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Development of Multi-Modal Cell Monitoring System and its Application in the Physioelectrochemical Characterization of Pluripotent Stem Cell Behaviors

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Development of Multi-Modal Cell Monitoring System and its Application in the Physioelectrochemical Characterization of Pluripotent Stem Cell Behaviors

A Thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Bioengineering

by

Lauren Yukwa Wong

June 2015

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ABSTRACT OF THE THESIS

Development of Multi-Modal Cell Monitoring System and its Application in the Physioelectrochemical Characterization of Pluripotent Stem Cell Behaviors

by

Lauren Yukwa Wong

Master of Science, Graduate Program in Bioengineering
University of California, Riverside, June 2015
Dr. Jin Nam, Chairperson

Stem cells exhibit unique characteristics of self-renewal and differentiation, the ability to produce unlimited daughter cells and transform into various cell types, respectively. Current biochemical-based analytical techniques for monitoring cellular behaviors are accurate, yet destructive, limiting their use as end-point analyses. In order to continuously monitor the same population of the cells, a novel multi-modal system that encompasses both quartz crystal microbalance (QCM) and electrochemical impedance spectroscopy (EIS), as well as having an optical visualization capability, has been developed to characterize physioelectrochemical changes of the cells during self-renewal and differentiation in real-time. Human induced pluripotent stem cells (IPSCs) were utilized to validate the functionality of the device. An equivalent circuit model was then designed from experimental impedance data to correlate physical changes of the cells to electrical circuit components throughout the progression of stem cell self-renewal and differentiation. Overall, the combination of the quantitative information from the device and electrical circuit modeling collectively offers a means for an in-depth understanding of physical
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Chapter 1: Introduction and background

1.1 Motivation

The present work aims at developing a device for monitoring stem cell behaviors. Characteristic stem cell behaviors include 1) self-renewal (proliferation without spontaneous differentiation), and 2) differentiation (the specialization of a cell from progenitor to mature cell types) (3). Current methods for monitoring such cellular behaviors through biochemical analysis of gene and protein expression are destructive end-point assays that only offer a snapshot of cells at that instant. Considering highly transient biochemical and morphological changes of the cells during self-renewal and differentiation, however, it is necessary to monitor such changes in situ in real-time to better understand the fundamentals of stem cell behaviors. Furthermore, a non-destructive method would provide an opportunity to 1) track a population of the cells removing heterogeneity in experiments and 2) to subsequently use the characterized cells. In this regard, development of a novel device that enables non-destructively monitoring cellular behavior in situ would provide a unique opportunity to further advance our understanding in stem cells and their translational applications.

1.2 Background

1.2.1 Pluripotent stem cells

Pluripotent stem cells (PSCs) exhibit the characteristics of long-term self-renewal without senescence, and pluripotency, the capability to differentiate into the cell types of all three germ layers (4). Human embryonic stem cells (hESCs) are one of the most well-characterized PSCs. The advances in stem cell biology enabled the scientific field to differentiate hESCs to multiple somatic
cell types, which positioned them as a promising cell source for regenerative medicine. However, derivation of hESCs from the human embryo sparked ethical controversy, limiting their usages in translational applications. In 2006, Takahashi and Yamanaka introduced the idea of induced pluripotent stem cells (IPSCs), by reprogramming adult fibroblast cells to pluripotent cells using a set of transcription factors (5). IPSCs possess many of ESC characteristics including long-term self-renewal capability and pluripotency. Thus, the derivation of IPSCs entirely removed ethical concerns regarding the use of hESCs while providing genomically patient-matching PSCs for clinical applications in regenerative medicine including cell therapy and tissue engineering. Due to their broader applications, therefore, IPSCs are primarily focused in the remainder of this study.

**Induced Pluripotent Stem Cells (IPSCs)**

Recently, the advances in IPSC research have greatly motivated the field of regenerative medicine, due to its potential to give rise to all cell types in the body (6). IPSCs are derived from adult somatic cells, e.g., skin cells from a biopsy, using a set of transcription factors, also known as Yamanaka factors. These factors consist of c-Myc, Sox2, Klf4, and Oct4, which will reprogram the cells to acquire pluripotency (5). Using IPSCs for patient-specific therapy predicts to reduce the possibility of immune rejection (7). In addition to their application as a cell source for regenerative medicine, these cells also provide a platform for understanding disease mechanisms and to carry out *in vitro* drug screening for potential therapeutics. These diverse utilities of the cells may allow for advancement in cell-replacement therapy and drug discovery (Figure 1.1) (8). For example, IPSCs have been used to model diseases such as amyotrophic lateral sclerosis (ALS), Parkinson’s disease, sickle-cell anemia, and type I diabetes (8, 9). These disease models enabled researchers to investigate the patient-specific pathogenesis and identify drug candidates for treating
diseases (1). Therefore, fundamental understanding of mechanisms underlying IPSC behaviors will significantly contribute to the growing field of regenerative medicine.

**Differentiation of Induced Pluripotent Stem Cells (IPSCs)**

The important advantage of using IPSCs is their differentiation potential, which is the transformation of IPSCs into any adult cell type. The cellular microenvironment and different biochemical factors determine the process of IPSC differentiation. Recently, the time-dependent addition of exogenous growth factors to mimic the developmental stages of differentiation has been shown to induce differentiation with greater efficiencies towards specific target lineages (10, 11). Once IPSCs undergo epigenetic modifications by these growth factors, their phenotype changes according to alterations in cell genotype (12). In addition to biochemical factors, physical interactions with extracellular matrix (ECM) also contributes to stem cell fate (13-15). For example, the micro- and nanoscale geometry of ECM topography can trigger cell morphology changes. Surface features may affect number of focal adhesions, subsequently altering the structure of the cytoskeleton, and cell shape, which can influence stem cell differentiation (16).

To determine stem cell differentiation, several methods are available for analyzing lineage-specific markers. Common methods for evaluating differentiation-associated gene and protein expression of cells include polymerase chain reaction (PCR), immune-blotting, and immunocytochemistry (17, 18). These established techniques are reliable and accurate, but cells are irreversibly damaged in the process of extracting RNA and DNA. PCR requires destruction of the cell membrane while blotting techniques use gel electrophoresis to separate target molecules (DNA, RNA, or protein) by size or charge. Immunocytochemistry also requires membrane permeabilization for antibodies to bind to proteins within the cell (19). Furthermore, these methods
for assessing gene and protein expression are semi-quantitative, requiring secondary measures to fully describe the state of cells.

1.2.2 Quantitative methods for monitoring cellular behaviors

Real-time measurements of stem cell behaviors provide valuable information on the state of the cells during self-renewal and differentiation. Quantifying cells’ physical properties offers insight on cellular morphology, and ultimately the function of the cells. Two techniques that offer real-time, non-invasive cell measurements are Quartz Crystal Microbalance (QCM) and Electrochemical Impedance Spectroscopy (EIS).

