Chapter 6

Microfluidic Arrayed Lab-On-A-Chip for Electrochemical Capacitive Detection of DNA Hybridization Events

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Abstract

A microfluidic electrochemical lab-on-a-chip (LOC) device for DNA hybridization detection has been developed. The device comprises a 3 × 3 array of microelectrodes integrated with a dual layer microfluidic valved manipulation system that provides controlled and automated capabilities for high throughput analysis of microliter volume samples. The surface of the microelectrodes is functionalized with single-stranded DNA (ssDNA) probes which enable specific detection of complementary ssDNA targets. These targets are detected by a capacitive technique which measures dielectric variation at the microelectrode–electrolyte interface due to DNA hybridization events. A quantitative analysis of the hybridization events is carried out based on a sensing modeling that includes detailed analysis of energy storage and dissipation components. By calculating these components during hybridization events the device is able to demonstrate specific and dose response sensing characteristics. The developed microfluidic LOC for DNA hybridization detection offers a technology for real-time and label-free assessment of genetic markers outside of laboratory settings, such as at the point-of-care or in-field environmental monitoring.

Key words Dielectric spectroscopy, Electrochemical impedance spectroscopy, Lab-on-a-chip, Microfluidics, DNA hybridization sensing, Label-free detection, Faradaic redox reaction, Electrical equivalent model, Biosensor

1 Introduction

There are many types of biosensors for deoxyribonucleic acid (DNA) hybridization detection that are based on various transduction mechanisms, such as optical and electrochemical detection [1, 2]. Within the electrochemical regime, there are three types of biosensing techniques that are used for DNA hybridization detection: amperometric, potentiometric, and capacitive [3]. Most of these techniques are based on a three-electrode electrochemical cell that includes working, counter, and reference electrodes (Scheme 1). These techniques monitor physical and chemical variations at the electrochemical interface of the sensor (i.e., working electrode) and the tested solution (i.e., electrolyte) due to the hybridization

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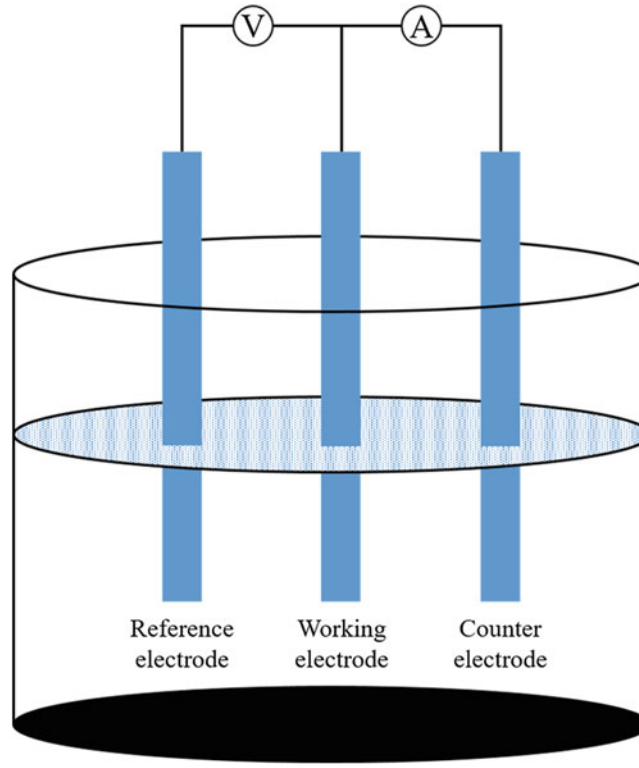


Scheme 1 A three-electrode configuration of an electrochemical cell. The potential is held between the working and the reference electrodes while the current is measured between the working and the counter electrodes

events. During amperometric detection the electrode is held at an electrochemical potential that is higher than the standard reduction potential (E_0) of an electro-active species in the sample (i.e., over-potential conditions), resulting in redox-based current (i.e., faradaic current). In the case where the species undergo an oxidation reaction the electrode's potential is held at a value that is more positive than E_0 while in a reduction reaction the value is more negative. Most common analytical amperometric technique is cyclic voltammetry (CV), where the potential of the working electrode is cycled at a constant rate between oxidation and reduction potential edge points. These scans enable CV to measure redox currents from multiple electro-active species with different E_0. The ability to detect DNA hybridization events with amperometry is dependent on the influence of the event on the measured electro-active species. For example, upon hybridization event the increased negative charge at the surface of the electrode (due to the negatively charged phosphate in the DNA) increases the repulsion forces between the hybridized DNA and negatively charged electro-active species in the bulk (e.g., ferricyanide). These repulsion electrostatic forces impede the charge transfer kinetics of the

