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Monitoring Electroporation-Induced Changes in Action Potential Generation in Genetically Engineered tet-on Spiking HEK Cells

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Abstract

Excitable cells such as neuronal and muscle cells can be primary targets in rapidly emerging electroporation-based treatments. However, they can be affected by electric pulses even in therapies where they are not the primary targets, and this can cause adverse side effects. Therefore, to optimize the electroporation-based treatments of excitable and non-excitable tissues, there is a need to study the effects of electric pulses on excitable cells, their ion channels, and excitability *in vitro*. For this purpose, a protocol was developed for optical monitoring of changes in action potential generation due to electroporation on a simple excitable cell model of genetically engineered tet-on spiking HEK cells. With the use of a fluorescent potentiometric dye, the changes in transmembrane voltage were monitored under a fluorescence microscope, and relevant parameters of cell responses were extracted automatically with a MATLAB application. This way, the excitable cell responses to different electric pulses and the interplay between excitation and electroporation could be efficiently evaluated.

Introduction

In electroporation, high-voltage electric pulses cause an increase in plasma membrane permeability for molecules that are otherwise poorly permeant[1]. Electroporation-based techniques are nowadays widely used in applications in medicine[2], biotechnology[3], and food technology[4].

Excitable cells such as neuronal and muscle cells are among the primary targets in rapidly emerging electroporation-based treatments in the heart, brain, and skeletal muscles[5]. Moreover, they can be affected by electric pulses even in therapies where they are not the primary targets, and this can cause adverse side effects such as pain, muscle twitching, and nerve damage[6]. Namely, electric pulses can cause electrical stimulation of excitable cells: they activate voltage-gated ion channels and trigger action potentials (AP). However, at higher electric fields, the cells' plasma membrane becomes permeabilized, and additional ionic current through

pores/defects occurs[7]. This additional ionic current affects cell excitability in a complex interplay between excitation and electroporation. Therefore, to optimize the electroporation-based treatments of excitable and non-excitable tissues, there is a need to study the effects of electric pulses on excitable cells, their ion channels, and excitability *in vitro*.

In a recent study[8], we explored genetically engineered human embryonic kidney (HEK) cells developed by Cohen et al.[9,10], as a valuable tool for studying electroporation in excitable cells. In these otherwise non-excitable cells, a minimal complement of sodium and potassium channels ($Na_V1.5$ and $K_{ir}2.1$) needed for excitability is expressed. The expression of $K_{ir}2.1$ in these cells is controlled by a doxycycline-induced tet-on system that allows the establishment of two cell variants: excitable spiking S-HEK (containing $Na_V1.5$ and $K_{ir}2.1$) and non-excitable non-spiking NS-HEK cells (containing only $Na_V1.5$ channels). This allows us to compare the effects of electric pulses on excitable and non-excitable variants of the same type of cells. Compared to isolated primary excitable cells, excitable cells differentiated from embryonic or induced pluripotent stem cells, and cell lines derived from excitable tissues, S-HEK cells have several advantages: they are easy to handle, cultivate and propagate, have well-defined sodium and potassium channels, and generate robust APs[8].

We designed a protocol for monitoring changes in transmembrane voltage (TMV) triggered by electric pulses of different amplitudes with the use of a fluorescent potentiometric dye. This way, we can study how electroporation affects AP generation in S-HEK cells and other changes in the TMV in both S-HEK and NS-HEK cells. Briefly, the S-HEK or NS-HEK cells are exposed to a single 100 µs electric pulse every 2 min, and relative changes in fluorescence are recorded for roughly 3 s around each pulse application. The applied pulses are of increasing voltage, first triggering APs in cells and, at a higher electric field, electroporation. The 2 min interval between pulse application was chosen to minimize the possible cumulative effects of consecutive pulses while keeping experiments short enough not to deteriorate the cells. The image processing was automated with a custom MATLAB application to extract relevant parameters of the fluorescence responses of the potentiometric dye, hence evaluating the changes in TMV (APs and sustained depolarization due to electroporation) in S-HEK and NS-HEK cells.