Quartz Crystal Microbalance

The Quartz Crystal Microbalance (QCM) is a technique capable of measuring a miniscule mass change on the order of 1 ng/mm² (20). The underlying mechanism of the QCM is the piezoelectric property of the quartz crystal. Piezoelectricity is defined as the generation of AC voltage when material is subjected to mechanical forces (21). QCM utilizes the inverse piezoelectricity by applying AC voltage to the crystal via the electrodes present on both sides of the quartz crystal, causing the crystal to resonate. The most common quartz crystal resonator is the AT-cut, where the quartz crystal is cut at the angle 35°10’ to the optic axis of the crystal. AT-cut quartz crystals are commonly used due to their low temperature coefficient, allowing for stable crystal performance during cell culture-related applications (22). By measuring the crystal’s resonant frequency, the QCM can detect minute changes in mass. When mass is present on the front electrode, the resonant frequency shifts proportionally to the mass change (23). Using the QCM’s capability of real-time measurements to detect mass change, extensive studies have
investigated cellular behaviors, such as cellular adhesion, and cell-substrate interaction kinetics in-situ (24-28).

Cellular adhesion plays a role in regulating cell behavior, such as migration, proliferation, and differentiation. Wegener et al. used the QCM to monitor the effects of seeding densities of three cell lines: strains I and II of the epithelial cell line, MDCK, and Swiss-3T3 fibroblasts (26). The frequency response of the QCM was governed by the number of cells that spread on the crystal surface. Modin et al. studied the attachment and spreading of pre-osteoblastic cells and their response to metal oxide surfaces, in which tantalum and chromium films were deposited (28). Results showed a positive correlation between QCM frequency shift and area of spread cells, measured by cryo-scanning microscopy. Soumetz et al. examined the cellular response to biomimetic coatings, containing fibronectin, on nickel/titanium and silicon samples for bone-tissue engineering (25). QCM measurements revealed the number adherent cells cultured on functionalized materials exceeded that of negative controls. Utilizing the QCM to study cell-substrate interaction shows great promise for tissue engineering applications.

Cellular kinetics related to the cell adhesion process can also be determined by observing the effects of substrate surface treatment (27). Hong et al. evaluated the effect of positively charged poly-D-lysine (PDL) coating, RGD peptides, and steady-state adhesion of endothelial cells. Correlation of QCM measurements and progression of cell adhesion demonstrated that PDL coating enhanced electrostatic interaction for initial contact because the rate of resistance change was greater to that of controls. The presence of RGD peptides prevented cell integrin binding, which showed a slow increase in motional resistance. For studying steady-state adhesion, heparan sulfate proteoglycan (HSPG) plays an important role in facilitating focal adhesion formation. The use of heparinase III, an enzyme that degrades HSPG, reduced the steady state of cell resistance,
which diminished long-term cell adhesion. Thus, QCM can be used for many applications, ranging from understanding cell-substrate interaction to cellular kinetics.

**Electrochemical Impedance Spectroscopy and Applications**

Electrochemical impedance spectroscopy (EIS) is an analytical method to measure electrical responses from matters in electrolytes. EIS commonly utilizes a three electrode setup, consisting of the working, reference, and auxiliary electrode. This technique applies a small AC voltage perturbation to the electrochemical cell and detects current response. The use of EIS allows determination of real surface areas *in situ*, including interfacial processes regarding redox reactions, geometric effects such as porous electrodes, and biological membranes (29, 30). Previous research have used EIS to monitor various cellular behavior, such as cellular attachment, adhesion, and morphological changes associated with toxin exposure and differentiation (31-36).

In an electrochemical cell composed of biological components such as ECM, cells and cell culture media, cells act as insulating materials, obstructing current flow between the ventral surface of the cells and substrate. When cells adhere and spread on a substrate surface, this causes fluctuations in the impedance measurement. The frequency response of AC impedance provides important information regarding cell structure to analyze changes in cell morphology (37, 38).

By this mechanism, Giaever and Keese demonstrated the use of EIS to monitor effect of cell surface coverage on impedance measurements (39-41). It was observed that the initial peak in impedance was due to cell attachment and fluctuations, followed by impedance changes due to cell motility (2, 40). Wegener et al. analyzed the use of EIS to monitor cell reaction to different extracellular matrix protein coatings: fibronectin, laminin, vitronectin, and bovine serum albumin (BSA) (42). Monitoring spreading of cells in real-time allowed for investigation into how cells
interact with the different protein coated surfaces, based on the changes in impedance and cell membrane capacitance.

Other research have focused on assessment for cytotoxicity on various cell lines using EIS. Toxin exposure to cells leads to changes in cell structure (31-33, 43). Primiceri et al. used EIS to observe effects of copper ions on two carcinogenic cell lines, neuroblastoma and HeLa cells (43). Increased resistance indicated that cytoskeletal proteins were affected by the presence of copper salts, inducing morphological changes prior to cell death. Opp et al. examined the effect of cytochalasin B concentrations, in which exposure of cytochalasin B to endothelial cells showed decreased junctional resistance between cells, increased membrane capacitance, and reduction of cells' micromotion. The presence of this toxin affected cellular networks that connected cell-cell and cell-substrate interaction, which was reflected in the impedance measurements (31).

Impedance response can also be dictated by alterations in actin cytoskeleton, and cell membrane properties (32, 33, 36, 44). Altered cell morphology is a qualitative indicator of differentiated cells. The physical change in cell morphology are correlated to variations in impedance measurements from the organizational modifications in the cell layer (45). Bagnaninchi and Drummond investigated differences in dielectric properties between cell lineages using EIS. The differentiation of adipose-derived stem cells (ADSCs) were induced toward osteoblasts and adipocytes. Monitoring changes in impedance at specific frequency resulted in differences of cellular resistance and capacitive reactance, indicators of cell differentiation (36). Research have highlighted the flexibility of the EIS technique to monitor cellular behavior by performing real-time impedance measurements.
1.3 Conclusion

Previous research have shown the feasibility to quantitatively monitor real-time cellular behaviors \textit{in situ}. Specifically, QCM and EIS have been utilized for many applications, including investigating cellular adhesion, cell-substrate interaction, and cell morphology changes (24, 26, 37, 38). The main advantage of using these techniques for monitoring cellular behaviors is the non-invasive, real-time capability. However, prior research have not been able to directly correlate such measurements in mass and impedance changes to physioelectrochemical changes of the cells during stem cell proliferation and differentiation. In this regard, we aim to identify characteristics specific for stem cell self-renewal and differentiation by combining quantitative measurements from a novel QCM-EIS cell monitoring system and by comparing them to optical observation of cell morphology changes associated with cellular behaviors.
Figure 1.1. Applications of induced pluripotent stem cells (iPSCs) in regenerative medicine, disease modeling, and drug screening. After iPSCs are reprogrammed from somatic, patient-derived cells, the cells can be differentiated into any adult cell type (1).
Figure 1.2. A schematic diagram showing applied current flow interacting with adherent cells during tissue culture. Solid line represents resistance from electric current flow underneath and between the cells. Broken line represents capacitive electric current flow through the cell membranes (2).
Chapter 2: Development of QCM-EIS system for in situ monitoring of stem cell behaviors

2.1 Introduction

Regenerative medicine is a growing area of research that aims to replace damaged or lost tissues in the human body. Adult stem cells have been a source for exploring therapeutic treatments because they are relatively well characterized and can be obtained from individual patients, removing immunogenic concerns (46). However, these adult stem cells may not be efficient for treating age related degenerative diseases due to their limited potential for cell expansion and possible loss of cell potency accompanied by cell aging. In this regard, iPSCs offer an alternative cell source to those who have limited cell quantity/quality from their own autologous adult stem cells (47, 48).

