

INDIUM TIN OXIDE MICRO-ELECTRODE ARRAY FOR NEURONAL STIMULATION AND RECORDING

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Abstract

Influence of applied electric field on biological cells has been widely investigated particularly for medical application, for example, to stimulate excitable neurons. Indium tin oxide (ITO), a transparent conducting semiconductor with biocompatible property, is used for electrical stimulating and electrochemical sensing the cell on the platform. In this work, ITO platform was developed by lithography micron-patterning and modified surface with protein coating. Surface morphology of the treated ITO surface was examined and biocompatibility was investigated by long period cell culture. SH-SY5Y, a human-neuroblastoma, was grown and subjected to 50 mV/mm of steady state direct current electric field applying through the protein functionalized ITO platform for 30 min. The electrical treated cells exhibited morphological changes by extending neuronal processes length to $10.75 \pm 30.96 \mu\text{m}$ ($n = 43$) longer than the non-electrical treated cells. After electrical treatment, cell alignment following the electric field direction can also be observed. This work can demonstrate the biocompatibility and possibility use of treated ITO electrode for inducing cells morphological changes and controlling cell alignment. Furthermore, the fabricated ITO electrode can also demonstrate additional benefit of observing cellular activity by the means of electrical signal detection during the cellular progressions.

Keywords: Indium tin oxide electrode, neuronal cell, electrical stimulation, protein functionalized surface

Introduction

Tissue development *in vivo* is a complex processes and dependent on various factors, for instant, surrounding cells, electric field (EF) or dipole, and hormone. Bioelectricity had widely studied for several decades due to its involvement in tissues in several aspects, especially for the electrical

conducted cells such as neuron, heart cell, and muscle cell. Although electricity can be a lethal cause of cell death (Pakhomova *et al.*, 2013) it also triggers several cellular activities, for example, apply electricity to restore the heart abnormal rhythm. The EF stimulation even from the

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alternative current or directed current source was reported to induce the mammalian cell such as PC12 -a rat neuroblastoma cell line (Kimura *et al.*, 1998; Chang *et al.*, 2013) and human neuronal precursor cells (Zhao *et al.*, 2015) morphological changes by extending neurite outgrowth. Moreover, EF has been reported to cause electrotaxis -a directional migration cause by electric field (Feng *et al.*, 2012; Zhao *et al.*, 2015; Hayashi *et al.*, 2016; Li *et al.*, 2017), to increase wound healing (Messerli and Graham, 2011), and to treat nervous injury (de Lima-Pardini *et al.*, 2018). However, understanding of the cellular mechanisms and the side effect of EF are still intensively investigated, which requires more sophisticated tools for observing.

In order to study cellular activities, which those cells are isolated from the body -called *in vitro* or *in situ*, the appropriated surface for cell adhesion is required for primary processing of cell culture. Current technologies allow the fabrication of sophisticated surfaces for detecting and observing cell response to several stimulations including EF stimulation. Among those techniques, the planar electrode for cell culture platform received more attentions due to its superior advantages of direct observation and allowing researchers to do further experiments in the same culture. Therefore, material selection is the fundamental development of the culture surface. Up to date, glasses, polymers such as polystyrene, and metal such as titanium, are well documented biocompatible material and are widely used for implanting devices. Synthesized semiconductor such as titanium dioxide (Canillas *et al.*, 2017) and indium tin oxide (ITO) (Zhao *et al.*, 2012; Tanamoto *et al.*, 2015) also received attention due to its various physical properties i.e. low reactivity, electrical conductivity, transparency, high thermal resistivity as well as processing ability, which are very attractive for development of the culture platform.

Transparent semiconductor provides not only the culture surface but also allow the further additional experiments operating on such platform. Among those semiconductor materials, ITO presents several attractive features of good electrical conductivity, transparent, non-toxic, and biocompatible property. Thus, several researchers pay attention and development of ITO electrode as a culture platform for studying of cellular activities (Kisler *et al.*, 2012; Zhao *et al.*, 2012; Choi *et al.*, 2015; Ryyanen *et al.*, 2018). In order to improve cell adhesion on the surface, various modifying methods, for instant, functionalized with biocompatible molecules of extracellular matrix component (ECM) (Liberio *et al.*, 2014) i.e. poly-L-lysine, laminin and collagen is widely used. Those molecules adsorbed on the targeted surface creating covered layer ranging from very thin to several

micrometer thicknesses providing anchoring sites for cell binding without electrical interferences (Wood and Willits, 2009; Wang *et al.* 2018).

