Porous Substrate-Based Electroporation with Transepithelial Electrical Impedance Monitoring

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Abstract

Porous substrate electroporation (PSEP) is an emerging method of electroporation that provides high throughput and consistent delivery. Like many other types of intracellular delivery, PSEP relies heavily on fluorescent markers and fluorescent microscopy to determine successful delivery. To gain insight into the intermediate steps of the electroporation process, a PSEP platform with integrated transepithelial electrical impedance (TEEI) monitoring was developed. Cells are cultured in commercially available inserts with porous membranes. After a 12 h incubation period to allow for the formation of a fully confluent cell monolayer, the inserts are placed in transfection media located in the wells of the PSEP device. The cell monolayers are then subjected to a user-defined waveform, and delivery efficiency is confirmed through fluorescent microscopy. This workflow can be significantly enhanced with TEEI measurements between pulsing and fluorescent microscopy to collect additional data on the PSEP process, and this additional TEEI data is correlated with delivery metrics such as delivery efficiency and viability. This article describes a protocol for performing PSEP with TEEI measurements.

Introduction

Electroporation is a technique in which cells are exposed to an electric field, creating temporary pores in the cell membrane through which cargos, including proteins, RNA, and DNA, can pass[1,2]. The most widely used version is bulk electroporation (BEP). BEP is performed by filling a cuvette with an electrolyte containing millions of cells, exposing the electrolyte to high voltage, and allowing cargo to enter the cells through diffusion or endocytosis[1]. There are many advantages to BEP, including high throughput and numerous commercially available systems. However, there are limitations to the BEP delivery. Inconsistent cell positioning relative to the electrodes and electric field shielding from adjacent cells causes significant variability in electric field exposure during BEP[3,4]. The high voltage required for BEP also has a significant negative impact on cell viability[5]. Since its inception in 2011[6], there has been growing interest in an electroporation method called porous substrate electroporation (PSEP), though it is sometimes referred to by other names, including localized electroporation and nano- or micro-electroporation[1,7,8]. In contrast to the cell suspension of BEP, PSEP is conducted on cells that are adherent to a porous substrate. Not only is an adherent state preferred for the majority of human cell lines[9], but the pores in the substrate also focus on the electric current, localizing the transmembrane electrical potential (TMP) to specific regions of the cell membrane[10,11]. This localization allows for a significant reduction in applied voltage, decreasing damage and increasing cell viability. This combination of effects helps control cell membrane pore development, resulting in a more consistent and efficient delivery[1,5,12].

A recent study introduced a PSEP device with a six-well, gold-plated electrode array for holding commercially available porous membrane inserts[13] (**Figure 1A,B**), a practice that was first introduced by Vindis et al.[14]. The device can apply pulses and measure the electrical impedance across the cell monolayer, known as the transepithelial electrical impedance (TEEI), in real-time[13]. The user interface of the device allows complete control over the electroporation waveform and polarity. Importantly, real-time impedance measurements can be used to predict delivery outcomes without the need for expensive reagents or fluorescent markers, a concept known as label-free delivery[15].

The PSEP platform consists of two major custom electrical components: the main body of the device, which houses the pulse generator and TEEI measurement equipment, and the electrode array, where the porous substrates are inserted, and the electroporation occurs. Diagrams for all custom electronics and 3D-printed components can be found at GitHub: https://github.com/YangLabUNL/PSEP-TEEI. In addition to the custom electronics, a computer is also required for the platform to function properly. The custom software requires MATLAB (version 2021a or later) to run, and Microsoft Excel to store and access data for analysis. The program controls the custom electronics and provides the graphical user interface (GUI) for adjusting settings. These programs were also made available at GitHub: https://github.com/YangLabUNL/PSEP-TEEI.

Preliminary data suggests this process is possible for different types of adherent cells (**Figure 1C**), but this article will only discuss the preparation of A431 cells using parameters that were found to be optimal for this cell line by Brooks et al.[13]. Additionally, because the propidium iodide (PI) cargo is cytotoxic, two experiments are performed, the first with a high concentration PI transfection media to quantify delivery efficiency, and the second with only cell culture media to measure TEEI over longer timescales. These experiments use identical electroporation waveforms, allowing the results to be correlated (**Figure 1D**).