The pluripotency of iPSCs makes them a suitable candidate for use in regenerative medicine research. However, due to the numerous cell types iPSCs can transform to, procedures must be followed closely to prevent differentiation to undesired cell types. Differentiation protocols typically require addition of biochemical supplements, influencing a variety of signaling cascades that ultimately determine phenotype of the cell, thus affecting cell functions. During this well-defined process, cells often obtain phenotype-specific morphology, e.g., elongated morphology for fibroblasts and spherical morphology for chondrocytes (49, 50). Such changes or maintenance of phenotype-specific morphology have been shown to affect cell functions, like the production of tissue-specific proteins.

The behaviors of iPSCs, i.e., self-renewal (proliferation) and differentiation, are very dynamic, leading to difficulties in monitoring cellular behaviors (3). These cells require cell-to-cell contacts for the maintenance of pluripotency in vitro, and therefore, they proliferate in very compact cell colonies. On the contrary, during differentiation from one cell type to another, the
cells exhibit changes in cell morphology (50). Cell morphology changes include structural reorganization of the cytoskeleton components and adhesion to substrates. Monitoring the differentiation process based on such structural changes is critical for therapeutic applications of iPSCs. Therefore, the real-time determination of the degree of differentiation will not only provide a means to optimize the process, but also eliminate the need of sacrificial samples for destructive analysis to determine end phenotype. However, currently there is no available device capable of monitoring cellular behaviors in situ.

In this regard, we aimed to develop a device to quantitatively monitor stem cell behaviors by utilizing quartz crystal microbalance (QCM) to measure mass change due to proliferation, and electrochemical impedance spectroscopy (EIS) to monitor impedance change of the cells due to cell morphology changes typically associated with differentiation. Additionally, an optical capability was incorporated to visually observe the cells by utilizing an indium tin oxide (ITO) transparent QCM crystal. Riv9, an iPSC line, was used to validate the optical, physical, and electrochemical capabilities of the device.

2.2 Materials and methods

2.2.1 QCM crystal preparation

The ITO QCM crystal (Microvacuum, Budapest, Hungary) was cleaned according to the protocol from the manufacturer (Inficon, USA). The crystal was then subjected to five minutes of air plasma treatment (Harrick Plasma, Ithaca, NY), followed by sterilization with 70% ethanol for one hour, and subsequent UV-sterilization for 30 minutes. A commercially available basement membrane matrix, Geltrex® (Life Technologies, Carlsbad, CA), was coated onto the crystal surface overnight. The Geltrex® layer allows for better iPSC attachment and adhesion.
2.2.2 IPSC culture

After overnight Geltrex® coating on the ITO QCM crystal, cells were seeded at approximately 75,000 cells/cm². The cells were then incubated for 12 hours at 37°C and 5% CO₂ with mTeSR1 media (Stem Cell Technologies, Vancouver, Canada) and Rock inhibitor (1:1000, Reagents Direct, Encinitas, CA) to ensure cell survival. After the 12 hour incubation time, the cell seeded ITO QCM crystal was then assembled into the device.

Rock inhibitor was taken out and cells were cultured in mTeSR1 maintenance media to monitor cell proliferation. Cells were approximately 20% confluent when the crystal was assembled in the device, and cells were cultured until reaching 100% confluency. The time duration for cells to reach 100% confluency was approximately 60 hours. Media was changed daily and optical images, using phase contrast microscope (Nikon Eclipse), were taken immediately after media change, to qualitatively observe cell growth and morphology change.

2.2.3 Mass and impedance monitoring during cell culture

The QCM instrument continuously monitored cell mass throughout the cell culture period. The EIS was set to measure impedance by applying an AC perturbation of 10 mV using a potentiostat (CH Instruments, Austin, TX). The frequency sweep ranged between 0.1 to 2000 Hz with 12 measurements performed per decade. The resulting data provided impedance values (real, imaginary, and magnitude), and phase shift that correspond to each frequency measurement. Impedance measurements were taken every 12 hours to quantitatively monitor cellular behavior.
2.3 Results

2.3.1 Device development

During proliferation, cells exhibited physical changes, such as cell quantity increase and changes in morphology (Figure 2.1). To non-destructively and quantitatively monitor such changes in real time, a QCM-EIS device was developed using a three-electrode setup (Figure 2.2). This setup incorporated the working, reference, and auxiliary electrode to work on behalf of both QCM and EIS. The bottom casing is made of polytetrafluoroethylene (PTFE), and houses two gold working electrodes, which is in contact with the QCM crystal. PTFE was selected due to its high temperature resistance, which allows for sterilization by autoclaving (51). The working electrodes delivered an alternating potential to resonate the crystal at its resonant frequency. The working electrodes on the top surface of the QCM crystal were alternatively used as the voltage perturbation source when the device was used in the EIS mode. An ITO QCM crystal was used due to its transparency, which allows for visual observation during cell culture.

The top casing was also made of PTFE and holds the Ag/AgCl reference electrode and platinum wire auxiliary electrode. The auxiliary electrode was coiled around the interior of the top casing. A glass window was placed immediately above the top casing to provide optical pathway and secured by a stainless steel ring.

An adapter made of acrylonitrile butadiene styrene was fabricated using a 3D printer (Stratasys Dimension Elite). The device, including all three components, was held together with coiled springs that latched from the bottom component to the three notches on the top component (Figure 2.2 C).
2.3.2 Device validation

The proliferation of IPSCs was optically observed through the glass window. The optical images show appropriate adhesion and colony formation of the cells at the earlier stage, indicated by extended cell colony edges (Figure 2.3). Larger colonies developed on the crystal surface by hour 36. The cells reached 100% confluency approximately at 60 hours post-seeding, apparent from complete cell surface coverage on the ITO QCM crystal. Control samples were characterized by Nanog immunocytochemistry, a marker for pluripotency (Figure 2.3 B). Nanog expression demonstrated that IPSCs maintained their pluripotent behavior throughout the 4 day culture period.

During IPSC culture in the device, the mass change was continuously monitored using the QCM (Figure 2.4). The mass increased during the culture due to cell growth. Specifically, the mass change exhibited two proliferation phases, initial slow increase followed by exponential growth, which is typical for the proliferation of adhesion-mediated cells (52). In agreement with the optical observation, the mass change was saturated at hour 60, indicating cells reached 100% confluency.

