MULTIMODAL INTELLIGENT TRANSWELL SYSTEM

P.Ramiah Rajasekaran¹, A. Chapin², D.N. Quan², S.H. Jang³,
J. Herberholz⁴, L. Hu³, W.E. Bentley², and R. Ghodssi,^{1,2,3*}

¹Department of Electrical and Computer Engineering, Institute for Systems Research,

²Department of Bioengineering, ³Department of Materials Science and Engineering,

⁴Department of Psychology in Neuroscience and Cognitive Science

University of Maryland, College Park, Maryland, USA

ABSTRACT

This work is the first demonstration of a 3D printed, modular, multimodal electrochemical-sensor-integrated, transwell system for monitoring tissue culture and molecular signaling, *in vitro*. Integral to the design of this platform is a porous membrane hosting an *in vitro* tissue culture with cell-interfaced electrochemical sensors. Interfacial -electrical cell surface impedance sensing (ECIS) electrodes and -cyclic voltammetric electrodes (CV) are used to monitor epithelial barrier formation and basolateral molecular release respectively. Such interfacial sensor-integrated tissue culture platform enables unprecedented access and provides temporal information that conventional transwells cannot furnish. We use this platform to uncover signaling events occurring along the gut-microbiome-brain-axis (GMBA).

INTRODUCTION

The gut-microbiome-brain-axis (GMBA) is a proposed signal transduction pathway between the gut microbiome and the brain. Human and animal studies have implicated that pathogenic bacteria in the gut play a role in development of both gastrointestinal and neural disorders, such as inflammatory bowel disease and depression, respectively [1]. While most commensal gut bacteria colonize the gut mucosa harmlessly, some commensal and non-commensal microbes degrade the mucosal layer and invade the epithelial barrier to infect deeper tissues [2]. This may negatively affect the brain by altering serotonin signaling of epithelial cells, the signaling method proposed to underlie the GMBA. Specifically, the gut epithelium contains enterochromaffin cells that can sense bacterial metabolites in the lumen (e.g. butyrate) and in turn secrete serotonin on their basolateral side, which stimulates enteric nerves that communicate with the brain [3]. Measuring the changes in serotonin signaling patterns due to different bacterial species may reveal the mechanism of bacterial influence on the brain, but the inaccessibility of the basolateral epithelium makes it difficult to measure this serotonin signaling *in vivo*. Therefore, a new multimodal sensor integrated *in vitro* tissue culture platform is required to provide measurements of basolateral serotonin from these cells and correlate them with measurements of bacterial pathogenicity and epithelial barrier integrity.

Gut-on-a-chip (GOC) platform is one such state-of-the-art *in vitro* platform for studying the dynamics of the gut epithelium in response to various molecular and cellular cues. GOC platforms are designed after conventional transwells. These *in vitro* systems allow for better control over cell environment constituents compared to complex *in vivo* tissues. However, current GOC systems mostly rely on downstream chemical analysis, post mortem histology analysis, and trans-epithelial electrical resistance (TEER) probes to monitor cellular behavior [4]. These systems often require tedious fabrication protocols and provide limited information on molecular mechanisms occurring *in vitro*. Though commercial tissue culture monitoring systems can provide pertinent information, they are expensive, limited in functionality, and lack customization [5].

While *in vitro* cell cultures are invaluable tools for studying biological mechanisms in controlled environments, real-time

hierarchical signal transduction is difficult to study with current GOC platforms. Tissue culture sample analysis occurs mostly *ex situ* (e.g. ELISA [6], mass spectrometry [7]), requiring several processing steps, limiting the time resolution of molecular sensing from cells.

Electrochemical methods provide a solution, with the capability for *in situ* electrode integration utilizing simple fabrication protocols for real-time, direct, and label-free detection of molecular and cellular events. MEMS fabrication techniques enable development of customizable electrochemical electrodes on a variety of substrates as bio-interfacial sensors for research and diagnostic use [8].

