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Impedimetric Detection for DNA Hybridization Within Microfluidic Biochips

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Summary

A fully integrated biochip for the performance of microfluidic-based DNA bioassays is presented. A microlithographically fabricated circumferential interdigitated electrode array of 1- to 5-μm critical line and space dimensions, with associated large area counterelectrode (1000 × WE) and reference electrode (Ag/AgCl), has been developed as a four-electrode system for the electrochemical detection of DNA hybridization using any of the techniques of amperometry, voltammetry, potentiometry, and impedimetry. This is presented as an alternative to optical detection with an emphasis on label-free impedimetric detection of hybridization. A micro total analysis system (μTAS) is presented, using fluidic channels to connect integrated reaction domains with downstream electrochemical detection. This is accomplished by bonding a patterned poly(dimethylsiloxane) (PDMS) substrate to the biochip or by adhesive bonding of the chip to channels fabricated within glass and plastic microfluidic cards, adding increased functionality to the device.

Key Words: DNA hybridization; electrochemical impedance spectroscopy; DNA diagnostics; oligonucleotide; silanes; DNA immobilization; biochips; poly(dimethylsiloxane); micro total analysis system (μTAS).

1. Introduction

Low-density arrays that directly detect DNA hybridization have tremendous potential for applications in human health care ***(1,2)***, forensics ***(3)***, and national security. Current technology for DNA hybridization detection is most commonly based on optical measurements and generally uses fluorescence ***(4)***. These optical methods require costly labeling strategies that use bleachable fluorescent probes and expensive optical equipment. Electrochemical methods (amperometry, voltammetry, potentiometry, and impedimetry) for the detec-

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tion of DNA hybridization are being developed as a way to reduce cost, eliminate fluorescent labeling of DNA targets, and reduce the overall complexity and size of the instrument footprint ***(5)***. Specifically, electrochemical impedance spectroscopy (EIS) has been demonstrated as an effective analytical technique for detecting and monitoring DNA hybridization at an electrode/electrolyte interface ***(5–7)***. We have previously demonstrated significant differences in impedance measurements before and after hybridization of 30- and 50-mer oligonucleotides, suggesting EIS as an effective method for detection in fully integrated Lab-on-a Chip (LOC) systems.

EIS is a radiofrequency technique that is widely used for the characterization of charge transfer kinetics at electrified interfaces and for the study of transport of ions in electrolytes. The technique uses a sinusoidally varying voltage that is both nonperturbating and interrogating (typically 20–50 mV p-t-p). The voltage is applied between a pair of opposing working and counterelectrodes that span the medium of interest. The inclusion of a third or reference electrode and an appropriate potentiostat allows the sine wave to be superimposed on the quiescent or other offset potential of the working electrode. Under these circumstances, the ensuing current informs on both the coupled mass transport and charge transfer kinetics of electrochemically dischargeable species and of the movement of ions under the influence of the oscillating electric field. When potentials are selected that do not electrochemically discharge redox active moieties of DNA (such as guanine) and possess a single frequency that is larger that the heterogeneous charge transfer rate of typical electrochemical reactions associated with the medium under interrogation, then the technique of electrochemical impedance (EI) measures the ability of DNA to support ionic mobility. EI has been demonstrated as an effective analytical technique for understanding the way in which charge migration is impeded or conducted through an interface that is decorated with oligonucleotides. Illustrated schematically in **Fig. 1**, EIS seeks to measure the change in ion density before and after probe hybridization with its complementary target. The inclusion of a fourth, large-area (>100 times) counterelectrode enables the implementation of the interrogation technique of the Electroactive Polymer Sensor Interrogation System (EPSIS) ***(8)*** and the use of electroactive polymer layers to amplify impedance signal associated with hybridization ***(9,10)***.

A convenient format for the immobilization of oligonucleotide probes and for the delivery of the interrogating sine wave is the co-planar interdigitated microsensor electrode (IME) shown in **Fig. 2** (*see also* **Fig. 4**). IMEs are fabricated using state-of-the-industry microlithography techniques and present a well-defined fringing electric field from which impedance changes may be measured. Direct measurements of DNA hybridization by electrochemical detection have shown much greater sensitivity to smaller sample quantities compared to optical methods ***(11)***.

