

Comparison of Flow Cytometry, Fluorescence Microscopy and Spectrofluorometry for Analysis of Gene Electrotransfer Efficiency

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Abstract In this study, we compared three different methods used for quantification of gene electrotransfer efficiency: fluorescence microscopy, flow cytometry and spectrofluorometry. We used CHO and B16 cells in a suspension and plasmid coding for GFP. The aim of this study was to compare and analyse the results obtained by fluorescence microscopy, flow cytometry and spectrofluorometry and in addition to analyse the applicability of spectrofluorometry for quantifying gene electrotransfer on cells in a suspension. Our results show that all the three methods detected similar critical electric field strength, around 0.55 kV/cm for both cell lines. Moreover, results obtained on CHO cells showed that the total fluorescence intensity and percentage of transfection exhibit similar increase in response to increase electric field strength for all the three methods. For B16 cells, there was a good

correlation at low electric field strengths, but at high field strengths, flow cytometer results deviated from results obtained by fluorescence microscope and spectrofluorometer. Our study showed that all the three methods detected similar critical electric field strengths and high correlations of results were obtained except for B16 cells at high electric field strengths. The results also demonstrated that flow cytometry measures higher values of percentage transfection compared to microscopy. Furthermore, we have demonstrated that spectrofluorometry can be used as a simple and consistent method to determine gene electrotransfer efficiency on cells in a suspension.

Keywords Fluorescence microscopy · Flow cytometry · Spectrofluorometry · Gene electrotransfer

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Introduction

Electroporation, a method of introducing molecules into cells by electric pulses, was first described by Neumann and Rosenheck almost four decades ago (Neumann and Rosenheck 1972). Various applications of electroporation have been developed since then, among them are electrochemotherapy (Mir et al. 1991; Belehradec et al. 1993; Heller et al. 1999; Marty et al. 2006; Mali et al. 2013) and gene electrotransfer (Neumann et al. 1982; Hojman et al. 2007; Favard et al. 2007; Daud et al. 2008; Gehl et al. 1999; Bureau et al. 2000; Marjanovic et al. 2010).

Among different theoretical descriptions of electroporation, transient aqueous pore model is most widely accepted. According to this model, hydrophilic pores are formed in membrane lipids of the cell membrane exposed to external electric pulse (Neumann et al. 1989; Weaver and Chizmadzhev 1996); therefore, we will use this term to

stress theoretical background. The phenomenon of electroporation can be described also as an increase in membrane permeability defined as electroporabilization (Teissié et al. 1999; Sukhorukov et al. 1995). In this paper, we will use the term electroporation to refer to biophysical phenomenon, term electroporabilization for the observations of transport of small molecules and term gene electrotransfer for complex process of gene delivery into the cell. Current description of gene electrotransfer defines several steps of: (i) electroporabilization of the cell membrane, (ii) contact of the plasmid with the cell membrane (formation of a complex), (iii) translocation across the membrane and (iv) transfer to and into the nucleus and gene expression (Golzio et al. 2002; Faurie et al. 2010).

Understanding of the mechanism underlying gene electrotransfer (Neumann et al. 1982; Favard et al. 2007; Kanduser et al. 2009; Faurie et al. 2010; Marjanovic et al. 2010; Pavlin et al. 2010; Haberl et al. 2010) and influence of different parameters on its efficiency is important for implementation of gene electrotransfer for gene therapy and for DNA vaccination (Heller et al. 1996; Rols and Teissié 1998; Mir et al. 1999; Gehl et al. 1999; Bureau et al. 2000; Daud et al. 2008; Rosazza et al. 2011; Spanggaard et al. 2013).

Therefore, a large number of experiments of gene electrotransfer were done *in vitro* on cells in suspensions, and different methods were used to quantify its efficiency in terms of percentage of transfected cells (%TR), fluorescence intensity (FL) or both, where fluorescence intensity directly correlates with the amounts of expressed transgene. In order to compare gene electrotransfer efficiencies from different studies, it is of interest to systematically compare different methods that are regularly used *in vitro*.