To validate the EIS component of the device, impedance measurements from using a platinum pseudo-electrode and Ag/AgCl reference electrode were compared. The frequency sweep ranged from 0.1 to 100 kHz, and the sample used for impedance measurements was a bare ITO QCM crystal with cell culture media. The Bode magnitude/phase and Nyquist plots show a discrepancy in impedance measurements greater than 2000 Hz, indicating an artifact is present above this frequency (Figure 2.5). Thus, impedance analysis will be performed within the 0.1 to 2000 Hz frequency range for all subsequent experiments.

Impedance measurements for ITO QCM crystal with cells are shown in Figure 2.6. Bode magnitude plot showed impedance magnitude increase with a shoulder formation at approximately 40 Hz, starting at the 36 hour time point (Figure 2.6 A). The shoulder formation was more evident
in the Bode phase and Nyquist plot (Figure 2.6 B, C). The magnitude increase and shoulder formation may be contributed from increased cell surface coverage during proliferation.

2.4 Discussion

The device developed in this study demonstrated its utility as a robust tool for monitoring cellular behaviors in situ by combining analytical techniques including, QCM, EIS, and optical microscopy to characterize physical changes of the cells. The device was able to monitor cell mass change by QCM, while simultaneously observe impedance differences due to cell coverage and morphology changes during IPSC proliferation. In addition, the utilization of the transparent ITO QCM crystal permitted optical inspection of the cells such as spreading and morphology changes, enabling a link between quantitative measurements to physical changes of the cells.

QCM and EIS provide quantitative information on mass and cell/colony morphology changes, respectively. The continuous monitoring of the changes in resonance frequency of QCM crystal, which is directly correlated to adherent cell mass change, provides not only a quantitative measure of cell proliferation, but also kinetics of such cell behavior from rate change. On the other hand, EIS measures the change in electric current flow around/through the cells, which is closely related to the cell surface coverage of the electrode and morphology of the cells. When cells spread across the electrode surface, they act as insulating matter, obstructing current flow between the electrode and electrolyte (34, 41, 42, 53, 54).

The frequency response of impedance provides important information regarding cellular morphology. During proliferation, cells exhibit transient changes in cell morphology, depending on their environment. Cells initially tend to elongate to sense their surroundings. Such physical changes of the cells cause fluctuations in impedance measurements at specific frequencies.
Therefore, the developed device would be very useful to quantitatively study the differentiation of IPSCs, which is typically characterized by cell morphology changes (55-57).

Prior studies have monitored the dynamic aspect of cell behaviors by applying an electric field. Xiao et al. analyzed the use of EIS to study attachment and spreading of fibroblasts on fibronectin and ovalbumin coated electrodes (34). It was observed that spherical cells initially exhibited a small change in impedance, however, when cells later attached and spread on the surface, impedance largely increased due to its flat morphology. This trend was evident in the validation study, when impedance measurements increased starting at hour 36. Arndt et al. investigated apoptosis-induced changes in endothelial cell shape (58). Impedance results showed the disassembly of intercellular junctions between endothelial cells occurred before changes in cell-cell and cell-substrate contacts prior to apoptosis. The changes in impedance measurements corresponding to the changing cell morphology throughout proliferation was present in the validation study. Impedance profiles for monitoring cell-cell and cell-matrix interactions pave the way for understanding cellular processes that govern cellular behaviors.

2.5 Conclusion

The multi-modal device developed in this study offers a powerful tool for real-time monitoring of cellular behaviors in situ by incorporating three analytical techniques, QCM, EIS, and optical microscopy. The combination of QCM-EIS aspects demonstrate the capability of simultaneously obtaining quantitative information about cellular mass and morphological changes. In addition, the capability for optical microscopy allows linking cell/colony shape changes to physioelectrochemical measurements. This novel device provides a powerful tool for an in-depth understanding of cellular processes in a non-destructive quantitative manner.
Figure 2.1. A design schematic showing changes in cell coverage on a substrate and cell morphology during initial adhesion to the substrate and subsequent proliferation. Cells initially elongate and spread to establish focal adhesions on the substrate. The cells then develop and grow in colonies, increasing surface coverage with transient changes in cell morphology.
**Figure 2.2.** A design schematic of the multi-modal QCM-EIS device: (A) an assembly view of the device labeled for the various components, and (B) an assembled view of the device. (C) A photograph of the prototype QCM-EIS device.
Figure 2.3. (A) Representative optical images taken every 24 hours during the course of iPSC culture in the device. Red arrows indicate the presence of cell colonies (scale bar = 100 µm). (B) Nanog stained fluorescence images, indicating presence of pluripotent cells before and after 4 days of culture in mTeSR1 maintenance media (scale bar = 50 µm).
Figure 2.4. Mass change of iPSCs was continuously measured by QCM during iPSC culture. A representative data from three independent samples is shown.
Figure 2.5. A comparison of EIS data, including (A) Bode magnitude, (B) Bode phase, and (C) Nyquist plots, measured from acellular sample (Geltrex®-coated QCM crystal with cell culture media) with Ag/AgCl electrode or platinum pseudo-electrode.
Figure 2.6. (A) Bode magnitude, (B) Bode phase, and (C) Nyquist plots of EIS measurements during IPSC culture in the device. Nyquist plot inset shows real and imaginary impedance at lower frequencies. The measurements were conducted every 12 hours. The control measurement for the Bode magnitude and phase plots is Geltrex®-coated QCM crystal without cells.
Chapter 3: Physioelectrochemical characterization of pluripotent stem cell self-renewal/differentiation

3.1 Introduction

IPSCs, derived from individual patients, provide an ideal cell source for personalized regenerative medicine (59). The use of IPSCs for patient-specific therapies, such as cardiomyopathies, greatly reduces the possibility of immune response/rejection (1, 60). IPSCs present an excellent platform for understanding pathological and molecular mechanisms of disease progression by providing a disease-specific in vitro model. For example, IPSCs have been used to study the pathology of amyotrophic lateral sclerosis (ALS) by reprogramming patient’s fibroblasts to IPSCs and differentiating into functional motor neurons. Other neuronal diseases, such as Parkinson’s disease and spinal muscular atrophy, have been successfully modeled (61, 62). In addition to these basic studies, IPSC-derived in vitro models also provide excellent platforms for drug screening for potential therapies. (1, 8). To fully utilize these diverse potentials of IPSCs, it is necessary to quantitatively characterize cellular behaviors during self-renewal and differentiation to specific lineages.

Traditionally, stem cells have been characterized by biochemical techniques such as PCR and immune-blotting based on changes in gene and protein expression during the proliferation and differentiation of the cells. Although these traditional techniques provide semi-quantitative measures for cellular behaviors, they are destructive in nature, preventing tracking of specific populations of the cells throughout biological processes. In this regard, physical changes of the cells, i.e., mass and morphology, provide another means to assess cellular behaviors. Cell proliferation is distinguished by increased cell quantity, which corresponds to changing cell mass. Cellular differentiation is described by changes in cell morphology from genetic alterations which
can be qualitatively characterized by size, shape, and structural features (49, 63, 64). For this reason, albeit semi-quantitative at best, optical microscopy has been a routine method to observe cellular behaviors via morphology characterization.