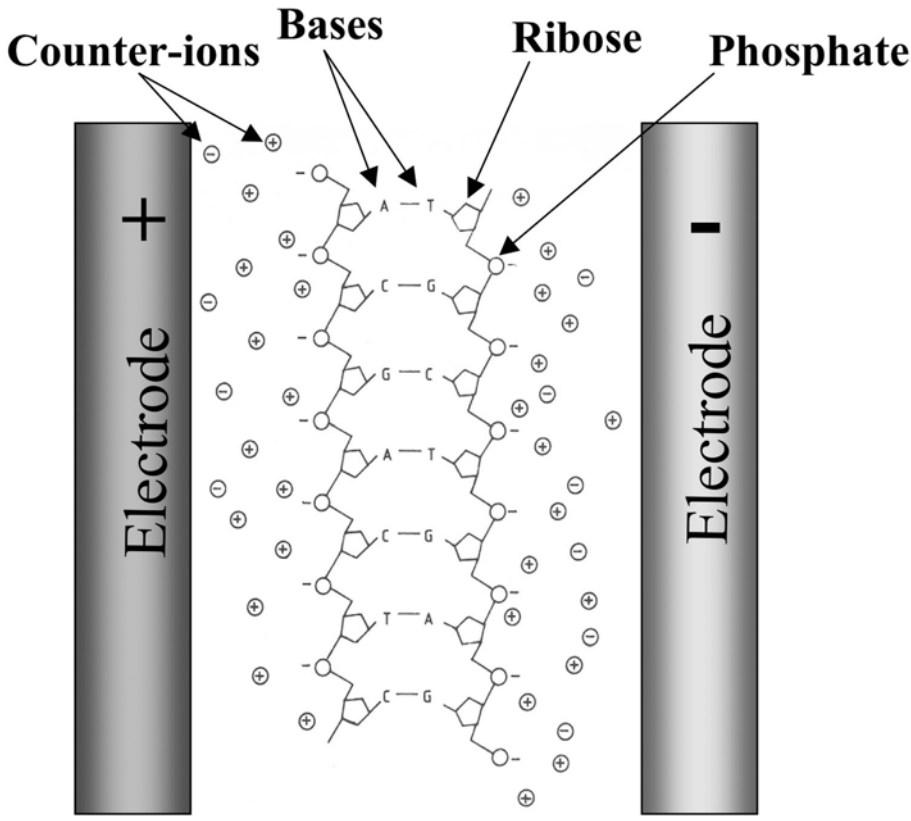

Fig. 1. Representation of DNA hybridization between a pair of electrodes. Increased impedance following hybridization is associated with a reduction of the density of water molecules and ions within the electric field.

DNA hybridization is based on the hydrogen bonding between single strands in a manner consistent with the DNA complementarity principle described by Watson and Crick in the 1950s. For detection based on EI or EIS, synthetic oligomeric probe DNA sequences taken from the 3' end that represent specific genes are covalently immobilized to the chip surface between opposing interdigitated electrodes, as shown in **Fig. 2** ***(5)***. Diffusive mass transfer is used to transport the target DNA (polymerase chain reaction [PCR] or reverse transcriptase product) to the immobilized probe on the substrate. Hybridization results in an overall increase in impedance because of the reduction of ionic conductivity surrounding the double-helical DNA relative to the single-stranded counterpart. The influence of the ionic hybridization buffer is minimized by the fact that the fringing electric field is within the diffusion boundary layer.

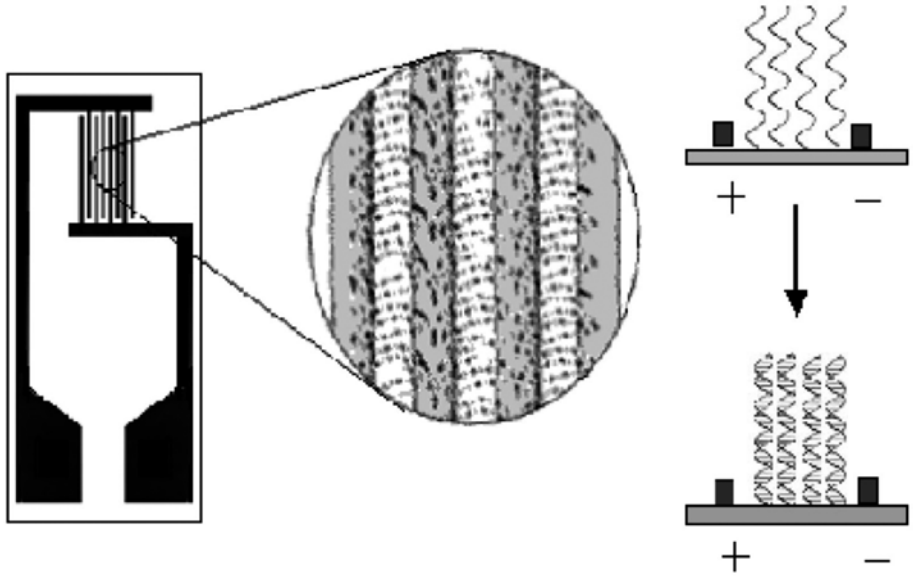

Fig. 2. Interdigitated microsensor electrode device showing the lines of force of the fringing electric field and of hybridization between interdigitated electrodes.

In solution, electric fields are typically created by the application of a voltage between a set of immersed electrodes ***(12)***. The electric field drives the transport of ions through the solution, generating an electric current. At small field strengths, the current generated is directly proportional to the strength of the electric field. The proportionality constant is known as the electrical conductivity and represents an intrinsic property of the electrolyte that is dependent on the concentration, valence state, and electrophoretic mobility of the ions present in solution. EI and EIS are based on applying an alternating sinusoidal voltage ($V = V_{max}$ sine ωt), rather than a fixed potential, over a wide frequency range, measuring the ensuing current ($I = I_{max}$ sine $\omega t+\theta$), and extracting the transfer function (V/I) ***(10)***.

Impedance is a complex electrical property comprised of two components: the real component, also known as the resistance, R, and the imaginary component, also known as the reactance, X, and is often presented as a Nyquist plot ***(13)***.