Gene electrotransfer efficiency *in vitro* is usually determined by quantifying the fluorescence intensity of reporter genes such as GFP (green fluorescent protein) or by determining percentage of transfected (fluorescent) cells. In order to analyse efficiency of gene electrotransfer, different methods that enable quantification in terms of percentage of transfected cells, total fluorescence intensity or both are used. Currently, there are two widely used methods for analysing gene electrotransfer efficiency. The first method, fluorescence microscopy, is based on acquiring micrographs of the sample, and quantification of transfected cells is obtained by counting the transfected (e.g. GFP-expressing) and non-transfected cells in the treated sample (Golzio et al. 2004; Rebersek et al. 2007; Kanduser et al. 2009). With this method, one can obtain relative percentage of transfected cells and, with additional image analysis, also fluorescence intensity. In addition, by counting the cells in sample not exposed to electric field and the sample of cells exposed to electric pulses, viability

and absolute percentage of transfected cells can be obtained. The majority of authors represent their results as relative percentage of transfected cells (Golzio et al. 2001, 2004; Kanduser et al. 2009; Pavlin et al. 2010; Haberl et al. 2010, 2013). Fluorescence microscopy is simple and reliable but time-consuming method (Usaj et al. 2011). The second method is flow cytometry (Faurie et al. 2004; Marjanovic et al. 2010; Haberl et al. 2010), which measures the percentage of transfected cell as well as fluorescence intensity and enables fast analysis of a large number of cells. The flow cytometer uses a computer-assisted discrimination of non-transfected and transfected cells by manual gating of fluorescence signal providing two information: emission histogram for each single cell and number of fluorescent cells.

Alternatively, spectrofluorometry as the third method can be used, since it is also used for detection of reporter genes such as GFP in several studies across different fields of life sciences (Degelau et al. 1995; Schmid et al. 2001; Paillusson et al. 2005; Torrado et al. 2008; Tesic and Cemazar 2013). Spectrofluorometry measures the total fluorescence intensity of the observed sample. Therefore, applicability of spectrofluorometry for quantification of gene electrotransfer was also evaluated in our study.

Even though all the three methods were already used for analysis of gene electrotransfer efficiency (Golzio et al. 2001; Kanduser et al. 2009; Haberl et al. 2010; Tesic and Cemazar 2013) until now, no systematic comparison of all the three methods was performed.

Therefore, the aim of this study was to systematically compare and analyse the three different methods for quantifying gene electrotransfer efficiency: fluorescence microscopy, flow cytometry and spectrofluorometry, and additionally to evaluate applicability of spectrofluorometry for analysis of gene electrotransfer efficiency *in vitro*.

Methods

Cell Culture

Chinese Hamster Ovary (CHO-K1; European Collection of Cell Cultures, Great Britain) and mouse melanoma (B16F1; European Collection of Cell Cultures, Great Britain) cells were used in our *in vitro* experiments. CHO cells were grown in F-12 HAM (Dulbecco's modification of Eagle's Minimum Essential Medium; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), while B16 cells were grown in EMEM (Eagle's Minimum Essential Medium, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) culture medium. Both culture media were supplemented with 10 % fetal bovine serum and 0.15 mg/ml L-glutamine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany)

and antibiotics penicillin/streptomycin and gentamycin. Cell cultures were grown as plated monolayers at 37 °C in a humidified 5 % CO₂ atmosphere in the incubator in 25-cm² culture dishes (TPP, Switzerland). A 10 mM isoosmolar phosphate buffer (Na₂HPO₄/NaH₂PO₄, pH 7.4, electrical conductivity = 0.127 S/m) with 1 mM magnesium chloride (MgCl₂) and 250 mM sucrose were used for gene electrotransfer.

Plasmid DNA Preparation

Plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) which encodes green fluorescent protein (GFP) was amplified in *Escherichia coli* Top10 strain and isolated with a Hi Speed Plasmid Maxi Kit (Qiagen, Hilden, Germany). The plasmid DNA concentration was determined by spectrophotometer at 260 nm and also confirmed by gel electrophoresis. Plasmid was dissolved in pure water (Aqua B. Braun, Braun Melsungen, Germany).

