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Measurements of the induced transmembrane voltage with fluorescent dye di-8-ANEPPS

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Duration of the experiments: 60 min
Max. number of participants: unlimited
Location: Online ONLY
Level: Basic

PREREQUISITES

No specific knowledge is required for this laboratory practice.

The aim of this laboratory practice is to measure the ITV on a spherical cell by means of a fluorescent potentiometric dye di-8-ANEPPS.

THEORETICAL BACKGROUND

When a biological cell is placed into an external electric field an induced transmembrane voltage (ITV) forms on its membrane. The amplitude of the ITV is proportional to the amplitude of the applied electric field, and with a sufficiently strong field, this leads to an increase in membrane permeability – electroporation [1]. Increased permeability is detected in the regions of the cell membrane where the ITV exceeds a sufficiently high value, in the range of 250–1000 mV, depending on the cell type and the sensitivity of the detection method [2,3]. In order to obtain an efficient cell electroporation it is therefore important to determine the distribution of the ITV on the cell membrane. The ITV varies with the position on the cell membrane, is proportional to the electric field strength, and is influenced by cell geometry and physiological characteristics of the medium surrounding the cell [4]. For simple geometric shapes the ITV can be calculated analytically (e.g. for a spherical cell, using Schwan's equation). For more complicated cell shapes experimental and numerical methods are the only feasible approach to determine the ITV [5].

EXPERIMENT

Potentiometric fluorescent dyes allow observing the variations of the ITV on the membrane and measuring its value [1,2,6,7]. Di-8-ANEPPS is a fast potentiometric fluorescent dye, which becomes fluorescent when it binds to the cell membrane, with its fluorescence intensity varying proportionally to the change of the ITV [8]. The dye reacts to the variations in the ITV by changing the intramolecular charge distribution that produce corresponding changes in the spectral profile or intensity of the dye's fluorescence.

Protocol: The experiments follow the protocol in [9] and are performed on Chinese hamster ovary cells (CHO-K1) grown in Lab-Tek chambers (Nunc, Germany) in culture medium HAM-F12 supplemented with 10% fetal bovine serum, L-glutamine (all three from Sigma-Aldrich) and antibiotics. When cells attach to the cover glass of a Lab-Tek chamber (usually after 2 to 3 hours to obtain attached cells of spherical shape), carefully replace the culture medium with 1 ml of SMEM medium (Spinner's modification of the MEM, Sigma-Aldrich) containing 30 µM of di-8-ANEPPS and 0.05% of Pluronic (both Life Technologies). After staining for 12 min at 4°C, wash the cells thoroughly with pure SMEM to remove the excess dye. After washing leave 1.5 ml of SMEM in the chamber. Place the chamber under a fluorescence microscope (Zeiss AxioVert 200, Germany) and use ×63 oil immersion objective. Position two parallel Pt/Ir wire electrodes, with a 4 mm distance between them, to the bottom of the chamber. Set a single 40 V, 50 ms pulse on the programmable square wave pulse generator. This will result in a voltage-to-distance ratio of ~100 V/cm. The pulse must be synchronized with the image acquisition. Set the excitation wavelength to 490 nm and use ANEPPS filter to detect fluorescence (emission 605 nm).

Find the cells of interest. Acquire the control fluorescence image and subsequently the image during a pulse application, using a cooled CCD camera (VisiCam 1280, Visitron) and MetaFluor 7.7.5 (Molecular Devices). Apply four pulses with a delay of 4 s between two consecutive pulses. For each pulse, acquire a pair of images, one immediately before (control image) and one during the pulse (pulse image) (Figures 1A and B).

Open the images in MetaMorph 7.7.5 (Molecular Devices). To *qualitatively* display the ITV on the cell membrane, convert the acquired 12-bit images to 8-bit images. For each pulse, obtain the difference image by subtracting (on a pixel-by-pixel basis) the control image from the pulse image. Add 127, so that 127, i.e. mid-gray level, corresponds to 0 V, brighter levels to negative voltages, and darker levels to positive ones (Figure 1C). Average the three difference images to increase the signal-to-noise ratio.