Here we have propose a transwell-like GOC system incorporating membrane-integrated, cell-interfaced sensors. The housing for the platform is 3D printed and designed to sandwich a porous cell culture membrane with electrochemical sensors fabricated on top and bottom of the membrane. A representative model of the gut epithelium is cultured on ECIS electrodes on the top. The bottom CV electrode electrochemically senses the biomolecule of interest released by the tissue upon stimulation. The system showcased here has the ability to detect, the evolution of tissue growth over the porous membrane via ECIS and, redox molecules diffusing through the membrane via CV, mimicking basolateral cellular secretion. We have developed off the chip carbon nanotube (CNT) modified gold electrode to sense physiologically relevant serotonin concentrations that can be incorporated into the membrane-fabricated electrode design. Ultimately, this platform will be used to investigate the correlation between epithelial barrier degradation by co-cultured pathogenic bacteria, and basolateral serotonin release profiles, enabling the understanding of GMBA signaling.

MATERIALS AND METHODS

Development of a Sensor-integrated Cell Culture Housing

Figure 1 shows a schematic of the 3D printed transwell system with the sensor-integrated porous tissue culture membrane. Unlike off-the-shelf transwells (Corning® Transwell®) with concentric wells, the proposed modular 3D design consists a top and bottom chamber sandwiching a sensor-integrated tissue culture membrane. The diameter of the electrode fabricated tissue culture membrane is 15 mm, which was chosen to be analogous to the area of a 24 well plate (diameter 15.6 mm). The platform was designed in Autodesk® Inventor™ and 3D printed with Stratasys Objet500 Connex3 3D printer with biocompatible MED610 photopolymer using photopolymer jetting (Polyjet) technology. The electrodes on the membranes were fabricated with e-beam evaporation (20 nm Ti/ 100 nmAu and 500 nm Ag) on track-etched polyester (PETE) membranes (thickness-11µm; porosity-2x10⁷ pores/cm² and pore radius-500 nm, Figure 2a) through laser-cut paper shadow masks. Ag/AgCl reference electrodes were fabricated *in situ* by electrolessly oxidizing Ag to AgCl by exposing it to 50 mM FeCl₃ for 45 seconds. Interdigitated fingers of width 500 µm and spacing 500 µm function as ECIS electrodes (face UP). The three-electrode electrochemical system (face DOWN) consists of a working

electrode and adjoining reference and counter electrodes was used for CV. The electrodes were extended to fabricate contact pads with an inter-pad spacing of 2.54 mm, so that it can be interfaced with any standard off the shelf contact pins. The sandwiched sensor-fabricated membrane is flexible, compressed, and has a wrinkled architecture that mimics the gut landscape. Sterility of the device is of paramount importance to prevent bacterial or fungal contamination of this *in vitro* platform. All of the individual parts are assembled and the complete device can be sterilized by autoclaving at 121°C for 30 minutes and stored for later use, similar to a traditional transwell.

Cell Culture, TEER and Histology

The cells cultured to model the human gut epithelium *in vitro* were chosen to represent the simplified human gut to the best approximation to stimulate GMBA events. The co-culture consists of three types of cells, (1) Enterocytes (Caco-2), that forms the skeletal framework holding all the other cell types in the model gut. (2) Goblet cells (HT29-MTX) that produce mucus; the foremost protective layer covering the entire gut from mouth to colon. (3) Enterochromaffin cells (ECCs) (RIN14B), the most important cell type responsible for generating serotonin, the cellular metabolite (redox active neurotransmitter) released from the basolateral side involved in signal transduction to the brain. Caco2-, HT29-MTX and RIN14B cell lines were purchased from ATCC®, thawed, passaged and plated on the device in the ratio 4:2:1. The cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (DMEM + 10% FBS) at 37°C and 10% CO₂ in an incubator. The schematic of the 3D printed platform, membrane interfaced electrode and the tissue culture are shown in Figure 1.