In this study, planar electrodes as a cell culture platform is developed in order to generate electric field and observe cellular responses due to electrical stimulation. Small finger interdigitated electrode was fabricated using simple in-house lithography followed by wet etching technique. Surface-functionalized electrode was achieved by protein immersion and was characterized by scanning electron microscope (SEM) and Fourier-transform infrared spectroscopy (FTIR). A dopaminergic human neuroblastoma SH-SY5Y cell line, which is widely used as a model for studying of degenerative nervous system (Lopes *et al.*, 2010; Kovalevich and Langford, 2013), was subjected to electric stimulation and cellular activities was observed. This fabricated planar culture platform could accommodate growing of several cell types. Therefore this technology has potential to satisfy many purposes involving stimulation of stem cell in regenerative medicine, cell controlling in tissue engineer and implanted devices.

Experimental Procedures

Fabrication of Indium Tin Oxide Electrode

Commercial ITO thin film coated on glass substrate with a thickness of 135 nm and resistant of 10-15 ohm/sq (Biotain, Fujian, China) was cut into a 1 inch² and thoroughly clean before patterned by lithography. Negative photoresist dry film (HT-100T, Etertec, Kaohsiung, Taiwan) was laminated on the ITO surface. The pattern mask with equal electrode width and gap of 100 μ m was transferred onto the laminated ITO surface by exposing to UV light creating a pattern of reactive and non-reactive polymer zone according to the patterned mask. Sodium hydroxide, NaOH (Ajax Finechem, Wollongong, Australia), solution was used to remove the non-reactive zone until it reached the ITO surface. Then this exposed ITO region was etched by hydrochloric acid, HCL, (RCI Labscan, Bangkok, Thailand) until it reached the glass substrate except the region that was covered by reactive photorisorist dry film. After that, the reactive dry film was removed from the ITO surface leaving the two regions of ITO electrode and glass substrate following the designed electrode pattern from the mask. ITO electrode was assembled with the cut-glass tube with 10 mm length and 14 mm inner diameter (Pyrex, Tewksbury, USA) and sealed with polydimethylsiloxane (Sylgard^(R) 184, Saint Loius, USA) to form a well for cell culture with the culture area of 154 mm². In order to apply electrical

stimulation through the ITO electrode surface, aluminum wire was connected to the ITO pad with electrical conducted silver paste before sterilization.

Characterization of Electrode Surface

Protein functionalized ITO electrode surface

To prepare the surface for cell culture, the culture well was soaked overnight in diluted cleaning solution (7x-O-matic solution, Solon, USA) and sonicated for 30 min. The fabricated well was then rinsed 3 times with reverse osmosis (RO) water followed by 95% ethanol before drying at 60°C. The culture well was sterilized by autoclave at 121°C for 20 min and dried in oven. The high-glucose Dulbecco's Modified Eagle Medium, DMEM (Gibco, Grand Island, USA), was supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Biochrom, Berlin, Germany). Two hundred microlitre of 10% FBS-DMEM based media was added into the ITO culture well and immersed for overnight in 5% CO₂ at 37°C then washed several times with sterile Dulbecco's Phosphate Buffered Saline, D-PBS (Biochrom, Berlin, Germany), and finally dried in laminar hood 1 hour before cell seeding.

SEM characterization

The protein functionalized ITO surface was prepared for SEM imaging as describe following. After surface modification, surface was rinsed several times with D-PBS and fixed with 2% paraformaldehyde in D-PBS for 30 min then rinsed several times with D-PBS. Samples were dehydrated by a series of ethanol substitution at 30, 50, 70, 80, and 90% in ultrapure water and absolute ethanol for 2 repeating times with 3 min per each. Then, samples were dried with critical point dryer and sputter coated with Pt/Pd alloy. SEM (TM 1000, Hitachi, Japan) was used to investigate both bare ITO electrode and the protein functionalized ITO surface at accelerating voltage of 10 kV.

