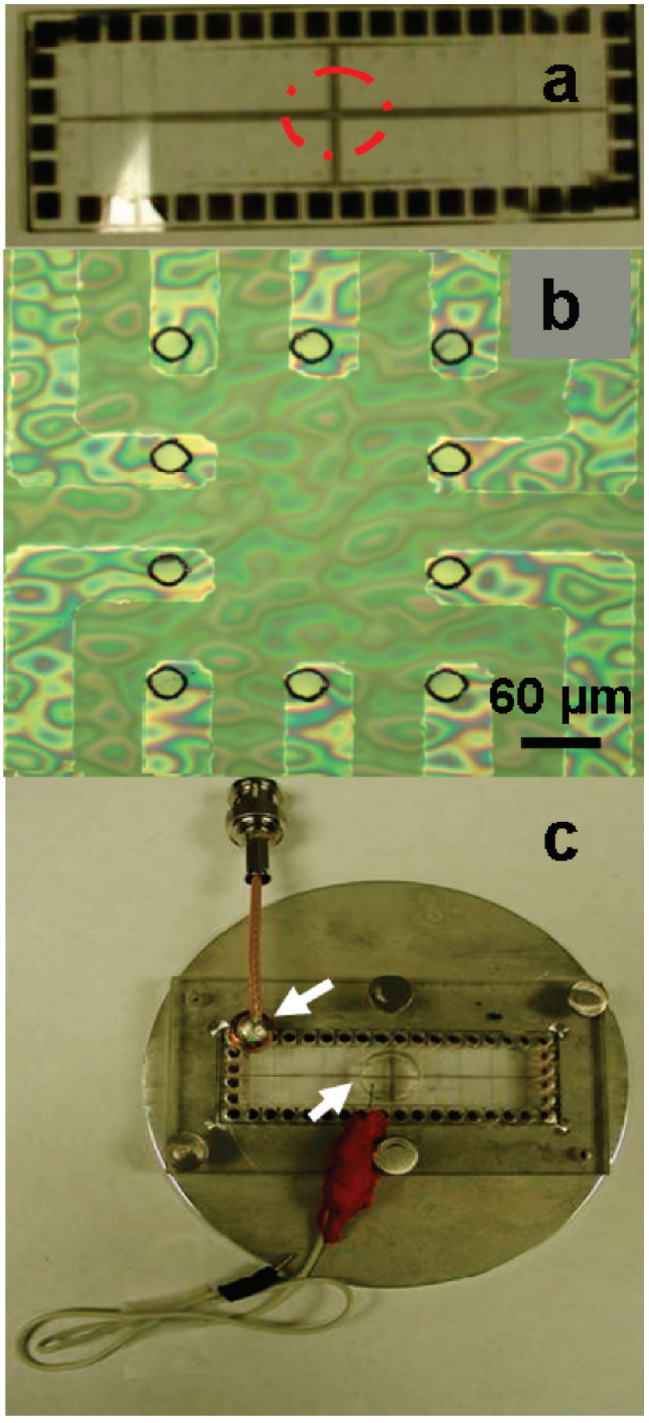

Figure 2. Photos of the device and recording configuration. (a) Microfabricated device of dimensions 25 × 75 mm with 40 microelectrodes in the center of the device (dashed circle). Connection to the chip is made via the connection pads arranged around the circumference of the chip. (b) Photomicrograph of the center of the chip depicting one of the four sets of 10 microelectrode/docking sites. (c) Photo of the chip in the custom-made holder, which facilitates the connection of the amplifier input to a connection pad (upper arrow) and insertion of a Ag/AgCl ground/reference electrode in the drop of solution confined to the middle part of the chip (lower arrow). The chip plus holder is placed on an inverted microscope to observe cell docking to electrodes.