Impedance profiles also may be described by the resultant magnitude, $|Z|$, and phase angle, θ, of the real and imaginary components, as shown in **Fig. 3**. Bode plots are used to present the relationship of $|Z|$ to θ. For a pair of co-planar interdigitated electrodes bearing oligomeric DNA immobilized between them and immersed in a hybridization buffer, such an electrified system may,

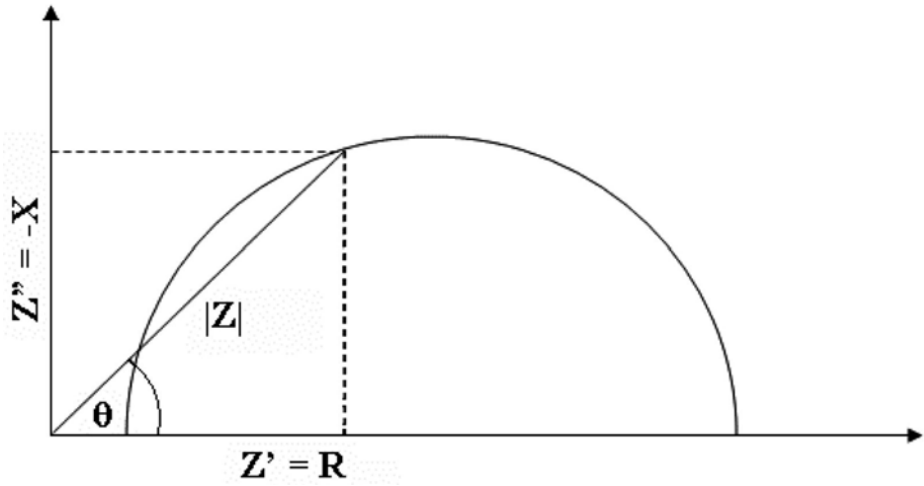

Fig. 3. Nyquest plot of frequency dependent real (*x*) and imaginary (*y*) components of the complex impedance and the corresponding magnitude, |*Z*|, and phase, θ, at given a single frequency.

in its simplest response, behave like a parallel resistor-capacitor (R-C) network. The reactance term then describes that part of the system that behaves as a capacitor while the resistance term describes that part of the system that behaves as a resistor ***(14)***. Frequency-dependent EIS has as one of its goals the identification of an appropriate frequency to be used with time-dependent EI from which the kinetics and equilibrium hybridization characteristics of probe–target and probe–mismatch pairs may be obtained.

Figure 4 shows a microfabricated electrochemical cell-on-a chip (ECC IME) developed by ABTECH Scientific, Inc. (Richmond, VA) for integration and use within microfluidic channels of micro total analysis systems (μTAS). This chip is available with several different conductors—gold, platinum, and indium tin oxide—the latter being used for its transparency and hence compatibility with optical detection systems. The available lines and spaces of interdigitation include 1, 5, 10, 15, and 20 μ. These ECC IME devices may be decorated with oligomeric DNA probes that are covalently bound to the interdigit space of the device shown in **Fig. 5**. To achieve DNA immobilization, the ECC IME devices must first undergo surface activation, surface modification, and then derivatization with the DNA probe before integrated bioanalytical measurements are performed. Surface activation of the borosilicate glass is required to expose the hydroxyl functional groups and is typically performed by using a brief and mild alkaline etch treatment (RCA clean: 1- to 10-s immersion in 5:1:1 solution of H_2O:NH_4OH:H_2O_2 followed by immediate,

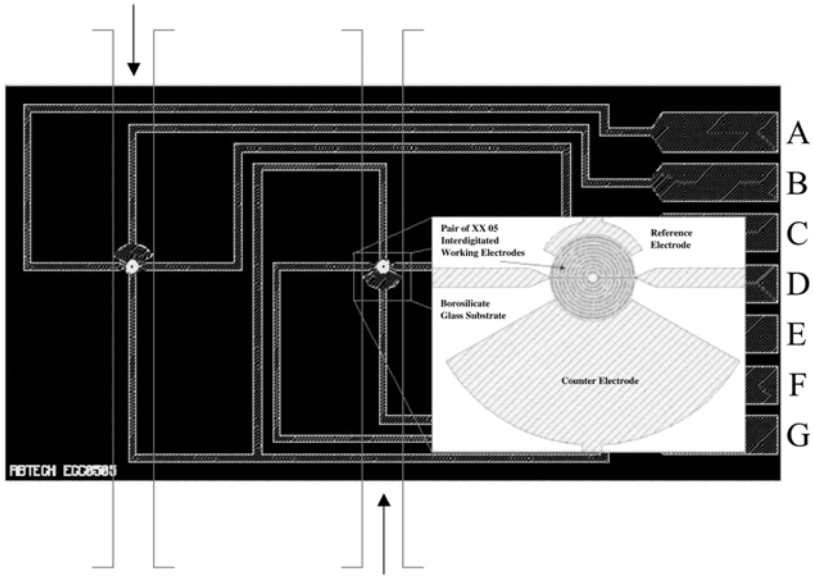

Fig. 4. Schematic illustration of a microfabricated, dual-channel, electrochemical cell-on-a-chip interdigitated microsensor electrode (ECC IME) device showing the fluid flow over the microelectrode arrays: **A** = C2W2, **B** = C2CE, **C** = C2W1, **D** = C1W1, **E** = C1CE, **F** = C1W2, **G** = REF. C2, Cell 2; C1, Cell 1; W, working electrode; CE, counterelectrode; RF, reference electrode.