Exposure to Electric Pulses

Gene electrotransfer experiments were performed at 24 h after trypsinization when cells are in the early logarithmic growth phase (Kanduser et al. 2009; Marjanovic et al. 2010; Pavlin et al. 2010; Haberl et al. 2013) on CHO and B16 cells. On the day of experiment, cell cultures were removed from the incubator, and suspension was prepared by 0.25 % trypsin/EDTA solution (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and centrifuged for 5 min at 1,000 rpm (180×g) and 4 °C (Sigma, Germany). The cell pellet was resuspended in an electroporative medium (isoosmolar phosphate buffer) at a concentration of 2.5×10^6 cells/ml. Electroporation was performed on cell suspension in cuvettes with incorporated aluminium electrodes (distance between the electrodes: $d = 4$ mm—Eppendorf, Hamburg, Germany). The volume of the sample placed in each cuvette was 100 µl. Plasmid DNA (pEGFP-N1; Clontech, USA; 4,649 base pairs), which encodes green fluorescent protein (GFP), was added to the cell suspension in a concentration of 40 µg/ml. After incubating the DNA with the cells at room temperature for 2–3 min, electric pulses were applied to the samples using the Cliniporator™ (IGEA, Carpi, Italy). Each sample was exposed to four electric pulses of 200 µs with a repetition frequency of 1 Hz and of different applied electric field strength amplitudes (Kanduser et al. 2009) $E = 0.6, 0.8, 1.0, 1.2, 1.4$ and 1.6 kV/cm with corresponding applied voltages $U = d E = 240, 320, 400, 480, 560$ and 640 V. No pulses were applied to cells in the control.

After pulse delivery, 25 µl of fetal bovine serum was added. Treated cells were then incubated for 5 min at 37°C for cell membrane resealing to take place, and then 1 ml of

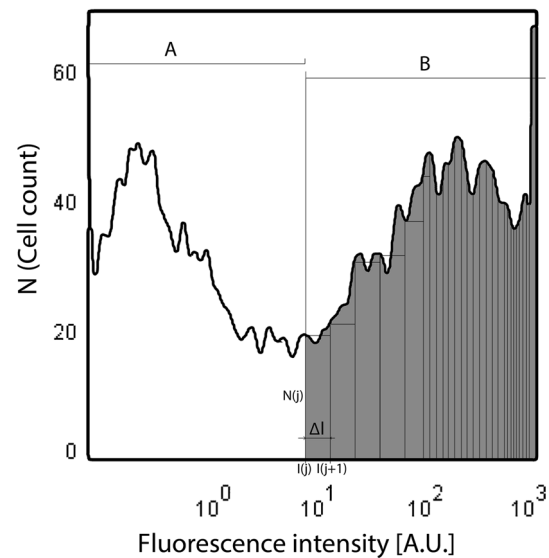


Fig. 1 Flow cytometer histogram of cells exposed to 4×200 µs pulses with 1.6 kV/cm electric field strength amplitude. The abscissas axis presents logarithmic scale of fluorescent intensity (arbitrary units), while the ordinate axis represents the number of events (counted cells). Section *a* represents non-transfected cells, and section *b* (grey) represents transfected cell. In section *b*, we schematically represent how integration of fluorescence intensity was performed in order to obtain the total fluorescence intensity *FL* (see [Methods](#))

complete culture media supplemented with 10 % fetal bovine serum was added. The cells were grown for 24 h at 37 °C and 5 % CO₂ in 24-well plates with the growing surface area/well of 1.9 cm² for microscopy or microplate reader analysis and in 25-cm² culture dish in five ml of the culture media for flow cytometry analysis of gene electrotransfer efficiency. For this period of cell culture, cell density did not affect the final result.

Flow Cytometry

After 24 h in the incubator, the cells were trypsinised; trypsin was removed by centrifugation for 5 min at 1,000 rpm (180×g) at 4 °C. Supernatant containing the residual trypsin was discarded while cell pellet was resuspended in phosphate buffered saline (PBS) at a concentration of 1×10^6 cells/ml for analysis by flow cytometry. The Coulter EPICS Altra flow cytometer (Beckman Coulter Electronics) was used to determine the percentage of transfected cells (GFP expressing) in each sample by setting the excitation at 488 nm, emission at 525 nm and recording of 10,000 events per each sample. The flow cytometer measures fluorescence intensity histogram that presents number of events at given fluorescence intensity. The total fluorescence intensity and %TR were obtained from flow cytometer histogram data as explained below.