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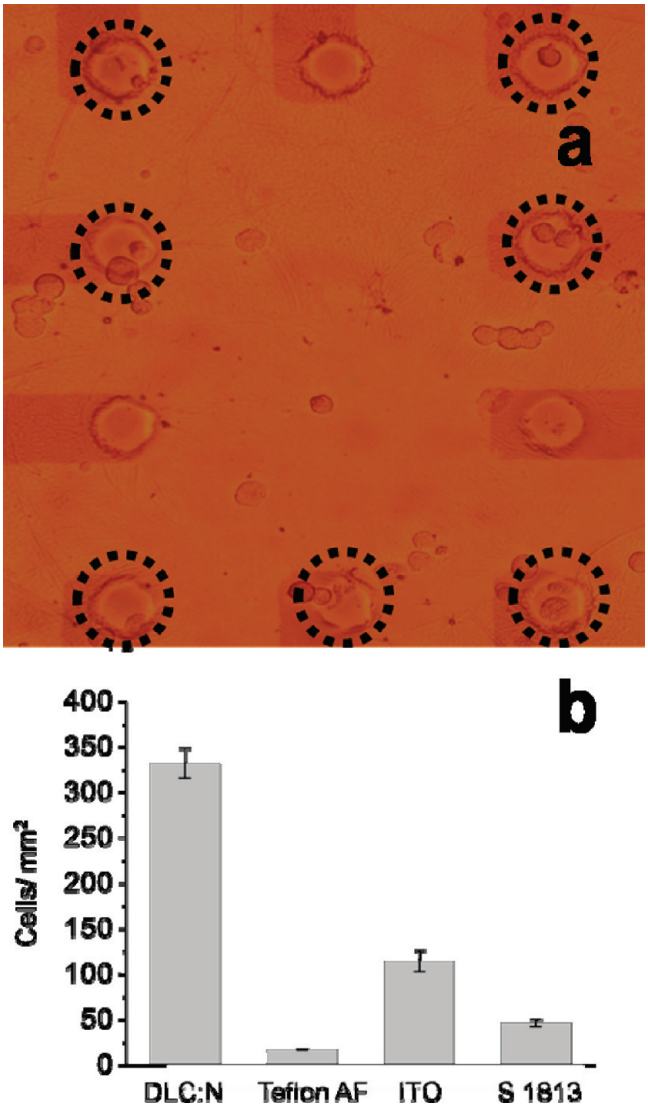

Figure 3. Cells are successfully targeted to electrodes using the DLC:N/Teflon AF device. (a) Sample photomicrograph depicting 7 out of 10 electrodes occupied by cells on a DLC:N/Teflon device. (b) Bar graph quantifying the density of cells adhered to the DLC:N electrodes versus those on the Teflon AF insulating film. The density of cells measured on similar devices with ITO electrodes and S1813 insulating films are also depicted for comparison. The error bars are the SEM measured from 14 electrode arrays for DLC/Teflon devices and 21 electrode arrays for the ITO/S1813 devices.

electrode material and S1813 photoresist to serve as the insulating material. We loaded and washed chromaffin cells under identical conditions and quantified cell attachment on 21 arrays of 10 electrodes. We found that only 47 of the 210 electrodes (22%) were occupied by cells. Figure 3b presents our quantification of cell density on the two materials and demonstrates that fewer cells stick to ITO compared to DLC:N, whereas S1813 is more cytophilic than Teflon AF. Thus, targeting of cells to electrodes is quite sensitive to the choice of materials.

