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ORIGINAL PAPER

Fluorometric Assay to Compensate for Non-viable Cells during Electroporation

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Abstract A fluorometric assay is described that allows adjustment for non-viable cells that result during electroporation. The technique, unlike others, relies on only one dye, requires a single instrument, and eliminates the need for a separate cell counting step. Murine melanoma (B16-F10) cells were electroporated using electric fields ranging from 400 to 2500 V/cm in the presence of SYTOX®-green. Compensation for the fluorescence resulting from non-viable cells was facilitated by a correction curve established by lysing a known number of cells in the presence of SYTOX®-green. In uncorrected data, an applied electric field of 2500 V/cm increased dye delivery but also reduced cell viability significantly. Compensating for the fluorescence of non-viable cells showed that changing the field strength to 800 V/cm or 2500 V/cm from 400 V/cm had only marginal effects on membrane pore formation. The fluorometric assay was used to compare electroporation in high conductivity (PBS) and a low conductivity medium (LC-PBS). Statistically significant increases of 10 to 30-fold were observed for cells electroporated at 400 V/cm and 800 V/cm in LC-PBS.

Keywords Electroporation · Fluorometric assay · Media conductivity · Electric field

Introduction

Delivery of biomolecules across the cell membrane is of great interest for medical therapies. In this context, electroporation has gained widespread acceptance for in vivo as well as in vitro scientific studies [1–6]. In the electroporation (EP) process, exposure of cell membranes to an electric field causes oppositely charged species to accumulate on either side of the membrane. The attraction associated with these accumulated charges causes the membrane to become thin and subsequently, defects are created [3, 4, 7, 8]. These defects, or pores, allow transport of material from the extracellular environment into the cytoplasmic space [4, 5, 9].

To use electroporation for biomolecular delivery, a protocol must first be established because optimal electrical parameters are a function of the cell size, electrode type, and other physical conditions. Development of a protocol invariably involves experimentation. During the development, it is common to utilize a tracer molecule such as a model fluorescent dye instead of a bioactive molecule. Dyes, such as SYTOX®-green or propidium iodide, minimize the waste of expensive therapeutic molecules [10–15] but also introduce a major challenge. A major issue with use of dyes is the inability to distinguish between fluorescence from irreversibly electroporated (IRE) cells and reversibly electroporated cells. More specifically, permanently compromised cells can allow more external material to transport across the membrane ultimately resulting in higher cellular fluorescence. Reversibly electroporated cells, on the other hand, limit dye transport time and the subsequent fluorescence due to the resealing process [16]. Correcting for the IRE cells is, therefore, critical for establishing EP protocols and determining whether molecular delivery using reversible electroporation is promising or not.

In the scientific literature, a few approaches have been commonly used for fluorescence correction [14, 15, 17–19]. In one technique reported by Wang et al. [15], two sets of EP experiments were performed. After each experiment a DNA stain was added and the fluorescent cells were counted. In the first experiment, data was representative of both viable and non-viable cells. In the second experiment, only non-viable cells were represented. To obtain the fraction of viable transfected cells, the values from the second experiment were

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subtracted from the first. This approach is depicted in 1a. A shortcoming to using this subtraction method is that two electroporation experiments are necessary thereby making the process tedious and time consuming because two samples have to be manually analyzed.

Figure 1b shows a slightly different approach by Homhuan et al. [20]. In this method only one EP experiment was carried out but two DNA stains were used. After the cells were pulsed the first stain was added and cells were incubated for 30 min. A second dye was then added and the cells were visually counted using a hemocytometer. The use of a second dye created a contrast between the viable and dead cells during the cell counting step, which eliminated the need for a second electroporation step. However, in this approach, the addition of a second dye creates the additional challenge of making certain that the binding from the first dye does not hinder the binding of the second dye. A third approach by Michie et al. used flow cytometry (depicted as Fig. 1c) [18]. Here the cells were pulsed, a fluorescent dye was added and the fluorescence was measured with a flow cytometer. The use of this instrument minimizes both the time and the inaccuracies associated with counting using a hemocytometer. However, this method necessitates the use of a separate instrument that may not be readily available. It also requires an additional cell removal step when using adherent cell types [17, 18].