Protocol

1. Preparation of reagents and cell culture

1. Prepare the cell culture medium by supplementing Dulbecco's Modified Eagle's Medium-high glucose with 10% fetal bovine serum, 2 mM glutamine, penicillin-streptomycin (penicillin 100 U/mL, streptomycin 100 µg/mL), 2 µg/mL puromycin, 5 µg/mL blasticidin, and 200 µg/ mL geneticin.
2. Prepare phosphate-buffered saline (PBS) solution with 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4. Filter it through a sterilizing membrane filter with a 0.22 µm pore size.
3. Prepare Tyrode solution with 2 mM KCl, 125 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 30 mM glucose, pH 7.3. Prepare low potassium Tyrode solution with 0.5 mM KCl, 126.5 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 30 mM glucose, pH 7.3. Filter both solutions through a sterilizing membrane filter with a 0.22 µm pore size.

4. Prepare 1 mg/mL poly-L-lysine stock solution by adding 5 mL of PBS in a vial containing 5 mg of poly-L-lysine.
5. Prepare 2 mg/mL doxycycline stock solution in distilled water and filter it through a sterilizing membrane filter with a 0.22 µm pore size.
6. Prepare 4 mM potentiometric dye ElectroFluor630 stock solution in pure ethanol (add 25 µL of pure ethanol to the tube containing 100 nM - 73 µg of the dye).
7. Culture genetically engineered HEK cells in a T25 flask in 6 mL of the cell culture medium as previously described[8]. The ingredients of the culture medium are light sensitive; thus, keep the medium protected from light (wrap the medium flask in aluminum foil) and perform passaging in low light conditions.
8. Passage the cells every 3-4 days by seeding 3 x 10^5 or 5 x 10^5 cells to a fresh T25 flask. Count the cells using a hemocytometer. Maintain cells in a master flask as non-excitable NS-HEK cells without doxycycline addition because they do not tolerate sustained $K_{ir}2.1$ expression. Use cells from passages lower than 14 in the experiments.

2. Sample preparation

1. Poly-L-lysine-coating of cover glass chamber slides
    1. Pipette 1 mL of PBS to one well of the 2-well cover glass chamber slides, add 10 µL of sterile 1 mg/mL poly-L-lysine solution and mix well with a pipette.
    2. Incubate for 1.5 h at room temperature in sterile conditions in a laminar flow hood. Remove the PBS with poly-L-lysine without any further washing.
2. Seeding the cells
    1. Prepare a 1:10 dilution of doxycycline stock solution in a culture medium in a 1.5 mL tube. For each well 30 µL of 1:10 doxycycline dilution is needed (3 µL of doxycycline stock solution and 27 µL of culture medium), prepare the final volume depending on the total number of experimental wells.
    2. Remove the culture medium from the T25 flask and trypsinize the cells with 2.5 mL of Trypsin-EDTA solution in a CO_2 incubator at 37 °C for 2 min.
    3. Add 2.5 mL of fresh culture medium and, by gentle pipetting, detach the cells from the surface. Mix the cell suspension well.
    4. Count the cells with a hemocytometer and determine the density of cells in suspension.
    5. Calculate the volume of trypsinized cell suspension needed to establish the culture. For 2-day culture, seed 2.5 x 10^5 cells, and for 3-day culture, seed 1 x 10^5 cells per well. Adjust the exact number of cells if needed.
    6. Calculate the volume of the culture medium by subtracting the previously calculated cell suspension volume from 1470 µL. The remaining 30 µL represents the volume of 1:10 doxycycline dilution. The total volume in each well will be 1500 µL (cells, doxycycline, and culture medium).
    7. For preparing samples with excitable S-HEK cells, pipette the calculated volume of culture medium and trypsinized cell suspension into one well and add 30 µL of 1:10 doxycycline dilution. Use only one well of the two-well chambers since the experiment takes a long time (around 40 min). Mix well by gentle

pipetting just before placing the chambers into the CO_2 incubator.

8. For preparing samples with non-excitable NS-HEK cells, pipette the calculated volume of culture medium into a well and add 90% of the calculated volume of cell suspension for S-HEK cells without doxycycline. NS-HEK cells grow slightly faster than S-HEK cells.

9. Incubate the chambers in a 5% CO_2 incubator at 37 °C for 2-3 days prior to the experiment. For samples with doxycycline, this incubation time is sufficient to render the cells excitable.