FTIR characterization

Attenuated total reflectance Fourier transform infrared, ATR-FTIR, spectroscopy (Nicolet 6700, Thermo Scientific, USA) was used to investigate the transmitting property of the protein functionalized electrode surface. Data were collected and processed by Omnic software. Samples were prepared with the same processes as for protein functionalized ITO electrode surface.

Cell Culture

Neuroblastoma SH-SY5Y cell line (CLS, Eppelheim, Germany) with selected passage number 30-35 were cultured in T25 flask (Nunc, USA) to reach 75% confluent in a 10% FBS supplemented DMEM with 1% antibiotic at 37°C,

5% CO₂ ambient. The culture was sub-cultured with 0.25X trypsin/EDTA in D-PBS and transferred to the functionalized ITO culture well at seeding density 100 cell/mm². During electric field treatment, the media was renewed every day, just immediately after applied EF. The experiment was performed in three duplicate wells.

Electrical Stimulation

Electric field treatment

SH-SH5Y cells were cultured on the functionalized ITO culture well for 3 days and then subjected to electric field of 50 mV/mm by applying DC voltage (TR6120A, Advantest, Japan) on the ITO electrode for 30 min per day and continuously for 3 days. Images of cell respond to electric field were captured under light microscope at 24 hours after the previous stimulation but just before the next stimulation.

Amperometric detection

A programmable electrometer (Keithley 617, Keithley Instruments Inc., USA) was used to detect the cellular electrochemical changes cause by electrical stimulation. Amperometric detection was controlled by Labview software. After cell seeding, SH-SY5Y cell was allowed to grow on the ITO platform for 3 days and the media was changed to D-PBS just before the experiment. The cell culture was stimulated by a 100 mV and electrochemical changes due to cellular response to electric stimulation was recorded and expressed as a current vs. time. For the non cell seeding recording, the electrode surface was prepared the same method as for cell culture.

Results and Discussion

ITO Culture Well Configuration

Interdigitated electrode with 16 pairs of the active site was designed and successfully fabricated by using in-house lithography followed by wet etching method (Figures 1(a) and 1(b)). The electric signal can be directly transferred to the electrode pad by aluminum wire, which be glued with silver paste. SEM was used to examine the size of electrode fingers showing that the electrode finger size and the interspacing gap is about 100 µm (Figure 1(c)). This small interspacing gap between electrode fingers was designed to accommodate a limited number of cells and to provide a localized detection of cellular electrochemical activities. The culture area and the media containing volume was limited to the maximum of 800 µl by the cut-glass tube and sealed outside with silicone elastomer (Figure 1(d)). Therefore, it can reduce expense of exaggerating media use and the cost for performing experiment.

This culture well configuration can be sterile by moist heated or autoclave with no material degradation or liquid leakage. Furthermore, the fabricated culture well showed a good biocompatibility for growing long period cell, in this work, the cells were cultured for 6 days with no significant of biological toxicity or limited cell growth noticed.

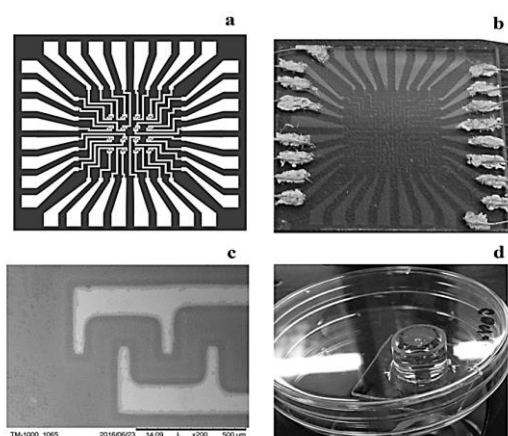


Figure 1. ITO electrode geometry of (a) the electrode pattern, (b) etched ITO electrode, (c) SEM image of the interdigitated ITO electrode, and (d) ITO electrode based culture well