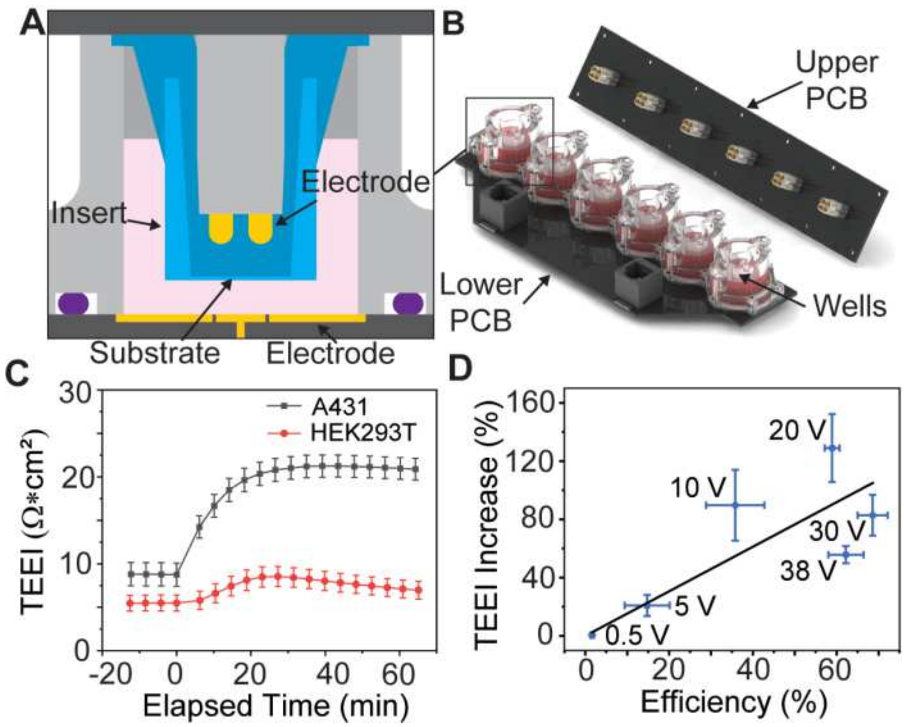

Figure 1: Electrode array assembly diagram and foundational data. (**A**) CAD model of an insert inside a well of the electrode array. (**B**) CAD model of the electrode array. (**C**) Impedance increase due to PSEP for select cell lines, n = 3 per cell line. Error bar: standard error of the mean. (**D**) Delivery efficiency *vs*. TEEI increase correlation data. Delivery efficiency was calculated by dividing the number of cells labeled in both PI and calcein images from delivery experiments by the total number of cells identified with Hoechst. Cell count was determined using a custom CellProfiler pipeline, n = 6 per voltage. Error bar: (x- and y-axis) standard error of the mean. This figure is reproduced from Brooks et al.[13] with permission. Please click here to view a larger version of this figure.

Protocol

The details of the reagents and the equipment used in the study are listed in the **Table of Materials**.

1. Preparation of reagents and cell culture

1. Prepare the cell culture media by adding 50 mL of fetal bovine serum (FBS) and 5 mL of penicillin-streptomycin to a 500 mL container of Dulbecco's Modified Eagle Medium (DMEM). Produce eleven 50 mL aliquots to reduce the risk of contamination and refrigerate at 4 °C.
2. Create 1 mL of 25 µg/mL human plasma fibronectin in phosphate-buffered saline (PBS) stock solution according to the manufacturer's instructions.
3. Create 15 mL of 0.1 mg/mL propidium iodide in DMEM stock solution to allow for experiments with varying cargo concentrations.

4. Culture A431 cells in a T75 flask containing 12 mL of the prepared cell culture media. Cells were passaged every 1-2 days to maintain 50% confluency.