Analysis of Results of Flow Cytometry

In Fig. 1, typical histogram measured by flow cytometer is presented. The abscissas axis presents logarithmic scale of fluorescent intensity (arbitrary units), while the ordinate axis represents the number of events (counted cells). On the start of all sets of experiment, side- and forward-scatter data of the control sample were used to determine the region of dead cell fraction and viable cells similarly as described in Haberl et al. (2010). The gate for GFP positive cells was determined by control sample at the start of experiments, and non-transfected cells were labelled as group A, while transfected (GFP positive) cells were labelled as group B. %TR was automatically obtained by flow cytometer software as $100 \times N_B/N$, where N is the total number of all counted cells and N_B is the number of cells in group B (GFP positive cells).

In order to analyse, the results from flow cytometer in a manner that they would be comparable to results obtained by spectrofluorometer, an integral of fluorescence intensity of transfected cells (group B in Fig. 1) was performed on histogram data from flow cytometer for every sample for all fluorescent cells in group B. The integral of fluorescent intensity was calculated numerically as described below (Fig. 1).

In our experiments, flow cytometer measures 2^{10} different fluorescent intensity levels I_j presented as histogram. In order to obtain the total fluorescent intensity FL (R.F.U.) of transfected cells (group B in Fig. 1), the following formula was used:

$$FL(\text{flow cytometer}) = \sum_j^{1024} N(j)\Delta I_j, \quad (1)$$

where j starts at the first fluorescence intensity measurement at the beginning of group B, $N(j)$ represents the number of cells at a given j and ΔI_j is corresponding fluorescent intensity difference (interval):

$$\Delta I_j = I(j+1) - I(j). \quad (2)$$

Using Eq. 1 FL (flow cytometer) was calculated for every measured sample.

Fluorescence Microscopy and Spectrofluorometry

Twenty-four hour after pulse delivery, fluorescence intensity of GFP (excitation wavelength: 488 nm, emission wavelength: 525 nm) was evaluated with a microplate reader (Tecan infinite M200, Tecan Austria GmbH) using the Magellan program (Tecan Austria GmbH). During the cell culture, cells were attached to the bottom of the dish and the fluorescence intensity was evaluated in the culture media. The fluorescence optics of microplate reader was set

