L4
Comparison of flow cytometry and spectrofluorometric measurements in cell permeabilization experiments

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Duration of the experiments: 90 min
Max. number of participants: 4
Location: Cell Culture Laboratory 2
Level: advanced

PREREQUISITES
Participants should be familiar with Laboratory safety (S1). The basic knowledge of handling with cells is required for this laboratory practice.

The aim of this laboratory practice is the comparison of two different methods of permeabilization detection using fluorescent dye propidium iodide.

THEORETICAL BACKGROUND
When cells are exposed to high electric fields, non-permeant molecules can cross the cell membrane. A commonly used way of detecting cell membrane permeabilization is by using fluorescent dyes such as propidium iodide [1]. When the cell is permeabilized, the propidium ion enters the cell, binds to nucleic acids in the cytosol and nucleus, and upon excitation starts to emit 20-times higher fluorescence than in the unbound state. Cell membrane permeabilization can be determined using different methods, e.g. fluorescent microscopy, spectrofluorometric measurements, flow cytometry, or clonogenic test after electroporation with chemotherapeutics [2-4]. In this lab work, we will compare spectrofluorometric measurements and flow cytometry.
Spectrofluorometric measurements allow for the analysis of a large number of cells at different wavelengths, but the exact number of permeabilized cells cannot be extracted. Namely, as a result, we obtain the sum of the fluorescence intensities of all cells which can conceal subpopulations of differently permeabilized or even non-permeabilized cells [2].
Flow cytometry, on the other hand, gives information on the shape, size, internal structure, and fluorescence of each separate cell, and thus offers possibility to detect subpopulations which differ in any of the measured parameters. Cells (or any other particle) move through a laser beam and refract or scatter light in all directions. Forward scatter (FSC) is the light that is scattered in the forward direction as laser strikes the cell while side scatter (SSC) is the light that is scattered at larger angles. The magnitude of FSC is roughly proportional to the size of the particle and SSC is indicative of the granularity and the internal structural complexity. Fluorescence can be measured at different wavelengths, and the measured signal is proportional to the amount of the emitted fluorescence. After measurements, the analysis is done by gating to separate different cell subpopulations (Figure 1) [2-4].

EXPERIMENT

We compare the fluorescence detected by flow cytometry (Life Technologies, Attune NxT, USA) and by the spectrofluorometer (Tecan Infinite 200, Tecan, Austria) after one of the two electroporation protocols. First protocol will consist of 50 bipolar pulses consisting of 1 µs long positive and negative pulses with 1 µs delay between them, delivered in 8 bursts at repetition frequency of 1 Hz. The second protocol will consist of monopolar pulses of parameters traditionally used in electrochemotherapy (8×100 µs pulses, 1 Hz repetition frequency) [5]. In both protocols the total duration of pulses will equal 800 µs. To apply the pulses, we use the laboratory prototype pulse HF-IRE pulse generator (University of Ljubljana) based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA), and we monitor the pulses by an oscilloscope and current probe (both LeCroy, USA).

