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Electrofusion of B16-F1 and CHO cells: The comparison of the pulse first and contact first protocols

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ABSTRACT

High voltage electric pulses induce permeabilisation (i.e. electroporation) of cell membranes. Electric pulses also induce fusion of cells which are in contact. Contacts between cells can be established before electroporation, in so-called contact first or after electroporation in pulse first protocol. The lowest fusion yield was obtained by pulse first protocol (0.8% ± 0.3%) and it was only detected by phase contrast microscopy. Higher fusion yield detected by fluorescence microscopy was obtained by contact first protocol. The highest fusion yield (15%) was obtained by modified adherence method whereas fusion yield obtained by dielectrophoresis was lower (4%). The results are in agreement with current understanding of electrofusion process and with existing electrochemical models. Our data indicate that probability of stalk formation leading to fusion pores and cytoplasmic mixing is higher in contact first protocol where cells in contact are exposed to electric pulses. Another contribution of present study is the comparison of two detection methods. Although fusion yield can be more precisely determined with fluorescence microscopy we should note that by using this detection method single coloured fused cells cannot be detected. Therefore low fusion yields are more reliably detected by phase contrast microscopy.

1. Introduction

High voltage electric pulses induce the permeabilisation of the cell membranes [1]. This dramatic phenomenon, known as electroporation, is used in cell and molecular biology, biotechnology and nowadays in medicine [2]. Moreover the electroporation also induces cells to fuse, a phenomenon known as electrofusion [3–6]. Namely an electroporated cell membrane is for a limited time after exposure to electric pulses in "fusogenic" state and it can fuse with another membrane when they are in close contact.

The fusogenic state correlates with the permeabilised state of the membrane [7]. It is known that the cell membrane permeabilisation occurs only in areas where the induced transmembrane voltage (ITV) exceeds a threshold value. The theoretical description of the transmembrane voltage induced on a spherical cell exposed to electric field is known as Schwan's equation and was treated in depth elsewhere [8]. It is well established that the induced transmembrane voltage and therefore the electroporation occurs predominantly at the poles of the cell exposed to the electric field facing the electrodes.

Nevertheless the permeabilised area can be enlarged without reducing a cell survival by changing the electric pulse direction during the pulse application [9]. From the theory of electroporation and performed experiments it follows that applying pulses to cells in different directions causes the permeabilisation of the larger area of the cell membrane. The increase in the total permeabilised area increases the overall electrofusion efficiency by 20–30% compared to pulses delivered in one direction [10,11].

In addition to the fusogenic state, a close membrane contact has to be established in order to obtain the cell fusion. A physical contact between cells can be achieved by different ways: i) mechanically by using the specific fusion chamber [12], filters [13], centrifugation [14–16], simple sedimentation [17,18] or confluent cell cultures [5,11,19–21]; ii) dielectrophoretically by using an alternating electric field resulting in cell migration and pearl chains formation [6,22]. Nowadays special microfluidic devices or chips based on dielectrophoresis are being developed [23].

The two conditions needed for electrofusion i.e. the electroporation and cell contact can be applied in two different time sequences, commonly known as the pulse-first or the contact-first protocol. Although in most electrofusion studies researchers have used an experimental protocol where first the contact between cells have been established and then high voltage electric pulses have been applied (the contact-first protocol) also the reverse order of these two critical conditions (the pulse-first protocol) have been used [16,24]. The advantage of the pulse-first protocol is a possibility to separately electroporate different cell types that require different electric pulse parameters for bringing their membranes in fusogenic state [16].

Besides the electrofusion protocols, the cell type used in the experiments and the methods used for their evaluation affects the reported final electrofusion yield. The differences related to the cell type can be attributed to *i)* different electroporation parameters required for

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efficient cell permeabilisation [25] and *ii)* to biological characteristics of the treated cells [26,27].

It is also important to stress that the final result of the electrofusion depends also on the method used for its evaluation. When comparing the results of different electrofusion protocols it can be noted that different methods such as flow cytometry [28], phase contrast [5] and fluorescence microscopy [29] as well as spectrofluorimetry [30] are used to determine the fusion yield.

Taken all together, we can say that according to the state of the art in the field of the electrofusion achieving efficient yield still requires further optimisation studies and/or new protocol developments [10,31–33]. We believe that it is crucial for fusion yield evaluation and comparison of different electrofusion protocols to present the obtained results as objectively as possible. Thus we systematically compared pulse-first and contact-first electrofusion protocols using the same evaluation methods. The cell contact was achieved by four different methods. In the pulse-first protocol it was achieved by *i)* sedimentation and *ii)* centrifugation; while in the contact-first protocol by *iii)* modified adherence method and *iv)* dielectrophoresis. Special attention was also dedicated to the determination of the fusion yields using the phase-contrast and fluorescence microscopy. Thus we compared the fusion yields obtained by the pulse-first and the contact-first electrofusion protocols by using the same determination and calculation methods.

2. Materials and methods

2.1. Chemicals, cell culture media

Eagle's minimal essential medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), Ham's Nutrient Mixtures (F-12 HAM), foetal bovine serum (FBS), L-glutamine, sucrose, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), trypsin and EDTA were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany). Antibiotics (crystacillin and gentamicin) were purchased from Lek (Lek, Slovenia). Hoechst 33342 nucleic acid stain, CMFDA and CMRA cell trackers were purchased from Molecular probes (Invitrogen, USA).

