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Lab-on-chip device for single cell trapping and analysis

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Abstract Traditional cell assay gives us an average result of multiple cells and it is assumed that the resultant is the outcome of all cells in population. However, single cell studies have revealed that individual cells of same type may differ dramatically and these differences may have important role to play in cells functionality. Such information can be obscured in only studying cell population experimental approach. To uncover biological principles and ultimately to improve the detection and treatment of disease, new approaches are highly required to single cell analysis. We propose to fabricate a lab on chip device to study high throughput single cell nanotoxicity analysis. The chip incorporates independently addressable active microwell electrodes for cell manipulation and analysis. We employed positive-dielectrophoresis approach to quickly and efficiently capture single cells in each wells with having control over individual microwells. We examined change in impedance properties to verify cell capture in microwell and its health and present a novel model of single cell assay for nanotoxicity, and drug testing.

Keywords Single cell analysis · Lab-on-chip · Dielectrophoresis · Cell trapping · Electrical impedance sensing

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1 Introduction

Traditional cell assay techniques study a population of cells in order to understand cell proliferation, differentiation, protein or gene expression, drug response and toxicity assays, etc. and the result is assumed to be average of the whole population. However, cellular heterogeneity in isogenic cell population is often found (Graf and Stadtfeld 2008; Irish et al. 2006). Variety of techniques has been developed to study behavior of a single cell, such as optical, patch clamp, needle electrode and lab on chip based devices. Analyzing individual cells with higher spatiotemporal resolutions will provide more accurate representation of cell-to-cell variation in an isogenic population instead of the stochastic average masked by bulk measurements (Wang and Bodovitz 2010).

To analyze single cell, sorting of a single cell from population of cell is necessary. One of the most frequently used method to quickly and efficiently sort, count and/or measure the characteristics of single cells in large volume (large throughput) is flow cytometry (FCM). Cells can be tagged with different fluorescent markers and simultaneous measurement of multiple fluorescent signals, as well as light scatter–induced illumination of single cells or microscopic particles in suspension can also be detected (O'Connor et al. 2001). However, this technique cannot support real time measurements of cells in their natural environment. Capacitance based patch clamp is a very sensitive technique to detect dynamic cell signaling, however it interferes with cell membrane and requires complex set-up (Eilers et al. 1995). Needle electrode based technique is also very sensitive approach for spatio-temporal single cell analysis, however, like patch clamp, it also requires complex and time consuming set-up, need a trained professional to operate and manipulate precise positioning of electrodes and pipettes, and has low throughput (Prabhulkar and Li 2010; Wightman et al. 1991; Li et al. 2011).

Today, several lab-on-chip (LOC) cell immobilization and manipulation methods have been developed, such as, microwell, microchambers, dams, traps or single cell adhesion through functionalized surfaces. These LOC devices employed acoustic (Franke et al. 2010), magnetic (Lee et al. 2008; Pamme and Wilhelm 2006), optical (Chiou et al. 2005; Wang et al. 2005), hydrodynamic (Chabert and Viovy 2008; Chen et al. 2010), mechanical (Chung et al. 2011; Di Carlo and Lee 2006), and electrical (Kim et al. 2011; Murata et al. 2009) approaches to aid trapping of cells. Acoustic, magnetic and optical cell sorting techniques require additional labeling with antibody conjugated micro/nano particles for cell sorting. However, additional labeling of cells may induce changes in physiological property of cells. Hydrodynamic approach is a good passive approach for cell sorting where cells are flown through the micro channel at a controlled flow rate. The main challenge in hydrodynamic capturing is that it requires a precise microfluidic control of multiple streams to employ cell sorting. Mechanical cell sorting approaches are based on the microfabricated structural filters where cells are separated or captured based on its morphology. The filter structures can be blocked and intensive surface interactions during the filtration process can cause significant shear forces on sorted cells (Fritzsch et al. 2012) in such designs. Surface functionalization to capture high-throughput array-based single cell employs modification surfaces with cytophilic and cytophobic materials to attract and repel cells, respectively. Dielectrophoretic is an effective and noninvasive technique to efficiently manipulate single cells. It allows label free, shear stress–free, and strong deflection as well as fast response times. Microwell structure can support time elapsed study of single cell. Dielectrophoresis integrated with microwell structure has been used before (Kim et al. 2011; Murata et al. 2009), however, it was applied in an array format and control of an individual electrode was not functional. Here, we present an active microwell lab-on-chip device to capture and electrochemically analyze cells without any additional detection mechanism.