Physical changes in cells cause corresponding differences in their electrical properties as the cell acts as both a resistor and a capacitor (65-68). Many morphological features such as cell spreading, roundness, and compactness as well as cell-cell and cell-substrate junctions, affect resistive and capacitive properties of the cell layer. These electrical properties of cells were recently analyzed by electrochemical impedance spectroscopy (EIS) during the differentiation of stem cells (36, 44, 66). This pioneering work has demonstrated a clear correlation between the degree of differentiation of stem cells and impedance, but did not quantitatively relate the physical (cell quantity and morphology) properties to electrical (resistive and capacitive) properties of the cells. Understanding such a relationship will provide a means to quantitatively characterize changes in cellular behaviors in a non-destructive manner.

In this study, we utilized a novel device, which combines quartz crystal microbalance (QCM) to measure mass and EIS to measure impedance to quantitatively monitor stem cell behavior in situ during proliferation and differentiation of iPSCs. Using the QCM-EIS real-time monitoring system, the changes of cell quantity and shape were correlated to impedance change. Furthermore, electrical circuit modeling was utilized to further dissect resistive and capacitive electrical properties of the cells to reveal changes in the proliferation/differentiation-dependent cell morphology.
3.2 Materials and Methods

3.2.1 QCM crystal preparation

The ITO QCM crystal (Microvacuum, Budapest, Hungary) was cleaned according to the protocol from the manufacturer (Inficon, USA). The crystal was then subjected to five minutes of air plasma treatment (Harrick Plasma, Ithaca, NY), for surface cleaning. Sterilization with 70% ethanol for one hour and subsequent UV-sterilization for 30 minutes was then performed. Geltrex® (Life Technologies, Carlsbad, CA) is a commercially available basement membrane used to coat the crystal surface. The Geltrex® membrane allows for better iPSC attachment and adhesion.

3.2.2 iPSC Culture

Geltrex® was coated on the ITO QCM crystal overnight, and cells were seeded at approximately 75,000 cells/cm². The cells were then cultured in an incubator for 18 hours in 37°C and 5% CO₂ with mTeSR1 media (Stem Cell Technologies, Vancouver, Canada) and Rock inhibitor (1:1000, Reagents Direct) to ensure cell survival and attachment. After the 18 hour incubation, the cell-seeded ITO QCM crystal was then assembled into the device.

iPSC behavior was monitored according to the experimental plan shown in Figure 3.1. Cells were cultured until reaching 100% confluency (Figure 3.1 A), or treated under differentiation conditions at 70% confluency (Figure 3.1 B). Differentiation media was exchanged daily with appropriate growth factors according to an established protocol (49). Growth factor concentrations are presented in Table 3.1 with day 1 corresponding to the start of differentiation. The differentiation media consisted of (1% DMEM:F12 (Lonza), 1% ITS (Corning), 1% NEAA, 90μM β-mercaptoethanol, 2% B27(Gibco)).
Control samples cultured on tissue culture plates were fixed at every 12 hour time point with 4% paraformaldehyde (Fisher Scientific, USA) for immunocytochemistry. Fixed cells were stained for NANOG, a pluripotent marker, and Goosecoid (GSC), a mesendodermal marker (R&D Systems) to confirm for presence of differentiated cells. 4’,6-Diamidino-2-Phenylindole (Vector Laboratories) and Phalloidin (Life Technologies, Carlsbad, CA) staining were used to identify nucleus and actin, respectively.

The shape descriptors feature from ImageJ was utilized to quantify cell area, perimeter, circularity, and roundness from optical and fluorescence images (69). Circularity is defined with 
\[4\pi \times \frac{(\text{Area})}{(\text{Perimeter})^2}\] with a value of 1.0 indicating a perfect circle, and a value approaching 0 representing an elongated shape. Roundness is calculated by \[\frac{a}{b}\], with \(a\) and \(b\) as the width and length of the minimum bounding (the smallest rectangle enclosing the selection), respectively (69).

3.2.3 Mass and impedance monitoring during cell culture

Cell mass was continuously monitored by QCM (Inficon, USA) throughout the culture duration, with the exception of the short periods when media was exchanged. Optical images were taken immediately after media exchange. EIS measured impedance every 12 hours by applying a 10 mV AC perturbation using a potentiostat (CHI600C, CH Instruments). The frequency sweep ranged from 0.1 to 2000 Hz with 12 measurements performed per decade. The QCM data presented are an average of three independent sample measurements with error bars signifying standard deviation, and representative impedance data is presented.

3.2.4 Fitting and Simulation

EIS Analyser software was used to fit the experimental data with the proposed equivalent circuit model (70). The circuit model was created using the graphical user interface and the Powell
algorithm was used to fit the data. The local minimum between the experimental and fitted data was selected using the Powell algorithm (71). The fitted results produced information for circuit values. After fitting was performed, simulation was carried out for the program to compute theoretical data using circuit values from the circuit model. Both proliferation and differentiation studies were used for equivalent circuit modeling.

3.3 Results

3.3.1 Proliferation of IPSCs

The proliferation of IPSCs in the QCM-EIS device was optically monitored until the cells reached 100% confluency, for approximately 60 hours. Representative optical images were taken every 3 hours during the course of proliferation (Figure 3.2). Initial attachment showed sharp edges, possibly due to the influence of Rock inhibitor (72), which was used to enhance IPSC survival upon seeding. During device assembly, Rock inhibitor was taken out and cells were cultured in mTeSR1 media for the remainder of the proliferation period. As proliferation progressed, the cell colonies gained round edges, which are characteristic of pluripotent stem cell growth. In addition to the macroscopic morphological changes of the cell colonies, the changes in individual cell morphology were characterized by ImageJ (Figure 3.3). Cell coverage area and cell perimeter measurements peaked at the 24 hour time point, indicating cell spreading on the substrate (Figure 3.3 A, B). Cell morphology at the hour 24 time point in the optical images showed the greatest distinction of cell junctions. In contrast, a relative minimum value was observed for circularity and roundness at the same time point, suggesting that cells were elongated while spreading (Figure 3.3 C, D).
The optical observations in cell growth via measuring cell colony coverage on the substrate were compared to the quantitative mass measurements determined by QCM (Figure 3.4 A). In general, the surface coverage change closely represented the mass changes during proliferation (Figure 3.4 A, B). The QCM results, as measured mass change and its conversion to cell quantity, showed a gradual increase in cell number during the first 30 hours and a sudden increase until cells reached 100% confluency at approximately 60 hours (73) (Figure 3.4 B, C). The cell growth rate was calculated from the change in cell quantity (Figure 3.4 D).

Culture duration-dependent impedance changes are presented in the form of Bode magnitude/phase plots or the Nyquist plot in Figure 3.5. The Bode magnitude plot showed an increase in impedance magnitude at approximately 40 Hz after 36 hours of cell culture (Figure 3.5 A). Information on the characteristic frequency at which the largest impedance change occurs is presented in the normalized magnitude plot (Figure 3.5 B). The Bode phase and Nyquist plots also showed a shoulder formation at hour 36 (Figure 3.5 C, D). Collectively, the shift in impedance and phase angle may indicate the changes in cell morphology and surface coverage throughout the course of proliferation. The inset seen in the Nyquist plot showed the real and imaginary impedance at the low frequency range.