2.2. Cells

All cell lines were cultured in humidified atmosphere at 37 °C and 5% CO_2 (Kambič, Slovenia) in following culture media: Murine melanoma (B16-F1) in DMEM supplemented with 10% FBS, antibiotics (gentamicin, crystacillin) and L-glutamine; Chinese hamster ovary cells (CHO) in F-12 HAM supplemented with 10% FBS, antibiotics and L-glutamine. Cell lines were grown in 25 cm^2 culture flask (TPP, Switzerland) until they reached 80–90% confluence.

2.3. Electrofusion buffers

Iso- and hypo-tonic buffers (potassium phosphate buffer — KPB, [K_2HPO_4/KH_2PO_4] = 10 mM, [$MgCl_2$] = 1 mM, [sucrose] = 250 or 75 mM) of osmolarities 260 and 93 mOsm (mOsmol/kg), conductivity 1.62 mS/cm and pH 7.2 were used as in refs. [33,34]. The osmolarity of solutions was determined with Knauer vapour pressure osmometer K-7000 (Knauer, Wissenschaftliche Gerätebau, Germany). For experiments with dielectrophoresis low conductive buffers have to be used. Hypotonic KPB with low conductivity was prepared by dilution of hypotonic KPB of "normal" conductivity (1.62 mS/cm) with hypotonic sucrose solution ([sucrose] = 75 mM). The conductivity of the buffer was measured by conductometer (Metrel, Slovenia) to obtain desired buffer conductivity 120 μS/cm.

2.4. Cell preparation and labelling protocols

For detection of the fused cells by fluorescence microscopy two different combinations of fluorescence dyes were used. Cells were labelled with green and red cell trackers [33] or green cell tracker and blue DNA stain. Cells, which were grown in two 25 cm^2 culture flasks, were washed and labelled with green CMFDA (excitation/emission = 492 nm/517 nm) or with red CMRA (excitation/emission = 548 nm/576 nm) or blue DNA stain Hoechst 33342 (excitation/emission = 350 nm/461 nm). Loading cell trackers solutions ([5-chloromethylfluorescein diacetate] = [9′-(4-(and 5)-chloromethyl-2-carboxyphenyl)-7′-chloro-6′-oxo-1,2,2,4-tetramethyl-1,2-dihydropyrido[2′,3′-6]xanthene] = 7 μM) were prepared in bicarbonate-free Krebs–Hepes buffer [35]. Cell nucleus was labelled by Hoechst ([2,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-/ 23491-52-3 2] = 2 μg/ml) in serum free EMEM. Cells were stained for 45 min at 37 °C and then washed with culture media and maintained at 37 °C for another hour. Cells were then washed again with culture media and trypsinised with 0.25% trypsin/EDTA solution for 1–1.5 min to obtain homogenous cell suspension. Cells in proportion (1:1) of green and red cells or green cells and cells with blue nucleus were then mixed.

2.5. Determination of electrofusion yield

The fusion yield was determined by dual colour fluorescence microscopy [36] and by phase contrast image analysis.

2.5.1. Fluorescence microscopy

For fluorescence microscopy we used emission filters; for CMFDA at 535 nm (HQ535/30 m) and for CMRA at 605 nm (D605/55m) or for Hoechst at 461 nm (D460/50 m), all from Chroma (Chroma, USA) and monochromator Polychrome IV (Visitron, Germany). Cells were observed by inverted microscope AxioVert 200 (Zeiss, Germany) under 20× objective magnification. Three images (phase contrast, green, red or blue fluorescence) were acquired from five randomly chosen fields for each sample using cooled CCD video camera VisiCam 1280 (Visitron, Germany) and PC software MetaMorph 7.1 (Molecular Devices, USA).

Three channel images (Fig. 1A, B) were created from each image triplet (phase contrast, green and red or blue fluorescence) in image processing software ImageJ (NIH Image, USA). In order to improve visual quality of images three pre-processing steps were applied to original fluorescence images: a) background subtraction, b) contrast enhancement (both already implemented in ImageJ) and c) image smoothing by Sigma filter plus plug-in (object edges are preserved). Finally three channel images were composed using ImageJ plug-in RGB to Grey. On each image cells were manually counted using ImageJ plug-in Cell Counter. A fusion yield was determined by measuring the fraction of the cells with green and red cytoplasm, i.e. the fraction of the double labelled cells (DLCs):

$$f(\mathrm{DLCs}) = N_{\mathrm{DLCs}}/N, \tag{1}$$

where N_{DLCs} denotes number of double labelled cells and N number of all cells in a given sample. The fraction of the double labelled cells equals fraction of fused cells for the given conditions detected by fluorescence microscopy $f(\mathrm{DLCs}) = f(\text{fusion yield})$, therefore

$$\text{Fusion yield}(\%) = f(\mathrm{DLCs}) \times 100. \tag{2}$$

Using dual colour fluorescence microscopy only double labelled fused cells can be detected. Fused cells of the same colour, however, are not detected.