Impedance based sensors has been in studies for many applications, such as detection of cell migration (Schiller et al. 2010; Wang et al. 2008), cell growth and proliferations (Xiao and Luong 2003), cell health (Campbell et al. 2007; Keese et al. 2002), cytotoxicity (Asphahani et al. 2012), nanotoxicity (Hondroulis et al. 2010), drug effects (Asphahani and Zhang 2007), and circulating tumor cell detection (Arya et al. 2012). Here, we describe a novel concept of microfluidic device containing array of (2×4 here but it can be practically fabricated for any size) electroactive microwells that performs pDEP based single-cell trapping in controlled manner with subsequent electrical impedance sensing to confirm the trapping of single cells on top of an electrode inside the microwell. Individual control of each microwell on the chip allows capturing of a single cell inside a chosen microwell or all microwells simultaneously in less than 30 s. Sensitive microelectrodes allow the concept to be used in for high throughput applications with precise and easy control for single cell analysis in drug screening, and cytotoxicity/nanotoxicity studies.

2 Experimental section

2.1 Chemicals and reagents

Hydrogen peroxide (H_2O_2), Sulfuric Acid (H_2SO_4), Acetone, Methanol, and Isopropanol all were purchased from Fisher Scientific Inc. and used as received. 1X Phosphate Buffered Saline (PBS) (pH=7.4), L-Cysteine, F-12 K with L-glutamine Medium (ATCC, VA), fetal bovine serum (FBS) (Gibco), penicillin, Sucrose (BDH).

2.2 Cell culture and solution

Rat lung epithelial cells (CCL-149) were obtained in a vial from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in a humidified atmosphere containing 5 % CO_2 in an incubator. F-12 K supplemented with Fetal Bovine Serum (10 %, Gibso), and penicillin (1 %, Sigma Chemical Co.) was used as cell culture medium. The culture medium was changed at every 48 h. Once the cell culture was confluent (after 2–3 days), they were trypcinised and centrifuged to collect the pallet of cells and re-suspended it in 0.2 M sucrose buffer for pDEP. It is very important to have high resistive buffer for pDEP application. Cells in the culture medium were centrifuged at 1,700 rpm for 5 min. We gently removed the culture medium and added 0.2 M sucrose buffer. Final cell concentration was about 2×10^5 cells/mL. The average diameter of cells was approximately 15 μm.

2.3 Design and fabrication of the microfluidic chip

Two rows of four electrodes were designed such that they maintain a distance of 200 μm between them to avoid any cross talk. A bigger size reference electrode of 400 μm was created on chip to avoid any need of external electrode in microfluidic chip. The size reference electrode was big enough compared to working electrode (20 μm) to avoid any capacitive effects. Schematic representation of electrode placement is shown in Fig. 1b.

Fabrication process is depicted in Fig. 1a. The double sided, polished, 4 in. quartz glass wafer (University wafers, USA) piranha cleaned in 3:1, H_2SO_4:H_2O_2 (Sigma-Aldrich) at room temperature for 30 min. The wafers were rinsed thoroughly in running DI water for at least 5 min and dried with Nitrogen gas. After cleaning; it was baked on hot plate at 115°C for 5 min to remove any moisture available on the