Demonstrated here is a method to distinguish between the fluorescence associated with non-viable and viable cell populations present after electroporation. In this method, cells were pulsed, SYTOX®-green was immediately added, and the fluorescence was quantified. Cell viability was then determined using a standard MTT colorimetric assay. In a separate experiment, cells of known concentrations were lysed, SYTOX®-green was added, and the fluorescence was determined. The data from the latter experiment were used to establish the fluorescence of non-viable cells only, which could then be used to correct the overall fluorescence to yield fluorescence data representative of only the viable cells. There are several advantages associated with this technique. First, only one instrument (fluorescence microplate reader) and one DNA stain are needed to carry out the method. Second, the method does not require a tedious visual counting step. It is also important to note that this protocol can be used with any DNA stain. In this paper, the novel analytical technique is used to further show statistically significant differences in EP with varying field strengths in either PBS or a low conductivity medium (LC-PBS). Results from the

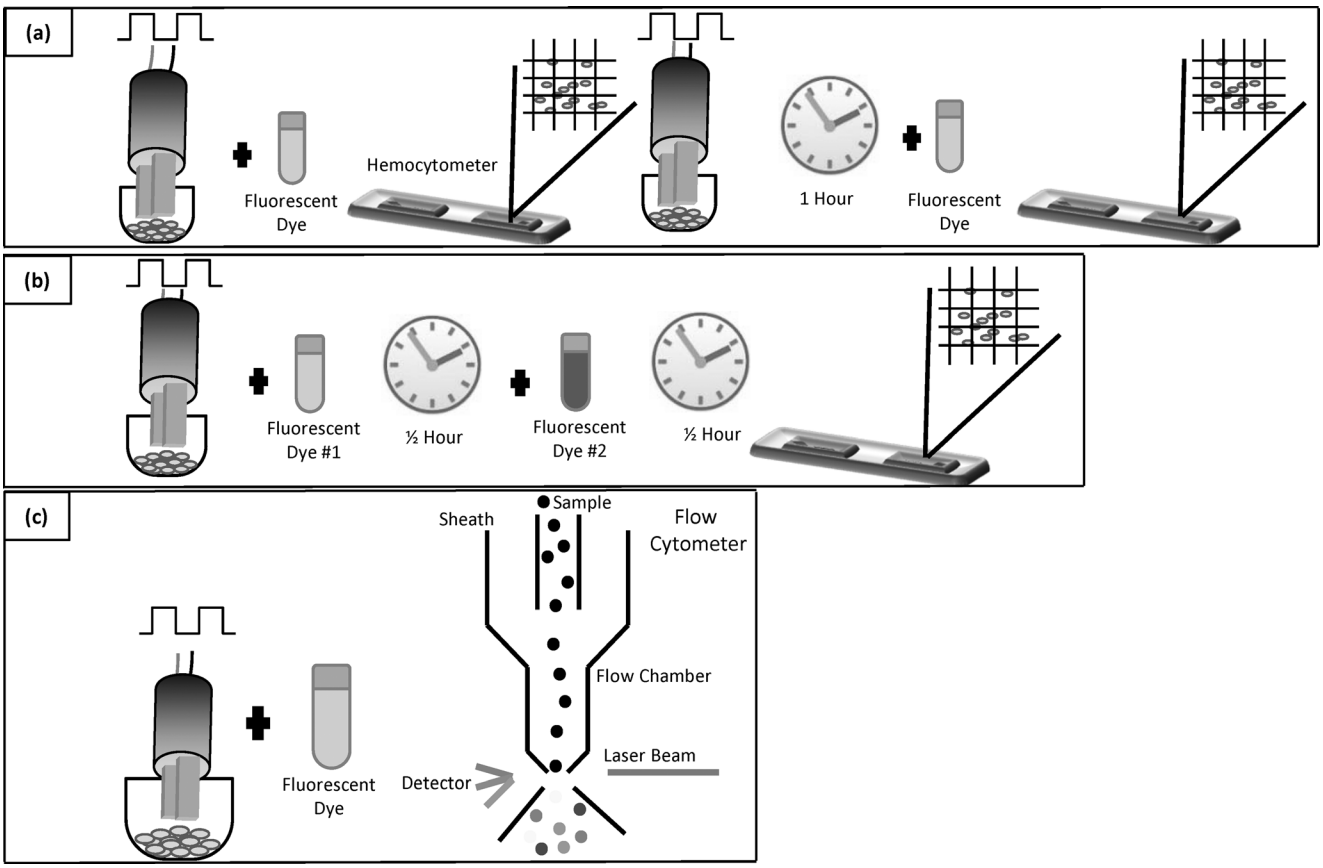


Fig. 1 Illustration of previously reported fluorescence correction methods (**a-c**). See text for description

experiments reported here demonstrate that neglecting the correction for non-viable cells can have a detrimental impact on establishing an effective EP protocol.