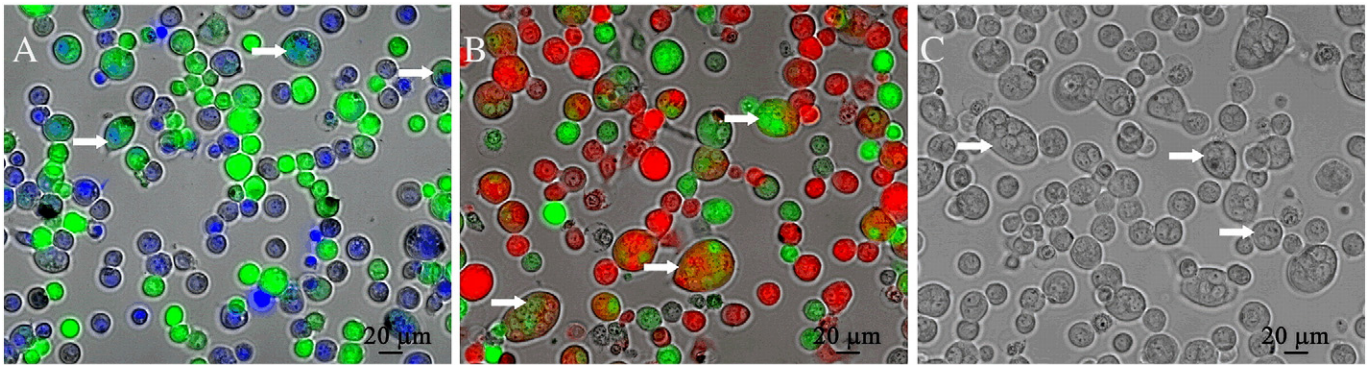

Fig. 1. Three channel fluorescence microscopy images and phase contrast microscopy image of fused B16-F1 cells. The images were captured 10 min after being exposed to a train of 8×100 μs pulses with repetition frequency 1 Hz and electric pulse amplitude of 600 V resulting (see Eq. (7)) in electric field strength 1.2 kV/cm at room temperature (T=22 °C). (A) Cell nuclei were stained with Hoechst (blue) and cell cytoplasm with CMFDA (green). (B) Cells were stained with CMRA (red cytoplasm) and CMFDA (green cytoplasm). Overlapping of both colours and phase contrast image enables easy detection of double labelled fused cells (arrows). (C) Fused cells can also be detected in a phase contrast images only (arrows). In order to keep images clearer, not all of fused cells are marked with arrows.

2.5.2. Phase contrast microscopy

Phase contrast images were obtained using the same microscopy system and procedure as described above. To determine fusion yield using phase contrast images (Fig. 1C) we manually counted fused cells. Fused cells are those with larger cell area and with two and more nuclei. A fusion yield was determined by measuring the fraction of such polynucleated cells (PNCs):

$$f(\text{PNCs}) = N_{\text{PNCs}}/N, \tag{3}$$

where N_{PNCs} denotes a number of polynucleated cells and N denotes a number of all cells in a given sample. Since certain amount of polynucleated cells is always present in the unsynchronised cell culture we also determined the fraction of the polynucleated cells in control sample (PNCs, CS):

$$f(\text{PNCs, CS}) = N_{\text{PNCs,CS}}/N, \tag{4}$$

where $N_{\text{PNCs, CS}}$ denotes a number of polynucleated cells in control sample and N a number of all cells in a given sample. The subtraction of the above fractions of polynucleated cells equals fraction of electrofused cells for the given conditions and detected by phase contrast microscopy,

$$f(\text{DFCs}) - f(\text{PNCs, CS}) = f(\text{fusion yield}),$$

therefore

$$\text{Fusion yield}(\%) = (f(\text{PNCs}) - f(\text{PNCs, CS})) \times 100. \tag{5}$$

All experiments were repeated at least three times on different days. Results from different repetitions of experiments were pooled together and are presented as a mean and standard deviation (STD) of the mean.

2.5.3. Coefficient of variation (CV)

Additionally we evaluated fluorescence and phase contrast microscopy by comparison of the means and standard deviations and we calculated the coefficient of variation (CV) in each data point (Table 1). CV was calculated from the mean and standard deviation (STD) as:

$$\text{CV}(\%) = \text{STD}/\text{Mean} \times 100. \tag{6}$$

Then, the average of four data points obtained with fluorescence microscopy and the average of three data points (control data point included) obtained with phase contrast microscopy served as final information about the variation of the results in each detection method.

2.6. Electrofusion protocols

We studied the efficiency of electrofusion using two different protocols. We fused cells by using the pulse-first protocol, where cells in suspension were electroporated and then cell contacts were obtained by sedimentation or centrifugation. Whereas in the contact-first protocol we used slightly attached cells and dielectrophoresis. In addition we compared two different detection methods: the phase contrast and fluorescence microscopy.