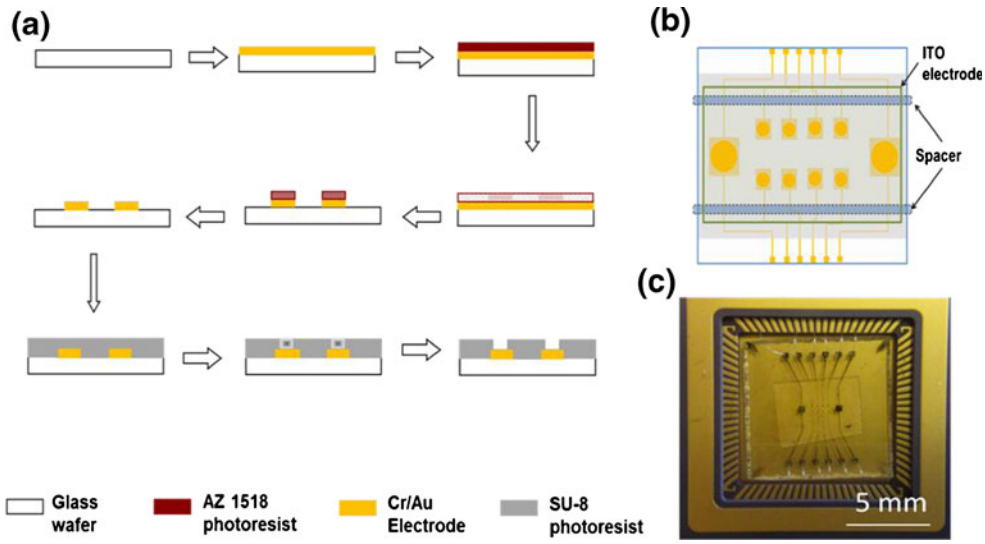

Fig. 1 **a** Step by step representation of lithography process to fabricate microchip; **b**) Schematic representation of a chip assembly; **c**) microchip wire-bonded to PLCC adapter

surface before any further processing. I) 25 nm of Cr adhesion layer and 250 nm of Au thin film was deposited on the cleaned glass wafer using ion beam evaporator (JEOL, Japan) available in the AMERI facility at FIU. The electrode pattern created using lithography process and wet chemical etching. Briefly, ii) AZ1518 (MicroChem Co.) positive photo-resist of 1.5 μm thickness spin coated on wafer and iii) exposed it through a pattern glass-chrome mask using mask aligner (OAI 800). Iv) Photoresist was developed in developer (4:1. DI water:AZ400) for 50 s to define the pattern on wafer. V) Undesired Au and Cr were etched chemically through Au etchant and Cr etchant, respectively. Vi) After defining the electrode pattern on wafer, it was cleaned in Acetone, Methanol and DI water for 5 min under sonication at each step and finally plasma cleaned in reactive oxygen chamber. Vii) Passivation layer of negative photoresist SU-8 2025 (MicroChem Co.) was spin coated for 30 s at 3,500 rpm to achieve 25 μm thickness using spin coater and was viii) exposed under UV light through another pre-defined glass-chrome mask using mask aligner. Exposed wafer was hot baked and developed using SU-8 developer. Development time and method was optimized using sonication to clearly defined SU-8 pattern. The wafer was cleaned using ashing in plasma chamber to remove any uncross-linked SU-8 and organic contamination from the surface. The SU-8 was hard baked at 150 °C for 30 min. Thickness of SU-8 was measured by a profilometer (Alpha step). The Su-8 pattern was created such that only the sensing part (20 μm cell capturing well) and connection (bond pad) were exposed while the rest of the layer serves as dialectic passivation layer to avoid any cross talk. The fabricated gold micro-electrodes were characterized for resistance and roughness and found to possess a sheet resistance of 5 μΩ-cm.

2.4 Surface modification

Electrode surface was modified with 10 mM L-cysteine by allowing self-assembled monolayer (SAM) formation on Au electrode for 1 h. –SH group of cysteine will covalently bind to the gold surface and develops SAM. While the free amine group is positively charged at pH 7.4 will favor cell attachment of normally negatively charged cell membrane (Heiskanen et al. 2008).

2.5 Microfluidic channel

The fabricated microchip was assembled with a top ITO electrode (Delta-technologies Inc, USA) using double sided tape in order to construct a fluidic channel. Prepared microchip was then wire-bonded to the PLCC adapter to provide a robust platform and easy connections to analyzer and signal generator. The final assembled chip is depicted in Fig. 1c.