Figure 1: Schematic of the 3D printed device with multimodal electrochemical sensors. Sensors were fabricated on track-etched PETE membranes. Cells were grown directly on impedimetric, sensors (face UP) and opposite face of the potentiometric, sensors (face DOWN). C, R and W represent the counter, reference, and working electrodes respectively. The cross-section on the right shows the position of cells relative to the electrode position.

Cells were cultured over the course of 21 days on the porous membrane (Figure 2a) in the device, growing into a confluent monolayer with high expression of tight junctions. Fluorescent staining shown in Figure 2b demonstrates a classic method to confirm tight junction formation in an epithelial monolayer. In order to fix and stain the cells, the membrane with cells was removed and fixed in 4 % paraformaldehyde for 30 min and washed thrice with phosphate buffered saline (PBS). It was stained with 1mg/ml of DAPI (blue), DNA stain. For staining tight junctions, the membrane was treated with 1% (bovine serum albumin) BSA for 2 hours to block non-specific binding, rinsed and incubated with primary antibody, rabbit anti-rat polyclonal antibody against occludin

(1:100). The primary antibodies were incubated overnight at 4 °C. The membrane was washed and incubated with donkey anti-rabbit IgG secondary antibody, and Alexa Fluor 488 conjugate (1:100) to tag tight junctions. This membrane was rinsed and imaged with Zeiss LSM 710 confocal microscope.

Figure 2: a) SEM of the porous PETE membrane on which the electrodes were fabricated and gut cells were cultured. b) Superimposed bright-field and confocal image of a 21-day co-culture. The Dark area represents the placement of the gold electrode. The co-culture was stained with DAPI (blue) to show the nucleus and an anti-occludin antibody (green) to show the formation of tight junctions.

Impedance monitoring of cell culture growth

Among electrochemical impedance measurements, (ECIS) has evolved to become the preferred tool for label-free and noninvasive sensing of adherent cells. As cells adhere and spread on the electrode surface, the gradual coverage of the electrode surface impedes the flow of electrical current between the electrodes. Subtle responses of cells to chemical, biological or physical stimuli/stressors, affect the cellular monolayer, which can be monitored in real-time with this non-invasive methodology. Impedance values for a confluent monolayer depends upon the given cell type, cell morphology, specific architecture of cell-cell and cell-matrix contacts. When the cells on the electrode change their shape, or if the ECM composition is changed in response to a stimulus, there is a corresponding change in the measured impedance. These physical changes can be interpreted by comparing impedance changes with an equivalent electrical model. Impedance measurements were recorded with a Biologic VSP 300 bipotentiostat by applying a sinusoidal AC voltage with an amplitude of 10mV_{rms} and a frequency of 10Hz to 10MHz. The media was changed once every 2 days during the course of the experiment. The obtained data was analyzed by equivalent circuit modeling with inbuilt EC-lab software with Z-fit module (Biologic Inc.).

TEER is complementary and classic method to measure the confluence of cell monolayers. TEER was measured with EVOM2™ Epithelial Voltmeter using chopstick TEER electrodes. TEER was used as a control to validate ECIS measurements of cell growth and coverage of the sensor-integrated membrane. Impedance and TEER measurements were done in sterile conditions in a biosafety cabinet.

Electrochemical sensing of serotonin

The performance of the gold electrochemical electrodes fabricated on PETE membranes was assessed by characterization with CV by detecting 2 mM ferrocene dimethanol (FDM) in 0.1 M phosphate buffered saline (PBS) pH = 7.4. CV was performed by applying a voltage scan from 0 – 350 mV at 300 mV/s and resulting current response was measured. FDM was allowed to diffuse from the top well into the bottom well for CV detection, giving a measurement of both the redox activity and the diffusion rate of small molecules across the membrane mimicking basolateral serotonin release. The Biologic VSP-300 potentiostat was employed for all electrochemical measurements.