3. Labeling the cells

1. Before the experiment, pipette 3 µL of potentiometric dye stock solution to a 1.5 mL tube and leave the open tube at room temperature in a laminar flow hood to allow the ethanol to evaporate and the potentiometric dye to dry (approximately 15 min). Add 997 µL of cold culture medium (from a refrigerator at 4 °C) to the tube and dissolve the dye to a final concentration of 12 µM.

2. Remove the culture medium from the well and add 1 mL of potentiometric dye solution to the cells. Refrigerate them at 4 °C and incubate for 20 min.

3. Remove the potentiometric dye solution and save it in the tube for further reuse (for up to six sequential experiments in 1 day).

4. Gently wash the cells 3x with Tyrode solution, and after the last wash, pipette 1 mL of low potassium Tyrode solution into the well.

3. Electrostimulation and electroporation

1. Configure synchronization of the pulse delivery with image acquisition using a TTL signal from the microscope system that triggers the pulse generator. For generation of high-voltage pulses, use a custom pulse generator[11] or any pulse generator that can be triggered externally with a TTL signal.

   NOTE: In the presented experimental configuration, LAS X software is used for image acquisition, which can be configured to send a TTL signal at a preselected time during a time-lapse acquisition.

2. Place two parallel Pt/Ir wire electrodes, with 0.8 mm diameter and 5 mm distance between inner edges, at the bottom of the chamber and fix the chamber under the microscope.

3. Connect the electrodes to the pulse generator. Connect the output of the pulse generator to an oscilloscope using a voltage and a current probe to enable verification that the pulses were correctly delivered during the experiment.

4. Focus the cells in brightfield. Image the cells that are located roughly in the middle between the electrodes.

5. Start the first acquisition of fluorescence images in time-lapse mode with a 40x objective, recording the fluorescence signal in the whole field of view. The camera should be fast enough to acquire a clear time course of an action potential (ideally higher than 25 frames/s).

   1. Acquire 80 images in a time-lapse mode, with at least 25 frames/s to resolve the real-time course of the action potential. The total duration of image acquisition results in around 3 s. During image acquisition, illuminate the cells with an excitation

wavelength of 635 nm, exposure time of 10 ms, and detect the emission at around 700 nm. In this first acquisition, do not apply any pulse - this acquisition will be used to correct the subsequent acquisitions for dye photobleaching. After this acquisition, wait for 2 min.

NOTE: This experiment uses 27.8 frames/s, i.e., 36 ms period, which is the time from the beginning of the first image acquisition to the beginning of the next image acquisition in the time-lapse recording).

6. Every 2 min, record a time-lapse in the same way as in step 3.5. For these acquisitions, apply a single 100 µs pulse at the time of acquisition of the 10th image (at 324 ms). Acquire images with the following pulse voltages: 63 V, 75 V, 88 V, 100 V, 125 V, 150 V, 175 V, and 200 V. Estimate the electric field to which the cells are exposed as the applied voltage-to-electrode-distance ratio (E = 126, 150, 176, 200, 250, 300, 350, and 400 V/cm).

   NOTE: At lower electric fields, the pulses will trigger action potentials; at higher electric fields, they will trigger electroporation, which manifests as a prolonged depolarization. The duration of the experiment on each sample of cells is approximately 40 min (20 min for labeling and 20 min for microscopy).