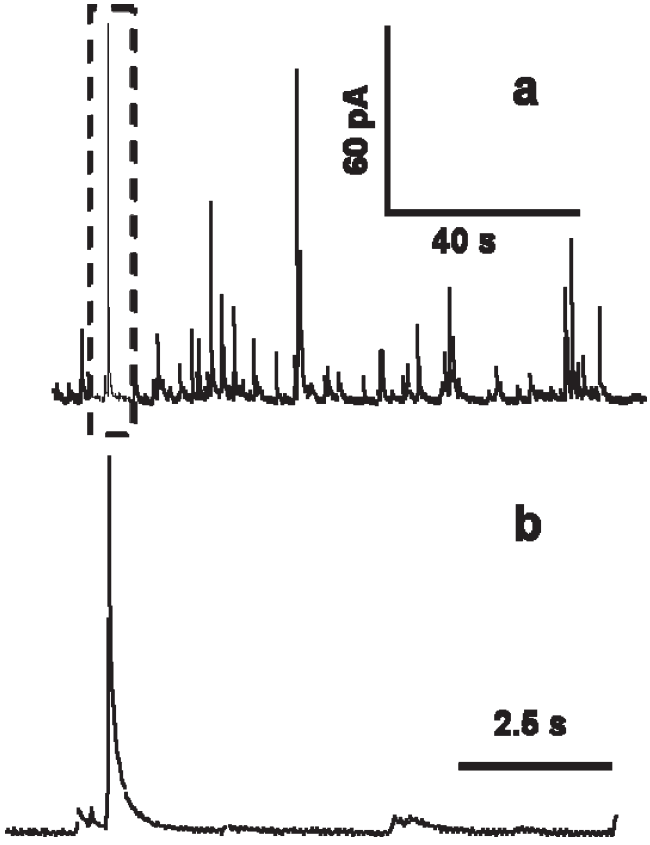

Figure 4. Amperometric detection of quantal exocytosis of catecholamines from chromaffin cells using a DLC:N on an ITO electrode. (a) Typical recording of amperometric spikes elicited by a depolarizing high K^+ solution over a 120 s time period. (b) Expanded view of time interval indicated by the dashed box in part a.

Cell Stimulation and Recording

Elevation of the extracellular K^+ concentration causes cell depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and Ca^{2+}-triggered exocytosis. We therefore added ~100 μL of a high-K^+ solution to the cell reservoir to stimulate exocytosis. Exocytosis of a catecholamine-containing granule from the surface of a cell adjacent to a working electrode results in a spike of faradaic current as catecholamines are oxidized on the electrode surface. Figure 4 depicts a sample recording containing amperometric spikes with amplitudes and time courses similar to what is typically observed with carbon-fiber electrodes.

We quantified several parameters from amperometric spikes using the software of Segura et al (*23*). I_{max} is the amplitude of the amperometric spike, whereas Q is the charge, and $t_{1/2}$ is the duration that the spike exceeds the half-maximal value and is thus is a measure of the time course of release from the vesicle. How these parameters are descriptive of single-granule fusion events is reviewed in ref *3*. Figure 5 depicts histograms of these three parameters obtained from 493 spikes recorded from 24 cells. The median values are 26.9 pA

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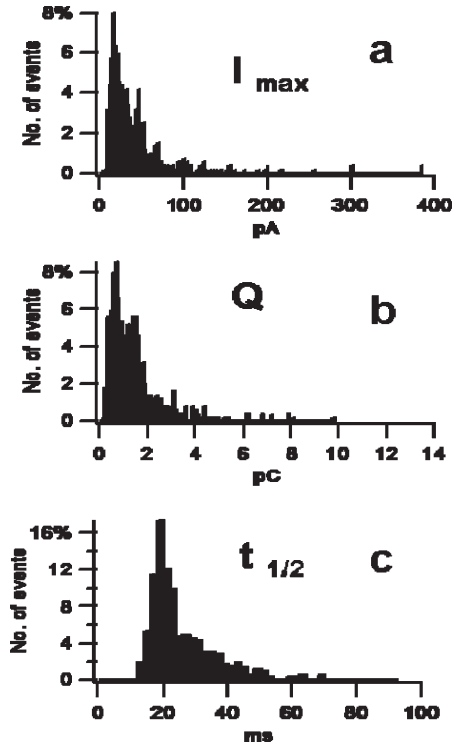

Figure 5. Quantitative analysis of 493 spikes recorded from 24 cells. Histograms depict (a) spike amplitude (I_{max}). (b) Spike area or charge (Q). (c) Duration that the spike exceeds the half-maximal amplitude ($t_{1/2}$).

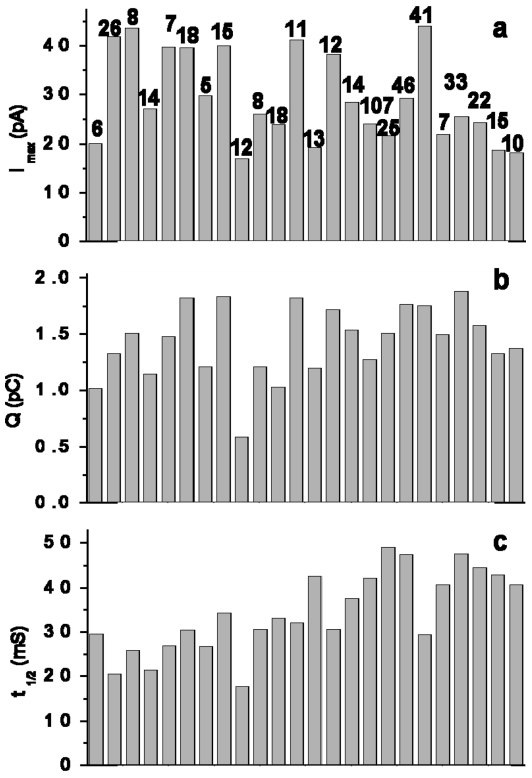

Figure 6. Quantification of the consistency of measured spike parameters between different cells and electrodes. Each bar indicates the median value of each parameter for each of the 24 cells. The number above each bar denotes the number of spikes recorded from that particular cell.