2. Sample preparation

1. Fibronectin coating
    1. Select twelve inserts and two 24-well plates. Place the inserts into one well plate, creating two rows of six. Set the second well plate aside until later.
    2. Create 1,300 µL of 1 µg/mL fibronectin solution by mixing 52 µL of fibronectin stock solution and 1,248 µL of PBS in a 1.5 mL tube.
    3. Distribute 100 µL of the fibronectin solution into each insert. Incubate the inserts in the well plate at 37 °C for 3 h.
2. Adjusting the cell concentration for optimized cell density
    1. Around 1 h before the fibronectin incubation is complete, remove the T75 flask of A431 cells from the incubator for cell extraction.
    2. Remove the media in the flask with an aspirator and wash with 5 mL of PBS. Remove the PBS in the same fashion and add 3 mL of Trypsin. Incubate for 3-4 min before tapping the side of the flask to completely detach cells.
    3. Add 6 mL of cell culture media to the flask, mixing vigorously with a pipette to detach any remaining cells, and transfer contents to a 15 mL centrifuge tube. Centrifuge at 100 x *g* and 20 °C for 5 min.
    4. Remove the cell culture media and Trypsin from the centrifuge tube using an aspirator, being careful not to disturb the cell pellet. Add 1 mL of media to the centrifuge tube and pipette back and forth (without producing bubbles) to break up the cell pellet and resuspend the cells.
    5. Pipette 10 µL of the cell suspension, 40 µL of cell culture media, and 50 µL of trypan blue dye into a 200 µL tube, using a pipette to mix thoroughly.
    6. Remove 10 µL of the dye mixture and inject it into a hemocytometer. Count the cells using the 10% dilution of the dye mixture to estimate the total cell count in the 15 mL centrifuge tube.
    NOTE: For this protocol, assume a concentration of 5,000,000 cells/mL.
    7. Multiply the desired seeding density by the insert membrane's surface area, divide by the counted cells/mL in the suspension, and multiply by 1,000 to calculate the required microliters of cell suspension per insert.
        1. To find the total quantity of cell suspension required, multiply this figure by 10 (to ensure enough cells for 9 samples, as 3 of the 12 inserts will be cell-free controls), and round up to the nearest whole number. In this case, a total of 135 µL of the cell suspension is required for this experiment.
    8. Create 2,000 µL of adjusted cell solution by mixing the previously calculated 135 µL of the cell suspension with 1,865 µL of cell culture media in a separate 15 mL centrifuge tube.
3. Seeding cells
    1. Remove the excess fibronectin from each insert once the fibronectin incubation is complete.
    2. Wash the inserts twice by adding 100 µL of sterile distilled water to each insert. Remove the water

following the same order as it was added to ensure a consistent wash time between inserts.

3. Wash the insert again by adding 100 µL cell culture media to each insert. Remove the media following the same order as it was added to ensure a consistent wash time between inserts.
4. Cell sample inserts
    1. Seed cells by pipetting 200 µL of adjusted cell solution into each insert. To ensure consistent confluency between inserts, mix the cell solution in the centrifuge tube prior to distribution and mix again within each insert after distribution.
5. Negative control inserts
    1. Pipette 200 µL of cell culture media into each insert. To remain consistent with the cell sample inserts, use the pipette to mix the cell culture media within each insert.

4. Labeling and incubation
    1. Draw a line dividing the second well plate into two columns that are three wells wide (for conditions run in triplicate) using a permanent marker. Separate each column into rows. Label each region in the grid with relevant parameters.
    2. Add 1 mL of cell culture media to every well to receive an insert for the experiment. Transfer the inserts from the preparation well plate to their appropriate location in the labeled experiment well plate and incubate at 37 °C for at least 12 h.

3. Experimental procedure

1. Delivery experiment
    1. Pipette 1.5 mL of the 0.1 mg/mL PI solution into each well in the electrode array. Place an insert into each well in the electrode array, fitting the feet of the insert into the alignment grooves so the insert is flush with the upper surface of the well (**Figure 1A,B**).
    2. Screw the top electrode printed circuit board (PCB) to the top of the electrode array wells and connect the electrode array to the PSEP device.
    3. Place the electrode array in the 37 °C incubator for at least 1 h to allow the temperature to equilibrate.
    4. Click the drop-down next to "Membrane" in the top left corner of the GUI and click on **400 nm GBO**. Repeat this step for "Electrolyte", "Cells", "Cell Seeding Density", and "Cell Duration", selecting DMEM, A431, 200, and 12, respectively.
    NOTE: These values are for record-keeping purposes only, and do not impact the function of the device. Please ensure to adjust these values as necessary for correct data tracking.
    5. Type **1** into the **Post Pulse Time Duration (min)** edit field on the right side of the GUI to change the default post-pulse measurement time to 1 min. Leave all other settings in the default state.
    NOTE: Default pulse parameters create a square waveform with 30 volts, 20 Hz, 1 ms duration, and 200 pulses. Default TEEI measurement parameters are 0.5 volts and 100 Hz, 1,000 Hz, 10,000 Hz, and 100,000 Hz.
    6. Click on the **Run** button and enter appropriate names for wells 1-3 and 4-6 when prompted. Click on **OK** to start the experiment.
    7. Remove the electrode array from the incubator and transfer the inserts back into the original locations