4. Data analysis

1. Export the time-lapse recordings to tiff format.
2. Place time-lapse recordings of all the applied voltages during an individual experiment (one sample of cells) into a single folder. Rename the time-lapse recordings in such a form that electric field E can be recognized by the MATLAB application: Vcm (e.g., 126Vcm for 126 V/cm). The remaining parts of the name can be arbitrary. For the acquisition at the beginning of the experiment where no pulse is applied, rename using the phrase 000Vcm. An example of the time-lapse recording names: 2022_1_20 1DOX 000Vcm, 2022_1_20 1DOX 126Vcm, 2022_1_20 1DOX 150Vcm, etc.
3. Open the application, which can be downloaded from https://github.com/bor-kos/fluorescenceActionPotential. Click on **Select Data Folder** to choose the folder containing time-lapse recordings of one experiment.
4. Select the pixels to be used for further data analysis. To do this, use two sliders to determine the low threshold for a clear image of the membranes (in purple) and the high threshold for eliminating debris (large spots with high fluorescence) from the analysis (in green).
5. Select **Analyze Data.** In the Analysis tab, a table with extracted parameters appears. On the right side of the table, graphs of relative fluorescence in time appear for each E used. Select graphs for a given E from the drop-down menu on the left side above the table.
6. Examine all the graphs and perform manual correction in the case of false peak detection. Select **Clear AUTO Peaks** at the bottom in the Analysis tab, and the peaks that were detected automatically are cleared. Click on the **Graph** to manually determine a new peak time point.
7. Select **Rerun AUTO Peak Detection** at the bottom in the Analysis tab to perform automatic peak detection again. The extracted parameters will now be adjusted to the new peak.
8. Select **Export Data** at the bottom right in the Analysis tab to export data to the same folder in the form of a .mat file, spreadsheet file, and PNG images for each E used.

Representative Results

Changes in TMV in excitable cells *in vitro* triggered by electric pulses can be optically monitored with this protocol, and the fluorescence signals can be analyzed to extract their relevant parameters.

The experimental setup is shown in **Figure 1A**. A typical signal of 100 µs electric pulse used in experiments is shown in **Figure 1B**. Cells in the chambers that are placed under the microscope typically look like the cells in **Figure 1C** (brightfield image) and **Figure 1D** (fluorescence image). We perform an experiment where every 2 min, an electric pulse with increasing voltage is applied to the same cells. Note that the 2 min interpulse time may not be sufficiently long to allow for full resealing and recovery of the cell membranes, especially when using pulses of the highest amplitude, which are associated with prominent electroporation. Thus, some cumulative effects of the pulses can be expected. To avoid these cumulative effects, the interpulse delay can be extended, but at the expense of reducing the number of the tested pulse amplitudes and avoiding keeping the cells on the microscope for too long.

After capturing fluorescence images in experiments, analyze them with the custom MATLAB application. First, determine the pixel thresholds (**Figure 2A**); by adjusting the low threshold, we get a clear image of the membranes around the cells (in purple), and by adjusting the high threshold, we remove the signal of debris (green). The application then determines the average fluorescence intensity of all threshold pixels for each delivered consecutive pulse to obtain a time-dependent signal of the relative change in fluorescence F/F0. This signal is corrected for dye photobleaching by subtracting the slope of the linear decrease of F determined from the control time-lapse recording in which no pulse was applied (**Figure 2B**). After correcting the signal, the parameters of responses are extracted in the form of an in-app table and a graph of the corrected fluorescence signal (**Figure 2C**). In rare cases, the algorithm does not detect an obvious peak or detects spurious noise signals as false positives. For these cases, the user has the option to manually select the corresponding point in the fluorescence signal as a peak or to remove the detected false peak (**Figure 2D**-removal of a small peak that was anomalously detected before the electric pulse was applied).

By using the MATLAB application, we can get images of TMV responses to electric pulses in both excitable S-HEK (**Figure 3A**) and non-excitable NS-HEK cells (**Figure 3B**). The following response parameters are extracted (**Table 1**): number of peaks (NoPeaks), average peak-to-peak time (when more than one peak is detected, MPPT), maximum response (MR), time to first peak (TtFP), and time from peak to 25%, 50%, 75%, and 90% recovery (T25, T50, T75, T90, respectively). These parameters enable the evaluation of responses to different electric pulses (e.g., determine the number of peaks triggered by electric pulses of different E, **Figure 3C**) and the interplay between excitation and electroporation.