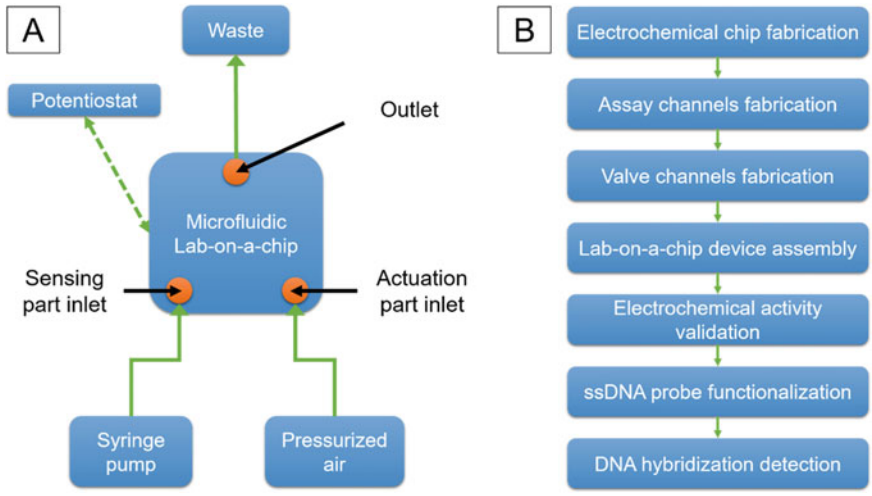
electro-active species oxidation reaction, resulting in decreased electrochemical currents. The same detection elements are true also for capacitive detection where instead of the generated electrochemical current, impedance variations at the electrode–electrolyte interface (i.e., the electric double layer) are measured. These variations are usually detected by perturbing the interface with a small amplitude (5–50 mV) sinusoidal potential signal in different frequencies, and measuring changes in the amplitude and the phase of the resulted electrochemical current (i.e., electrochemical impedance spectroscopy—EIS). The Nyquist plot is the most common representation of these changes that plots the imaginary part of the impedance as a function of the real part. This representation emphasizes overall resistive (bigger changes in the *x*-axis direction) or capacitive (bigger changes in the *y*-axis direction) changes in the system. The changes can be further analyzed with equivalent electrical models of the electrochemical system, calculating major parameters (e.g., charge transfer resistance and double layer capacitance). Changes in the double layer capacitance and other parameters that define the overall potential of the electrode–electrolyte interface can be also detected with potentiometry. While maintaining the electrochemical current in the cell at a specific value (most common is 0 V vs. the counter electrode—Open circuit potential conditions) any accumulated charge will affect the measured electrochemical potential, such as during hybridization events.

The specificity of the sensor is achieved using ssDNA oligomers that functionalize the surface of the electrode, and act as probes for complementary ssDNA targets in the tested solution. Upon interaction of the probe with a complementary target hybridization event occurs. These events change the total electric charge present at the electrode–electrolyte interface due to the negatively charged phosphate groups at the backbone of the ssDNA. Hybridization events can be detected by monitoring physical and chemical components in the electrochemical system that either involve non-faradaic reactions such as the double layer capacitance or faradaic reactions such as the charge transfer resistance. In order to induce faradaic currents, electro-active species that are affected by the DNA hybridization event are introduced. Hence, for higher number of hybridization events a lower electrochemical current is measured. Other parameters that may affect the resulted electrochemical current (and hence can be measured) are the length of the ssDNA oligonucleotide (probe and target) and point mutations in the ssDNA sequence. These variations induce smaller changes in the electrochemical system and require sensors with improved specificity and sensitivity.

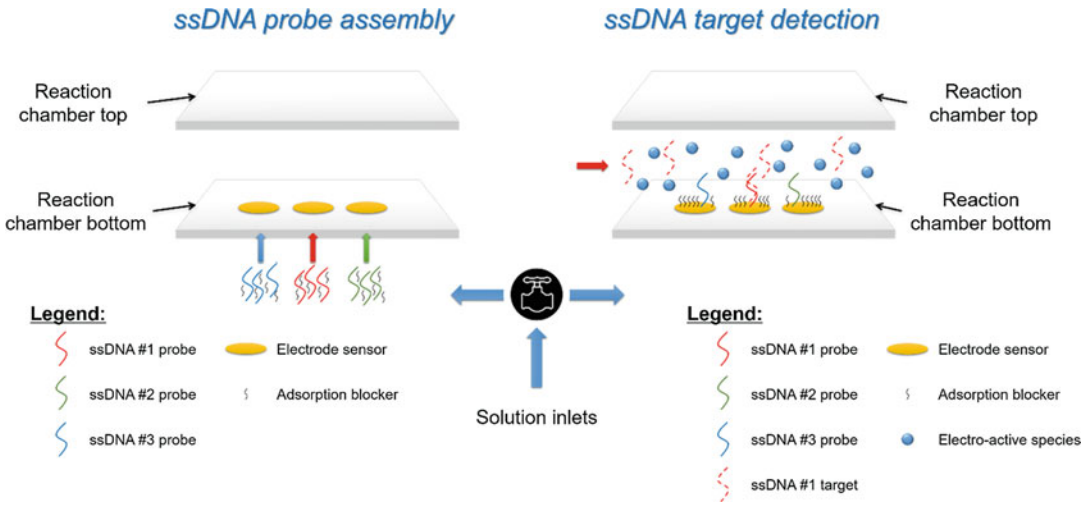
The last decade has witnessed an unprecedented convergence of biological, physical, chemical, and engineering sciences that allows the construction of novel hybrid biodevices. These

biodevices are utilized in various applications such as clinical diagnostics, environmental monitoring, and biomedical research. One of the most common examples of these biodevices are microfluidic lab-on-a-chip (LOC) microsystems. These microsystems have the potential for integration with other technologies and miniaturization, leading to portability, high-throughput usage, and low-cost mass production. These translational technologies hold the potential for improving the resolution, regulation, sensitivity, flexibility, and cost savings over more traditional approaches, bringing benchtop methods to the point-of-care. Integration of biosensors allows for label-free detection of various biomolecules and drastically reduces reagent volumes and response time required for analysis [4, 5]. There are many advantages when using electrochemical signals to perform biological and chemical detection in microfluidic systems. The fabrication is inherently less complicated since these sensors typically only require patterned electrodes to operate. In addition, electrical signals can be directly interfaced with most measurement instruments. However, new challenges are presented as these systems are miniaturized. The most important challenges to overcome include: sample preparation and mixing of fluids (due to the low sample volume and Reynolds number), physical and chemical effects (including capillary forces, surface roughness, chemical interactions between construction materials and analytes), and low signal-to-noise ratio (produced by the reduced surface area and volume) [6].