Characterization of Protein Functionalized ITO Electrode Surface

Pre-immersion of ITO electrode platform with FBS based DMEM media, a simple technique, was used to functionalize the surface and to improve cell-surface adhesion. After incubated for overnight, the functionalized ITO electrode surface was characterized by FTIR and SEM. It is clearly observed from the FTIR spectra that surface of both bare ITO and bare etched ITO surface showing a high intensity peak of about 880-890 cm^{-1} , which indicated the Si-O bonding (Oh, 2010) from the glass as a substrate of the electrode platform, as illustrated in Figures 2(a) and 2(b). The absorption peak of the trace element, such as a vibration peak at about 3,600 cm^{-1} from the O-H and hydrogen bonding (El-Batal *et al.*, 2010) and peak range of 1,500-1,700 cm^{-1} from C=C can also be noticed in the spectra. Additionally, the inserted SEM images also showed the plain surface morphology compliance with the FTIR spectra.

It is well known that FBS consist of several protein and is used as a protein and nutrient source for cell culture (Zheng *et al.*, 2006) thus it is a good choice for modifying surface to enhance the cell-surface adhesion. Overnight pre-immersion of FBS based DMEM media allowed absorption of proteins on the platform surface, which is clearly observed from the FTIR spectra. Figures 2(c) and 2(d) demonstrated the FTIR spectra of the FBS

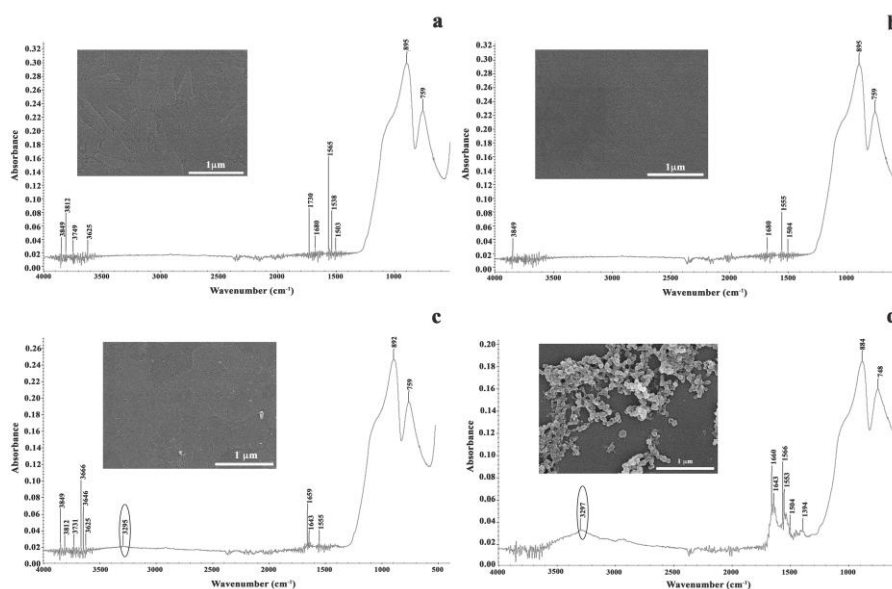


Figure 2. FTIR spectra of (a) bare ITO surface, (b) bare etched ITO surface, (c) FBS pre-immersion on ITO surface, and (d) FBS pre-immersion on etched ITO surface. The SEM inserted image represented surface morphology according to the electrode surface treatment. The marked peak at 3,300 indicated the N-H stretching

pre-immersion platform and displayed a vibration spectra at about 3300 cm^{-1} from the N-H stretching and the other interesting peak was the peak at $1,643\text{ cm}^{-1}$, which indicate amide I compound (Servagent-Noinville *et al.*, 2000; Della Porta *et al.*, 2016). Those two characteristic peaks demonstrated that there were proteins absorbed on the platform surface. It was noticed that the peak intensity obtained from the etched ITO surface is higher than the non-etched surface. According to the Beer-Lambert law, it can be implied that, from this FTIR result, a high peak intensity reflecting the increasing of the protein amount on the surface. This result also coincided with the SEM images showing that there were small amount of protein attached to the ITO surface, while on the etched ITO surface it was found a large amount of protein strongly adhered on the surface even it was washed several times during preparation processes.