Electrochemical detection of serotonin is highly dependent on

electrode materials and the applied cyclic voltammetric (CV) waveform. Therefore, serotonin sensing was optimized on commercial gold disk electrodes before optimizing serotonin sensing on PETE-integrated porous gold electrodes. Working electrodes were created by spray-painting single wall carbon nanotubes (SWCNTs) onto the gold disk surface, following surface polishing with alumina powder. Serotonin solutions were measured by CV at various concentrations (100 nM – 10 M) in DMEM + 10% FBS. CV was performed by measuring current across an applied voltage range of 100 – 450 mV with a scan rate of 50mV/s, using an Ag/AgCl reference electrode and a platinum coil counter electrode. *In vitro* ECCs were also stimulated to produce serotonin in separate cell culture (75 mm² surface area) by incubation with 100μM butyrate in 7mL DMEM + 10% FBS for 2-3 h. CV of the resulting supernatant was performed to detect serotonin from ECC cell culture.

RESULTS AND DISCUSSIONS

Membrane-integrated sensor performance

Figure 3: a) The exponential decrease in impedance measured at 10 Hz, representing an increase in double layer capacitance due to increase in the ECM protein ($N=3$). b) Nyquist plot showing the impedance at 3 stages of cell culture. Inset shows the phase contrast image of the coculture on day 1 and day 21. Scale bar 100 μm.

Impedance values derived from the bode plot show that the normalized change in impedance measured at 10 Hz decreases exponentially and reaches a steady state over the time course of the tissue culture (Figure 3a). This is representative of the initial log phase where the cells grow exponentially, followed by a stationary phase where the cells become confluent and stop multiplying. The observed decrease in impedance corresponds to the increased capacitance from accumulation of ions from cell-associated extracellular matrix (ECM) on the surface of the electrode. The corresponding nyquist plots of three cases, (i) No cell (bare electrode), (ii) Day 1 (seeding) and, (iii) Day 21 (confluent monolayer) are shown in Figure 3b. They show a drop in real vs imaginary impedance at low frequency which is dominated by the double layer capacitance of the ECM covered electrode-cell interface. Inset shows the phase contrast image of the coculture on day 1 and day 21.

Figure 4a shows the equivalent circuit model used to fit the data, simulated with Z-fit module from EC-lab. The model is a combination of constant phase element and Warburg impedance that dominates the current at cell-free state and cell-covered state respectively. The direction of current flow in each case is shown by the double-sided arrow. Figure 4b, shows the fitting between the real data (patterns) and the simulated data (solid line) by the model shown in Figure 4a. A close fit and a similar trend between the real and simulated data shows the validity of equivalent circuit model. It can be seen from the experiment and the model that as the cells reach confluence, the system shift to a charge transfer resistance dominated state, characterized by the emergence of a semicircular

profile in the nyquist plot. The charge transfer resistance stems from tightly packed monolayer of cells covering the surface of the electrode, resisting any nonspecific charge transfer reactions occurring at the electrode-tissue interface. This state is also characterized by a corresponding high TEER value for day 14 (708 Ω.cm²), day 16 (1105 Ω.cm²) and day 18 (1615 Ω.cm²).

Figure 4: Data Fitting. a) Equivalent circuit model of the bare electrode (top) and cell covered porous electrode (bottom). R_s , R_{CT} , Q , α , CPE , C_{dl} and W are charge transfer resistance, solution resistance, constant phase element (CPE) parameter, CPE exponent, double layer capacitance, and Warburg element respectively. The arrows indicate that CPE dominates the bare electrode case and R_{CT} dominates cell-covered case b) The dot patterns represent the data points obtained in the experiment and the solid lines show the fitting of equivalent circuit to the data. The data also shows the transition of the system from a diffusion limited state to a charge transfer limited state as the cell culture progresses

In-situ electrochemical detection of model metabolite.