for I_{max}, 1.49 pC for Q, and 33.0 ms for $t_{1/2}$ and are similar to the values typically reported for recordings from bovine chromaffin cells using carbon-fiber electrodes (*1*, *4*, *24*, *25*).

In order to demonstrate the reproducibility and consistency of recording on our microfabricated devices, we plot the median values of the three spike parameters for 24 cells in Figure 6. Data are only reported for cells where at least 5 spikes were recorded (range 5 to 107). This level of cell-to-cell variability (typical coefficient of variation ~1) is also observed in recordings using carbon fiber electrodes (*26*).

Conclusions

Our microfabricated devices overcome a bottleneck by providing a simple and effective surface-chemistry approach to target cells to an array of electrochemical electrodes. Teflon AF is used both to insulate inactive areas of the surface and to resist cell attachment and thus facilitate cell movement to electrode docking sites; therefore, cell targeting to electrodes is self aligned. Cell handling is simpler and much gentler than with microfluidic approaches for cell targeting, and the cells are accessible for probe techniques such as atomic force microscopy and patch-clamp electrophysiology. Standard batch microfabrication approaches are employed; therefore, fabrication costs per electrode will be orders of magnitude lower than that with the current manual methods of fabricating carbon-fiber microelectrodes. Whereas we recorded from only one cell at a time due to hardware limitations, the use of multichannel amplifiers with our electrode arrays will enable hundreds of cell recordings to be carried out in a day.

A final positive feature of transparent electrodes of this type is that it allows simultaneous cell fluorescence measurements, e.g., to quantify the intracellular Ca^{2+} concentration or image docking and fusion of fluorescently labeled vesicles (*29*, *30*). The transparent electrode can be used to amperometrically measure the release from the same vesicles imaged at the bottom surface of the cell using total internal reflection fluorescence microscopy. In contrast, it is impossible to simultaneously image vesicles and detect the release of their contents using carbon-fiber electrodes.

Methods

Fabrication Process

Borosilicate/glass slides, 25 by 75 mm, were cleaned with acetone, isopropanol (all obtained from the MU chemistry store), and DI water. The substrate was then cleaned using sulphuric acid and phosphoric acid in a ratio of 4:1 for 10 min.

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The substrate was blow dried with air and left on a hot plate for 2 min to remove the moisture.

Indium tin oxide (ITO, target from Kurt J. Lesker, Pittsburgh PA, USA) was sputter-deposited (ATC 2000-V, AJA international, Inc., MA, USA) using 180 W RF power, with inflow of argon at 20 sccm and a pressure of 4 mTorr for 20 min at 50 °C to obtain a film thickness of ~100 nm. Without breaking the vacuum, diamond-like carbon (DLC), was subsequently deposited using 300 W DC power, a pressure of 2 mTorr, Ar flow of 10 sccm, N_2 flow of 2 sccm, and a temperature 200 °C for 1 h. The thickness of the film is ~110 nm. In some cases, the ITO-coated slides were purchased commercially (Sigma-Aldrich, St. Louis, MO USA).

The conductive films were patterned using photolithography. S1813 positive photoresist (Rohm & Hass, PA,USA) was spin coated (CEE Model 100, Brewer Science, MO, USA) onto the substrate at 3000 rpm with an acceleration of 1100 rpm for 30 s. The substrate was then heated at 115 °C on a hot plate for 2 min. A transparency photomask (photoplotstore.com and/or outputcity.com) was used with a mask alligner (OAI Model 200 IR San Jose, CA, USA) and illuminated with ~10.5 mW/cm^2 UV light for 14 s. The photoresist was developed with MF 321 (Rohm & Hass, PA,USA), washed with DI water, and blown dry with compressed air. The photoresist was subsequently postbaked on the hot plate for about 3 min.