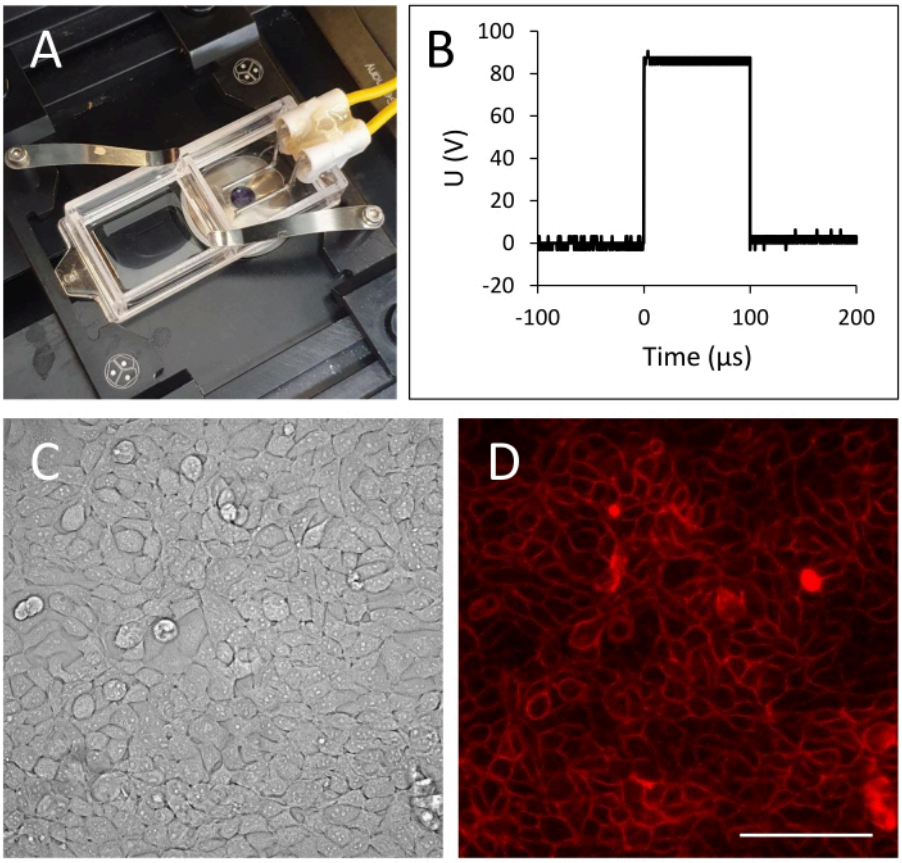

Figure 1: The setup, waveform, and S-HEK cells. (**A**) The image of the chamber on the microscope stage. (**B**) Example of the waveform of a pulse used in the study (voltage (U) over time). (**C, D**) The image of S-HEK cells ((C): brightfield, (D): fluorescence), scale bar: 100 µm. Please click here to view a larger version of this figure.