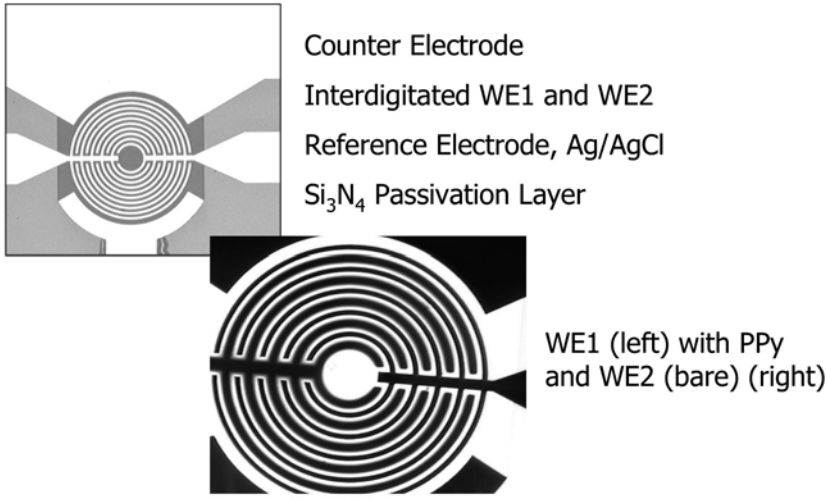

Fig. 5. Micrographs (×50) showing one channel (Cell 1) of the microfabricated, electrochemical cell-on-a-chip interdigitated microsensor electrode (ECC IME) device. **(A)** Various electrodes of the individual cell; **(B)** C1W1 with electropolymerized polypyrrole (PPy)—C1W2 serves as an opposing driving electrode.

profuse washing with deionized [DI] water) of the IME device. Following surface activation, the hydroxyls are modified using an organosilane. Silanization of the borosilicate glass has proven to be a simple and effective method for DNA immobilization ***(14,15)***. Common silanizing agents include 3-aminopropyltrimethoxysilane (APS), 3-mercaptopropyltrimethoxysilane (MPS), and 3-glycidoxypropyltrimethoxysilane (GPS), with terminal amino, mercapto, and epoxy moieties, respectively, which react and are coupled through the modified terminal group on the nucleotide. For GPS, the terminal amino group of the NH_2-C6-DNA probe reacts with the epoxy functionality of the silane compound, resulting in covalent attachment ***(16)***, and has been shown to be superior to other methods of direct covalent immobilization ***(17)***. Because organosilanes form siloxane polymers that deposit coherently and indiscriminately over the complete device, the electrodes (digits) must be subsequently cleaned to remove the polysiloxane. Subsequent covalent attachment via derivatization of the ω-terminus of the silane is a particularly effective method of DNA immobilization, and one that ensures effective probe alignment and surface coverage ***(6,7)***.

Following the necessary surface modifications, hybridization occurs when the complementary strand of DNA binds to the immobilized probe under the appropriate conditions. EIS measurements are performed before, during, and after hybridization, with the sensitivity of the detection determined by the effective change in the temporal impedance measurement ***(6,7)***. Both single- and multiple-frequency measurements can be made based on EIS detection. Single-frequency measurements are more rapid but require specific knowledge of an appropriate and effective frequency for measurement of the system of interest.

In order to incorporate the hybridization array into an integrated device that contains additional sample preparation steps (e.g., cell sorting, DNA extraction, polymerase chain reaction ampification, etc.), it is necessary to pattern additional flow channels and chambers that must be fluidically sealed. Much success has been reported with sealing PDMS to glass surfaces—this can be performed either reversibly or irreversibly (with additional surface treatment). PDMS is an ideal substrate because it is nontoxic, can be reproduced with high fidelity by replica molding, cures at low temperatures, is elastomeric, and its surface can be adapted for a range of chemistries.

2. Materials

2.1. Electrochemical Cell-on-a-Chip Interdigitated Microsensor Electrode: Cleaning, Silverization, and Platinization

1. Electrochemical Cell-on-a-Chip interdigitated microsensor electrodes (ECC IMEs) and STC 7 Test Clip (ABTECH Scientific Inc., Richmond, VA) consisting

of five pairs of opposing platinum electrodes several micrometers wide and several millimeters long (*see* **Note 1**).
2. Trichloroethylene (Sigma Aldrich, St. Louis, MO).
3. Acetone (Sigma Aldrich, St. Louis, MO).
4. Isopropyl alcohol (Sigma Aldrich, St. Louis, MO).
5. Ultraviolet (UV)/Ozone Cleaner (Boekel Industries, Inc.)
6. Alkaline etch cleaning solution: 5:1:1 solution of H_2O:NH_4OH:H_2O_2 (30% solution).
7. Acidic activiation solution: 4:1 concentrated H_2SO_4:H_2O_2 (30% aqueous solution).
8. DI, distilled water.
9. Silverizing solution: Silver Cy-less II Ready-to-Use (http://www.technic.com) electroplating solution (Technic, Inc.).
10. Platinizing solution: YSI 3140 (YSI, Inc., Yellow Springs, OH).
11. Potentiostat/Galvanostat PAR Model 283 (AMETEK Princeton Applied Research).
12. Ag/AgCl, 3 *M* Cl^- reference electrode (RE 803). (ABTECH Scientific, Inc., Richmond, VA),

2.2. Surface Modification via Silanization of ECC IME Device

1. Silanizing agent: 3-glycidoxypropyltrimethoxysilane as 0.1% by volume in anhydrous toluene (Sigma Aldrich, St. Louis, MO) (*see* **Note 2**).
2. Anhydrous toluene (Sigma Aldrich, St. Louis, MO).
3. Water bath (42°C).
4. Vacuum oven (120°C).

2.3. Cathodic Cleaning of ECC IME Device

1. Phosphate-buffered saline (PBS) (0.15 *M* NaCl, 0.1 *M* NaH_2PO_4 at pH 7.4).
2. Potentiostat/Galvanostat PAR Model 283 (AMETEK Princeton Applied Research).

2.4. DNA Probe Immobilization

1. Spotting buffer (0.5X standard sodium citrate [SSC] buffer, 1.5 *M* betaine, pH 5.4).
2. 50-mer NH_2-C6-Oligonucleotide and complementary sequence (Integrated DNA Technologies, Coralville, IA).
3. Water bath (42°C).
4. Controlled atmosphere oven (50°C, 50% RH) (Custom Built).