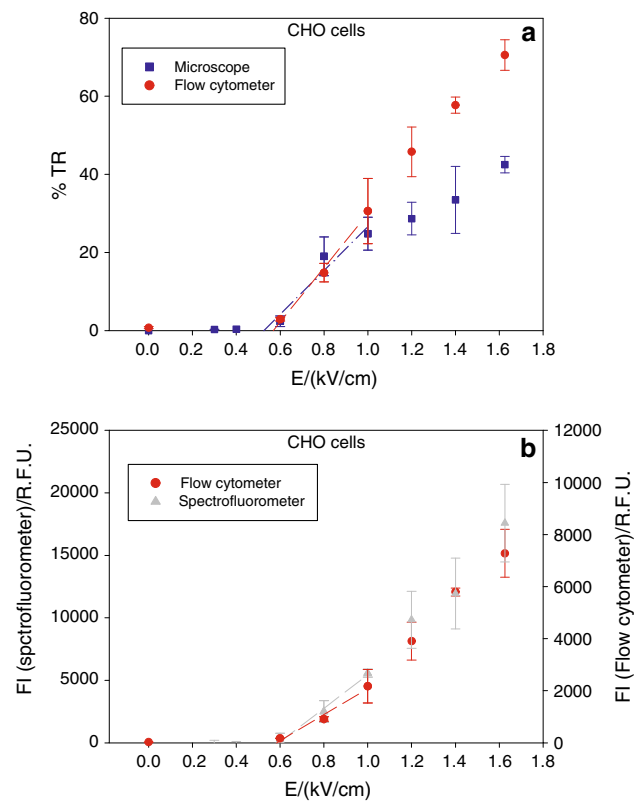


Fig. 2 The effect of the electric field strength on gene electrotransfer efficiency (GFP expression) for CHO cells in a suspension, measured by **a** fluorescence microscope and flow cytometer (% TR) and **b** spectrofluorometer and flow cytometer (total fluorescence intensity—see [Methods](#) section). $0.4 \times 200 \mu\text{s}$ pulses with repetition frequency 1 Hz were used for gene electrotransfer. The presented values are means of two independent experiments measured by flow cytometer and three independent experiments measured by fluorescence microscope and spectrofluorometer. Please note the double y axis in Fig. 2 (b), and that the relative fluorescence units (R.F.U.) of flow cytometer and spectrofluorometer are not the same

to the bottom of the well, and multiple reads per well were performed for each sample. With the spectrofluorometer, approximately 5×10^4 – 1×10^5 of cells per sample were investigated. Values of control samples—wells with cells not exposed to electric field, were used to subtract any fluorescence that might be present in the culture media or due to auto fluorescence of the cells.

In parallel, the same samples were analysed in the micrographs obtained by a fluorescence microscope (Axiovert 200, Zeiss, Germany). Bright field and fluorescence micrographs were acquired by 12-bit CCD-cooled camera (Visicam 1280, Visitron, Germany,) and analysed in MetaMorph imaging software (Visitron, Germany). The transfection efficiency was determined on fluorescence and bright field micrographs acquired 24 h after pulse delivery by manually counting of all the cells in bright field and fluorescent images. The percentage of transfected cells (% TR) in a given sample was determined as the ratio between

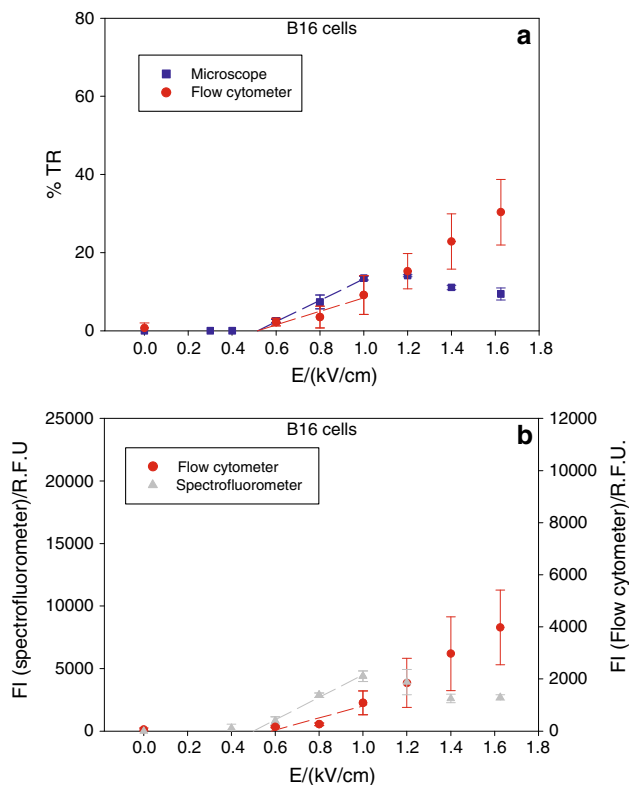


Fig. 3 The effect of the electric field strength on gene electrotransfer efficiency (GFP expression) for B16 cells in a suspension, measured by **a** fluorescence microscope and flow cytometer (% TR) and **b** spectrofluorometer and flow cytometer (total fluorescence intensity—see [Methods](#) section). $0.4 \times 200 \mu\text{s}$ pulses with repetition frequency 1 Hz were used for gene electrotransfer. The presented values are means of two independent experiments measured by flow cytometer and three independent experiments measured by fluorescence microscope and spectrofluorometer. Please note the double y axis in graph **(b)** and that the relative fluorescence units (R.F.U.) of flow cytometer and spectrofluorometer are not the same

the number of fluorescent cells expressing GFP (N_{GFP}) counted in the fluorescence images and the number of viable cells (N) counted in the corresponding bright field images (Kanduser et al. 2009; Pavlin et al. 2012):

$$\%TR = 100 \times N_{GFP}/N. \quad (3)$$

All experiments were repeated two or three times on different days, and at least seven bright field and seven fluorescent micrographs were acquired per each sample. Results from different experiment repetitions were pooled together. Error bars in Figs. 2 and 3 present mean values with standard deviation.