Figure 5: FDM diffusion through PETE membrane, detection by porous gold CV electrodes fabricated on the membrane. a) Cyclic voltammogram detecting diffusion in real time, peak height is proportional to FDM concentration. Scan rate = 300mV/s, 2.3s/cycle. b) Linear fit of peak anodic current vs. $t^{1/2}$ as a measure of diffusion rate: slope = $4.6576 \mu A s^{-1/2}$.

Figure 5 shows quantifiable detection of FDM at the PETE-integrated gold electrochemical electrodes (without cells). The Nernst equation describes ideal behavior of a redox molecule at the electrode occurs when the anodic and cathodic peak heights are approximately equal, and when the difference between anodic and cathodic potential is ~59 mV for an one electron transfer reaction [9]. These conditions are met for FDM detection ($i_{pa}/i_{pc} \sim 0.8$, $E_{pa}-E_{pc} = 56$ mV) indicating that the fabricated electrodes are reliable. Figure 5a shows increasing anodic and cathodic peaks over the duration of FDM diffusion, saturating after 60 seconds. The linear relation of anodic peak current and square root of time gives a measure of diffusion rate on the order of $5 \mu A s^{-1/2}$ (Figure 5b). These results demonstrate that molecular detection is possible with time resolution in the order of a few seconds, though full diffusion

through the membrane limits time resolution to 1 minute.

Ex-situ electrochemical serotonin detection

Figure 6a shows the ability of CNT-modified gold electrodes to detect serotonin in prepared solutions and samples collected from cells. As shown by the calibration curve of serotonin detection at these electrodes, the linear range of detection spans potential physiological concentrations (100 nM – 10 μ M) with a sensitivity of 140 μ A/mM. The sensitivity achieved here is higher than both gold and glassy carbon electrodes, owing to the higher surface area and more ideal carbon structure provided by the CNT film. However, the repeatability standard deviation (RSD) of these measurements is in the range 15-40%, indicating relatively poor repeatability over successive trials, especially at lower concentrations. This variation at low concentrations limits the resolution of the technique to 45 nA (3 σ) and the limit of detection to ~300 nM serotonin in cell media. Further experiments detecting cell produced serotonin in cell supernatant (Figure 5b) show that this limit of detection may still be reasonable for our system. In comparison with an electrode-specific calibration curve performed before the detection of cell produced serotonin, the peak shown in Figure 5b corresponds to ~600 nM serotonin. These results provide a proof of concept that CNT-coated gold is an ideal electrode material for serotonin detection in cell media and for serotonin produced directly by cultured cells. The inset on left shows bare gold microdisc electrode (diameter- 1.6 mm) and CNT ink spray painted electrode. The inset on the left shows SEM of the CNT ink covering the gold electrode.

Figure 6: a) Calibration of CV detection of serotonin, showing linear range from 0.1 – 10 μ M serotonin in cell media (DMEM + 10% FBS). Inset: Zoom in on 0.1 – 1 μ M. Error bars denote standard deviation (N=4). $R^2 = 0.9866$. b) Cyclic voltammogram of serotonin detection from ECC supernatant, after 2h of stimulation with 100 μ M butyrate (pink) vs. controls (black: DMEM, red: DMEM + 100 μ M butyrate, blue: ECC DMEM supernatant with no butyrate). Serotonin peak occurs at $E_{pa} = 0.323$ V vs. Ag/AgCl. Inset: gold disk electrode vs CNT-ink-coated gold disk electrode (1.6mm radius), and an SEM image of the CNT film (scale bar = 200 nm).