2.5. DNA Target Hybridization Detection With EIS

1. Hybridization buffer (20 µL phosphate-buffered KCl solution and 100 µL MWG hybridization buffer).
2. MWG Hybridization buffer comprised of 50% formamide, 6X SSC, 0.5% sodium dodecyl sulfate, 50 m*M* Na_3PO_4, and 5X Denhardt's reagent at pH 8.8 (MWG Biotech Inc.).

3. Synthetic, single-stranded, 50-mer oligonucleotides complementary, noncomplementary (random) and single, double, and multiple mismatch sequences (MWG Biotech Inc.) (*see* **Note 3**).
4. PBS (0.1 *M* KCl, 0.33 m*M* NaH_2PO_4 at pH 7.2).
5. DI, distilled water.
6. Perkin-Elmer Princeton Applied Research M283 Potentiostat/Galvanostat.
7. Solartron Schlumberger 1260 Frequency Response Analyzer (FRA).

2.6. Fabrication of a Flow-Through Device

1. Silicon monitor wafer (100 mm) (MEMC Electronic Materials, St. Peters, MO).
2. Silanizing agent: 1,1,1,3,3,3-hexamethyldisilazane (HMDS) (Acros Organics, Morris Plains, NJ).
3. Photoresist: NANO SU-8 25 (MicroChem, Newton, MA) (*see* **Note 4**).
4. Spin coater: WS-400-6NPP-LITE (Laurell Technologies Corporation, North Wales, PA).
5. Photomask (Pixels, Charlottesville, VA) (*see* **Note 5**).
6. UV lamp.
7. Developer: 2-(1-methoxy)propyl acetate (PGMEA) (Acros Organics, Morris Plains, NJ).
8. PDMS elastomer: Sylgard 184 base and curing agent (Dow Corning, Midland, MI).
9. Plasma cleaner/sterilizer (Harrick Scientific, Ossining, NY).

3. Methods

3.1. Cleaning of Microfabricated ECC IME

*3.1.1. Standard Wash for Grease and Solvent Removal (*see ***Note 6****)*

1. Set up a water bath to accommodate wash solvents.
2. Immerse ECC IME chip in boiling trichloroethylene (TCE) for 3 min.
3. Immerse ECC IME chip in boiling acetone for 3 min.
4. Ultrasonicate the ECC IME chip in 2-propanol for 3 min
5. Wash the ECC IME chip in flowing DI water for 3 min.

3.1.2. Activation of Borosilicate Glass Surface and Removal of Residual Organic/Ionic Contamination

1. Heat the 5:1:1 solution of H_2O:NH_4OH:H_2O_2 (30% aqueous) to 75–80°C in a water bath.
2. Immerse the ECC IME device in the heated 5:1:1 solution of H_2O:NH_4OH:H_2O_2 for 1–10 s (*see* **Note 7**).
3. Wash immediately in flowing DI water for 3 min.

3.1.3. Clean the ECC IME Device Using UV-Ozone

1. Clean the ECC IME device for 10–25 min in an UV/Ozone Cleaner to remove adsorbed organics (*see* **Note 8**).
2. Wash the UV-cleaned IME device by ultrasonic washing for 1 min in 2-propanol.

3. Dry the device in an air-filtered convection oven at 80°C from 30 min to 1 h for the complete evaporation of water from the surface.

3.2. Surface Activation of Metallic Digits (Au and Pt)

1. Immerse ECC IME device in 4:1 concentrated H_2SO_4:H_2O_2 (30% aqueous solution) at 80°C for 1–10 s (*see* **Note 9**).
2. Wash immediately in flowing DI water for 3 min and dry in the air-filtered convection oven.

3.3. Silanization of ECC IME Devices

1. Immerse the ECC IME device in 0.1% by volume 3-glycidoxypropyl-trimethoxysilane (GPS) in anhydrous toluene at room temperature for 2 h (*see* **Note 10**).
2. Wash the ECC IME device three times with anhydrous toluene.
3. Oven-cure the polysiloxane on the ECC IME device for 10 min at 110°C in the air-filtered vacuum oven.

3.4. Cathodic Cleaning

1. Set up a three-electrode electrochemical cell arrangement with the ECC IME connected via the STC-7 Test Clip, an external platinum mesh counterelectrode, and the external Ag/AgCl, 3 *M* Cl reference electrode to the PAR 283 Potentiostat/Galanostat.
2. Using the Multimeter, check that the electrode bonding pads on the chip are in contact with the leads on the STC 7 Test Clip. The working electrode to be cathodically cleaned is the shorted version of all the electrodes (A–F) of the ECC IME device (*see* **Note 11**).
3. Submerge the two active regions of the ECC IME device into the pH = 7.4 PBS solution at room temperature.
4. Cycle the electrodes between –1.2 and –2.0 V at a scan rate of 50 mV/s for 5–8 min. For an 8-min cycling, 15 cycles should occur.
5. Gently tap the electrode handle to dislodge any bubbles attached to the electrode surface.
6. Rinse the electrodes with DI, distilled water.

3.5. Silverization of ECC IME Reference Electrodes by Galvanostatic Deposition

1. Set up a three-electrode electrochemical cell arrangement with the ECC IME connected via the STC-7 Test Clip and the external Ag/AgCl, 3 *M* Cl to the PAR 283 Potentiostat /Galanostat.
2. Using the Multimeter, check that the electrode bonding pads on the chip are in contact with the leads on the STC 7 Test Clip. Set the electrode to be silverized (G = REF) as the working electrode and short the large area electrodes (B = C2CE and E = C1CE) as the counterelectrode and place the Ag/AgCl, 3 *M* Cl to serve as the external reference electrode.