2.6.1. Pulse-first protocol

For electrofusion of cells we used special pipette tip with integrated platinum electrodes [37,38] which allows application of electric pulses in different orientations. The diameter of electrodes is 1.4 mm. The opposite electrodes are 2 mm apart. We used similar experimental protocol as described in our recent paper [37]. Briefly, the pipette tip was sterilised before experiments in 70% ethanol for 10 min and rinsed thoroughly in sterile electroporation buffer before the first sample was treated. Cells in suspension were prepared by tripsinisation and mixing of red and green labelled cells. Cells were then centrifuged (270×g, 5 min, 4 °C), supernatant was removed and cells were re-suspended in hypotonic buffer with conductivity 1.62 mS/cm to obtain a cell density of $\rho = 5\times10^6$ cells/ml. For each experiment 100 μl of cells in suspension was aspirated into the tip. We used orthogonal single polarity (OSP) electric field protocol, where single polarity electric pulses were applied between two orthogonal pairs of electrodes (⇨ ⇩), 4 pulses in each direction. [10,37]. For control cells no pulses were delivered. Treated cells

Table 1
The effect of electric field strength and time duration of dielectrophoresis on cell fusion of B16-F1 cells for contact first protocol. The fusion yield as a function of different electric field strengths E and time duration of dielectrophoresis t_{DEF} was determined by fluorescence microscopy (detection of double labeled green (CMFDA) and red (CMRA) cells, see Eq. (3)). The contact between cells was established by dielectrophoresis (E_{max} = 0.34 kV/cm, frequency 2 MHz) before and after electroporation. Cells were exposed to a train of 8×100 μs pulses with repetition frequency 1 Hz at room temperature (T=22 °C). Values represents means ± standard deviation (STD) from 3 independent experiments. Asterisks represent statistically significant differences (*$P<0.05$, **$P<0.01$) regarding experiment #1.

E (kV/cm)	Detection method	Dye used	Fusion yield (%)
0.8	Fluorescence microscopy	CMFDA&Hoechst	10.8 ± 3.8
0.8		CMFDA&CMRA	11.4 ± 4.9
1.2	Fluorescence microscopy	CMFDA&Hoechst	15.8 ± 1.4
1.2		CMFDA&CMRA	14.8 ± 6.1
0.8	Phase contrast microscopy	/	20.3 ± 12.3
1.2		/	26.4 ± 12.6

were exposed to 8 electric pulses with pulse duration 100 μs and repetition frequency of 1 Hz. The electric pulse amplitudes were 225 V for CHO cells and 300 V for B16-F1 cells. Since the distance between the applied electrodes was 2 mm this resulted in $E = 1125$ V/cm and E = 1500 V/cm, respectively, according to Eq. (7). Applied electric field is estimated as

$$E = U_{appl}/d, \quad (7)$$

where U_{appl} denotes applied voltage and d electrode distance.

The contact between cells immediately after the application of pulses was obtained by sedimentation [17] or by centrifugation [10,16]. In the first method electroporated cells were plated from electrode pipette tips into 6-well plate (TPP, Switzerland) and incubated for 10 min at room temperature. During the incubation cells settled at the bottom of the well making spontaneous contact. Then 2 ml of culture media was added and fused cells were determined or cells were grown for additional hour or 24 h for later analysis. In the second method contacts between cells were established by centrifugation ($270\times g$, 5 min, 4 °C) within 30 s after pulses were applied when the membranes are still in their fusogenic state. Cells in pellet were left for another 5 min at room temperature, after which they were plated into 6 well plate (TPP, Switzerland) containing 2 ml of culture media and the fused cells were either determined 10 min after the electroporation or were grown for additional 1 or 24 h.

2.6.2. Contact-first protocol

For the contact-first protocol cell contacts were established before electric pulses were delivered by modified adherence method (Fig. 2A and B) or by dielectrophoresis (Fig. 2C).

In the case of the modified adherence method, we determined cell number that was appropriate to establish a monolayer of spherical cells in a close contact. For these experiments a cell density of $\rho = 2.5\times10^5$ cells/ml was used. 1 ml of cells in suspension was plated into a 24-well multiplate (TPP, Switzerland) and incubated for 20 min. During the incubation cells formed spontaneous contacts but still preserved their round shape. Before electroporation cells were washed with isotonic buffer and hypotonic buffer was added. 2 min later, 8 rectangular electric pulses (pulse duration 100 μs, repetition frequency 1 Hz) were delivered by electric pulse generator Cliniporator (IGEA, Italy) using two parallel wire platinum electrodes (wire diameter was 1 mm). The applied voltages were 400 V and 600 V. The distance between electrodes d was 5 mm and according to Eq. (7) this resulted in 0.8 kV/cm and 1.2 kV/cm E, respectively. For control treatment no pulses were applied. Cells were then left undisturbed for 10 min when fusion yields were determined.