2.6 pDEP for single cell capture

DEP is generated due to interaction between any dielectric particle's dipole movement and spatial gradient of the electric field. DEP phenomenon can be used to move and manipulate polarizable micro-particles such as cells, markers, etc. suspended in liquid medium (Jones 2003). The time averaged dielectric field, F_{DEP} exerted on cells suspended in liquid may be approximated by following equation;

$$F_{DEP} = 2\pi R^3 \varepsilon_m Re[f_{CM}(\omega)]\nabla E^2 \quad (1)$$

Where R = radius of cell; ε_m = permittivity of suspending medium, ε_c = permittivity of cell; ∇E = rms value of applied A.C. field, ω = angular velocity of the applied field,

Re[$f_{CM}(\omega)$] is the real part of the "Clausius–Mossotti" factor (polarization factor) given by the equation;

$$f_{CM}(\omega) = \left(\varepsilon_c^* - \varepsilon_m^*\right)/(\varepsilon_c + 2\varepsilon_m) \quad (2)$$

Where, ε^*_m and ε^*_c are the complex electrical permittivity of suspended medium and cell, respectively. $\varepsilon^* = [\varepsilon - j\sigma/\omega]$, where σ is electrical conductivity and $j = \sqrt{-1}$. The exerted dielectric field on the cell can be either positive or negative and the real part of Re[$f_{CM}(\omega)$] suggests that, it can be controlled by adjusting the conductivity of suspending medium and frequency of the applied field.) Cell will be attracted or repelled from the electric field region depending on real part of the polarization factor Re[$f_{CM}(\omega)$] value. When Re[$f_{CM}(\omega)$]>0 ($\varepsilon_c > \varepsilon_m$), it refers to attraction; if Re[$f_{CM}(\omega)$]<0 ($\varepsilon_c < \varepsilon_m$), it corresponds to repulsion.

We suspend our cells (~ 2×10^5 per ml) in 0.2 M sucrose buffer, which is very resistive in nature to get effective resultant of positive-DEP by making electrical permittivity of cell greater than that of suspended medium ($\varepsilon_c > \varepsilon_m$).

2.7 Cell viability

Cell viability measurements were carried out using trypan blue. 10 μl mixtures of 1:1 PBS buffer and trypan blue was inserted into the microchannel to replace sucrose buffer. Trypan blue solution was allowed to rest for 5 min in order to stain dead cells and then washed and replaced by 10 μl fresh PBS buffer for 2 times. Cells were analyzed under the microscope to verify the viability based on dead cells was stained blue due to trypan blue accumulation in their cytoplasm. Trypan blue measurement was only carried out to verify the viability of cells under pDEP field and was not carried out before cell impedance measurements.

2.8 Impedance spectrometry

For EIS testing, the impedance detection set-up consisted of a test chip, connection wires, and an impedance measurement system (CH Instruments, Texas) controlled by a computer. The impedance was measured from individual working electrodes and the on-chip counter/reference electrode at fixed 0.015 V. The frequencies ranged from 1 Hz to 10^5 Hz and 60 data points (12 points per decade) were recorded and analyzed for each measurement. All readings were taken in PBS solution (pH=7.4) at room temperature (~25 °C).

3 Results and discussion

3.1 Optical characterization of microelectrodes

Figure 2a and b show the optical bright and dark field images, respectively of electrodes and SU-8 pattern. The exposed circular area (cell capturing/sensing well) is seen in the image as a contrast circle is responsible for contribute to the signal. Figure 2c and d show the bright and dark field images, respectively of a single cell capturing electrode.