3. Submerge the two active regions of the ECC IME device into the Technic Silver Cy-less II Ready-to-Use electroplating solution at room temperature.
4. Calculate the appropriate current (I) and time (t) for silverization based on the known or calculated working electrode area and the charge density of 6456 mC/cm^2 at a current density of 5.38 mA/cm^2.
   - I (current setting, mA) = Total REF electrode area of ECC IME XX05 (cm^2) * Current density (5.38 mA/cm^2)
   - Q (total charge, mC) = Charge density of 6,456 mC/cm^2 * Total REF electrode area of ECC IME XX05 (cm^2)
   - t (duration, s) = Q (mC)/I (mA)
   - For ECC IME 1005, t = 2,368.5 mC/cm^2 * 0.000454 cm^2/0.000454 cm^2 * 5.38 mA/cm^2 = 1200 s or 20 min (*see* **Note 12**)
5. Apply the calculated current for the calculated duration vs Ag/AgCl to achieve silverization.
6. Verify siliverization by checking the potential difference between the silverized REF electrode and the external Ag/AgCl, 3 *M* Cl^- using the Multimeter. The ΔE should be close to zero and steady.
7. Rinse the ECC IME device with DI, distilled water.

3.6. Platinization of ECC IME Working Electrodes by Galvanostatic Deposition

1. Set up a three-electrode electrochemical cell arrangement with the ECC IME connected via the STC-7 Test Clip and the external Ag/AgCl, 3 *M* Cl to the Potentiostat/Galanostat.
2. Using the Multimeter, check that the electrode bonding pads on the chip are in contact with the leads on the STC 7 Test Clip. Short (connect together) the electrodes to be platinized (WE_{TOT} = A + C + D + F, where A = C2W2, C = C2W1, D = C1W1, F = C1W2) as the working electrode, and short the large-area counter-electrodes (CE_{TOT} = B + E, where B = C2C2 and E = C1CE) as the counter-electrode and the recently silverized electrode (G = REF) as reference electrode.
3. Submerge the two active regions of the ECC IME device into the YSI 3140 Platinizing Solution at room temperature.
4. Calculate the appropriate current (I) and time (t) for platinization based on the known or calculated working electrode area and the charge density of 6000 mC/cm^2 at a current density of 59.0 mA/cm^2.
   - I (current setting, mA) = Total working electrode area (WE_{TOT}) of ECC IME XX05 (cm^2) * Current density (59.0 mA/cm^2)
   - Q (total charge, mC) = Charge density of 6000 mC/cm^2 * Total working electrode area (WE_{TOT}) of ECC IME XX05 (cm^2)
   - t (duration, s) = Q (mC) / I (mA)
   - For ECC IME 1005 (10 µm line and space), t = (6,000 mC/cm^2 * 2 * 0.000946 cm^2) / (2 * 0.000946 cm^2 * 59.0 mA/cm^2) = 102 s or 1.70 min (*see* **Note 12**)
5. Apply the calculated current for the calculated duration vs Ag/AgCl to achieve platinization.

6. Rinse the ECC IME device with DI, distilled water and dry in the air-filtered convection oven.
7. Verify there is no short-circuit of the electrode digits by checking the resistance between the digits with the Multimeter set to Ohms. The resistance should still correspond to open circuit. Verify the quality and distribution of the platinization by inspection under an optical microsope.

3.7. DNA Probe Immobilization

1. Prepare a 150-µ*M* solution of the oligonucleotide probe in a pH 5.4 spotting buffer (0.5X SSC buffer and 1.5 *M* betaine) (*see* **Note 13**).
2. Incubate single-strand oligonucleotide probes on GPS-modified ECC IME surface overnight at 50°C in pH 5.4 spotting buffer.
3. Wash for 15 min with DI, distilled water at 50°C to remove unreacted DNA probe.
4. Repeat the cathodic cleaning (**Subheading 3.4.**) to remove nonspecifically adsorbed oligonucleotide probes from the metallic digits.
5. For storage, dry the ECC IME device in a desiccator for several hours and store in a sterile DNAse-free container before use. To use stored DNA chips, rehydrate in hybridization buffer.

3.8. Preparing a Photoresist Master

The preparation of the master follows conventional photolithography methods for a negative SU-8 photoresist on a silicon wafer ***(18)***. The process is illustrated in **Fig. 4**.

1. The silicon wafer is blown with a stream of air to remove any dust on the surface and then placed in a sealed container containing HMDS to promote adhesion of the resist.
2. The wafer is then placed on the spin coater chuck and held in place with vacuum. SU-8 photoresist is added dropwise from a medicine dropper into the center of the wafer and spun at 1000 rpm for 40-µm thickness (*see* **Note 14**).
3. The wafer is then transferred to a hotplate and soft-baked for 15 min at 95°C to set the resist (*see* **Note 15**).
4. A photomask containing an image of the microfluidic pattern is placed directly on top of the resist-coated wafer and illuminated under UV light (*see* **Note 16**).
5. A postexposure bake is then performed at 95°C for 4 min (*see* **Note 17**).
6. The wafer is then placed in the developer for a recommended time of 6 min for 40 µm (*see* **Note 18**). Following development, the wafer is rinsed with 2-propanol and dried with a stream of air. The positive relief formed on the wafer acts as the master (*see* **Note 19**).