In the case of the dielectrophoresis we used the micro fusion chamber (Eppendorf, USA) with 0.5 mm electrode distance. The electrofusion was performed according to modified manufacturer instruction replacing Eppendorf low conductivity media with our buffer of known composition. Aliquots of 5×10^5 cells (half labelled with green CMFDA and half with red CMRA) were centrifuged ($270\times g$, 5 min, 4 °C) and kept at 4 °C. Aliquot was then re-suspended in low conductive hypotonic buffer which allows pearl chain formation. We used 20 μl of cells in suspension (with a cell density of $\rho = 1.6\times10^6$ cells/ml) and we applied AC (sinusoidal) electric signal with maximal voltage $U_{max} = 17$ V which resulted in 340 V/cm E (Eq. (7), electrode distance $d = 0.5$ mm) and frequency 2 MHz. We used the AC generator custom built in our laboratory. The duration of dielectrophoresis before electroporation was 30 or 60 s. Then AC electric field was switched off and cells were electroporated with 8 rectangular pulses (pulse duration 100 μs, repetition frequency 1 Hz) using high voltage generator Juan (CNRS, France). For electroporation in low conductive hypotonic buffer we adapted electric field strength [34]. The applied voltages were 30 V and 40 V which resulted in 0.6 kV/cm and 0.8 kV/cm E, respectively (Eq. (7), electrode distance $d = 0.5$ mm). The contact of electroporated cells was maintained with post pulse AC electric field application with the same parameters as before pulses. The duration of dielectrophoresis after electroporation was also the same as before electroporation, i.e. 30 or 60 s. Thus the cells were exposed to dielectrophoresis for 60 and 120 seconds (as the total duration of the dielectrophoresis). The lag between the cell electroporation and the re-application of the dielectrophoresis was at maximum one second since we used s custom made switch to change between dielectrophoresis and electroporation signals. Fusion yields were determined 10 min after electroporation.

Observed differences in electrofusion yields were statistically tested using independent samples t-test (SPSS Statistics; SPSS, Inc., USA).

3. Results

In this paper we performed electrofusion using two different approaches: the pulse-first protocol and the contact-first protocol. For the detection of the electrofusion we used two different methods: phase contrast and fluorescence microscopy.

3.1. Pulse-first protocol

In the pulse-first protocol cell contact was achieved after electroporation either by cell sedimentation ($1\times g$) or by centrifugation ($270\times g$).

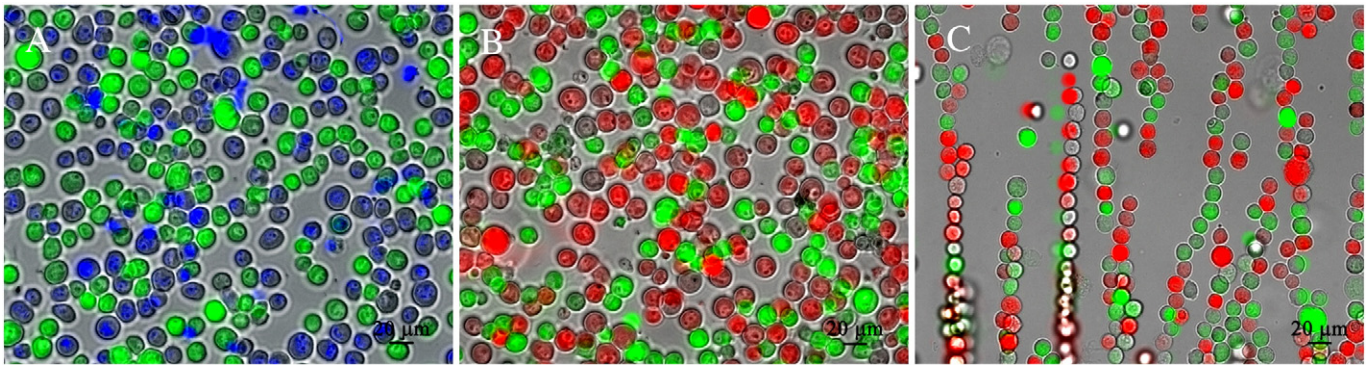

Fig. 2. Methods of cell contact achievement in contact-first electrofusion protocol using B16-F1 cells. (A) and (B) modified adherence method, (C) dielectrophoresis. In (A) cell nuclei were stained with Hoechst (blue) and cell cytoplasm with CMFDA (green) and (B) cells were stained with CMRA (red cytoplasm) and CMFDA (green cytoplasm). In (A) and (B) cells were plated in 24 multiwell plate where they slightly attached to a well surface while preserving their spherical shape. In (C) cells were exposed to alternating electric field ($U_{max} = 17$ V resulting in $E_{max} = 0.34$ kV/cm and frequency 2 MHz) for 30 s to obtain pearl chain formation before electroporation.