Integration of DNA biosensing in microfluidic LOC devices provides portable diagnostic platforms that enable new capabilities, such as rapid screening of markers at the point-of-care or detection of pathogens in the environment [7, 8]. These platforms have been developed in research where some have been translated to commercial biomedical products. A major advantage of LOC integrated DNA biosensors is the reduction in volume of sample and reagents. The reduced volume decreases costs of reagents and DNA sample preparation time, and provides rapid detection where only several copies of DNA are present. Here, we introduce the methods for fabrication and utilization of microfluidic LOC devices for the detection of DNA hybridization events. The device is composed of two main parts: an electrochemical sensing chip and a microfluidic sample manipulation chip. The design of the electrochemical chip contains an array of microelectrodes ordered in a grid [9, 10]. The microfluidic chip is a stack of two layers of microchannels: a bottom channel that is in contact with the electrochemical chip and acts as the assay channel, and a top channel that control the actuation of the valved system. Following fabrication of the chips and assembly of the device, a syringe pump, a pressurized air regulator, and a potentiostat are used to manipulate and sense DNA testing samples (Schemes 2a, b; Scheme 3 for controlled assembly and detection operations).



Scheme 2 (**a**) Flow diagram of the device operation. Solid arrows indicate solution and gas flow. Dashed arrow indicates electrochemical information recording. (**b**) Methods flow of the device fabrication, assembly, and testing



Scheme 3 Valved-based controlled lab-on-a-chip for improved DNA hybridization detection. *Left*: ssDNA probe assembly. *Right*: ssDNA target detection due to DNA hybridization event. Adjusted from [9] with permission from Elsevier

2 Materials

2.1 Microfabrication Tools and Materials

1. 4″ Silicon wafer (single side polished, p-type, boron doped, orientation 1-0-0, thickness 500 μm, resistance 10–20 Ω-cm; Ultrasil).
2. 4″ Borosilicate glass wafer (Promptar).
3. Hexamethyldisilazane (HMDS; Dow Corning).

4. Shipley 1813 photoresist (positive resist; MicroChem).
5. AZ 5214-E photoresist (image reversal resist; Hoechst Celanese).
6. AZ 9260 photoresist (positive resist; MicroChem).
7. SU-8 50 photoresist (negative resist; MicroChem).
8. Microposit 352 developer (Rhom and Haas Electronic Materials).
9. AZ 400K developer (Clariant).
10. SU-8 developer (MicroChem).
11. Gold etchant (no dilution; Transense).
12. Chromium etchant (no dilution; Transense).
13. Polydimethylsiloxane (PDMS) elastomer and curing agent (Dow Corning).
14. Deionized water (DI; Purity: 18 MΩ-cm).
15. Dermatological punching tool (Healthlink).
16. Plasma Enhanced Chemical Vapor Deposition (PECVD; Chamber pressure 1000 mTorr, Temperature 200 °C, RF power 20 W, Gasses: (a) N_2O flow rate 710 sccm; (b) a composition of 5% SiH_4 and 95% N_2 gasses flow rate 170 sccm, SiO_2 growth rate 690 Å/min; PlasmaLab System 100, Oxford Instruments).
17. DC sputtering unit (For Chromium: Chamber pressure, 10 mTorr, Argon flow rate 20 sccm, Supplied DC power 200 W, Sputter rate 10 nm/min. For Gold: Chamber pressure 10 mTorr, Argon flow rate 20 sccm, Supplied DC power 200 W, Sputter rate 36 nm/min; ATC 1800-V, AJA International).
18. E-beam evaporation system (For Titanium: Chamber pressure $<2 \times 10^{-6}$ Torr, Supplied power to the e-gun 7.5 kW, Filament current 150–200 mA, Evaporation rate 6–10 Å/s. For Platinum: Chamber pressure $<2 \times 10^{-6}$ Torr, Supplied power to the e-gun 7.5 kW, Filament current 150–200 mA, Evaporation rate 2–3 Å/s; Denton).
19. March Jupiter III Reactive Ion Etch (RIE) Oxygen Plasma system (Chamber pressure 400 mTorr, Supplied power 20 W, 30 s exposure time; Nordson March).

2.2 Device Operation Equipment

1. Tygon flexible tubing (OD 0.087″, ID 0.015″; Cole Parmer).
2. Tygon flexible adapter (OD 0.1875″, ID 0.0625″; Cole Parmer).
3. 1 mL syringe (Beckton-Dickenson).
4. Syringe pump (KDS230; KD Scientific).

2.3 Electrochemical Characterization Equipment

1. Potentiostat (660D; CH Instruments).
2. EC-Lab software (v10.33, Bio-Logic SAS).

2.4 Working Solutions

1. Phosphate Buffer (PB): Monobasic Potassium Phosphate, Potassium Phosphate Dibasic Anhydrous. Materials are mixed to yield pH 7.0.
2. Phosphate Buffer Saline (PBS): 10 mM PB, 100 mM Sodium Chloride (NaCl).
3. Ferrocyanide/Ferricyanide Electrochemical Testing Solution: 5 mM Potassium Hexacyanoferrate(II) Trihydrate (Sigma-Aldrich), 5 mM Potassium Hexacyanoferrate(III) (Aldrich), 10 mM PBS.
4. ssDNA Probe Incubation Solution: 10 mM PB, 100 mM Sodium Chloride (NaCl; Sigma-Aldrich), 10 μM Tris(2-carboxyethyl)phosphate (TCEP; Aldrich), 1 μM ssDNA Probe (*see* Table 1 for oligomers' sequences).
5. Blocking Solution: 1 mM 6-mercapto-1-hexanol (MCH; Aldrich), 10 mM PBS.
6. SSC Buffer: 20× concentrated saline-sodium citrate buffer (SSC; Sigma), DI. Components are mixed to yield a 4× concentrated SSC buffer.
7. Noncomplementary ssDNA Incubation Solution: 1 μM ssDNA oligomer (*see* Table 1 for oligomers' sequences), SSC Buffer.