Materials and Methods

Cell Culture Murine melanoma, B16-F10 cells (American Type Culture Collection) were sub-cultured for 4 days to 70–80 % confluency in McCoy's 5A media that was supplemented with 10 % (*v*/*v*) fetal bovine serum and 0.1 % (*v*/*v*) gentamicin sulfate (all from Corning Cellgro, Manassas, VA). Cells were grown in 75 cm^2 flasks, seeded at a density of 2×10^4 cells/cm^2, and incubated in a humidified environment at 37 °C containing 5 % CO_2. Cells were removed from culture using Trypsin/EDTA, 1X (Mediatech, Inc., Manassas, VA) and centrifuged at 200×g and 4 °C for 5 min. The supernatant was then discarded and the cellular pellet was dispersed in fresh media. Cells were counted using an Invitrogen (Eugene, Oregon) Countess automated cell counter and then seeded using 3×10^4 cells/well in 96 well plates. B-16 cells were seeded at cell density ranges of $1–5\times10^4$ per well and $0.5–20\times10^3$ per well for viability and fluorescence curve experiments, respectively. In all experimental cases, seeded plates were incubated overnight containing 200 μl of growth media per well to allow the cells to recover and adhere to the bottoms of the wells.

Electroporation of Cells Cells were electroporated using two different solutions. One was phosphate buffered saline (PBS, Corning Cellgro) without Ca^{2+} and Mg^{2+}, and the other was low conductivity PBS (LC-PBS). The latter solution was made by diluting PBS with 250 μM glucose in water solution [21], at a ratio of 1:8.8. Conductivities for PBS and LC-PBS were measured to be 1.4 and 0.14 S/m, respectively, using a portable conductivity meter (Fisher Scientific Medex Supply, Brooklyn, NY). SYTOX®-green (Invitrogen), a membrane impermeable nucleic acid stain, was used as the model uptake molecule. The stain was diluted separately in both solutions, PBS and LC-PBS, from 5 mM in DMSO to 2 μM.

After cells were incubated overnight the culture media was aspirated and 50 μl of EP media (PBS or LC-PBS) was added to each well. Cells were pulsed using a single parallel plate electrode. The electrodes were stainless steel 4×50×0.5 mm plates with an inter-plate distance of 0.36 cm. Wells were pulsed with a ECM 830 Square Wave Electroporater (BTX, Holliston, MA) in triplicate using 8 rectangular-wave DC pulses with 150 μs pulse duration and an interval of 1 s between pulses. For the purposes of demonstrating the fluorometric assay and studying the effect of media conductivity, three different field strengths were used: 400 (low), 800 (medium), and 2500 (high) V/cm.

Immediately after each well was pulsed, 50 μl of SYTOX®-green solution was quickly added to each well and the fluorescence was measured one hour later at 485 nm using a BioTek FLx800 plate reader. Each experiment was repeated a total of 3 times on different days using different passage numbers of the B-16 cell line. A separate set of control experiments were done to determine if changing the media only, without electroporation, caused any effect on fluorescence. In these experiments, plasmid DNA (gWiz™ High Expression Luciferase Vector) was mixed in DI water, PBS or LC-PBS along with SYTOX®-green and the fluorescence was measured. Fluorescence results from these experiments ranged between 17,000 and 19,000 and showed no statisticallly significant difference in the magnitudes based on media changes alone.