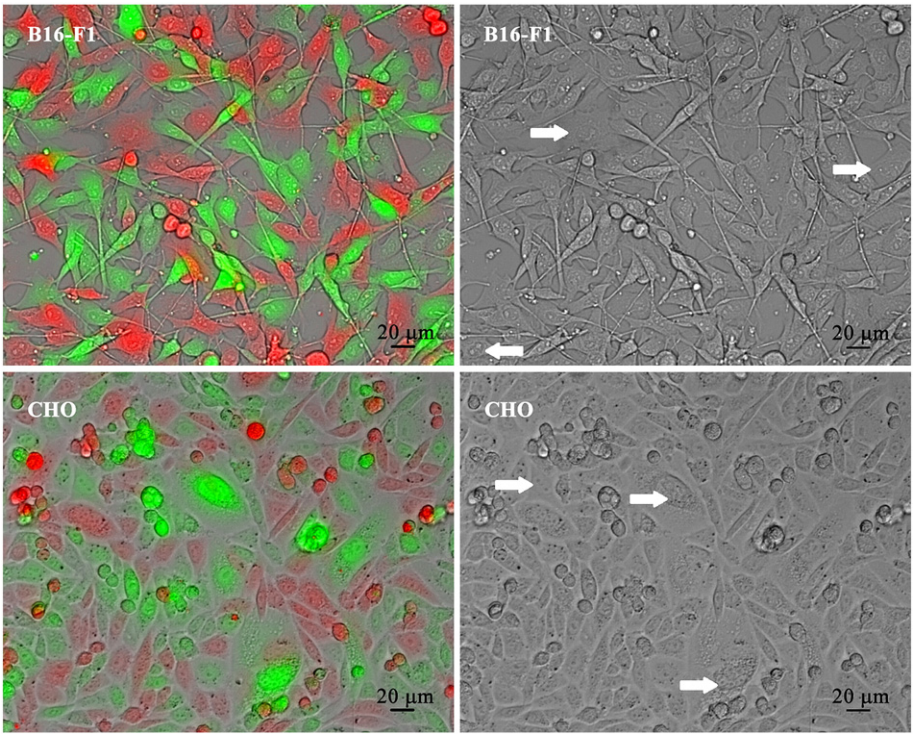

Fig. 3. Pulse-first electrofusion protocol. Three channel microscopy images (left), and phase contrast images (right) of B16-F1 and CHO cells 24 h after being exposed to electric pulses: 8×100 μs, repetition frequency 1 Hz and U = 300 V resulting in E = 1.5 kV/cm (B16-F1) or U = 225 V resulting (see Eq. (7)) in E = 1.125 kV/cm (CHO). Single polarity electric pulses were applied between two orthogonal pairs of electrodes (⇨ ⇩), 4 pulses in each direction. Contact between cells was established within 30 s after electroporation by centrifugation (270×*g*, 5 min, 4 °C). Cells were stained with CMRA (red cytoplasm) and CMFDA (green cytoplasm) and observed under 20× objective magnification. No double labelled cells were detected on fluorescence images. On phase contrast images fused cells were regularly detected (arrows). Bar represents 20 μm.

3.1.1. Sedimentation and centrifugation

In Fig. 3 results of cells electrofusion detected 24 h after electric pulses by phase contrast and fluorescence microscopy are shown. We did not detect double labelled cells with fluorescence microscopy and fusion yield detected with phase contrast microscopy was around 1% (0.8 ± 0.3%). Surprisingly all polynucleated cells appeared as single coloured on fluorescence images. Similar results were obtained for both cell lines. Centrifugation of cells within 30 s after electroporation did not improve cell fusion yield. Determination of fusion yield at different time intervals after electric pulses (10 min, 1 h, 24 h) gave similar results (data not shown).

3.2. Contact-first protocol

In contact-first protocol cell contact was achieved before electroporation either by our recently published modified adherence method [33] or by dielectrophoresis.

3.2.1. Modified adherence method

In Table 1 cell electrofusion of B16-F1 cells detected by phase contrast and fluorescence microscopy where cell contact was established by modified adherence method is presented. Both combinations of fluorescence dyes (green-blue and green-red) give comparable electrofusion yields. Cells were electroporated using two electric pulses of amplitudes 400 V and 600 V resulting in electric field strengths: 0.8 kV/cm and 1.2 kV/cm. Fusion yield increases with increasing electric field strength and reaches maximum values of 14.8 ± 6.1% (detected by combination of green and red cell trackers) or 15.8 ± 1.4% (detected by combination of green cell tracker and blue DNA stain) at 1.2 kV/cm. Differences between 0.8 kV/cm and 1.2 kV/cm are, however, not statistically significant. Fusion yields detected on phase contrast images are approximately two times higher and reach 26.4 ± 12.6% at 1.2 kV/cm.

Furthermore the difference in the standard deviations between detection methods was evaluated. While both fluorescence methods give considerably lower standard deviation values (maximum 6.1% for green and red cell trackers and 3.8% for combination of green cell tracker and blue DNA stain) standard deviation of phase contrast method is considerable higher (maximum 18.5%). The coefficient of variation (CV) is on average more than two times lower (32%) if we used fluorescence microscopy in comparison to phase contrast microscopy (CV = 78%). This suggests that more consistent results can be obtained with fluorescence microscopy.

3.2.2. Dielectrophoresis

Dielectrophoresis was used to form contact between cells in suspension before and after electroporation. Cell electrofusion was detected by dual colour fluorescence microscopy due to better detection resolution demonstrated in experiments in which modified adherence method was used. The fusion yields obtained with this method of the contact-first protocol were lower than yields obtained with modified adherence method (Table 2). For electroporation in low conductive hypotonic buffer we used electric pulses of 30 V and 40 V resulting in electric field strength: 0.6 kV/cm and 0.8 kV/cm. Dielectrophoresis was applied before and after the electroporation. To evaluate the effect of the cell contact duration we used two different time exposures of dielectrophoresis. We applied dielectrophoresis for total time duration (t_{DEF}) of 60 s or 120 s distributed equally before and after electroporation. The highest fusion yield was 3.8 ± 0.5% for electric field strength 0.8 kV/cm at total dielectrophoresis duration t_{DEF} = 60 s. Nevertheless similar fusion yield (3.5 ± 0.9%) was obtained with lower (0.6 kV/cm) electric field strength and longer total dielectrophoresis duration (t_{DEF} = 120 s). At this electric field strength shorter total dielectrophoresis duration resulted in significantly lower fusion yield (2.1 ± 0.7%).