SH-SY5Y Cell Electrical Stimulation

SH-SY5Y was cultured on the protein functionalized ITO culture platform for 3 days and was subjected to EF stimulation. A 16 site recording ITO electrode can locally detect media electrochemical changes cause by the nearby cells. Observing under light microscope, it was noted that the small cell amount was located in the interspacing gap as showed in Figure 3(a), which conformed the objective of the designed pattern. From the picture, cell adhered and spread well over the protein modified platform on both ITO and etched ITO region proving the biocompatibility property of the fabricated ITO culture platform. Triggering the cell culture by external electrical stimulation of 100 mV induced a huge potential change between the inside

and outside of the cell or referred as membrane potential change and consequently, releasing of the cellular electrolyte -ions leakage- can be detected by amperometric method. Figure 3(b). represented the amount of ions flow or current from the cells located near the electrode. From the graphs, it is worth to note that under the control experiment, amperometric detection of the cell loaded on electrode show dynamical changes and fluctuation over time during stimulation while the recording from the non cell loading electrode was plateau as illustrated in Figure 3(c). In this experiment, D-PBS, a well known PBS based solution without calcium (Ca^{2+}) and magnesium ion, was selected as a based electrolyte for amperometric detection, which was demonstrated as a non interference for amperometric detection (Zhao *et al.*, 2012). Besides, there were reports that Ca^{2+} may involve in cellular response to electrical stimulation (Huang *et al.*, 2010) therefore using D-PBS solution could reduce interference effect from the Ca^{2+} . According to the amperometric results, it should be marked that the magnitude of the recording current from the cell loading on ITO electrode is over 40 times higher than the non-cell loading ITO electrode. The large amount of current detection from the cell loading electrode may relate to the sum of the ions that released from several cells around the electrode. In addition to the number of the sensing cell, Zhao (Zhao *et al.*, 2012) reported the SH-SY5Y cell with neurite process tend to response to external stimulation better than the non process cell thus growing cell on the ITO platform for 3 days before stimulation would rise the chances for electrical responding cell those due to the cell would have superior morphology than the just seeding cell.

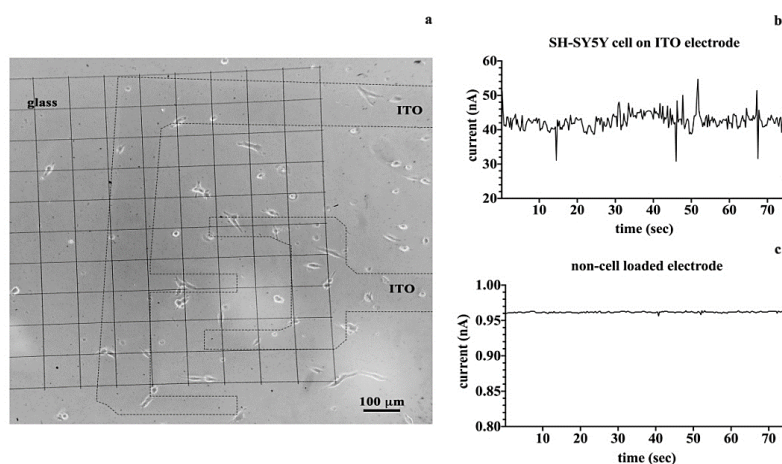


Figure 3. SH-SY5Y cells on localized ITO electrodeplatform (a) after culture for 3 days and amperometric detection of (b) SH-SY5Y cell on the same localized electrode and (c) non cell-loading electrode in D-PBS solution