3.2 Electrochemical characterization of microelectrodes

Electrochemical testing immediately before the measurement provides the opportunity to remove damaged chips or electrodes and improves reliability of experiments. Characterization of reproducibility and electrochemical activity was carried out by cyclic voltametry (CV) in potassium ferricyanide solution ($[Fe(CN)_6]^{3-/4-}$), prepared in PBS (pH=7.4) buffer. CV is the most widely used technique for acquiring qualitative information about electrochemical reactions. During the potential sweep, the potentiostat measures the redox current resulting from the applied potential using the Randles–Sevcik equation (Faulkner and Bard 2001);

$$i_p = \left(2.69 \times 10^5\right) n^{3/2} A C D^{1/2} v^{1/2} \quad (3)$$

where i_p is the peak current, n is the number of electrons, A is the surface area of the working electrode, C is the bulk

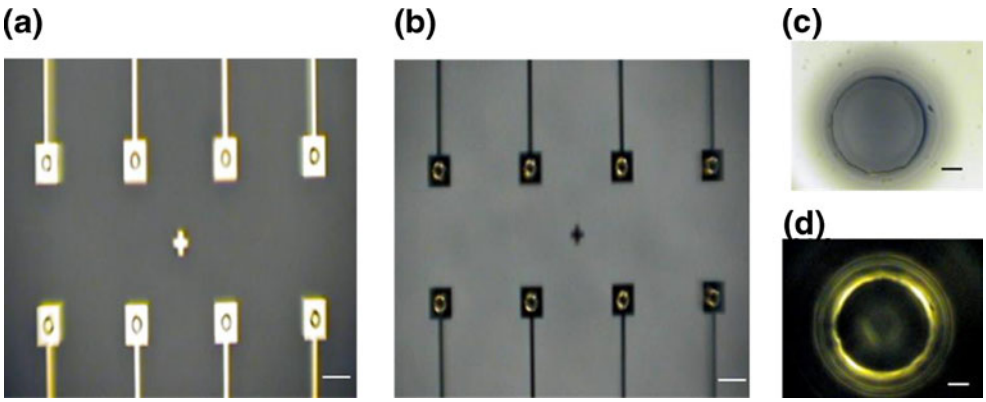

Fig. 2 Optical characterization of microchip; **a**) and **b**) bright and dark field image at 5X magnification, respectively; scale bar 100 μm. **c**) and **d**) bright and dark field images at 100X magnification, respectively; scale bar 5 μm

concentration of the electroactive species (5 mM), D is the diffusion coefficient of the electroactive species (~7.2×10^{-6} cm^2/s for potassium ferricyanide (Albillos et al. 1997)), and v is the scan rate of voltammograms.

Figure 3a shows the cyclic voltammograms of eight electrodes at 100 mV scan rate in potassium ferricyanide solution ($[Fe(CN)_6]^{3-/4-}$). It is evident from Fig. 3a that the shape and current intensity of all eight electrodes are very close to each other and shows a diffusion limited microelectrode behavior. Figure 3b shows the cyclic voltamogram of an electrode at various scan rates between 100 and 500 mV/s and a standard reversible behavior of ferricyanide solution was noted. Figure 3c shows Randles-Sevcik plot of the anodic peak and cathodic peak current intensities with respect to the square root of scan rate for the values of Fig. 3b and it is evident that the electrode posses a very good linearity and electron transfer is surface controlled.

3.3 Single cell trapping

We have demonstrated single-cell trapping using pDEP technique within a simple microwell array device. The cell suspension in 0.2 M sucrose media is introduced into the one end of microfluidic chip. Due to capillary force, the flow will be drawn into the micro channel towards the other end of the channel. When the DEP force is not applied, the capillary force is high enough to drag the cells along with it and the chances of a cell to be captured in a microwell is scared under constant flow. Cell trapping due to gravitation method usually takes several minutes and the cell retention rate in the microwell is also very low (Figueroa et al. 2010). Cell trapping using surface modification feature also takes few minutes to capture a cell on the electrode surface and it will not be selective for individual electrode. With DEP we can control cell capture on an individual electrode separately and simultaneously for selective trapping of a cell in a