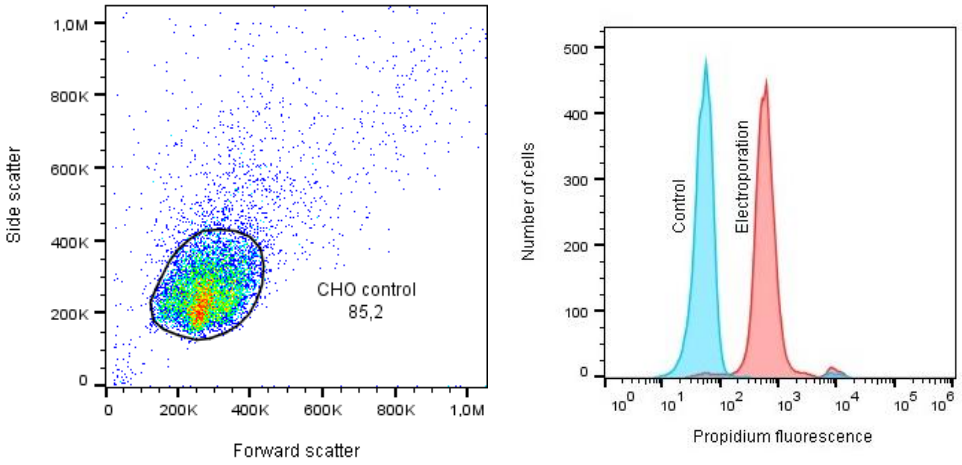

Figure 1: The analysis of the permeabilization data obtained by flow cytometry measurements in the software FlowJo (TreeStar, USA). Left: the viable cells are determined from the FSC-A and SSC-A dot diagram by gating. Right: histogram of measured fluorescence for control and pulsed cells. After electroporation, the cell fluorescence shifts for two decades which allows the discrimination between permeabilized and non-permeabilized cells. The peak at 104 are the dead and/or irreversibly permeabilized cells.

Protocol:

The experiments are performed on Chinese Hamster Ovary (CHO) cells. First, cells are detached by 10x trypsin-EDTA (Sigma-Aldrich, Germany), diluted 1:9 in Hank's basal salt solution (Sigma-Aldrich, Germany). Then, they are centrifuged (180g, 21°C, 5 min), the supernatant is removed and replaced with the low-conductivity KPB buffer (10 mM KH2PO4/K2HPO4, 1 mM MgCl2, 250 mM sucrose) in concentration 107 cells/ml. 100 µl of cell suspension is dispensed in 1.5 ml microcentrifuge tubes (Isolab, Germany). Immediately before pulse application, 10 µl of 1.5 mM propidium iodide (Life Technologies, USA) is added to the tube. Then, 100 µl of cell suspension with propidium iodide is pipetted between 2 mm stainless-steel electrodes. For the application of bipolar and monopolar pulses, we will use a laboratory prototype pulse. We will monitor the delivered voltage and current by an oscilloscope, a differential probe and a current probe. After the pulse application, 80 µl of cell suspension is transferred from between the electrodes to a new 1.5 ml tube. Two minutes after pulse application, the sample is centrifuged (1 min, 2000g, room temperature), the supernatant is removed and replaced by 500 µl of KPB buffer. The change of the buffer stops propidium influx in the cells and allows us to compare different parameters at the same time point. From each tube, 100 µl of the cell suspension is transferred to a 96-well plate in triplicates. 100 µl of the cell suspension is transferred to a 1.5 ml tube. Then, the protocol is repeated for the next sample. When all samples are prepared, we start with the measurements.

First, the fluorescence intensity is determined spectrofluorimetrically. We set the appropriate excitation (535 nm) and emission (617 nm) wavelengths. We measure at an optimal gain which prevents from signal saturation. The optimal gain is automatically determined by the software based on sensor sensitivity and the maximum signal intensity we are measuring. The average fluorescence intensity is calculated for each voltage from the triplicates. We plot the fluorescence intensity in dependence on the applied voltage. Second, we determine the number of fluorescent cells by flow cytometry. On the control cells, we set up the optimal measuring parameters at the lowest flow rate (12.5 µl/min). When optimal parameters are determined, we measure 10,000 events for each voltage with higher flow rate (200 µl/min). By gating, living cells and the percentage of permeabilized cells are determined for each voltage. We plot the cell permeabilization in dependence on the applied voltage for both measurements (spectrofluorometric and flow cytometry) and compare the results.

REFERENCES:

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[2] Marjanovič I., Kandušer M., Miklavčič D., Keber M.M., Pavlin M. Comparison of flow cytometry, fluorescence microscopy and spectrofluorometry for analysis of gene electrotransfer efficiency. *J Membr Biol,* 247:1259-1267, 2014.

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[5] Sweeney D.C., Reberšek M., Dermol J., Rems L., Miklavčič D., Davalos R.V. Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. *Biochim Biophys Acta BBA – Biomembr,* 1858:2689-2698, 2016.

NOTES & RESULTS

Bipolar/Monopolar	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar (C)	Monopolar
Voltage (V)							
Fluorescence intensity as measured with spectrofluorometer (a.u.)							
Percentage of fluorescent cells as determined by the flow cytometry (%)							

(C) – combination of negative control and highest voltage