Table 1
List of ssDNA probe and target oligomers

Name	Sequence
ssDNA probe #1	100 nmole DNA oligo, 5′-HS-$(CH_2)_6$-AAAGC TCCGATAGCGCTCCGTGGACGTCCC-3′ (Integrated DNA Technologies)
Complementary ssDNA target #1	100 nmole DNA oligo, 5′-GGGACGTCCACG GAGCGCTATCGGAGCTTT-3′ (Integrated DNA Technologies)
ssDNA probe #2	100 nmole DNA oligo, 5′-HS-$(CH_2)_6$-ACGCG TCAGGTCATTGACGAATCGATGAGT-3′ (Integrated DNA Technologies)
Complementary ssDNA target #2	100 nmole DNA oligo, 5′-ACTCATCGATTCG TCAATGACCTGACCCGT-3′ (Integrated DNA Technologies)
ssDNA probe #3	100 nmole DNA oligo, 5′-HS-$(CH_2)_6$-ACCTAG ATCCAGTAGTTAGACCCATGATGA-3′ (Integrated DNA Technologies)

8. Complementary ssDNA Incubation Solution: 0.01, 0.1, 1, or 10 μM of the ssDNA target (*see* Table 1 for oligomers' sequences), SSC Buffer.

3 Methods

The microfluidic valved-based arrayed lab-on-a-chip device is designed to have two operations—actuation and sensing. The actuation part is based on a top layer of microchannels and valves made of PDMS. The sensing part is the bottom layer with assay channels and a layout of electrodes array that is designed to provide individually addressable working electrodes. By actuating the top microfluidic channels vertical and horizontal configurations are achieved, that expose either rows or columns of electrodes. This controlled actuation configurations enables addressable assembly of ssDNA probes on specific electrodes followed by parallel introduction of ssDNA targets and DNA hybridization testing of multiple samples.

3.1 Microfluidic Valved-Based Arrayed Lab-on-a-Chip Fabrication

1. Electrochemical chip fabrication (microfabricated chip is shown in Fig. 1
2. An array of gold microelectrodes is fabricated using standard photolithography and thin film deposition technologies (*see* **Note 1**) using the following steps.
   (a) A 200 Å thick layer of chromium followed by a 2000 Å thick layer of gold are deposited on borosilicate glass wafers using a sputtering system.

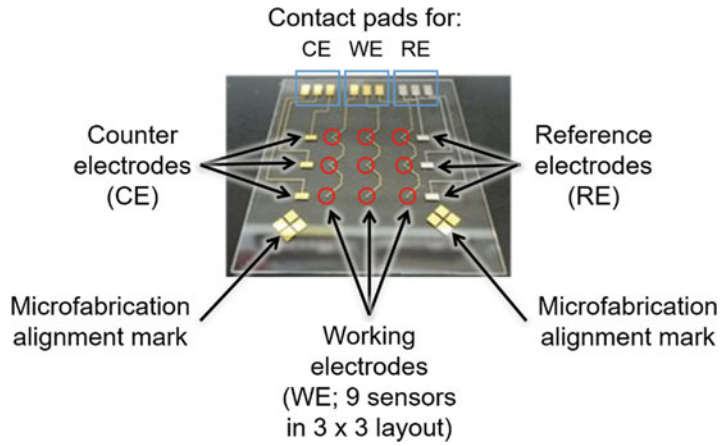


Fig. 1 Photograph of the fabricated arrayed electrochemical chip (3.5 × 4 cm). Micromanipulators are used to connect to each one of the reference, counter, and working electrode pads. Adjusted from [9] with permission from Elsevier

(b) A positive photoresist (Shipley 1813) is spun to create a uniform film ~1.6 μm thick (spinning parameters: 3000 rpm, 1000 rpm/s, 30 s).

(c) The wafer is pre-baked for 1 min at 100 °C on a hot plate.

(d) The wafer is exposed with 190 mJ/cm^2 at 405 nm wavelength through a photomask.

(e) The wafer is developed for 30 s in a 352 developer.

(f) Gold etchant is used to etch the gold layer for 1:30 min. Chromium etchant is used to etch the chromium layer for ~30 s.

(g) The photoresist is stripped by rinsing with acetone, methanol, and iso-propanol.

(h) The wafer is cleaned with 4:1 H_2SO_4–H_2O_2 mixture ("Piranha" clean) for 1 min (*see* **Note 2**).

3. A set of three platinum reference electrodes are patterned on the wafer.

(a) An image reversal photoresist (AZ 5214-E) is spun to create a uniform film ~1.4 μm thick (spinning parameters: 3000 rpm, 1000 rpm/s, 30 s).

(b) The wafer is pre-baked for 1 min at 100 °C on a hot plate.

(c) The wafer is exposed with 30 mJ/cm^2 at 405 nm wavelength through a photomask.

(d) The wafer is further baked for 45 s at 125 °C on a hot plate.

(e) The wafer is exposed with 1000 mJ/cm^2 at 405 nm wavelength without a photomask (Flood expose; *see* **Note 3**).

(f) The wafer is developed for 2 min in 6:1 AZ 400K developer and rinsed with DI.

(g) A 400 Å thick layer of titanium followed by a 1600 Å thick layer of platinum are deposited using an E-beam evaporation system.

(h) The photoresist is lifted off in an ultrasonicated acetone bath for 5 min followed by rinse with acetone, isopropanol, and DI.

3.2 Microfluidic Valved Chip Fabrication

The microfluidic valve-based chip is comprised of two layers of microchannels made of PDMS; a bottom layer with assay channels and a top layer with valves (Fig. 2). Two different PDMS processes are used to create the molds and the PDMS chips for the dual layer stack of microchannels.