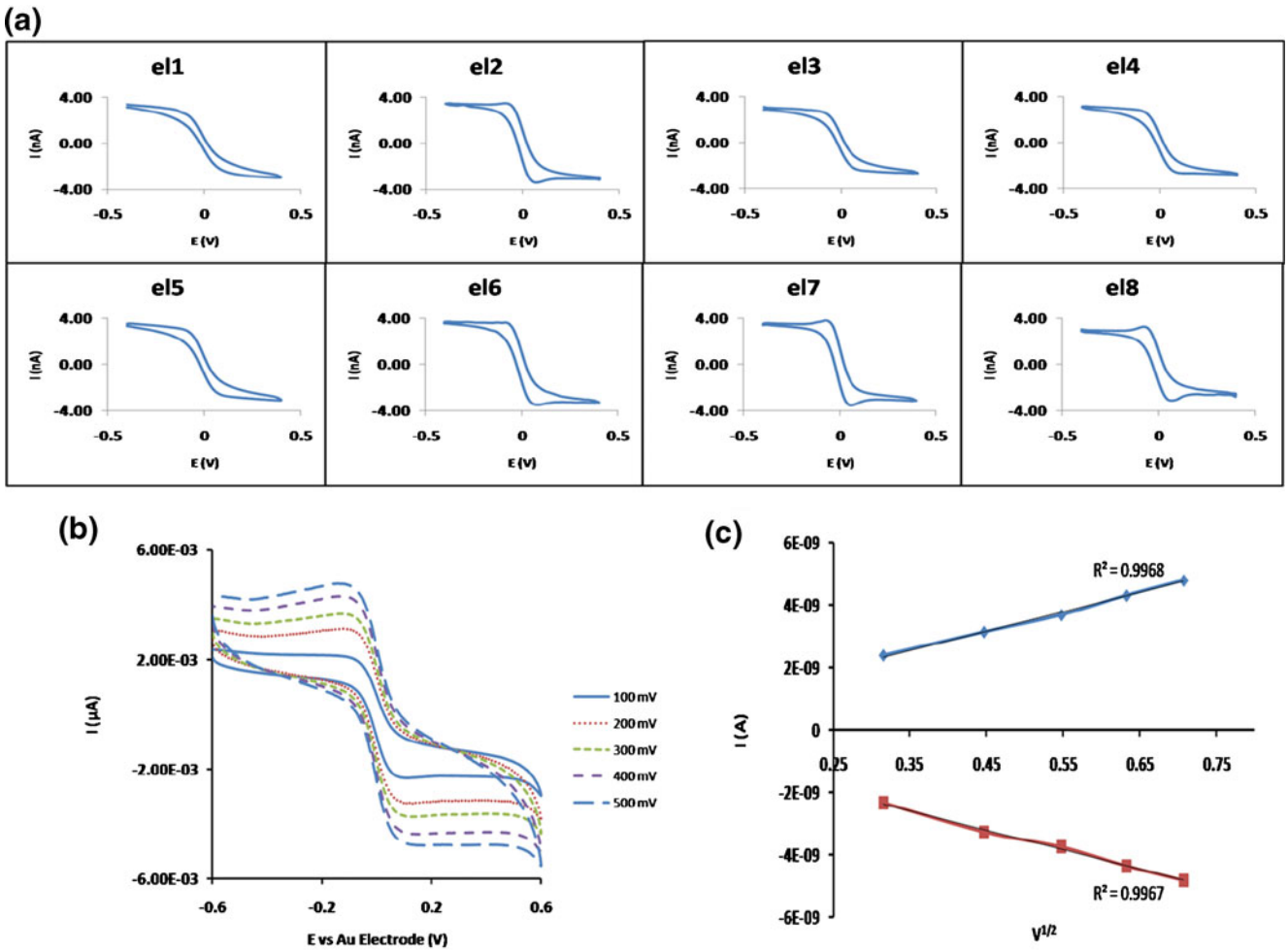

Fig. 3 Electrochemical characterization of microchip; **a**) Simultaneous cyclic voltametry of 8 electrodes **b**) Cyclic voltametry of an electrode at different scan rate from 0.1 V–0.5 V; **c**) Anodic and cathodic peak current vs square root of voltage scan rate