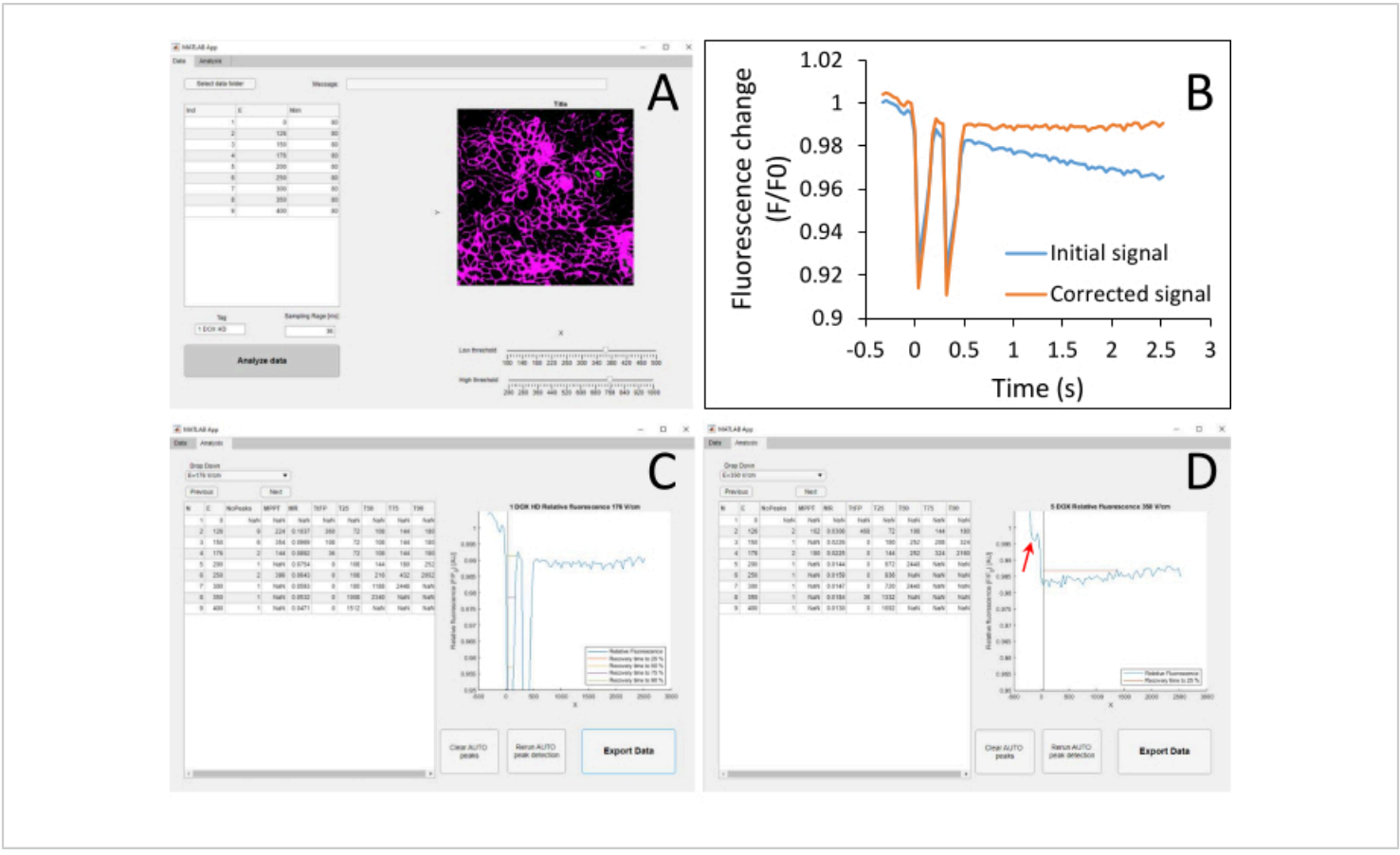

Figure 2: Data analysis application. (**A**) The image of the application interface - thresholding. (**B**) The graph of the raw and corrected signal of fluorescence change. (**C**) The image of the application interface - extracted data. (**D**) Manual correction of peak detection (removal of a small peak that was anomalously detected before the electric pulse was applied, marked with a red arrow). Please click here to view a larger version of this figure.

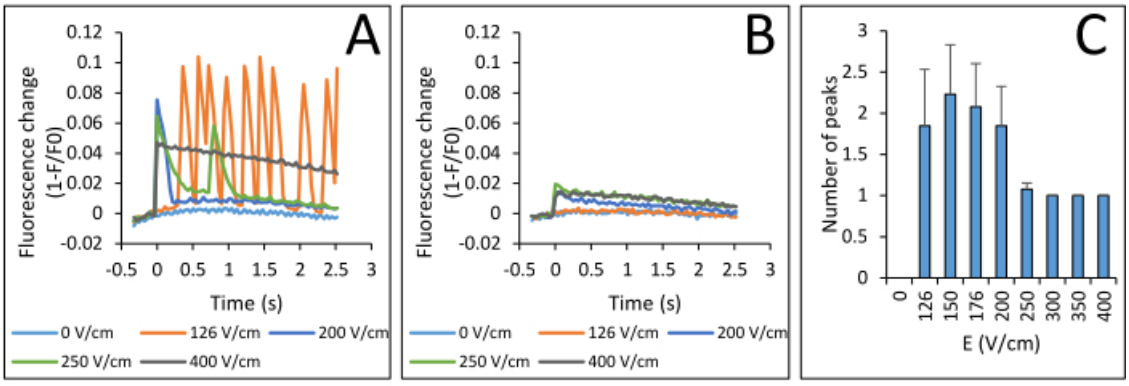

Figure 3: Representative results. (**A, B**) Triggering changes in TMV in (A) excitable S-HEK and (B) non-excitable NS-HEK with 100 µs electric pulses of different amplitudes, results from representative experiments. For clarity, only responses to selected pulses in the applied pulse sequence are shown. (**C**) The parameters of TMV responses triggered by the 100 µs pulses of different amplitudes: Example shows the number of peaks. The results are expressed as average ± SE, number of experiments: N = 13. Please click here to view a larger version of this figure.