Table 2
The effect of electric field strength on cell fusion of B16-F1 cells in contact-first protocol. The fusion yield as a function of different electric pulse amplitudes of 400 V and 600 V resulting (see Eq. (7)) in different electric field strengths of 0.8 kV/cm and 1.2 kV/cm was determined by fluorescence microscopy (see Eq. (2)). The fusion yield was also determined by phase contrast microscopy (see Eq. (5)). Contact between cells was established by modified adherence method. Cells were exposed to a train of 8×100 μs pulses with repetition frequency 1 Hz at room temperature (T=22 °C). Values represents means±standard deviation (STD) from at least 3 independent experiments.

Experiment #	E (kV/cm)	t_{DEF} (s)	Fusion yield (%)
1	0.6	2×30	2.1±0.7
2	0.8	2×30	3.8±0.5**
3	0.6	2×60	3.5±0.9*

4. Discussion

In this paper we systematically compared four methods for achieving cell contacts and two methods for determining the fusion yield. We compared two electrofusion protocols, so-called pulse-first and contact-first. For establishing of cell contacts in the pulse-first protocol we tested sedimentation and centrifugation, while in the contact-first protocol modified adherence method and dielectrophoresis were used. Special attention was dedicated to the quantification of the fusion yield. We used two methods: the phase-contrast and dual colour fluorescence microscopy as obtained final fusion yield is affected by the quantification method used.

4.1. Comparison of pulse-first versus contact-first electrofusion protocol

Both protocols have been already compared in studies [16,24] using two mammalian cell types, i.e. erythrocytes ghosts and L929 fibroblast like cells. However the final outcomes obtained by phase contrast microscopy are inconsistent, therefore we decided to use two different detection methods and to focus on critical factors that might affect the cell fusion.

In the pulse-first protocol the most critical factor is the delay between the electroporation and establishing of contact between cells [7]. The fusogenic state of the cell membrane responsible for cell fusion was detected within the first few seconds after cells were exposed to an electric field as reported in one of the earliest papers on electrofusion by Neumann and co-authors [4]. The duration of fusogenic state is not clearly defined yet and it is ranging from less than 1 min [7] to 10 min for CHO WTT clone cells and erythrocyte ghosts [16,24,39–41]. Therefore in our experiments special care was taken to perform the cell fusion when the membrane is in the fusogenic state. The delay between cell electroporation and subsequent establishing of the cell contact by centrifugation was minimised to 30 s. Despite that we obtained very low fusion yield (1%) indicating that fusogenic state of the membrane could be very short for cell lines used in our study (<< 1 min).

Another reason for a low fusion yield can be the fact that pulse-first protocol is not as effective as the contact-first protocol, at least for the cell lines used here. However some authors stated that pulse-first protocol gives similar results as contact-first protocol [16,24,41]. In the existing published literature on electrofusion we can find reports that fusion yield of erythrocyte ghosts dropped by a half when the pulse-first protocol was used compared to contact-first protocol [42]. Sukharev and co-workers also obtained negligible electrofusion yield on fibroblast-like cell line L-929 using pulse-first protocol [14,43]. The lower electrofusion yield obtained in pulse-first protocol was explained by misalignment between cell membranes during establishing of cell contact after electroporation [42]. Taking into account the role of misalignment we used the experimental design where we exposed cells to the electric field in two perpendicular directions. The larger area of the cell membrane in fusogenic state reduced negative effects of misalignment however the electrofusion yield remained low (Fig. 3).

Why pulse-first protocol is less efficient than contact-first can be explained by the existing theory and models of electroporation and electrofusion as well as by biological characteristics of the treated cells. Sugar and co-workers [44] propose an electrofusion model of two adjacent membranes that involves an electric field enforcement of tight contacts and pore formation leading to pore coalescence and finally resulting in one large fusion pore in the contact area [44]. Sukharev with co-workers proposed that not only pore formation but also pore coupling is a field-dependent process. Electric field applied to two neighbouring membranes in contact can create strong attraction between coaxial pore edges and thus promote establishing of fusion stalk leading to a pore formation and ending in cytoplasmic mixing [43]. The studies of the electroporation process can further explain why the contact-first electrofusion protocol gives higher fusion yield. It was proposed that the electroporation of the cell membranes starts with short-lived transient pore formation [45]. These short-lived structural changes in the cell membrane are present mostly during electric field exposure. Therefore it is possible that cell membrane during electric field exposure is highly fusogenic and as such enhances the cell fusion. All the studies mentioned above support our experimental data. Our results show considerably higher electrofusion yields when we used contact-first protocol. It is interesting to note that the maximum fusion yield was obtained by the modified adherence method (Table 1). The fusion yields obtained were comparable to the other published results as was already discussed in our previous study [33]. Maximum fusion yield obtained by dielectrophoresis was considerably lower (Table 2) but still comparable to the published data [32].

