Video Article

Cell Electrofusion Visualized with Fluorescence Microscopy

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Abstract

Cell electrofusion is a safe, non-viral and non-chemical method that can be used for preparing hybrid cells for human therapy. Electrofusion involves application of short high-voltage electric pulses to cells that are in close contact. Application of short, high-voltage electric pulses causes destabilization of cell plasma membranes. Destabilized membranes are more permeable for different molecules and also prone to fusion with any neighboring destabilized membranes. Electrofusion is thus a convenient method to achieve a non-specific fusion of very different cells *in vitro*. In order to obtain fusion, cell membranes, destabilized by electric field, must be in a close contact to allow merging of their lipid bilayers and consequently their cytoplasm. In this video, we demonstrate efficient electrofusion of cells *in vitro* by means of modified adherence method. In this method, cells are allowed to attach only slightly to the surface of the well, so that medium can be exchanged and cells still preserve their spherical shape. Fusion visualization is assessed by pre-labeling of the cytoplasm of cells with different fluorescent cell tracker dyes; half of the cells are labeled with orange CMRA and the other half with green CMFDA. Fusion yield is determined as the number of dually fluorescent cells divided with the number of all cells multiplied by two.

Protocol

I. Loading the cells with Cell trackers CMFDA and CMRA

1. Experiments were performed on previously prepared cells of mouse melanoma cell line (B16-F1). Cells are grown in two separated 25 cm^2 culture flasks (TPP, ZDA) to 70–80 % confluence in DMEM culture medium (Dulbecco's modified Eagle's medium) supplemented with 10 % fetal bovine serum, 0.15 mg/ml L-glutamine, 16 mg/ml gentamicin (all from Sigma-Aldrich, Germany), 200 units/ml crystacillin (Pliva, Croatia), and incubated in 5% CO_2 at 37°C.
2. Prepare two 10 mM stock solutions of Cell trackers (Invitrogen, USA) by adding 10.76 µl and 9 µl (for Green CMFDA and for Orange CMRA, respectively) of DMSO (Sigma-Aldrich, Germany) to 50 µg of the dye in the original Invitrogen vial. The stock solution can be stored in a refrigerator at 4°C for few months. Before starting the experiments, warm up the solution until the crystals of DMSO dissolve.
3. Prepare bicarbonate-free Krebs-Hepes buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.7 mM D-glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4). In two 15 ml Eppendorf tubes separately mix 2.1 µl of each stock solution (10 mM CMFDA or CMRA, respectively) in 3 ml of bicarbonate-free Krebs-Hepes buffer. This yields a "loading solution" containing approximately 7 µM CMFDA (or CMRA).
4. Rinse cells twice with bicarbonate-free Krebs-Hepes buffer and then insert loading solutions into the flasks. Incubate cells for 30 minutes in 5% CO_2 at 37 °C. During this first incubation reagents pass freely through cell membranes, but once inside the cell, the reagents are transformed into cell-impermeant fluorescent reaction products.
5. After first incubation, rinse and incubate cells with culture medium for another two hours in 5% CO_2 at 37°C.
6. Trypsinize cells in both flasks (loaded with CMFDA and CMRA) and mix red and green cells together in a ratio 1:1 in a 50 ml centrifuge tube (TPP, ZDA). Adjust cell concentration to 5 x 10^6 cells/ml by dilution with DMEM medium or by concentration with centrifuge. Place a 20 µl drop of cell suspension in each well of 24 multiwell plate (TPP, ZDA). Incubate cells in 5 % CO_2 at 37 °C for 20 min to allow them to slightly attach to the surface of the well and establish cell contacts.

II. Electrofusion

1. Prepare isoosomolar potassium phosphate buffer (10 mM KH2PO4, 10 mM K_2HPO_4, 1mM $MgCl_2$, 250 mM sucrose) and hypoosmolar potassium phosphate buffer (10 mM KH2PO4, 10 mM K_2HPO_4, 1mM $MgCl_2$, 75 mM sucrose).
2. Place the multiwell with cells onto the microscope stage, position the electrodes at the bottom of the well and connect them to the pulse generator.
3. Wash the cells with 1 ml of isoosomolar potassium phosphate buffer. Add 350 µl of hypoosmolar potassium phosphate buffer in order to induce cell swelling. The buffer should cover the electrodes.
4. Leave cells in hypoosmolar buffer for 2 minutes before applying electric pulses. During this time influx of water molecules into the cells due to osmotic imbalance between the interior and the exterior of the cells cause an increase of cell volume. Electric pulses should be applied when cells are close to their maximum volumes, before regulatory volume decrease start.
5. To achieve optimal electrofusion and maintain cell viability, optimal parameters of electric pulses should be used. These depend on cell line used [1]. In this experiment a train of 8 rectangular pulses (each with duration of 100 µs at 1 Hz) is applied to each sample, using electroporation device (in our case Cliniporator, IGEA, Italy). The pulses are delivered to two parallel Pt/Ir wire electrodes with 0.8 mm diameter and 5 mm distance between them, creating an electric field of approximately 1200 V/cm between the electrodes in each well, except for the control well.
6. Leave the cells undisturbed for 10 minutes after pulse delivery. Determine the fusion yield by means of fluorescent and phase contrast microscopy.