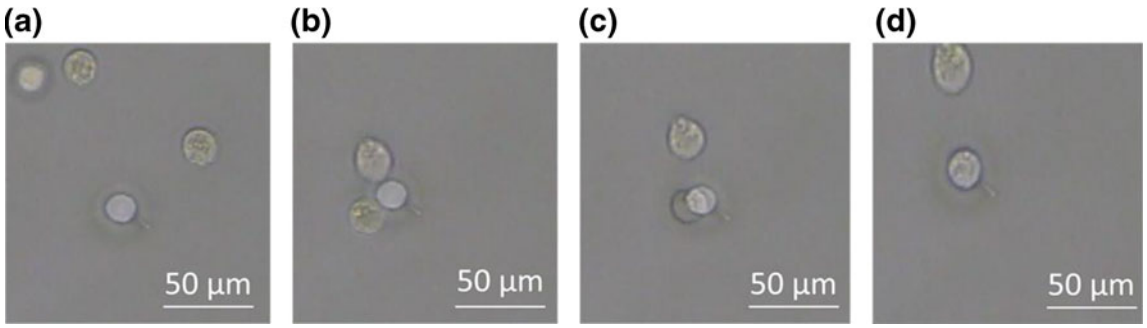

Fig. 4 Single cell capturing using pDEP technique; a) at 10 s, b) 15 s, c) 19 s and d) 20 s. Images were taken from a cell capturing video

particular microwell. For pDEP, 6 Vpp sinusoidal electric potential at frequency of 1 MHz is applied on sensing electrode and top ITO electrode, in 180° out of phase. Figure 4 shows the trapping positions of a cell taken from a video clip at different time interval. The cell will be trapped immediately under pDEP when it passes from top of the microwell. Within 30 s we can achieve a single cell capturing on individual electrodes with this technique. After achieving cell entrapment, the applied field was turned off and additional cells in the channel were removed by a slow flow-stream of PBS buffer.

Once the cell is trapped, it will rest at the edge of the microwell. When a single cell is captured in a 20 μm diameter microwell, another cell will not be able to get in the same microwell because there will be no physical space for another cell and the occupied cell will also reduce the pDEP force by blocking the electric field.

We used trypan blue in order to verify the cell viability after capturing cells using pDEP. 10 μl of trypan blue solution was inserted within 5 min of cell capturing. Cells captured in the microwells were confirmed alive under the microscope as they did not stained blue. Rare dead cells were observed outside the