The DLC:N was etched for ~30 s using oxygen plasma RIE with a power of 500 W and a flow of 50 sccm O_2 (Precision 5000 mark II Applied materials, CA, USA). The ITO thin film was then wet etched for ~100 s using 37% HCl. The substrate was thoroughly washed with DI water and then dipped in acetone to lift-off the photoresist and expose the patterned DLC:N on ITO.

An adhesion layer of FSM 600 (Cytonix, MD, USA), 5% by volume in ethanol, was spin-coated onto the device at 3500 rpm for 30 s and then baked on a hot plate for 10 min at 95 °C. Teflon AF-1600 pellets (DuPont, DE, USA) were dissolved (2 wt %/wt) in FC-75 or FC-770 (3M, MN, USA). Teflon pellets were sonicated in the solvent at 40 °C for 1 h or until the solution was totally clear. Alternatively, a 6% Teflon solution obtained from Dupont was diluted to 2%. The 2% Teflon solution was spin coated on the device at 3000 rpm for 30 s followed by baking on a hot plate at 115 °C for 15 min. Subsequently, the temperature was increased to 225 °C for another 15 min and then to 300 °C for 1 h. Following cooling, the Teflon-coated substrate was rinsed with acetone (MU chemistry store) and heated to 95 °C for 2 min.

The S1813 photoresist was spin coated on the Teflon-coated device and baked as previously described. Sometimes multiple coatings were required to totally cover the Teflon. A second photomask determined the pattern of openings in the Teflon that serve as electrode/docking site or connection pad. The photoresist was exposed through the mask for 14 s using the mask aligner and developed with MF 321. Openings in Teflon were made using RIE at 500 W, a pressure of 250 mTorr, and a gas flow rate of 50 sccm O_2. It took ~17 s to etch a 2% film of Teflon with an average thickness of 250 nm. Subsequently, the S1813 photoresist was removed using acetone, followed by washing with ethanol and DI water, and then blown dry with compressed air.

Fabrication of devices with ITO as the electrode material and S1813 as the insulation were carried out as described above except that the DLC:N and Teflon films were ommitted, and the patterned S1813 film remained as the insulation material.

Cell Preparation

Chromaffin cells were harvested from bovine adrenal glands as described (*24*). Harvested cells were placed into 25 cm^2 polystyrene cell culture flasks at a density of ~10^6 cells in 5 mL of culture media (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin). Cells were kept in a 37 °C incubator in a humidified environment with 5% CO_2 and used for a period of 1−2 days after preparation. Immediately before the experiment, cells were removed from the culture flask using rigorous pipet washing with the medium and put into a 10 mL plastic tube. After gentle centrifugation for about 5 min, the supernatant was removed, and the cells were gently resuspended in the standard bath solution. The standard bath solution consisted of (in mM) 150 NaCl, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 HEPES titrated to pH 7.2 with NaOH. The high-K^+ solution used to stimulate exocytosis consisted of (in mM) 100 KCl, 55 NaCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose. The final cell concentration in the standard bath solution was ~10^6 cells/mL. Fifty microliters of the cell suspension was loaded onto the microelectrodes on the chip for experimentation. About 100 μL of the high-K^+ stimulation solution is added to the reservoir after the cells wer allowed to settle for ~30 min.

Data Acquisition and Analysis

An EPC-9 patch-clamp amplifier with Pulse software (HEKA, Lambrecht, Germany) was used to record amperometric currents. Electrodes were held at a potential of +0.6 V relative to Ag/AgCl. Signals were low-pass filtered with a cutoff frequency of 2.9 kHz and sampled at 10 kHz. Analysis of amperometric spikes was performed using the software of Segura et al. (*23*) running on Igor Pro (Wavemetrics, Lake Oswego, OR).

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Author Contributions

S.B., K.D.G., and S.G. designed the devices and developed the fabrication process with the aid of J.C.M. and M.H. Devices were fabricated by S.B., X.L., J.C.M., and M.H. Authors S.B. and X.L. tested devices and carried out cell experiments. S.B., X.L., K.D.G., and S.G. analyzed data. K.D.G. wrote the paper with contributions from the other authors.

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