Upon closer observation at the individual peak present in the normalized impedance plot, an increase was evident between the 24 and 36 hour time point (Figure 3.6 A). The normalized peak frequency plot showed the peak impedance occurred at a relatively similar frequency at 46 Hz during IPSC self-renewal (Figure 3.6 B). Therefore, 46 Hz was the characteristic frequency chosen for the time-course impedance, characterizing cell proliferation (Figure 3.6 C).
3.3.2 Simultaneous proliferation and differentiation

Mesendodermal differentiation was induced at 70% cell confluency in this study because inducing differentiation at a lower cell confluency allows for better differentiation efficiency (74). GSC expression increased while cells still proliferated under differentiation conditions (Figure 3.7 A). The fluorescence intensity was quantified using ImageJ, and increased intensity supported qualitative observations (Figure 3.7 A). ImageJ was also used to quantify cell morphology, and it was observed that a minimum value was present at the 48 hour time point for both cell coverage area and perimeter (Figure 3.8 A, B). For circularity and roundness, the values decreased as differentiation progressed, indicating the elongation of the cells during the differentiation process (Figure 3.8 C, D).

Additionally, optical images of the cells in the QCM-EIS device were taken every 12 hours throughout the duration of cell culture (Figure 3.9). It was seen that the cell surface coverage increased and brighter spots emerged on the surface of the cell colonies, showing that some cells detached from the surface. By hour 72, cells attached onto the surface exhibited an elongated morphology, indicative of mesendodermal formation.

Surface coverage increased during IPSC differentiation (Figure 3.10 A). The cells reached 70% confluency by hour 24, but the cell quantity continued to increase because there was space available for the cells to expand (Figure 3.10 B). The cells approached confluency by hour 48, in which a constant cell quantity and growth rate were observed (Figure 3.10 C, D). The slight decrease in cell mass after 48 hours may be due to the cells undergoing apoptosis during differentiation selection process or overcrowding of the cells.

During differentiation, Bode magnitude and normalized magnitude plots showed gradual increased impedance as differentiation progressed (Figure 3.11 A, B). The magnitude increase began at the 36 hour time point. In parallel, Bode phase and Nyquist plots showed a shoulder
formation starting at the 36 hour time point (Figure 3.11 C, D). These changes in impedance were likely due to the combinatorial effects from cell coverage increase and cell shape change by proliferation and differentiation, respectively. The peak normalized value plot for individual measurements showed a progressive increase throughout this culture period (Figure 3.12 A). The peak normalized frequency plot showed that the characteristic frequency for impedance measurements was relatively constant (Figure 3.12 B). 37 Hz was the characteristic frequency for the time-course impedance plot (Figure 3.12 C).

### 3.3.3 Equivalent circuit modeling

Based on the EIS data presented, an equivalent circuit model was developed to describe cellular behaviors during proliferation and differentiation. Initially, Geltrex®-coating alone without cells was subjected to EIS and an equivalent circuit model was proposed to fit the data (Figure 3.13 A). $R_s$ signifies the media resistance. $C_e$ and $R_e$ represent the presence of extracellular material. The comparison between experimental and fitted data with the model showed relatively good fitting in the Bode magnitude/phase, and Nyquist plots (Figure 3.13 B-D). The minor mismatch at higher frequencies may be due to the porosity of Geltrex® layer, preventing its behavior as a perfect resistor.

Based on this electrical circuit model and observation of an emergence of impedance shoulder during cell culture, a parallel RC circuit was added to represent the cellular component of our system (Figure 3.14 A). The cellular RC circuit was nested inside the Geltrex® circuit to accommodate the observation that the current can flow directly through the Geltrex® to the media without interacting with the cell during proliferation. $C_c$ and $R_c$ denotes the capacitance and resistance of cellular components, including intracellular constituents and cell membrane, while $C_e$ and $R_e$ represent the capacitance and resistance of the extracellular components, such as Geltrex®.
and ECM deposited by the cells. Figure 3.14 B showed a representative fit between the experimental data and circuit model using EIS Analyser. The fitted values for C_c, C_e, R_c, and R_e from IPSC proliferation (Figures 3.2-3.6) were presented in Figure 3.14 C-F. C_c peaked at hour 24 (Figure 3.14 C), which coincides with the increase in cell coverage area and perimeter measured from optical images (Figure 3.3 A, B). In contrast, the C_e was inversely related to that of the C_c, which dipped at hour 24 (Figure 3.14 D). Changes in cell shape may be responsible for the changes in C_c and C_e. Similarly, circularity and roundness may also contribute to the surge in C_c and the dip in C_e as they both showed a valley present at hour 24 (Figure 3.3 C, D). On the other hand, R_c continuously increased during the course of proliferation, parallel that of mass change, due to the increased cell quantity, while R_e maintained similar values (Figure 3.14 E, F). The correlation between cell morphology measurements, mass, and circuit values was statistically significant (Table 3.1).

Similarly, the equivalent circuit model was applied to determine IPSC behaviors, which were subjected to differentiation at 70% confluency, allowing simultaneous proliferation and differentiation (Figure 3.15 A). The comparison between fitted data from circuit model and experimental data is shown in Figure 3.15 B. The resulting fitted data showed that C_c and C_e are inversely proportional to each other during proliferation, and saturated to plateaus (Figure 3.15 C, D). The initial decrease in C_c may be due to the cell size decrease and increased cell thickness. This is explained by the compact behavior of the cells during the latter part of differentiation (Figure 3.15 C). C_e initially increased during this culture period because changes in cell junction/adhesion during differentiation occurred (Figure 3.15 D). R_c (Figure 3.15 E) showed a similar trend to cell quantity change, reaching a plateau approximately at hour 40 (Figure 3.10 D). R_c increased throughout differentiation, unlike the relatively unchanged R_e during IPSC proliferation (Figure 3.15 F). The circuit value results for determining IPSC behavior during differentiation were
comparable to that of the proliferation. $C_c$ and $C_e$ values were attributed from cell morphology changes while $R_c$ and $R_e$ values were due to changes in cell quantity.

### 3.4 Discussion

Throughout the process of stem cell proliferation and differentiation, the cells undergo transient changes in cell shape and quantity. These physio-morphological changes are important markers to determine the proliferative or differentiated state of the cells. Typically, optical microscopy has been a choice of non-destructive analytical methods to monitor cellular behaviors. However, the optical analysis is semi-quantitative at best, and often subjective by observers. In this regard, the development of new methodology in monitoring cellular behaviors in real-time in a non-destructive manner would facilitate understanding stem cells by providing a quantitative analytical tool.