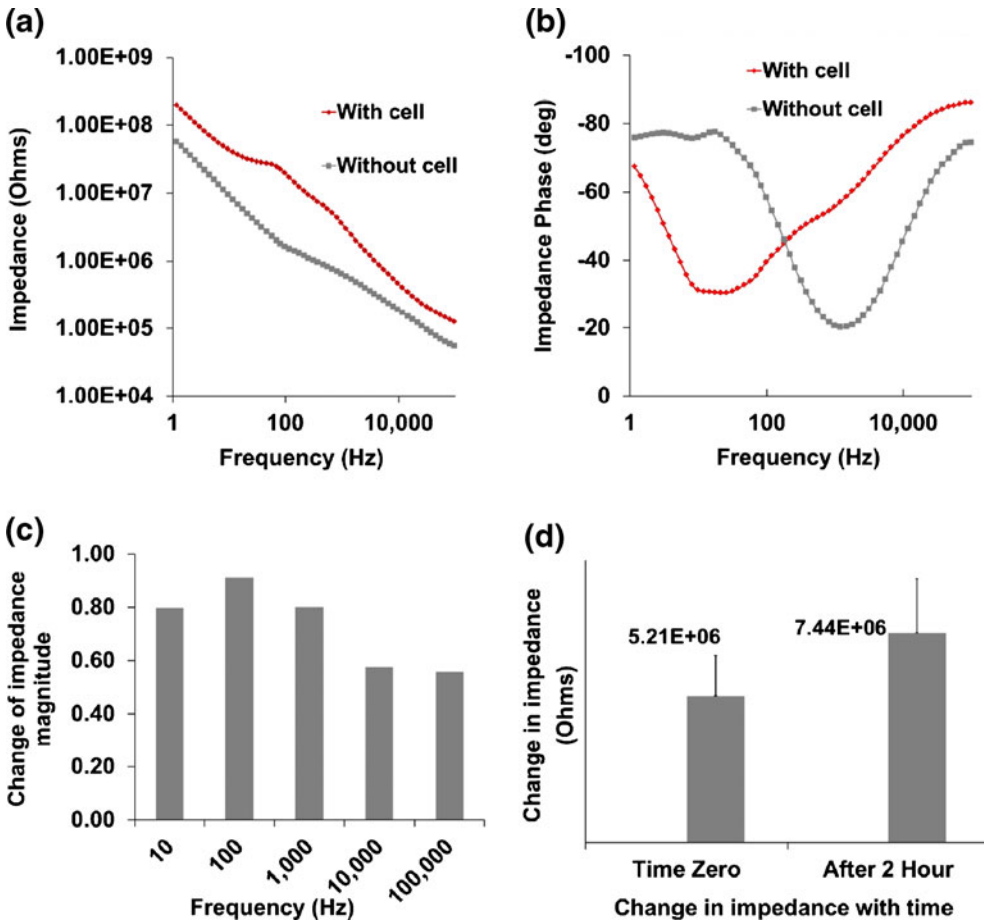

Fig. 5 **a** Bode plot of impedance spectrum in absence and in the presence of a single cell in microwell; **b**) Bode plot of phase spectum in absence and in the presence of a single cell in microwell; **c**) Magnitude of change in impedance comparision at various frequencies; **d**) Comparision of change in impedance compared with an empty electrode when the electrode has just captured the cell (time zero) and when the cell starts attaching and spreading on the electrode (after 2 h)

microwell in the microfluidic channel or outside the microfluidic channel on the chip.

3.4 Electrical impedance sensing of a single cell

Impedance has widely been used for the detection of cellular properties because of its simplicity of measurement and non-invasive nature. Based on ohms law, I = V/R, an immobilized cell on top of electrode would interfere with the flowing current via electrolyte media between the working and reference electrodes due to anchored plasma membrane on top of electrode surface (Giaever and Keese 1993). Figure 5a shows the impedance spectrum and Fig. 5b shows the phase spectrum of an electrode on chip from 1 Hz to 10^5 Hz frequency range, in the absence and in the presence of a cell on top of the electrode.

The impedance spectrum suggest that the difference in impedance at 100 Hz is significant compared to other frequencies in the spectrum. Figure 5c shows the comparison of the change in impedance magnitude at each decade of frequency range and was further confirmed that the change of impedance magnitude was greater at 100 Hz. The impedance was noted to be changed from 1.51 MΩ in absence of a cell to 17 MΩ in the presence of a single cell attached on the electrode surface.

The change in impedance is basically sensed in terms of changing capacitance or resistance at the electrode surface. As the cell proliferates and attaches to the electrode, the impedance increases. Any damage to the cell induces change in impedance measurement. The microwell size is slightly bigger than the size of a single cell and so for a slight change in the impedance was noted when the cell only sits on top of electrode and when it proliferates and covers the larger area of an electrode after 2 h, as shown in Fig. 5d. The impedance reading taken for time zero of an electrode was within 5 min of the cell capturing whereas the impedance measurement for increased size and attachment of the cell was taken after 2 h incubation of the cell.

4 Conclusion

In this study, a novel DEP based microfluidic array device was successfully used to capture single cells with subsequent assessment of their presence on top of electrode by sensing the change in impedance. Cyclic voltametry (CV) was used to characterize micro electrodes and linear diffusion controlled reaction was measured. The challenging task of single cell trapping showed a high success rate of precise control capturing of single cells in the chosen microwell in less than 30 s. Such new concept of active microwell array combined with highly sensitive electrodes promises high throughput assay of single cells in a controlled manner. The application of this lab-on-chip platform holds potential to be used in drug screening, biomarker detection and cytotoxicity analysis and it is currently under study for nanotoxicity measurement.

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