Determination of Critical Electric Field Strength for Gene Electrotransfer

In order to determine the critical electric field strength for gene electrotransfer— E_c (the electric field strength, above which gene electrotransfer is first detected), a linear

regression line was fitted to the first three amplitudes on all charts using Sigmaplot v11 (Systat Software Inc., USA) as described before (see Fig. 1 in reference (Marjanovic et al. 2010)). E_c above which transfection was observed was determined as a point where the linear fit crossed the electric field strength axis. The dependence of %TR on E is presented in Figs. 2 and 3. A linear curve was fitted to the experimental data:

$$\%TR = k \times E + y_0. \quad (4)$$

The critical electric field strength E_c was extracted from the intersection of the fitted curve with the x -axis: $E_c = -y_0/k$, from which we obtained the critical electric field strength in range from 0.5 to 0.6 kV/cm. Here we have to stress, that linear fit is just a method for determination of approximate E_c and not to suggest linear dependence of %TR or expression (fluorescence intensity) on E , since gene electrotransfer is a complex multistep process. Furthermore, the choice of function that we fit is somewhat arbitrary, it mainly serves as a way to consistently determine E_c , but also some other method/function could be used to determine the critical electric field strength (Puc et al. 2003).

It is important to note that the critical electric field strength for electroporation is directly related to the critical induced transmembrane voltage, while the critical electric field strength for gene electrotransfer is higher than the critical electric field strength for electroporation, since gene electrotransfer is a more complex phenomenon composed of different processes (Neumann et al. 1982; Wolf et al. 1994; Golzio et al. 2002; Kanduser et al. 2009; Faurie et al. 2010; Marjanovic et al. 2010).

Results and Discussion

The aim of this study was to compare the three different methods used for quantification of gene electrotransfer efficiency: fluorescence microscopy, flow cytometry and spectrofluorometry.

Since most of the measurements to date are performed either by flow cytometry or fluorescence microscopy, the knowledge of the correlation between these three methods is very important. In addition to test applicability of spectrofluorometry for quantification of gene electrotransfer as it is issued across different fields of science, it is also important to understand to what extent the results obtained by spectrofluorometer correlate with the other two methods.

All the three methods were compared in terms of percentage of transfected cells (percentage transfection - %TR) and total fluorescence intensity (FI/R.F.U.) for CHO cells in

Fig. 2 and for B16F1 cells in Fig. 3. In panels (a), percentage transfection obtained by fluorescence microscopy and flow cytometry is compared, while in panels (b), total fluorescence intensity obtained by flow cytometry and spectrofluorometry is shown. From Figs. 2 and 3, it can be seen that: (i) all the three methods determined similar critical electric field strength (E_c) and (ii) for both cell lines, E_c is similar: between 0.5 and 0.6 kV/cm.

When we analysed the percentage of transfected CHO cells measured by fluorescence microscope and flow cytometer (Fig. 2a), a similar linear increase of transfection with increased electric field strength was observed up to 1.0 kV/cm. At higher electric field strengths (1.6 kV/cm), higher percentages of transfection were obtained by flow cytometer compared to fluorescence microscope (71 vs 41 %).