Index	E-strength (V/cm)	No peaks (-)	MPPT (ms)	MR (-)	TtFP (ms)	T25 (ms)	T50 (ms)	T75 (ms)	T90 (ms)
1	0								
2	126	9	224	0.104	360	72	108	144	180
3	150	6	354	0.097	108	72	108	144	180
4	176	2	144	0.089	36	72	108	144	180
5	200	1		0.075	0	108	144	180	252
6	250	2	396	0.064	0	108	216	432	2052
7	300	1		0.059	0	180	1188	2448	
8	350	1		0.053	0	1008	2340		
9	400	1		0.047	0	1512			

Table 1: Parameters of TMV responses triggered by the 100 µs pulses of different amplitudes.

Discussion

S-HEK cells are a simple excitable cell model with well-defined sodium and potassium channels, $Na_V1.5$ and $K_{ir}2.1$[9]. This enables us to perform in-depth studies of how excitable cells respond to applied electric pulses and link the experimental data with mathematical models based on the well-known theoretical characteristics of the channels expressed in S-HEK cells. In our previous study[8], we described the responses of these cells to 100 µs pulses of increasing E both experimentally and theoretically. At lower E, the S-HEK cells respond with single or multiple action potentials, whereas at higher E, APs become prolonged and transform into a sustained depolarization. Since the sustained depolarization is clearly visible also in a non-excitable version of these cells (NS-HEK), it is attributed to electroporation and the associated increase in the nonselective ionic current.

The use of the fluorescent potentiometric dye proved to be a very effective way to study APs and electroporation in excitable cells. Compared to classical electrophysiological methods, such as patch clamp, optical measurements are much more convenient, time efficient, and do not require special technical skills. Moreover, the use of high-voltage electric pulses does not interfere with the measurements, which can become a problem in patch clamp measurements[12]. However, the major drawbacks of this protocol are related to the nature of the dye. Due to its photobleaching, the captured fluorescence signal needs correction. The dye spontaneously diffuses into the cell, so the signal from the membranes decreases with time. For this reason, the dye is suitable for monitoring action potentials and other short-term changes in TMV (~3 s in these experiments). Since the dye enables ratiometric imaging[13], such imaging could avoid some problems with dye diffusion, but at the expense of lower attainable imaging speed. In this experimental configuration, ratiometric imaging would be too slow to correctly capture the shape of action potentials. Moreover, the measured changes in the dye fluorescence provide qualitative information on the change in TMV; nevertheless, the dye can be calibrated to enable quantitative measurements.

The automated image analysis using a custom MATLAB application enables us to analyze a large amount of data and to efficiently extract valuable characteristics of TMV responses in a short period of time. The application is configured to capture the fluorescence signal from membranes of all cells in the field of view. This improves the signal-to-noise ratio but at the expense of losing information on how TMV changes at the individual cell level. In the given experimental configuration, the signal from individual cells was too noisy to enable useful measurements. Moreover, the membranes of adjacent cells were hard to distinguish. Nevertheless, HEK cells endogenously express gap junctions through which they are electrically connected. Mathematical modeling, presented in the previous study[8] demonstrated that due to their electrical connections, the cells within the field of view are close enough to trigger AP simultaneously. Furthermore, AP is propagated along the cell monolayer at high speed (roughly 34 mm/s, estimated based on imaging at 5x objective magnification)[8,9]. Considering the side length of the field of view (~0.33 mm), the AP would travel this length in roughly 10 ms, which is shorter than our image acquisition period. Thus, the AP propagation effect cannot be seen with this magnification and recording speed. Nevertheless, propagation of the AP from the electrodes to the field of view can cause a delay between the applied pulse and the captured AP[8].

In the previous study[8], as well as in this publication, the effects of pulses of 100 µs durations on the interplay between excitability and electroporation are described. However, this protocol can be adapted to study electric pulses with other parameters (of different duration, voltage, number, repetition rate or shape) or other excitable cells.

Disclosures

The authors declare no conflict of interest.

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