3.9. Making the PDMS Replica

The preparation of the replica follows conventional processing methods for PDMS ***(19)***. The process is illustrated in **Figs. 6** and **7**.

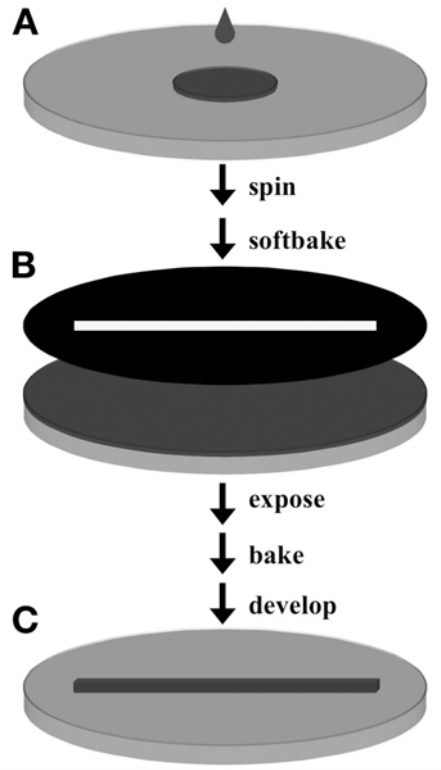

Fig. 6. Preparation of the master mold: **(A)** Photoresist added dropwise in the center of the silicon wafer. **(B)** After spinning the resist to achieve the desired thickness, photomask containing the desired features is brought into contact with the surface and the features are illuminated. **(C)** After removing the unexposed photoresist, the patterned features are left on the wafer as positive relief.

1. The PDMS elastomer base and curing agent are mixed in a 10:1 ratio as per manufacturer's instructions. The mixture is stirred thoroughly and poured over the master.
2. The master and elastomer are placed in a dessicator, and vacuum is applied to remove any air bubbles from the PDMS. The combination is then transferred to an oven for 45 min at 100°C to set the elastomer (*see* **Note 20**).
3. The resulting PDMS replica can then be peeled off from the master, yielding a negative relief containing the microfluidic network.

3.10. PDMS–Glass Bonding

The PDMS will form a reversible seal by bringing it into contact with a clean glass surface, and this may suffice. However, to ensure a good fluidic

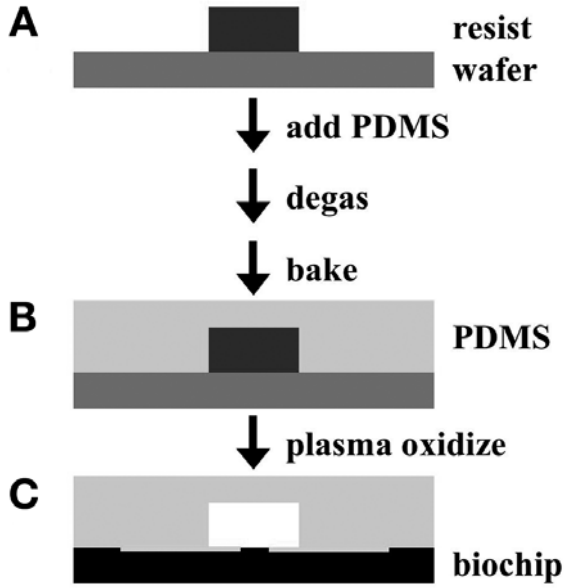

Fig. 7. Making the poly(dimethylsiloxane) (PDMS) replica: **(A)** End-on view of feature in resist master. **(B)** PDMS is poured onto the master, degassed, and baked to set. **(C)** Once the PDMS is set, it is removed from the master, plasma-oxidized, and bonded to the biochip.

seal and prevent leakage, the elastomer surface is plasma-oxidized to form an irreversible seal against the glass. The surface is exposed to the plasma for approx 1 min before being removed from the chamber and brought into contact with the array chip (*see* **Note 21**).

3.11. Integrated System Assembly and Test

1. Gently apply the PDMS fluidic chamber to the surface of the chip such that the electrochemical cells sit within and form the base of a designated channel.
2. Introduce buffer under pressure-driven or electroosmotic flow and check for leaks.

3.12. DNA Hybridization

1. Dissolve 8.22 µ*M* oligonucleotide target in hybridization buffer (20 µL phosphate-buffered KCl solution and 100 µL MWG hybridization buffer) for 4 h at 42°C.
2. Introduce target DNA into the microfluidic DNA detector and commence electrochemical impedance measurements.

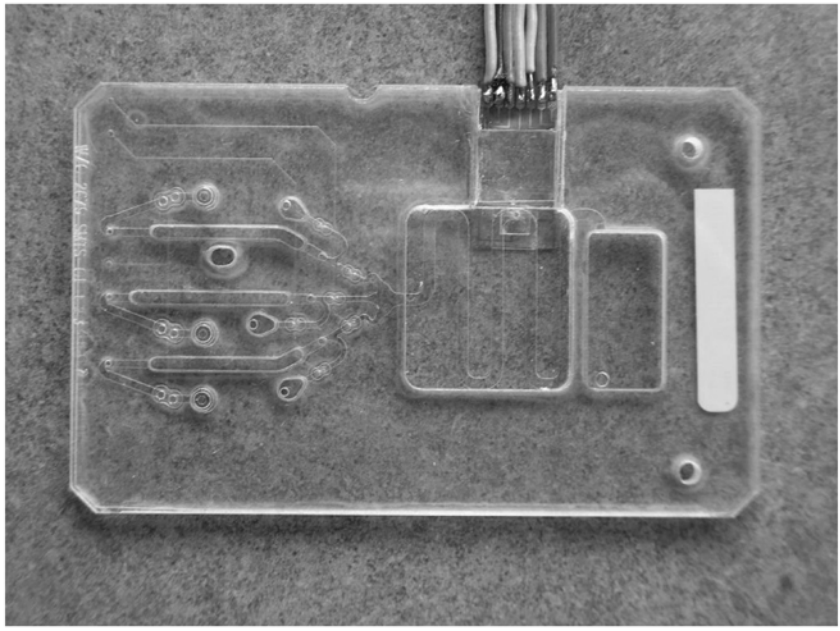

Fig. 8. An integrated biochip showing the microfabricated pattern of electrodes bonded and sealed into the Micronics' microfluidic T-cassette.