For B16 cells, percentages of transfected cells obtained from the fluorescence microscope and flow cytometer (Fig. 3a) exhibited a similar increase up to 1.2 kV/cm. At higher electric field strengths, a decrease in percentage of transfection by fluorescence microscope was obtained, while percentage of transfection was continuously increasing by flow cytometer. The maximum percentage of transfection determined was 14 % by fluorescence microscope and 30 % by flow cytometer. Lower maximum percentage of transfection obtained by fluorescence microscope can be explained by the differences between the two methods. Fluorescence microscope quantifies percentage of transfection by counting the number of transfected cells from recorded micrographs. Since the brightness of each image pixel corresponds linearly to the fluorescence intensity (and we are limited by the number of 4096 grey levels of a CCD camera), transfected cells that produce smaller amounts of GFP were difficult to detect and were subjected to counting error. The flow cytometer, on the other hand, analyses each cell individually and has much larger range of fluorescence intensity levels (channels) compared to microscopy and can thus detect even cells that produce small amounts of GFP (Haberl et al. 2010). Overall, the percentage of transfected cells obtained by flow cytometer tends to be higher for higher electric fields compared to results obtained by fluorescence microscope, due to lower detected %TR by microscopy and in our opinion also partially due to higher sensitivity of flow cytometer as obtained also by Degelau et al. (1995) and Gabrijel et al. (2004).

In Figs. 2b and 3b, the total fluorescence intensity determined by flow cytometer and spectrofluorometer is presented. For CHO cells, fluorescence intensity determined by flow cytometer and spectrofluorometer exhibited a similar linear increase with increased electric field strength up to the maximum value at 1.6 kV/cm (Fig. 2b).

For B16 cells, we obtained a similar electric field strength dependence of the percentage of transfection

obtained by fluorescence microscope (Fig. 3a) and of the spectrofluorometric results of fluorescence intensity (Fig. 3b). Both methods detected a decline of %TR or fluorescence intensity above 1.0 kV/cm. However, the results of flow cytometer showed different behaviour (Fig. 3b) since fluorescence intensity and %TR measured by flow cytometer increased continuously with E .

The discrepancy between the flow cytometric and results of the other two methods (spectrofluorometry and microscopy) at high electric field strengths for B16 cells could be explained by several factors: (i) due to higher %TR measured by flow cytometer compared to %TR obtained by microscopy due to different detection sensitivities of the two methods as explained above and (ii) the discrepancy of the results at high field strengths could also be explained by influence of secretion of melanin by B16 cells when viability is decreased. Namely, for bacteria, it was shown that melanin interacts with the DNA (Geng et al. 2010) and this interaction could lead to the quenching of the fluorescence intensity for higher electric fields, where, as we have shown, viability is significantly reduced (Marjanovic et al. 2010).

In general, results obtained with all the three methods consistently showed similar relation between B16 cells and CHO cells. Altogether, B16 cells consistently exhibited lower maximal percentage of transfection compared to CHO cells measured either by fluorescence microscope (41 % for CHO cells, 14 % for B16 cells) or by flow cytometer (71 % for CHO cells, 31 % for B16 cells). B16 cells also exhibited lower maximal total fluorescence intensity compared to CHO cells measured either by spectrofluorometer (17000 R.F.U. for CHO cells, 5000 R.F.U. for B16 cells) or by flow cytometer (7000 R.F.U. for CHO, 4000 R.F.U. for B16 cells). The obtained results are in agreement with previously published studies on gene electrotransfer (Golzio et al. 2001; Rebersek et al. 2007), where it was shown that an increase in the percentage of transfection is accompanied by an increase in the corresponding fluorescence intensity.

It is also important to note that our results demonstrate that flow cytometry, as already mentioned tends to measure higher percentage transfection and fluorescence intensity compared to the other two methods, probably due to higher detection sensitivity. Partially, the discrepancy could be also due to additional preparation of a sample (additional trypsinization and centrifugation for flow cytometry) since part of the cells (approximately 10 %) is lost; however, without further analysis, it is difficult to determine which method is the most accurate for measuring %TR (Gabrijel et al. 2004; Černe et al. 2013).