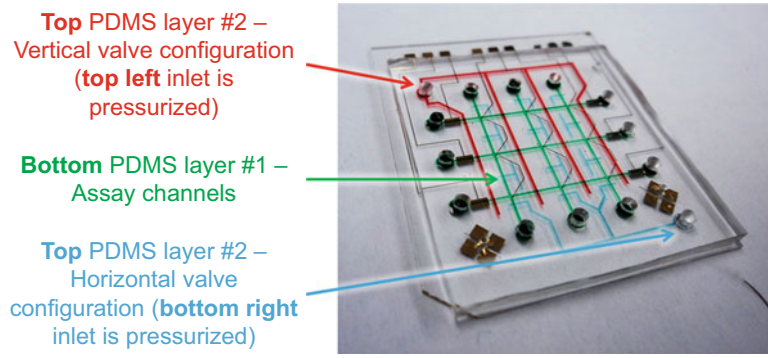


Fig. 2 Photograph of the entire assembled device. Assay channels are filled with green dye, and valve channels are filled with either blue or red dye for horizontal or vertical assay channels orientation, respectively. Adjusted from [9] with permission from Elsevier

1. Dual layer molds fabrication
   (a) Assay channels mold fabrication (bottom layer)
      - An adhesion promoter (HMDS) is spun on a blank silicon wafer (spinning parameters: 3000 rpm, 30 s).
      - A first layer of a positive photoresist (AZ 9260) is spun on the wafer to create a uniform film ~11 μm thick (spinning parameters: 2400 rpm, 800 rpm/s, 60 s).
      - The wafer is baked at 110 °C for 80 s on a hot plate.
      - A second layer of a positive photoresist (AZ 9260) is spun on the wafer to create a final uniform film ~24 μm thick (spinning parameters: 2100 rpm, 700 rpm/s, 60 s).
      - The wafer is baked at 110 °C for 210 s on a hot plate.
      - The photoresist is rehydrated at room temperature for 15 min (*see* **Note 4**).
      - The wafer is exposed with 1200 mJ/cm^2 at 405 nm wavelength through a photomask.
      - The wafer is developed (2-step development parameters: step one—4:1 AZ 400K developer, 10 min. Step two—3:1 AZ 400K developer, 5 min).
      - The profile of the photoresist is rounded by baking at 115 °C for 90 s on a hot plate.

   (b) Valves channels mold fabrication (top layer)
      - A negative photoresist (SU-8 50) is spun on blank silicon wafer to create a uniform film ~100 μm thick (2-step spinning parameters: step one—600 rpm, 120 rpm/s, 10 s. Step two—1150 rpm, 383 rpm/s, 27 s).

- The wafer is pre-baked on a hot plate (2-step baking parameters: step one—ramp up from room temperature to 65 °C at 300 °C/h and hold temperature for 10 min. Step 2—ramp up to 95 °C at 300 °C/h and hold temperature for 30 min).
- The wafer is exposed with 2500 mJ/cm^2 at 405 nm wavelength through a photomask.
- The wafer is post-baked by ramping up to 95 °C at 300 °C/h and holding temperature for 10 min on a hot plate.
- The wafer is developed for 10 min in SU-8 developer followed by rinse with iso-propanol and dry with nitrogen gun.

(c) Dual-layer microfluidic chip fabrication.

- The assay channels are formed using a 20:1 PDMS mixture (20 g of elastomer, 1 g of curing agent) that is completely degassed in a vacuum chamber for 20 min.
- The prepared 20:1 PDMS mixture is spun on the assay channels mold to create a uniform film ~55 μm thick (spinning parameters: 1000 rpm, 90 s; *see* **Note 5**).
- The spun PDMS is cured in a box furnace (2-step parameters: step one—5 min ramp to 80 °C. Step two—80 °C for 17 min).
- The valve channels are formed using a 5:1 PDMS mixture (20 g of elastomer, 4 g of curing agent) that is completely degassed in a vacuum chamber for 20 min.
- The prepared 5:1 PDMS mixture is poured over the valve channel mold and cured in a box furnace (2-step parameters: step one—5 min ramp up from room temperature to 80 °C. Step two—hold at 80 °C for 17 min).
- Individual pieces are cut from the PDMS using a razor blade and aligned over the PDMS that was spun on the assay channels mold to form a stack of two layers (*see* **Note 6**).
- The dual layer PDMS is cured in a box furnace at 80 °C for 3 h followed by cooling down for 3 h.

(d) Device assembly

- The dual layer PDMS stack is cut out using a razor blade and peeled away from the mold (*see* **Note 7**).
- A dermatological punch with a radius of 1 mm is used to punch holes in the PDMS stack to create both fluid and pneumatic inlets (*see* **Note 8**).

- The electrode chip is dipped in "Piranha" solution to clean the surface of any organic contaminants (Caution! *see* **Note 2**).
- The PDMS stack along with the electrochemical chip are placed face up in a RIE machine to expose each surface to oxygen plasma.
- After exposure, a few drops of methanol are placed on the chip and the PDMS stack is aligned carefully over the electrodes (*see* **Note 9**). The methanol evaporates and the bond is allowed to set for 48 h.
- Flexible tubing is connected to a plastic elbow or straight connector using a short flexible tube as an adapter. The connectors are attached to each of the hole-punched inlets in the device.
- *Assay channels inlets:* A syringe is connected on the other end of the tubing and place the syringe in a syringe pump. The set of a syringe, a tube, and a connector is alternatively changed according to the required procedure steps in Subheading 3.3–3.5 and their corresponding working solutions. *Valve channels inlets*: the channels are filled with water using a syringe pump and connected to a pressurized air regulator.