This sensing paradigms supports the integration of both impedance interdigitated electrodes and CNT-gold CV electrodes into the 3D printed cell culture device to directly interface with cells. The serotonin detection methods achieved with commercial electrodes can be directly translated to *in situ* measurements within the cell culture by modifying PETE-fabricated gold electrodes with CNT films. The potential to correlate changes in the epithelial barrier integrity with concentration-specific serotonin detection on the basolateral side may allow research into the direct molecular and cellular influences on GMBA signaling. Further, this modular platform could be redesigned to integrate other types of cell cultures which would benefit from real-time, multimodal, and automatable molecular and cellular sensing.

CONCLUSIONS

This work reports the initial results of the first electrochemical

electrode integrated 3D printed cell culture platform, aimed towards detecting signal transduction events within a model of the gut-microbiome-brain axis. Current GOC platforms lack sophisticated on-chip cellular and molecular measurement capabilities that promise to enhance quantification and correlation of biological events. The possibility of performing real-time, direct, automated measurements over the course of a cell culture lifetime is realized by MEMS fabrication of electrodes directly onto bio-interfacing substrates, such as a transwell membrane.

As shown here, gold electrodes can be fabricated onto both sides of a porous membrane with unique and customizable geometries to apply two different sensing modalities. Impedimetric sensing is capable of measuring the quality of cell coverage over the membrane, acting as an online method to compliment standard cell techniques (e.g. TEER, fluorescence microscopy). Electrochemical sensing is capable of measuring redox molecules such as serotonin. By interfacing these electrodes directly underneath the epithelial cell layer, measurement of basolateral serotonin release can be measured, which has not been achieved *in vivo* because of its inaccessibility.

Future measurements using MITS will use impedance to detect changes in epithelial and mucus integrity due to colonization of gut microbiota and simultaneously detect serotonin release from the epithelium. Since these measurements can occur in real-time over the duration of a cell culture lifetime, we can temporally correlate how certain bacteria affect both gut health and serotonergic nerve stimulation. This type of discovery platform may be used both as a research tool for understanding the bacterial influence on the GMBA and as a diagnostic tool to evaluate patient gut cells and microbiome for signs of altered serotonin signaling. Here, technological advancements may aid in connecting molecular events to incidence of disease in humans.

REFERENCES

- [1] M. Carabotti, A. Scirocco, M. A. Maselli, and C. Severi, "The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems", *Ann. Gastroenterol.*, 28, 203 (2015).
- [2] M. McGuckin, S. Linden, P. Sutton, and T.H. Florin, "Mucin dynamics and enteric pathogens", *Nature Review Microbiology*, 9, 4 (2011).
- [3] J. Zhu, X. Wu, C. Owyang, and Y. Li, "Intestinal serotonin acts as a paracrine substance to mediate vagal signal transmission evoked by luminal factors in the rat", *The Journal of Physiology*, 530, 3 (2001).
- [4] H. J. Kim, D. Huh, G. Hamilton, and D. E. Ingber, "Human gut-on-a-chip inhabited by microbial flora experiences intestinal peristalsis-like motions and flow," *Lab Chip*, 12, 2165 (2012).
- [5] xCELLigence RTCA HT Instrument from ACEA Biosciences, Inc. ECIS® ZΘ from Applied Biophysics, Inc.
- [6] V. Veregut, M. Florescu, and M. Badea, "Classic and Modern Methods for Detection of Serotonin", *Sensing in Electroanalysis*, 7 (2012).
- [7] J. Arnold, J. Roach, and M. Azcarate-Peril, "Emerging Technologies for Gut Microbiome Research", *Trends in Microbiology*, 24, 11 (2016).
- [8] T. Winkler, S. Lederer, E. Kim, H. Ben-Yoav, D.L. Kelly, G. Payne, and R. Ghodssi, "Molecular processes in an electrochemical clozapine sensor," *Biointerphases*, 12, 2 (2017).
- [9] A. J. Bard, and L. R. Faulkner, "Electrochemical Methods: Fundamentals and Applications", Wiley, New York (2001).

CONTACT

*R. Ghodssi, tel: +1-301-405-8158; ghodssi@umd.edu