Results of all the three methods (fluorescence microscopy, flow cytometry and spectrofluorometry) were further analysed by calculating Pearson correlation coefficients. For CHO cells very good correlation was obtained; Pearson

Table 1 Comparison of the three analysed methods—measured parameters (percentage transfection—%TR, fluorescence intensity -FL_{int}) and advantages/drawbacks of fluorescence microscopy, flow cytometry and spectrofluorometry for quantification of gene electrotransfer efficiency

	Measured quantity	Advantages	Drawbacks
Fluorescence microscopy	%TR and fluorescence intensity distribution of an image	Most direct observation of phenomena, for large number of acquired images very reliable in terms of both quantification and visualization, does not require additional preparation of samples, sample can be used for further experiments	Relatively slow, difficult to obtain good statistics, %TR has to be determined either with manual counting (slow) or with advance image processing FL _{int} per cell can be obtained only with additional image processing, total FL _{int} of whole images has large background signal
Flow cytometry	%TR, fluorescence intensity histogram—also total fluoresce intensity	Direct measurement of both %TR and FL _{int} (most complete information is obtained: average FL _{int} per cell and number of cells with given FL _{int}), relatively fast (with respect to number of analysed cells), very good statistics (large number of analysed cells)	Additional trypsinization of sample needed, contamination of sample
Spectrofluorometry	Total fluoresce intensity	Very simple and fast, does not require additional preparation of samples, sample can be used for further experiments.	Measures only total FL _{int} , no information on %TR can be obtained

correlation coefficients were $r = 0.993$ (spectrofluorometer vs. flow cytometer) and $r = 0.955$ (spectrofluorometer vs. fluorescence microscope). This is in agreement with the study (Degelau et al. 1995) on the detection of Poly- β -hydroxybutyrate (PHB) in *A. Eutrophus* bacteria, where the relationship between the results obtained by flow cytometer and spectrofluorometer was also found to be linear.

For B16 cells, the Pearson correlation coefficients were as follows: $r = 0.397$ (spectrofluorometer vs. flow cytometer) and $r = 0.974$ (spectrofluorometer vs. fluorescence microscope). We can see that there is poor correlation between spectrofluorometer and flow cytometer ($r = 0.397$) for results obtained on B16 cells. As already discussed, this can be explained by quenching of fluorescence intensity due to melanin. Pearson correlation coefficients were therefore high (strong correlation) for all combinations except for the relationship between spectrofluorometric and flow cytometric results for B16 cells.

In Table 1, we analyse advantages and drawbacks of the three studied methods. Using fluorescence microscopy, the %TR can be obtained straightforwardly (manually) but the variability of the results can be considerable since we are limited by the number of cells that can be counted; fluorescence intensity can be obtained only by additional imaging processing, e.g. by integration of fluorescence intensity to obtain total fluorescence intensity or by automatic cell detection to obtain intensity of each cell. However, the advantage is that microscopy enables most direct observation of phenomena both visually as well as quantitatively if advanced image processing is used.

Flow cytometry, on the other hand, enables fast determination of both percentage of transfected cells and intensity of fluorescent cells with high reliability, which is the main advantage over the other two methods. However,

the preparation of a treated sample requires additional trypsinization and centrifugation related to additional loss of the cells as already mentioned above (Černe et al. 2013).

Finally, spectrofluorometry enables fast measurement of samples and can accurately determine E_c . The advantage of this method is that it does not need additional trypsinization, and the sample also never comes into physical contact with equipment (also true for microscopy). However, major disadvantage is that only total fluorescence intensity can be measured and no information on %TR can be obtained, therefore, spectrofluorometry is more appropriate as a complementary method and not as a standalone method.

Therefore, depending on the type of application, one or another or combination of methods is appropriate. It is important to note that when results for different methods are compared, the difference, for e.g. in percentage transfection, can be also due to the choice of a specific method.

Conclusions

Altogether, all the three methods, fluorescence microscopy, flow cytometry and spectrofluorometry, detected similar critical electric field strength for gene electrotransfer. All the three methods also consistently obtained higher transfection efficiency for CHO cells compared to B16 cells. Furthermore, relatively good correlations of results of different methods were obtained except for B16 cells at high electric field strengths and even for these specific conditions results obtained with spectrofluorometry correlated well with microscopy. The results also show that flow cytometry tends to measure higher values of percentage transfection and fluorescence intensity compared to the

other two methods which is important for interpretation of results where different methods are used.

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Conflict of interest The authors declare no competing interests.

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