MTT Viability Assay To determine cell viability post electroporation, a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) colorimetric assay was implemented. Once cells were pulsed and the fluorescence was measured, 50 μl of EP media was added to all the control wells to bring their final volume to 100 μl, equivalent to wells containing SYTOX®-green. Culture media (100 μl) was then added to all of the wells to aid in cell recovery and to bring the final volume of each well to 200 μl. The MTT powder was dispersed in EP media at 5 mg / mL and 20 μl of this solution (1:10) were added to each well. Cells were incubated at 37 °C in a humidified environment containing 5 % CO_2 for 4 h. Next, media was aspirated from each well and 100 μl of DMSO was added to dissolve the purple formazan crystals. After 30 min the absorbance of was read at 540 nm with a reference filter at 630 nm. Applying this same procedure, the standard curve used to calculate the number of viable cells post electroporation was generated. In this case, cells were not pulsed but all other parameters, such as temperature and media content, were kept identical to the treated cell. Also, cells were seeded with different densities ranging from 1 to 5×10^4 cells/well.

Fluorescence Correction Curve Towards the goal of isolating the fluorescence of viable cells a fluorescence curve was. This curve was genearated by seeding and lysing a known number of cells. First, $X\times10^3$ (X=0.5, 1, 2.5, 5, 10, and 20), cells were seeded in triplicate in a 96 well plate and incubated overnight at 37 °C and 5 % CO_2. After cells were removed from incubation the culture media was aspirated and 50 μl of deionized water was added to lyse the cells. Cells in DI water were left at room temperature for 10 min before 50 μl of 2 μM SYTOX®-green diluted in DI water was added. Over a period of 15 min, cells were monitored using both phase contrast and fluorescence microscopy and after 60 min the fluorescence for each well was measured using a microplate reader. The fluorescence values obtained for different seeded cell counts were regressed to a quadratic equation.

Statistical Analysis Statistical results are demonstrated as grand means ± SE. The data was compared using an ANOVA test. A 95 % level of significance was used and the mean difference was considered significant at the 0.05 level.

Results and Discussion

Towards the goal of distinguishing fluorescence solely from viable cells after electroporation, correction curves such as the one shown in Fig. 2, were established. Here, a known number of lysed cells were exposed to SYTOX®-green and the fluorescence was recorded. This procedure was repeated after each EP experiment. Lysing the cells in the presence of SYTOX®-green allowed the fluorescence from a known number of non-viable cells to be determined. Figure 2 shows the results of fluorescence measured as a function of the lysed cell count. The MTT assay, after electroporation, provided the number of viable cells in a pulsed sample and the number of total cells in the untreated control sample. These two measurements were used to obtain non-viable cell count; the curve shown in Fig. 2 was then used to determine the fluorescence representative of the non-viable cells. Simple subtraction of the non-viable cell contribution from the fluorescence measured post-electroporation allowed determination of the fluorescence associated solely with the viable cells.

Figure 3 shows results obtained after implementation of the new fluorescence correction technique. The plots in Fig. 3a and b show the viability and fluorescence associated with cells pulsed in PBS using various electrical parameters. From Fig. 3a it was determined that that addition of fluorescent dye did not have an adverse effect on cell viability. Figure 3a shows a steady decrease in percent viability when an electric field was applied. Applying a field in low (400 V/cm), medium (800 V/cm), and high (2500 V/cm) ranges caused the viability to decline by 20, 30, and 86 %, respectively. Figure 3b illustrates the fluorescence measured directly after EP and the magnitude after correction for the fluorescence contribution of the non-viable cells. The open and filled bars indicate uncorrected and corrected data, respectively. The uncorrected data (open bars) in Fig. 3b indicate that cells in PBS alone exhibited no fluorescence. A slight intensification was observed when SYTOX®-green was added. The increase was possibly due to the presence of a small fraction of cells that did not survive the overnight seeding step. The open bars show that after pulsing, the fluorescence value increased as the field strength increased and the highest level of fluorescence was observed at 2500 V/cm. From this data, one can conclude that increasing the field strength enhances dye diffusion into the cells and thereby, the membrane pore formation. Because Fig. 3a indicated that the high field strength of 2500 V/cm gave less than 15 % cell viability, it can be concluded that the application of a medium field strength of 800 V/cm resulted in sufficient cell viability and also balanced it with signficant dye diffusion/membrane pore formation.