3.3 Microfluidic Valved Lab-on-a-Chip Operation

1. Hydraulic valve operation (Fig. 3a) is achieved using pressurized air (5 psi). The increase in pressure causes the PDMS membrane to bend downward closing the valve and sectioning the bottom assay channel, while releasing the pressure causes the valve to open (Fig. 3b).
2. Pressurized air is applied to the top left to form vertical microfluidic assay channels (Fig. 3c, top image), which results in three columns of three working electrodes, each can be functionalized with three different ssDNA probes.
3. Pressurized air is applied to the bottom right to form horizontal microfluidic assay channels (Fig. 3c bottom image), which results in a three-electrode system of three sensors, a counter electrode, and a reference electrode.
4. In all tested solutions, the assay channels are filled with the sample solutions at a flow rate of 30 μL/h using a syringe pump (*see* **Note 10**).

3.4 Electrochemical Activity Validation

1. Horizontal configuration is applied and the assay channels are filled with the reversible redox couple Ferrocyanide/Ferricyanide Electrochemical Testing Solution to characterize the Nernstian electrochemical response of the device.
2. Cyclic voltammetry technique is applied using a potentiostat at 25, 50, 100, 150, 200, and 250 mV/s scan rates for all nine working electrodes.

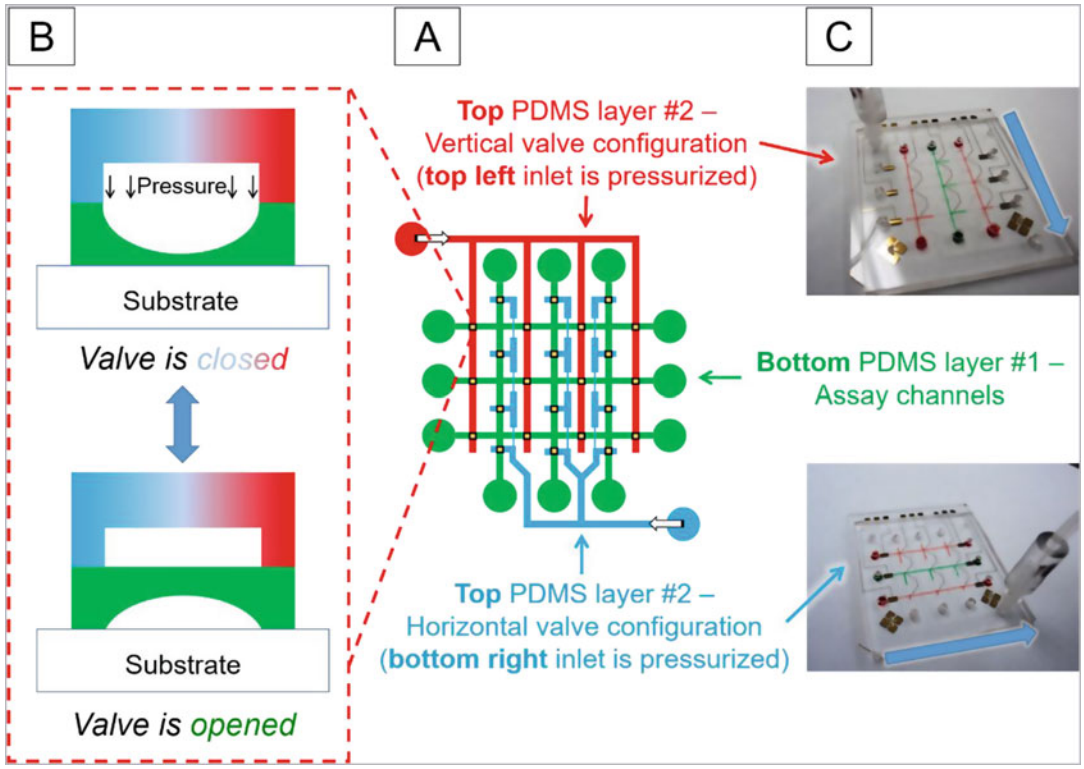


Fig. 3 Microfluidic valved-based device layout and operation. (**a**) Layout of the top valve configuration (*blue* or *red*) and the bottom assay (*green*) channels. Green circles are the punched inlet and outlet holes. (**b**) Schematic demonstrating valve actuation using hydraulic channel to pinch off the microfluidic assay channel below. *Bottom*—valve is opened when no pressure is applied. *Top*—valve is closed upon pressure application. (**c**) Photographs of the vertical (*top*) and the horizontal (*bottom*) valve configurations with the resulted assay channels orientation filled with red and green dyes. Thick arrows indicate fluid flow direction. Adjusted from [9] with permission from Elsevier

Electrochemical characterization with a known redox couple demonstrates reversible Nernstian characteristics for all nine electrochemical sensors (Fig. 4). By corresponding to Randles–Sevcik equation where a linear relationship is demonstrated between the current peak and the square root of the cyclic voltammetry's scan rate (Fig. 5) the electrochemical performance of the electrodes is validated.

3.5 ssDNA Probe Modification

Vertical configuration is applied and each of the three separate microchannels is functionalized with a different ssDNA probe sequence.

1. Each microchannel is filled with a different ssDNA Probe Incubation Solution and incubated for 3 h followed by rinsing with PBS (*see* **Note 11**). The incubation procedure functionalized the surface of the working electrode with the ssDNA probe.
2. Each microchannel is filled with a Blocking Solution and incubated overnight (~18 h) followed by rinsing with PBS.