The changes in cell surface coverage and cell morphology are the most characteristic features associated with the proliferation and differentiation of the stem cells (2, 41, 53). In this study, iPSCs exhibited an elongated morphology after cell seeding to establish focal adhesions for cell growth. As proliferation progressed, cell morphology became more rounded and compact as cell surface coverage increased by the formation and expansion of the cell colonies. Similarly, differentiation of iPSCs was marked by their transformation in cell morphology. Differentiated cells exhibited elongated behavior, and the formation of three-dimensional structures, as quantified by cell morphology characterization. Such morphological changes of the cells influenced impedance measurements. As expected, the proportional magnitude change of impedance to cell mass/quantity change was clearly observed in the normalized Bode plot with respect to the duration of proliferation. In addition, impedance change during simultaneous proliferation and
differentiation also exhibited a gradual increase. However, it is difficult to decouple the individual contributions to the overall impedance from changes in cell surface coverage and cell morphology.

In this regard, electrochemical impedance measurements provide additional information that can be extracted to characterize individual components comprising the electrical circuit (i.e., cell, Geltrex®, and cell culture media). Impedance includes resistance and capacitive reactance, presented in real and imaginary forms. The resistance and capacitive reactance can be decomposed from the overall impedance using an equivalent circuit model (68, 75). There were several studies that attempted to characterize stem cell differentiation via monitoring impedance measurements at a specific frequency, similar to the presented data (36, 44) (Figure 3.6 C, 3.12 C). However, these studies are limited to comparing impedance changes to biochemical changes during stem cell differentiation in a semi-quantitative manner, failing to link electrochemical properties to physical properties of the cells. In our knowledge, this study is the first to attempt correlating the electrochemical-physical relationship in order to characterize stem cell behaviors.

In our circuit model, $R_c$ increased proportional to cell quantity increase during proliferation as expected. The development and expansion of cell colonies caused a greater resistance for current to flow through, resulting in resistance increase. Changes in $C_c$ and $C_e$ were expected to depict the alterations in cell size and morphology. Similarly, the $R_c$ and $R_e$ showed a gradual increase and saturation, depicting cell quantity changes during simultaneous proliferation and differentiation. However, the capacitance changes were vastly different from those of proliferation alone; gradual changes for both $C_c$ and $C_e$ were observed, likely due to steady changes in cell size and shape until they reached a differentiated cell shape.

Stem cell differentiation is a complex process exhibiting transient cellular behaviors including cell quantity and morphology changes. Utilizing an equivalent circuit to model electrochemical responses of the cells based on EIS data provides quantitative measures to
determine cellular behaviors. Thus, our novel QCM-EIS system demonstrates to provide fundamental insight on physical changes of the stem cells during proliferation and differentiation.

### 3.5 Conclusion

The motivation driving the development of this device was to quantitatively and non-destructively monitor cellular behaviors in real-time. This novel multi-modal system combining both QCM and EIS present accurate quantification of cell measurements; QCM provides information with cell growth while EIS offers impedance measurements that deliver information regarding cell morphology. Additionally, the optical clearance of the device allows linking such electrical changes to the morphological changes in the cells. Therefore, the combination of these quantitative information and electrical circuit modeling collectively offers a means for an in-depth understanding of cellular processes during stem cell proliferation and differentiation.
Figure 3.1. A schematic describing the experimental conditions to monitor and determine iPSC behaviors using the multi-modal QCM-EIS system. The cells were (A) cultured until reaching 100% confluency, or (B) induced to differentiate after reaching 70% confluency, which allowed simultaneous proliferation and differentiation.
Table 3.1. A combination of growth factors added to differentiation media to direct iPSCs towards mesendodermal lineage.

<table>
<thead>
<tr>
<th>Day</th>
<th>Wnt3a (ng/μl)</th>
<th>Activin-A (μg/μl)</th>
<th>bFGF (ng/μl)</th>
<th>BMP4 (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>10</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>10</td>
<td>4</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 3.2. Representative optical images taken every 6 hours (before Hour 24) or 3 hours (after Hour 24) during the course of IPSC self-renewal. Red arrows indicate formation and expansion of cell colonies (scale bar = 200 μm).
Figure 3.3. Cell morphology characterization via image analysis: (A) area, (B) perimeter, (C) circularity, and (D) roundness cell morphology. ImageJ was used to quantify cell shape from optical images shown in Figure 3.2.
Figure 3.4. (A) Cell surface coverage, (B) cell mass change, (C) cell number change, and (D) growth rate of iPSCs during iPSC self-renewal. Cell surface coverage was estimated by optical image analysis. Mass change was continuously measured by QCM and calculated to cell number change using a conversion factor. Cell growth rate was then calculated from cell number change. Data are presented as averages of three independent sample measurements. Error bars represent standard deviation.
Figure 3.5. (A) Bode magnitude, (B) normalized magnitude, (C) Bode phase, and (D) Nyquist plots during IPSC culture in the multi-modal QCM-EIS device. Nyquist plot inset shows real and imaginary impedance at low frequencies. The measurements were conducted every 12 hours during IPSC self-renewal. The control measurement for the Bode magnitude and phase plots is that from an acellular sample. The normalized magnitude plot was produced by comparing impedance change with respect to initial impedance measurement with the cells. One representative data set from three independent samples is shown.
Figure 3.6. (A) Peak normalized magnitude to the initial impedance ($Z_0$), (B) normalized frequency to the initial frequency ($\psi_0$), and (C) time-course impedance at 46 Hz during IPSC self-renewal. The intensity at 46 Hz was chosen to observe time-dependent impedance change based on the emergence of characteristic impedance peak at this frequency. Data are presented as averages of three independent samples. Error bars represent standard deviation.
Figure 3.7. (A) Differentiation of iPSC after reaching 70% confluency, characterized by Goosecoid (GSC) immunocytochemistry. (B) Quantification of GSC immunofluorescence intensity measured by ImageJ.
Figure 3.8. Cell morphology characterization via image analysis: (A) coverage area, (B) perimeter, (C) circularity, and (D) roundness. ImageJ was used to quantify cell morphology from fluorescence images shown in Figure 3.7.
Figure 3.9. Representative optical images taken every 24 hours during the course of differentiation. Red arrows indicate formation and expansion of cell colonies (scale bar = 100 µm).
Figure 3.10. (A) Cell surface coverage, (B) cell mass change, (C) cell number change, and (D) growth rate of IPSCs during differentiation. Cell surface coverage was estimated by optical image analysis. Mass change was continuously measured by QCM and calculated to cell number change using a conversion factor. Cell growth rate was then calculated from cell number change. Data are presented as averages of three independent sample measurements. Error bars represent standard deviation.
Figure 3.11. (A) Bode magnitude, (B) normalized magnitude, (C) Bode phase, and (D) Nyquist plots of EIS measurement during IPSC culture in the multi-modal QCM-EIS device. Nyquist plot shows real and imaginary impedance at lower frequencies. The measurements were conducted every 12 hours during IPSC differentiation. The control measurement for the Bode magnitude and phase plot is that from acellular sample. The normalized magnitude plot was produced by comparing impedance change with respect to initial impedance measurement with the cells.
Figure 3.12. (A) Peak normalized magnitude to the initial impedance ($Z_0$), (B) normalized frequency to the initial frequency ($\psi_0$), and (C) time-course impedance at 37 Hz during IPSC differentiation. The intensity at 37 Hz was chosen to observe time-dependent impedance change based on the emergence of characteristic impedance peak at this frequency. Data are presented as averages of three independent samples. Error bars represent standard deviation.
Figure 3.13. (A) A schematic of an equivalent circuit model for the acellular system (Geltrex®-coated QCM crystal with cell culture media without cells). (B) Bode magnitude, (C) Bode phase, and (D) Nyquist plots are presented, comparing the experimental and fitted data.
Figure 3.14. (A) A schematic of an equivalent circuit model for iPSC self-renewal, consisting of Geltrex®, cell culture media, and cells, and (B) a representative fitted curve on an experimental EIS measurement. (C) Capacitance of cellular components ($C_c$), (D) capacitance of extracellular components ($C_e$), (E) resistance of cellular components ($R_c$), and (F) resistance of extracellular components ($R_e$) values obtained from the equivalent circuit model.
Figure 3.15. (A) A schematic of an equivalent circuit model for IPSC differentiation, consisting of Geltrex®, cell culture media, and cells, and (B) a representative fitted curve on an experimental EIS measurement. (C) Capacitance of cellular components (C_c), (D) capacitance of extracellular components (C_g), (E) resistance of cellular components (R_c), and (F) resistance of extracellular components (R_g) values obtained from the equivalent circuit model.
<table>
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<tr>
<th></th>
<th>Area</th>
<th>Perimeter</th>
<th>Circularity</th>
<th>Roundness</th>
<th>Mass</th>
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<tr>
<td>(C_c)</td>
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<td>0.913*</td>
<td>0.975**</td>
<td>0.953**</td>
<td>-0.362</td>
</tr>
<tr>
<td>(C_e)</td>
<td>-0.885*</td>
<td>-0.885*</td>
<td>0.966**</td>
<td>0.953**</td>
<td>0.484</td>
</tr>
<tr>
<td>(R_c)</td>
<td>-0.332</td>
<td>-0.353</td>
<td>0.403</td>
<td>0.191</td>
<td>0.858*</td>
</tr>
<tr>
<td>(R_e)</td>
<td>-0.251</td>
<td>-0.271</td>
<td>0.302</td>
<td>0.813</td>
<td>0.850*</td>
</tr>
</tbody>
</table>