In this experiment, the ITO culture platform capability was inspected beyond the electrochemical detection. EF was applied directly to the SH-SY5Y cell that culture on ITO platform and macroscopic of cellular response was observed under optical microscope. After culture cell on the ITO platform for 3 days, the cell morphology was imaged just before apply EF showing good and healthy cells adhering and spreading over the surface as in Figure 4(a). A small directed current EF strength of 50 mV/mm was applied to the culture for 3 days with duration of 30 min per a day. An attractive cellular response was noticed after the first EF injection (Figure 4(b)) that there were some of cells underwent morphological changes into a spindle-like shape. By measuring the angle between the cell longitudinal axis and the electrode based boundary, it was found that those spindle-like cells align

parallel to the EF direction. This phenomenon was dominant after the third EF injection (Figure 4(c)), which was corresponding to 6 culturing days for the non EF treated culture. It was noticed that cell proliferation under applying low EF stimulation risen the number of spindle-like cell and associated with extending cell process length, which did not found in the non EF treated culture (Figure 4(d)). The captured images also revealed that after applying EF for 3 times, the cells extended processes length to $24.34 \pm 25.88 \mu\text{m}$ whereas the processes length of the non EF treated cell was $11.17 \pm 21.41 \mu\text{m}$, which is $10.75 \pm 30.96 \mu\text{m}$ longer. Cell alignment is the other feature that was inspected in this experiment, it is clearly showed that the EF parallel cell, which degree alignment is between $85\text{--}95^\circ$ comparing to the electrode based line boundary, increased from 2.22% for the non treated cell to 30.23% for the EF treated cell as demonstrated in Figure 4(e).

Although the main mechanism of EF trigger cellular responses is still unclear, there were several evidences reported that, by applying of the low electrical treatment to the cell leading to the distribution interference of the actin filament which preferably enrich toward to the negative pole facing side of the cell (Li and Kolega, 2002). As a result, the actin filament aligns itself according to the negative pole of the electric field and reflecting as cell align parallel to the EF direction and makes the cell preferably extend the neurite to the negative side. This finding also consistent with the report from Koppes (Koppes *et al.*, 2014) that DC field only 50 mV/mm cause Schwann cell align parallel to the applied field direction with extending neurite length.

Conclusions

Effects of electrical treatment for cell growing on the patterned ITO electrode surface were demonstrated. From this experiment, several interesting cellular responses were observed including cell alignment, extending processes, and morphological changes. The site by site interdigitated electrode pair allowed the advantages of both stimulation and recording cellular activities through electrochemical detection. Protein absorption on the electrode surface plays a key feature role for cell anchoring site and enhancing cell growth for cellular detection but not limit the electrical signal. Besides that, applying the appropriated electrical stimulation through the ITO culture platform could control of cell migration, alignment, and differentiation, which further allow

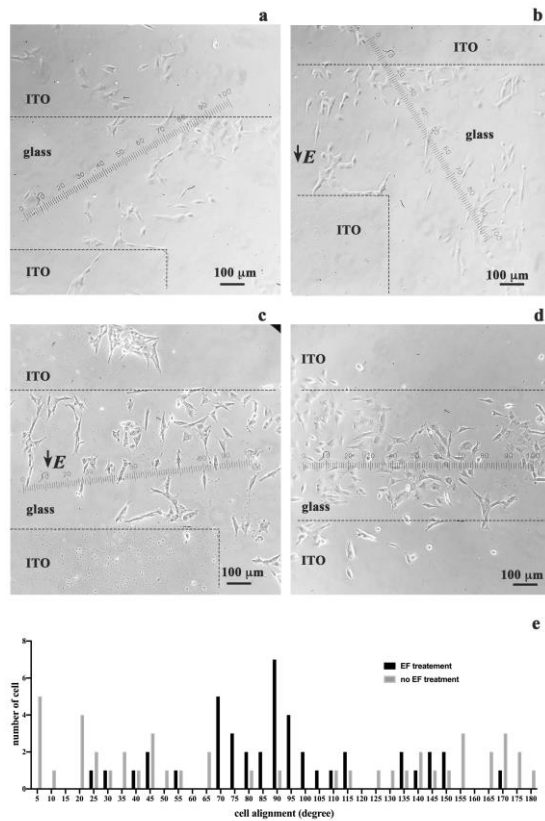


Figure 4. SH-SY5Y cell on ITO platform after (a) culture for 3 days and just before the first EF treatment, (b) culture for 4 days and just twenty-four hours after the first EF treatment, (c) culture for 6 days and just twenty-four hours after the third EF treatment, (d) culture for 6 days without EF treatment, and (e) frequency distribution of cell alignment. The electric field direction was assigned as E

the advancement in control of cell growth for tissue engineering technology.

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