3.13. Electrochemical Impedance Measurements

1. Set the two members of the interdigitated pair of electrodes of any one fluidic detector to the working electrode (e.g., WE = D = C1W1) and the counter-electrode (CE = F = C1W2) and connect the reference electrode to the silverized electrode (G = REF). **Figure 8** shows an example of an integrated biochip with a Micronics Microflow T-cassettte.
2. Check the quality of the connections using the Multimeter set at 40MΩ and activating the range with the audio on.
3. Electrochemical impedance measurements may be taken before, during, and following the hybridization event in hybridization buffer.
4. Impedance measurements should be performed in the hybridization buffer.
5. The frequency-dependent electrochemical impedance spectra may be obtained before (t = 0) and after (t = ~16 h) using a 10 mV amplitude sine wave over the frequency range of 10 mHz – 1.0 GHz at 20 ± 1°C using the Perkin-Elmer Princeton Applied Research M283 Potentiostat/Galvanostat coupled with a Solartron Schlumberger 1260 FRA.
6. Temporal impedance measurements are made at 10 mV amplitude sine wave at a frequency of 4 kHz.

4. Notes

1. Each borosilicate glass chip consisted of two discrete electrochemical cells over which channels were constructed. Each cell comprised a pair of circumferential presented interdigitated microsensor electrodes, a silverized reference electrode (0.1 × WE area), and a counterelectrode (10 × WE area). Each digit of the IME was 5 μm wide and was separated from its nearest opposing digit by 5 μm space. (Part number ECC IME 0505-Pt-U; ABTECH Scientific, Inc.)
2. The bottle of 3-glycidoxypropyl-trimethoxysilane must be degassed before storage by gently flowing nitrogen or argon gas over the top of the bottle for approx 5 min. Nitrogen or argon is used to displace air (moisture), which will initiate polymerization without displacement. Alternatively, a canular may be used.
3. Complementary, noncomplementary (random), single, and multiple mismatched target oligonucleotides are used to compare effects on EIS between specific and nonspecific hybridization.
4. In addition to the photoresist used in this work, different SU-8 formulations are available for a wide range of thickness with high aspect ratio features.
5. A local printing company was used to obtain a high resolution (3600 dpi) transparency. The microfluidic features were designed using Adobe Illustrator (San Jose, CA) software. When using a negative resist, the desired features are transparent in the mask.
6. This procedure is a general degreasing method that should be performed in sequence without much delay between steps.
7. This procedure is an aggressive alkaline etch of the glass; it removes trace organics and physically adsorbed monolayers of adventitious organics that are not removable by the procedure described in **Subheading 3.3.** *Caution*: Do not exceed the recommended 10 s because this etch may undercut the delicate microfabricated electrodes.
8. This procedure removes monomolecular layers of bound organics on metallic and oxide surfaces. This procedure removes chemisorbed and covalently bound self-assembled monolayers of adventitious organics, alkane thiols, and silane layers. This method is not applicable to thick organic films.
9. This procedure provides addition cleaning and activates the surface by producing a uniform concentration of hydroxyl groups at the surface.
10. Fresh solution should be made for each silanization reaction.
11. Cathodic cleaning is necessary to remove covalently attached GPS on the electrodes.
12. By adjusting the applied constant current to the area of the candidate electrode, the time to achieve the required charge density is always 1.7 min.
13. Spotting buffers are used in the fabrication of oligonucleotides microarrays.
14. The manufacturer recommends approx 1 mL of resist per 25 mm wafer diameter. An initial spread cycle is used to ensure uniform coverage, and it consists of a 100 rpm/s ramp to 500 rpm. To achieve the desired thickness, the wafer is ramped at 300 rpm/s to the appropriate spin speed and held for 30 s.
15. A prebake is recommended before the soft bake, although this will require either a second hotplate for temperature stepping or a programmable hotplate for

ramping. When a second hotplate is used, a 5-min prebake is recommended at 65°C. This allows the solvent to evaporate out of the resist film at a more controlled rate to ensure coating fidelity.

16. The manufacturer offers suggestions for wavelength and exposure dose as a function of desired thickness, but recommends using 350–400 nm.
17. A 1-min prebake at 65°C is recommended to minimize resist cracking.
18. Development time is approximate, as it will vary with such parameters as agitation and temperature.
19. While SU-8 has good mechanical properties, it may be desirable to perform a hard bake to preserve the integrity of the master. This will require a ramp or step to 150–200°C, with bake times of approx 30 min being sufficient.
20. The degassing step takes approx 30 min, and any bubbles remaining on the surface can be removed with a sharp utensil. As an alternative to the oven, the PDMS can be left out of the oven to cure overnight at room temperature.
21. If the glass–PDMS bond does not seal after oxidation, it may help to oxidize both of the surfaces to be bonded.

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