To *quantitatively* determine the ITV, open the acquired, unprocessed fluorescence images. Determine the region of interest at the site of the membrane and measure the fluorescence intensities along this region for the control and pulse image. Transform the values to the spreadsheet. Measure the background fluorescence in both images and subtract this value from the measured fluorescence. Calculate the relative changes in fluorescence ($\Delta F/F_C$) by subtracting the fluorescence in the control image F_C from the fluorescence in the pulse image F_P and dividing the subtracted value by the fluorescence in the control F_C; $\Delta F/F_C = (F_P - F_C) / F_C$. Average the relative changes calculated for all four acquired pairs of images. Transform the fluorescence changes to the values of the ITV ($\Delta F/F = -6\% / 100$ mV), and plot them on a graph as a function of the arc length (Figure 1D).

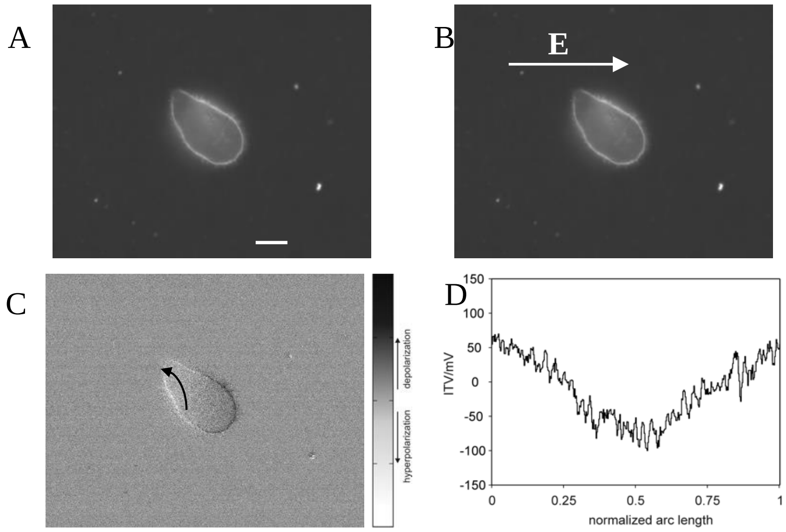

Figure 1: Measurements of the induced transmembrane voltage (ITV) on an irregularly shaped CHO-K1 cell. (A) A control fluorescence image of a cell stained with di-8-ANEPPS. Bar represents 10 µm. (B) Fluorescence image acquired during the exposure to a 35 V (~88 V/cm), 50 ms rectangular pulse. (C) Changes in fluorescence of a cell obtained by subtracting the control image A from the image with pulse B and shifting the grayscale range by 50%. The brightness of the image was automatically enhanced. (D) ITV measured along the path shown in C.

REFERENCES:

1. Kotnik T., Pucihar G., Miklavčič D. Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport. *J Membr Biol,* 236:3-13, 2010.

2. Hibino M., Itoh H., Kinosita K. Time courses of cell electroporation as revealed by submicrosecond imaging of transmembrane potential. *Biophys J,* 64:1789-1800, 1993.
3. Teissié J., Rols M.P. An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. *Biophys J,* 65:409-413, 1993.
4. Kotnik T., Bobanović F., Miklavčič D. Sensitivity of transmembrane voltage induced by applied electric fields – a theoretical analysis. *Bioelectrochem Bioenerg,* 43:285-291, 1997.
5. Pucihar G., Miklavčič D., Kotnik T. A time-dependent numerical model of transmembrane voltage inducement and electroporation of irregularly shaped cells. *IEEE T Biomed Eng,* 56:1491-1501, 2009.
6. Gross D., Loew L. M, Webb W. Optical imaging of cell membrane potential changes induced by applied electric fields. *Biophys J,* 50:339-348, 1986.
7. Loew L.M. Voltage sensitive dyes: Measurement of membrane potentials induced by DC and AC electric fields. *Bioelectromagnetics,* Suppl. 1:179-189, 1992.
8. Gross E., Bedlack R.S. Jr., Loew L.M. Dual-wavelength ratiometric fluorescence measurement of the membrane dipole potential. *Biophys J,* 67:208-216, 1994.

Video Article:

9. Pucihar G., Kotnik T., Miklavčič D. Measuring the induced membrane voltage with di-8-ANEPPS (Video Article). *J Visual Exp,* 33:1659, 2009.

NOTES & RESULTS