The corrected data (filled bars) in Fig. 3b also shows increase in fluorescence with field strength but the trend is significantly different. Application of 400 V/cm, increases the fluorescence from the unpulsed value by approximately 7-fold and little difference is observed when the field strength is doubled to 800 V/cm. The highest fluorescence value (51-fold increase) is still observed at 2500 V/cm but it is clear by comparing the corrected (open bars) and uncorrected (filled

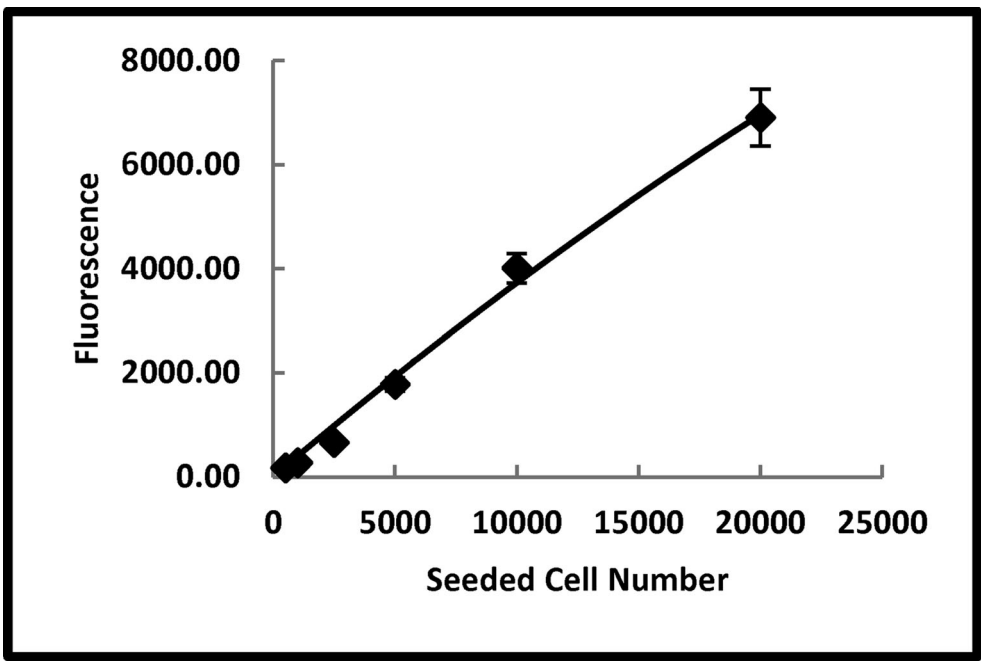
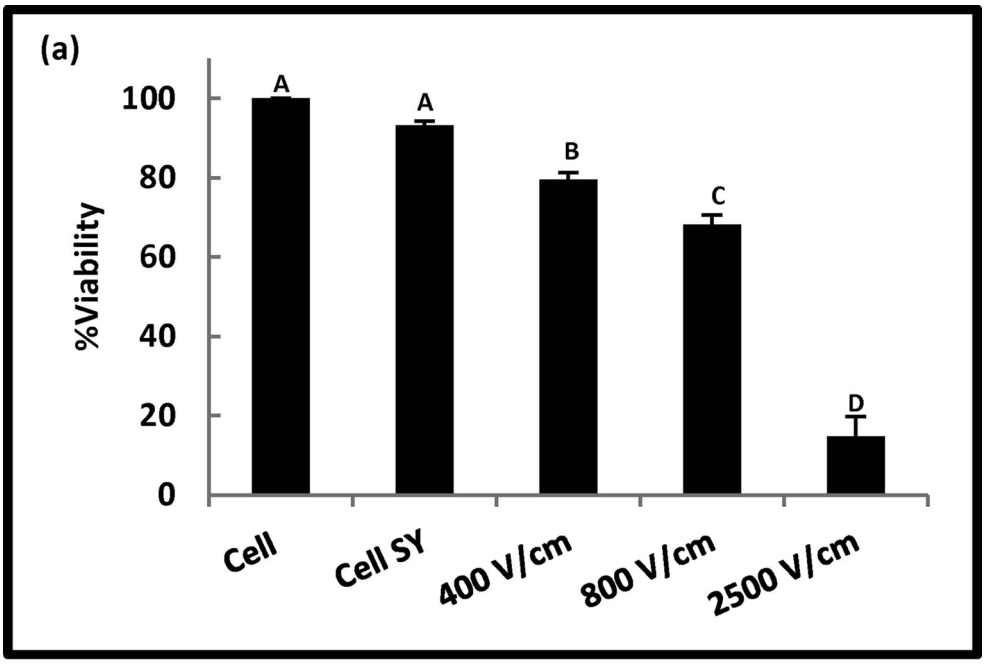