in the experiment well plate when the program has finished executing.

8. Mix 2 µL of Hoechst 33342 and 5 µL of calcein AM with 123 µL of cell culture media in a 200 µL tube.

9. Gently pipette 10 µL of the stain solution into each post-pulse insert and place the inserts back into the incubator for 5 min.

10. Transfer the well plate to the plate holder of a fluorescent microscope with a 5x magnification objective. Image using brightfield and the fluorescence of each stain. Center the insert over the objective before triggering the camera.

NOTE: The excitation wavelengths for PI, calcein AM, and Hoechst 33342 are 558 nm, 495 nm, and 353 nm, respectively. The emission wavelengths are 575 nm, 519 nm, and 465 nm, respectively.

2. TEEI measurement experiment

1. Pipette 1.5 mL of the cell culture media into each well in the electrode array. Place cell sample inserts into wells 1-3 and control inserts into wells 4-6, fitting the feet of the insert into the alignment grooves so the insert is flush with the upper surface of the well.

2. Screw the top electrode PCB to the top of the electrode array wells and connect the electrode array to the PSEP device.

3. Place the electrode array in the 37 °C incubator for at least 1 h to allow the temperature to equilibrate.

4. Click on the drop-down next to "Membrane" in the top left corner of the GUI and click on 400 nm GBO. Repeat this step for "Electrolyte", "Cells", "Cell Seeding Density", and "Cell Duration", selecting DMEM, A431, 200, and 12, respectively.

NOTE: These values are for record-keeping purposes only, and do not impact the function of the device. Please ensure to adjust these values as necessary for correct data tracking.

5. Leave all remaining settings in the default state.

NOTE: Default pulse parameters create a square waveform with 30 volts, 20 Hz, 1 ms duration, and 200 pulses. Default TEEI measurement parameters are 0.5 volts and 100 Hz, 1,000 Hz, 10,000 Hz, and 100,000 Hz.

6. Click on the **Run** button and enter appropriate names for wells 1-3 and 4-6 when prompted. Click on **OK** to start the experiment.

7. Remove the electrode array from the incubator and transfer the inserts back into the original locations in the experiment well plate when the program has finished executing.

8. Mix 2 µL of Hoechst 33342, 5 µL of calcein AM, and 10 µL of PI with 113 µL of cell culture media in a 200 µL reaction tube.

9. Pipette 10 µL of the stain solution into each post-pulse insert and place the inserts back into the incubator for 5 min.

10. Transfer the well plate to the plate holder of a fluorescent imaging microscope with a 5x objective lens. Image using brightfield and the fluorescence of each stain. Center the insert over the lens before triggering the camera.

NOTE: The excitation wavelengths for PI, calcein AM, and Hoechst 33342 are 558 nm, 495 nm, and 353 nm, respectively. The emission wavelengths are 575 nm, 519 nm, and 465 nm, respectively.

4. Data analysis

1. Analyzing image data with the CellProfiler pipeline
    1. Use the custom CellProfiler workflow that is provided at GitHub:https://github.com/YangLabUNL/PSEP-TEEI to process the delivery and TEEI measurement experiment images.
2. TEEI analysis
    1. Click on the **Analysis** tab in the GUI.
    2. Toggle the **impedance type indicator** to TEEI at the bottom of the GUI.
    3. Click on the arrow in the top left box to show all the experiment names in the data file. Select all **cell sample data** from the TEEI measurement experiment.
    4. Click on the arrow in the next box to the right to show all the experiment names in the data file. Select all **control insert data** from the TEEI measurement.
    5. Click on **Run**. A basic figure containing selected cell sample data at the lowest measurement frequency will appear.
    6. In the sample options box on the right-hand side of the GUI, click on the arrow to show all selected insert data. Outliers can be removed by selecting the appropriate data and clicking on **Remove** below.
    NOTE: Any data that was removed from the analysis by the last click of the **Remove** button can be retrieved by the **Undo** button.
    7. Click on **Done** to move on to the next figure when the desired data is shown in the figure.
    8. Repeat steps 4.2.6 and 4.2.7 for the remaining cell sample data and control data. When the final dataset has been confirmed by clicking "**Done**", the full analysis figure will appear.
    9. Save the analysis figure.