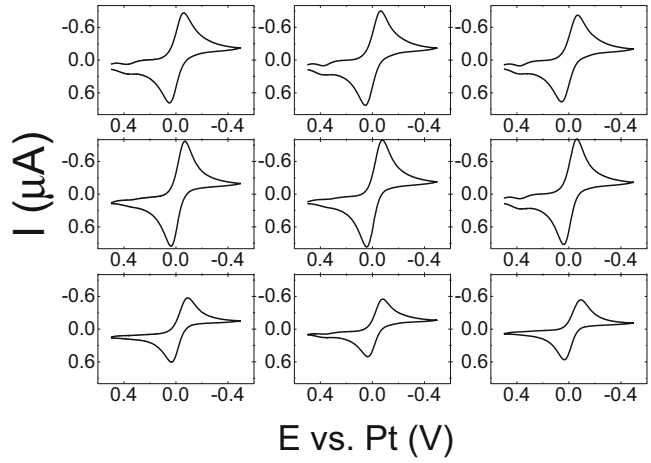


Fig. 4 Cyclic voltammograms for each of the nine working electrodes. The voltammograms are arranged in the same 3 × 3 grid as the physical sensor layout. Adjusted from [9] with permission from Elsevier

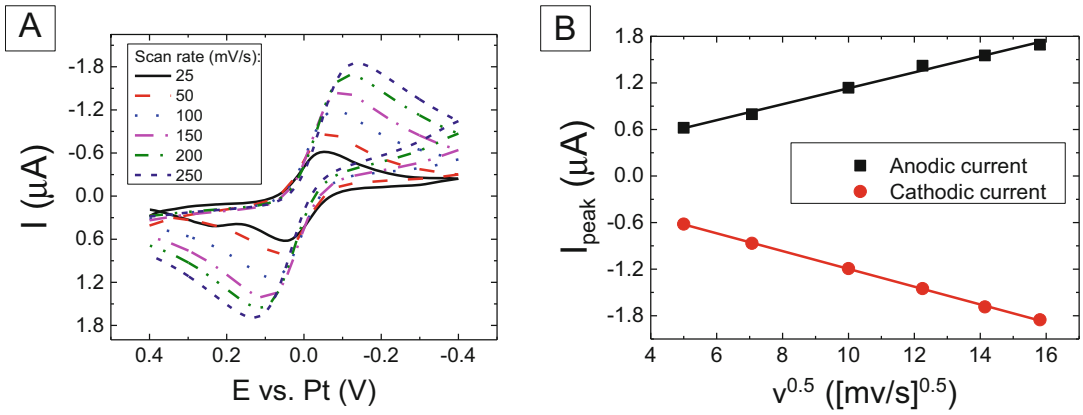


Fig. 5 (**a**) Cyclic voltammograms at 25, 50, 100, 150, 200, and 250 mV/s scan rates of a representative working electrode. (**b**) The influence of the square root of the scan rate on either the anodic (*black squares*) or the cathodic (*red circles*) peak currents according to the linear relation in Randles–Sevcik equation [11]. Adjusted from [9] with permission from Elsevier

3.6 DNA Hybridization Detection

Horizontal configuration is applied and each of the three microchannel rows is filled with the tested solution. Cross-contamination of ssDNA targets from different solutions is prevented by the alternated operation of the valves; leakage of fluids from one row to another is blocked by the closed configuration of the valves.

1. All three microchannels are filled with a control solution of SSC Buffer and incubated for 20 min (Background measurement).

2. Each microchannel is filled with Ferrocyanide/Ferricyanide Electrochemical Testing Solution and impedance values of the electrochemical system are recorded for different frequencies using a potentiostat connected to the working, counter, and reference electrodes (EIS parameters: frequency range from 100 kHz to 1 Hz, 12 frequency data points per frequency decade, 25 mV amplitude, 0 mV polarization versus the reference electrode; electrode connection configuration is described in Fig. 1). EIS measurements are repeated for each of the working electrodes three times (*see* **Note 12**).
3. Microchannels are filled with a noncomplementary ssDNA Incubation Solution and incubated for 20 min.
4. Electrochemical testing is done by filling each microchannel with a Ferrocyanide/Ferricyanide Electrochemical Testing Solution and performing EIS recordings (EIS parameters: frequency range from 100 kHz to 1 Hz, 12 frequency data points per frequency decade, 25 mV amplitude, 0 mV polarization versus the reference electrode). EIS measurements are repeated for each of the working electrodes three times (*see* **Note 12**).
5. Microchannels are filled with a Complementary ssDNA Incubation Solution and incubated for 20 min.
6. DNA hybridization measurements are done by filling each microchannel with a Ferrocyanide/Ferricyanide Electrochemical Testing Solution and performing EIS recordings (EIS parameters: frequency range from 100 kHz to 1 Hz, 12 frequency data points per frequency decade, 25 mV amplitude, 0 mV polarization versus the reference electrode). EIS measurements are repeated for each of the working electrodes three times (*see* **Note 12**).
7. EIS recordings (Fig. 6) are analyzed using an equivalent electrical circuit (Fig. 7a; *see* **Note 13**) and lumped components of the electrochemical system are calculated (Fig. 7b, c; *see* **Notes 14** and **15**).

4 Notes

1. Parameters of each of the process steps may change due to environmental effects (e.g., temperature and humidly), type of chemicals and tools (e.g., storage time and vendor brand), and the user (e.g., skills and experience). Therefore, these parameters should be optimized to fit the relevant environment.
2. CAUTION! This solution is a strong oxidizer and potentially explosive.