**Table 3.2.** Pearson’s correlation between the circuit parameters, and cell morphology or cell mass during IPSC self-renewal. \(P<0.05\) is indicated by * and \(P<0.01\) is indicated by **.
<table>
<thead>
<tr>
<th></th>
<th>Area</th>
<th>Perimeter</th>
<th>Circularity</th>
<th>Roundness</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cc</td>
<td>0.891*</td>
<td>0.751*</td>
<td>0.735</td>
<td>0.728</td>
<td>-0.954**</td>
</tr>
<tr>
<td>Ce</td>
<td>-0.841*</td>
<td>-0.675</td>
<td>-0.793*</td>
<td>-0.796*</td>
<td>0.965**</td>
</tr>
<tr>
<td>Rc</td>
<td>-0.585</td>
<td>-0.368</td>
<td>-0.861*</td>
<td>-0.923**</td>
<td>0.860*</td>
</tr>
<tr>
<td>Re</td>
<td>-0.476</td>
<td>-0.254</td>
<td>-0.871*</td>
<td>-0.905**</td>
<td>0.845*</td>
</tr>
</tbody>
</table>

Table 3.3. Pearson’s correlation between the circuit parameters, and cell morphology or cell mass during IPSC differentiation. P<0.05 is indicated by * and P<0.01 is indicated by **.
Chapter 4: Conclusions and recommendations

4.1 Conclusion

The present work has demonstrated the development of a device to quantitatively monitor cellular behaviors in situ. The self-renewal and differentiation of IPSCs were used as an in vitro model to validate the device by determining cellular behaviors. Specifically, the combination of quantification in cell mass and morphology change in addition to optical observations provided a precise method of determining the proliferative and/or differentiative stage of the cells. Furthermore, electric circuit modeling based on these quantitative measures allowed for an in-depth understanding of physioelectrochemical cellular processes that occur during stem cell proliferation and differentiation.

IPSCs may someday replace mesenchymal stem cells, the most clinically tested adult stem cells because the unlimited expansion and the full differentiation capacity makes these cells the most suitable candidate for stem cell based regenerative medicine. In this regard, optimization of expansion and differentiation protocols is of utmost importance to enhance economic efficiency and to safety concerns. Consequences of using incomplete differentiated cells for cell-replacement therapy may result in tumor formation, or produce false results for disease modeling and drug screening. Thus, monitoring the process of IPSC proliferation and/or transformation into terminally differentiated cells will greatly contribute to the field of regenerative medicine.

The reproducibility of stem cell therapy may start with proper maintenance of pluripotency during proliferation (self-renewal). In addition, stem cell differentiation is a complicated procedure in which supplementation of growth factors in a time-dependent manner is required for greater efficiencies. For such pluripotent stem cell expansion and stage-wise differentiation protocols, numerous samples need to be analyzed after each step to confirm degree of pluripotency
maintenance or differentiation before moving onto the next stage. The traditional biochemical analytical methods for verifying cells’ differentiation state are typically end-point assays that are destructive and semi-quantitative.

Using the developed multi-modal QCM-EIS device allows accurate monitoring of cellular behavior during IPSC self-renewal and differentiation. The real-time in situ capability provides an alternative means to continuously monitor cellular behaviors in a non-destructive manner. The use of an equivalent circuit to model cellular behaviors further allows decoupling of electrical components within a cellular microenvironment to fundamentally understand physioelectrochemical changes during IPSC self-renewal and differentiation. With the device’s high sensitivity and real-time measurements, establishing a standard for quantitative measurements associated with cell differentiation is achievable.

4.2 Recommendations for future work

Recommendations for future work include further analysis of ECM secretion from mesendodermal cells and its effects on QCM-EIS measurements. Secreted ECM proteins, such as collagen and aggrecan, may have influenced impedance measurements during the latter part of differentiation (49). Specifically, membrane-associated proteins may have significantly affected the capacitive behaviors of cellular component in the electrical circuit model. Detailed analysis of these additional measurements and modeling will further improve the usefulness of the QCM-EIS device.

In addition, various differentiation protocols can be tested for increasing cells’ differentiation efficiency. Impedance profiles can be created and be used to establish baseline standards for comparing future differentiation studies. For example, results from the
mesendodermal study carried out during this study may be used as a reference for comparing differentiation efficiency of other mesendodermal differentiation protocols.

Investigating different parameters that influence stem cell differentiation, such as cells’ microenvironment can also be performed. Stem cell differentiation is influenced by various factors, such as mechanical or biophysical cues (14, 76). In this regard, ECM plays a critical role in providing support for cell growth. Modification of ECM structure, such as changes in topography, compliance, or stiffness, will give insight with cellular response. These substrate variables can be easily incorporated into the QCM-EIS device. Understanding the impact of biophysical cues on cellular behaviors will contribute to differentiation studies by exploring combinatorial effects of mechanical and physical cues, which may result in better differentiation efficiency.
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