Representative Results

The given protocol establishes a method for using TEEI measurements to examine the intermediate processes of electroporation and make delivery predictions, specifically for the A431 cell line and PI cargo. While modification of this protocol is discussed further in the article, it is important to note now that while the specific values may change, general trends in the response remain consistent. For example, TEEI data that dips below the initial baseline corresponds with cell death, while the maximum increase in TEEI value above the minimum corresponds with delivery efficiency[13]. These general trends and their implications are explored below.

As shown in **Figure 2A**, a range of TEEI measurement and cell imaging trends can arise while using the PSEP platform. The ideal outcome of this protocol is to produce a curve similar to the optimized healthy data shown in **Figure 2Ai**. This is characterized as the ideal outcome, as there is no dip below baseline, indicating very little, if any, cell death. Additionally, the optimized healthy curve has the largest increase in TEEI from minimum, indicating a high degree of delivery efficiency[13]. These inferences are supported by the post-PSEP imaging of the cell monolayer, which reveals negligible cell death and a healthy, fully confluent cell monolayer (**Figure 2Aiii**). Furthermore, a successful delivery experiment using identical PSEP waveforms can be characterized by the images shown in **Figure 2B**. Proper electroporation waveform application and cargo concentration result in a high degree of delivery consistency and cell viability.

The health and confluency of the cell monolayer are critical to the successful application of TEEI-based delivery predictions[16,17]. Even with an optimized waveform, an unhealthy or incomplete cell monolayer results in a diminished TEEI response, as illustrated by the optimized unhealthy data in **Figure 2Aii**. However, note that the images for this outcome (**Figure 2Avi**) still correspond to the interpretation of the TEEI response given by Brooks et al.[13]. There is no dip below the baseline, indicating near-zero cell death (**Figure 2C,D**). Additionally, the reduced increase from the minimum corresponds to a negative impact on the delivery efficiency, as fewer cells in the monolayer reduce total PI delivery (**Figure 2C,D**).

If an unoptimized waveform is applied, it is possible to see even more significant decreases in TEEI response. Depending on the total energy and the timeframe in which it is applied, unoptimized waveforms can produce results ranging from decreased efficiency to near-total annihilation of the cell monolayer (**Figure 2Ai,iii,v**). Both the unoptimized and very unoptimized curves show a significant decrease from baseline, indicating substantial cell death. However, increasingly unoptimized waveforms impede cell recovery, resulting in diminished delivery efficiency.

Delivery efficiency was calculated by dividing the number of cells labeled in both PI and calcein images from delivery experiments by the total number of cells identified with Hoechst. Death was calculated by taking the cells marked with PI in TEEI measurement experiments and dividing by the total number of cells identified with Hoechst. Cell count was determined using a custom CellProfiler pipeline for both metrics.