III. Image acquisition and determination of the fusion yield

1. The cells are observed using a fluorescence microscope (in our case Zeiss AxioVert 200, Zeiss, Germany) equipped with an objective (×20) and a cooled CCD camera (VisiCam 1280, Visitron, Germany). The images are acquired in MetaMorph 7.1.1 (Molecular Devices, USA), but other similar acquisition software can also be used. CMFDA is excited with a monochromator (Polychrome IV, Visitron, Germany) at 492 nm and CMRA at 548 nm. The fluorescence of CMFDA and CMRA is acquired using two emission filters, one centered at 535 nm (HQ535/30m, for CMFDA) and the other centered at 510 nm (D605/55m, for CMRA, both Chroma, USA). The use of dichroic mirror (Q515LP) prevented the channel cross talk.
2. Acquire three images (phase contrast, red and green fluorescence) for five randomly chosen fields in each well. Create three channel images from each image triplet. In such image fluorescing cytoplasm can be seen together with the cell membranes. Fused cells can thus be easily determined [Figure 1].
3. Count all three types of cells (red, green and dually fluorescent) in each three channel image. Determine the percentage of dually fluorescent cells by dividing the number of dually fluorescent cells with the number of all cells in each image. Fusion yield is defined as the percentage of dually fluorescent cells multiplied by 2 since half of fused cells are not detected (when cells of the same color fuse).

Representative Results

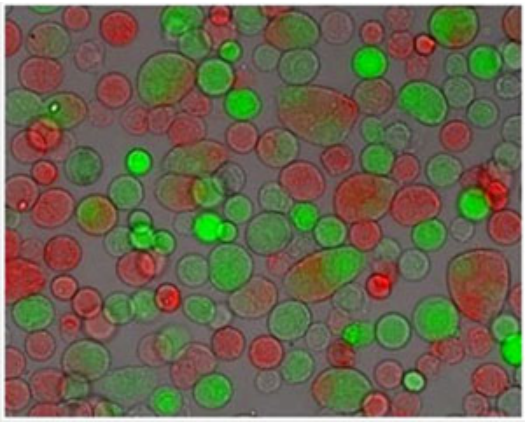

Figure 1. Three channel microscopy image of B16F1 cells after electrofusion: phase contrast, CMRA fluorescence (excitation at 548 nm) and CMFDA fluorescence (excitation at 492 nm), objective magnification 20x

Disclosures

No conflicts of interest declared.

Discussion

The ability of cell membranes to fuse non-specifically, e.g., by external electric fields, is important for biotechnology, medicine and research in biology. Such nonspecific fusion enables production of highly valuable hybrid cells and their products, such as monoclonal antibodies, and provides information about fundamental mechanisms of fusion [2]. Electrofusion is a potentially very effective method since it can be properly adjusted to different types of cells. Electrofusion is achieved when cells in close physical contact are brought into their fusogenic state (prone to fusion) by means of high-voltage electric pulses. The efficiency of electrofusion depends on various parameters that affect two parts of the electrofusion process. First part of the electrofusion process is achievement of the close physical contact between cells, which can be obtained with different methods [3-8]. Adherence method (growing cells to confluence) can be used efficiently due to spontaneously established cell contacts in large zones between cells; however, it produces very large fused cells with many nuclei. We are using the modified adherence method, where smaller cells (with 2 to 5 nuclei), which are more likely to survive and proliferate, are obtained (Figure 1). Contact between cells also benefit from osmotic swelling of cells, due to osmotic treatment used in the experiment [9]. Second part of the electrofusion process is the achievement of the fusogenic state of the cell membranes. Fusogenic state correlates well with electropermeabilized state of the membrane (cells are non-specifically permeabilized to molecules that normally cannot pass through intact membrane) and is governed by the same parameters of the electric pulses (amplitude, length, number and frequency) [10]. The values of electrical parameters needed for optimal electroporation [1] and electrofusion differ between different cells and depend on cells size and their biological properties. Electrical parameters thus need to be optimized for different cell lines, which are used as fusion partners, to obtain fusion.

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