A good electrofusion yield obtained with CHO WTT clone cells and erythrocyte ghosts with pulse-first protocol can be explained also with biological nature of the cells used in those studies. It is known that the electrofusion is cell line depended [20,33,46]. CHO WTT clone cells have peculiar actin cytoskeleton organisation with less stress fibres compared to parental anchorage depended CHO strain used in our study [47–49]. The absence of long and thick actin sheaths (stress fibres), according to some authors, enhances cell electrofusion [43]. In a similar way we can explain the results obtained with erythrocyte ghosts. The erythrocytes itself are the cells with a specific cell organisation and properties among which the specific and reduced cytoskeleton should be mentioned. Furthermore the ghost preparation requires an extensive cell manipulation such as the hypotonic haemolysis, alternation of cytoskeleton and pronase treatment which may change properties of the cell membrane. Besides that the erythrocytes ghosts not treated with electric pulses were shown to fuse with electroporated CHO cells (WTT clone) [50].

The differences in fusion yield obtained can be attributed also to the quality of the cell contact when comparing modified adherence method and dielectrophoresis, the two methods used in contact-first protocol (Tables 1, 2). When we focus on dielectrophoresis our results indicate that the prolonged time of the dielectrophoresis, i.e. a longer maintenance of cell contacts, enhances electrofusion yield (Table 2). One of the possible explanations for those observations could be as follows. When using the modified adherence method cells, slightly attached on the dish surface, formed spontaneous and tight contacts before the electroporation and maintain it for prolonged period of time after electroporation (10 min and more). Besides, the tightness of cell contact is enhanced due to the cell swelling caused by electroporation [34]. In contrast when we used dielectrophoresis on cells in suspension the duration of the contacts between the cells is significantly reduced to 2 min at the best case. Here it is important to take into account that presumably initial fusion pores (sites) are non-stable and can be disrupted by membrane gaping caused by cell shape relaxation after dielectrophoresis [43]. Furthermore the dielectrophoretic forces depend on membrane and cytosol conductivities, which are changed after the electric pulse application [51]. In that context the cell contact

obtained after electroporation by dielectrophoresis may not be of the same quality. It is also well known that for dielectrophoresis low conductivity media have to be used not only to prevent Joule heating but also to ensure an efficient dielectrophoretic force between cells. However the low media conductivity is associated with reduced ion content of the buffer used which can negatively affect the electrofusion. It was shown that divalent ions in millimolar concentration (e.g. Mg^{2+}) significantly increase electrofusion yield [5,52]. Since our buffer for dielectrophoresis was prepared by dilution of the parental buffer containing 1 mM $MgCl_2$ this can also explains the lower electrofusion yield obtained with dielectrophoresis.

4.2. Determination of fusion yield with two different microscopy methods

The detection method of the fused cells is a critical and important part of the electrofusion research. A review of the published literature shows that different authors used different electrofusion protocols, detection methods as well as quantifications of fused cells. Therefore, the results of different studies cannot be directly compared [12,13,32,36,53,54]. Among the techniques used in the field of electrofusion research the microscopy was proposed as the method of choice [55]. Therefore for the evaluation of the results in our study we chose the phase contrast and fluorescence microscopy. As we can see from our results less variability (as shown by the comparison of the CVs) is obtained by fluorescence microscopy. The selection of the dyes is not critical (Table 1).

It is important to note that the fluorescence microscopy gives expected lower electrofusion yields than what actual fusion yields are. This result is in agreement with the fact that only double labelled fused cells can be detected while fusion takes place also between cells stained with the same colour, which, however, are not detected. It is also worth to mention that the described approach for fusion yield determination does not distinguish between bi-nucleated cells as a result of the fusion between two cells and poly-nucleated cells as a result of multiple fusion events. From that point of view all our fusion yields are under evaluated since poly-nucleated cells were often obtained. Although the fusion yield can be much more precisely determined with the fluorescence microscopy the low fusion yields in the range of 1% can be more reliably detected by phase contrast microscopy. However it is important to note that this low number of the fused cells can still gives us viable hybridomas as reported in our previous study [10]. Thus when the overall fusion is very low, the fluorescence microscopy might not be the most adequate method to determine the actual fusion yield.

5. Conclusions

In summary we can conclude that for successful cell electrofusion it is advisable to use the contact-first protocol. However, the fusion yield strongly depends on the quality of the contacts between cells as can be seen from our results. By using the modified adherence method we obtained up to 15% of double labelled fused cells and up to 4% by dielectrophoresis. No double labelled cells were found in the experiments where the pulse-first protocol was used. However with the phase contrast microscopy we still determined the fusion yield to be around 1%. Based on our results and published literature we can conclude that the pulse-first electrofusion protocol efficiently works only in specific conditions and cannot be, at least for now, assumed equivalent to the contact-first protocol.

The findings of our paper establish the platform for further investigation of mechanisms involved in cell electrofusion. Detailed studies of membrane surface area changes, vesicle formation, kinetic of matter exchange between cells as well as visualisation of fusion pore formation can now be effectively performed.

Authors' contributions

Marko Ušaj performed all the experiments, analysed and interpreted the data and wrote the draft of the manuscript. Karel Flisar built the AC generator used for dielectrophoresis experiments. Damijan Miklavčič edited the manuscript and contributed to the interpretation of results. Maša Kandušer helped Marko Ušaj with the design of experiments and data interpretation and edited the manuscript. All authors read and approved the final manuscript.

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