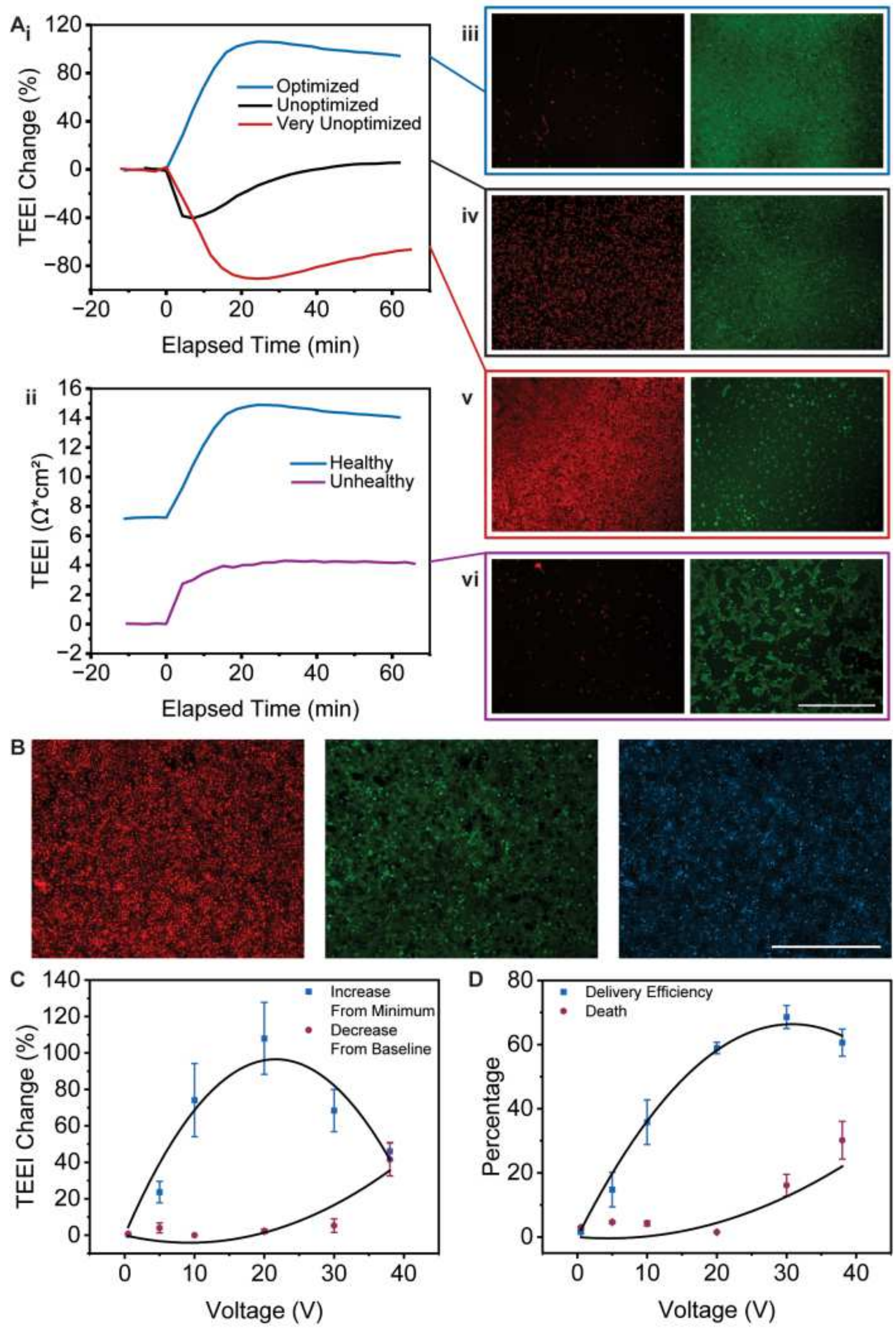

Figure 2: TEEI response curves and imaging for common conditions. (**A**) (**i**) TEEI response data illustrating percent TEEI change for optimized and unoptimized conditions. (**ii**) TEEI response comparison between healthy and unhealthy monolayers under optimized waveform conditions. (**iii-v**) Representative imaging of potential outcomes for optimized and unoptimized waveform conditions showing cell death (red) and living cells (green). (**vi**) Representative imaging of unhealthy monolayer after applying optimized waveform conditions showing cell death (red) living cells (green). (**B**) Images showing

successful PI delivery (red), living cells (green), and nuclei locations (blue). All images brightened for clarity. Scale bars: 1000 µm. (**C**) TEEI decrease from pre-PSEP baseline to minimum post-PSEP and increase from post-PSEP minimum to post-PSEP peak for given voltages. (**D**) Delivery efficiency and cell death percentages for given voltages. Error bars represent the SEM (n = 6). (**C,D**) reproduced from Brooks et al.[13] with permission. Please click here to view a larger version of this figure.