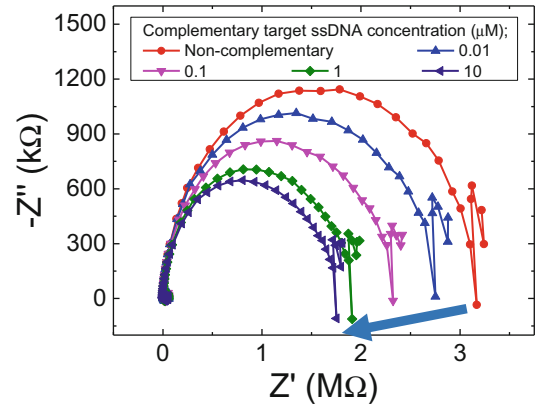


Fig. 6 Nyquist plot of impedance spectroscopy measurements demonstrating the influence of the concentration of the complementary ssDNA target on the biosensing mechanism (Arrow indicates increasing ssDNA target concentrations; 1 μM noncomplementary ssDNA target concentration). Adjusted from [9] with permission from Elsevier

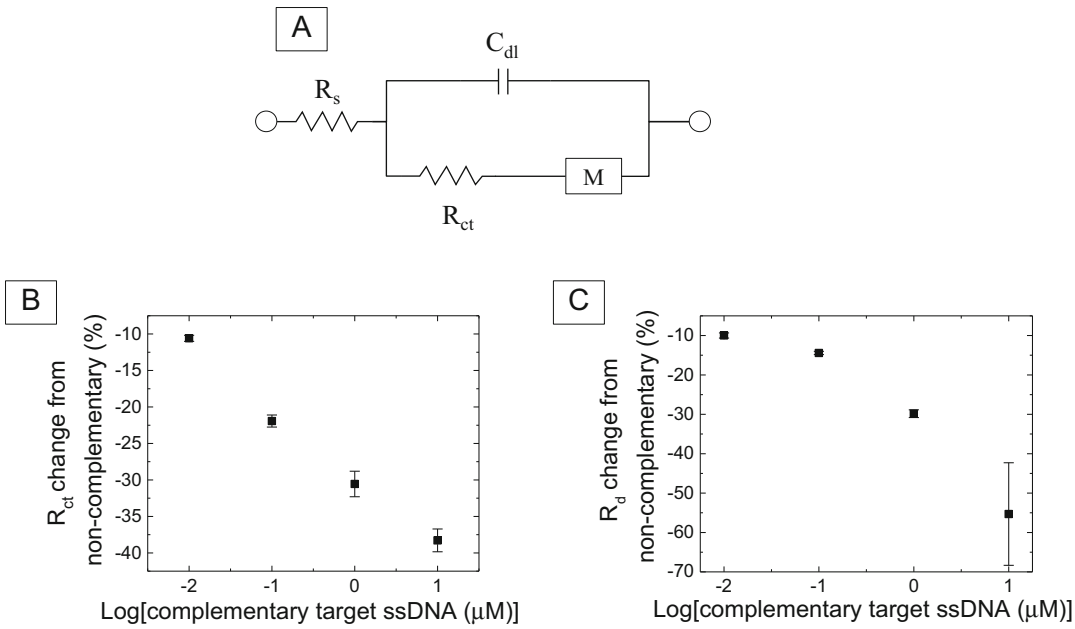


Fig. 7 (**a**) Diagram of a diffusion-restricted electrical equivalent circuit that is used for the analysis of the impedance spectroscopy measurements. R_s is the solution resistance, R_{ct} is the charge transfer resistance, C_{dl} is the electrode–electrolyte double layer interface, and M is restricted linear ordinary diffusion impedance element with a reflective boundary. The influence of the complementary ssDNA target concentration on the calculated change from noncomplementary target ssDNA of (**b**) charge transfer resistance—R_{ct} (coefficient of variation = 47%) and (**c**) restricted diffusional resistance—R_d (coefficient of variation = 75%) components. Mathematical expressions of the electrical components are listed in Table 2. Adjusted from [9] with permission from Elsevier

3. We experienced variable exposure time values on glass substrates due to their reflective properties and unintentional rehydration in high humidity environment.
4. Rehydration time is based on humidity and can vary between 15 min and 24 h.
5. PDMS mixture should be poured slowly to prevent formation of air bubbles in the spun PDMS.
6. Alignment marks can be drawn in the molds' masks to ease the alignment between the top and bottom PDMS layers.
7. The PDMS stack should be pilled slowly and gently to prevent tearing of the thin bottom layer. If the bonding between the bottom layer PDMS and the mold is too strong which prevents pilling off the dual layer stack, a modification step of the mold with trimethylchlorosilane that is evaporated under vacuum conditions can be added prior to spinning the PDMS (i.e., **step c** in Subheading 3.2.2a). The chemical agent covers the surface of the mold, a step that will ease the subsequent thin film PDMS release.
8. The dermatological tool can be twisted during the puncture of the inlet holes which helps preventing from partial removal of the dual layer punched PDMS.
9. Methanol allows the user to slide the PDMS over the chip and to provide better alignment.
10. Valve's membrane may stick to the glass substrate and prevent from filling the assay channels. Changing the flow direction in the syringe pump a couple of times helps releasing the membrane and filling the whole channel.
11. We experience improved coverage of the electrode with ssDNA probes for longer incubation periods.
12. The polarization potential between the working and the reference electrodes shall be optimized by application of different DC bias potentials that will yield high signal-to-noise ratio values.
13. The presented equivalent electrical circuit is used as an example and other circuits are available to model the chemical and physical reactions of the electrochemical system. In the example here the components of the circuit and their mathematical expressions are listed in Table 2.
14. Equivalent electrical circuit fitting is done with EC-Lab software.
15. A detailed video about the fabrication and testing methods of DNA hybridization detection can be seen in [12].

Table 2
The electrical equivalent circuit elements incorporated into the presented bioelectrical model and their corresponding impedances

Electrical element	Corresponding impedance expression
Solution resistance (R_s)	R_s
Double layer capacitance (C_{dl})	$1/jwC_{dl}$
Charge transfer resistance (R_{ct})	R_{ct}
Restricted linear diffusion with reflecting boundary (M). ω is the radial frequency, R_d is the diffusional resistance, and τ_d is the diffusional time constant.	$Z_M(\omega) = R_d \frac{\coth(\sqrt{\tau_d j\omega})}{\sqrt{\tau_d j\omega}}$

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