Discussion

Figure 2C demonstrates that TEEI increases from minimum and decreases from baseline are plotted for each PSEP waveform voltage. The TEEI increase creates a parabolic arc, peaking around 20 volts before reducing, while the TEEI decrease from baseline increases exponentially as voltage increases. The delivery efficiency and death percentages in **Figure 2D** mirror these trends, with delivery efficiency arcing parabolically, peaking around 30 volts, and death increasing exponentially as waveform voltage is increased.

One hypothesis for the underlying mechanism causing the TEEI increase is electro-osmosis through the negatively charged substrate microchannels, a phenomenon caused by the application of an electric field[18,19]. Whether the TEEI response is due to mechanical stimulus from cell swelling due to electro-osmotic fluid flow, a factor known to occur with electroporation[20], or due to the electrical stimulus of the waveform itself, it is clear health and completeness of the monolayer is paramount to achieving the proper voltage drop across the cell membrane required for electroporation. For this reason, the most critical steps in this method are the ones regarding cell seeding and ensuring proper cell monolayer formation. This can be confirmed by imaging the cell monolayer and by the baseline TEEI value. For A431 cells, the average TEEI is around 7 $\Omega \cdot cm^2$, whereas HEK293T cells average a slightly lower 5 $\Omega \cdot cm^2$ (**Figure 2Aii**), likely due to morphological differences causing differences in cell-cell junction area.

Due to the electric field required for porous substrate electroporation, electrolysis will occur, causing the electrodes to corrode[12,21]. This was especially evident for the bottom electrode, as it was positively charged in this experiment to deliver positively charged PI. Through experimentation, it was determined that the bottom PCB could be used approximately 20 times before significant negative effects require replacement[13]. To clean the electrode array for reuse, remove the remaining cell culture or transfection media from the chambers using an aspirator. Fill each chamber three-quarters of the way full of 70% ethanol and place the top electrode PCB onto the electrode array so the top electrodes are submerged. Leave the ethanol in the electrode array for at least 10 min before removing the ethanol and setting the electrode array aside to dry.

It is possible to reuse the purchased inserts by removing the substrates, sterilizing the insert, and replacing the substrate with one taken from another source. 6-well inserts with the same pore density and diameter are available commercially and can be used to harvest four 24-well insert-sized replacement substrates. Once the previously used inserts are sterilized, add 10 µL of ultraviolet-light-cured epoxy to a fresh Petri dish. Dip the substrate side of the insert into the pool of epoxy to coat the bottom surface, and carefully place a new substrate over the hole in the insert. Visually verify that the epoxy makes a complete ring to ensure there are no gaps in the connection. Cure under a UV light for 30 s and store the refurbished inserts in a clean 24-well plate to avoid damaging the new substrates before reuse.

As stated previously, while it is hypothesized that the observed TEEI increase will occur in multiple cell types, it has only been demonstrated with the A431 and HEK293T cell lines[13] (**Figure 1C**), both of which are adherent cells. The method can be modified by selecting different cell lines by selecting membranes with different pore characteristics, replacing the fibronectin coating with another extracellular matrix protein by adjusting the concentration, or by changing the cargo. However, if any changes are made to the experiment's setup, it may be necessary to reoptimize the waveform. To optimize the waveform, a TEEI measurement experiment can be conducted in which only one waveform parameter, such as voltage, is changed between each group of three samples. Select the optimal voltage by identifying the largest increase in TEEI over at least nine healthy samples. Repeat this process for each waveform parameter, using the newly optimized values when moving on to the next one. Remember there may be multiple local optima for waveform parameters (i.e., the optimal voltage for one pulse duration may not be the optimal voltage for another pulse duration, and so forth).

The benefits of porous substrate electroporation are wide-reaching. While other methods of intracellular delivery have existed for a considerable time, few have combined high throughput with a high degree of control that PSEP possesses[1,13]. Additionally, the platform's use of TEEI measurements provides a glimpse into the intermediary steps of the electroporation process. The TEEI readings tell the condition of the cells, guide the selection of electroporation parameters, and allow further insight into specific cell behaviors and mechanisms[13,17]. Through the TEEI measurements, the platform is also capable of label-free delivery[13], which allows for rapid optimization with a diminished need for expensive biomarkers and reagents every time an experiment is conducted. These contributions to the area of intracellular delivery make this a prime candidate as a delivery platform for fundamental biological research and biomedical applications.

Disclosures

The authors declare no conflict of interest.

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