Fig. 2 Fluorescence correction curve established by lysing a known number of cells



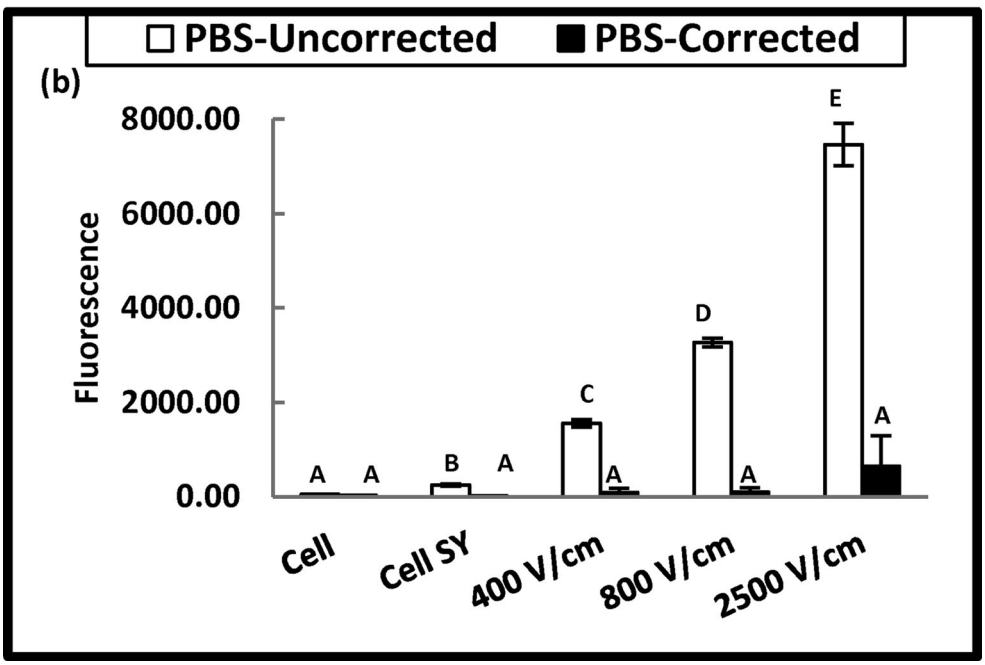


Fig. 3 **a** Viability of cells pulsed in PBS at low, medium, and high field strengths. **b** Uncorrected and corrected fluorescence of cells pulsed in PBS at low, medium, and high field strengths (SY = SYTOX®-green). Letters shared in common indicate no significant difference. Means with different letters are significantly different. For example, two groups that both possess the letter A indicate a p-value>0.05 between those two groups and for those that possess different letters such as A and B denote a p-value<0.05 between those two groups

bars) data that most of this fluorescence corresponds to non-viable irreversibly electroporated cells. From the corrected values it can be inferred that changing the field strength to 800 V/cm from 400 V/cm had marginal effect on membrane pore formation, as the fluorescence did not increase and the increase in field strength only decreased cell viability (Fig. 3a).

Greater molecular delivery was linked to the high field strength (2500 V/cm versus 400–800 V/cm) but the use of such intense field caused a large decrease in cell viability. Therefore, use of a lower conductivity medium was investigated with the same electrical parameters toward the goal of determining if high delivery could be maintained without compromising cell viability. Results for the effects of media conductivity on viability and molecular delivery, post-electroporation, are shown in Fig. 4a and b. In Fig. 4a, the viability associated with cells pulsed in low conductivity media (LC-PBS) is shown. As before, the viability decrease was minimal in the case of unpulsed cells with and without SYTOX®-green. Unlike electroporation in PBS, when cells were pulsed in LC-PBS using field strengths of 400 V/cm and 800 V/cm, the viability dropped only by 4 % and 15 %,

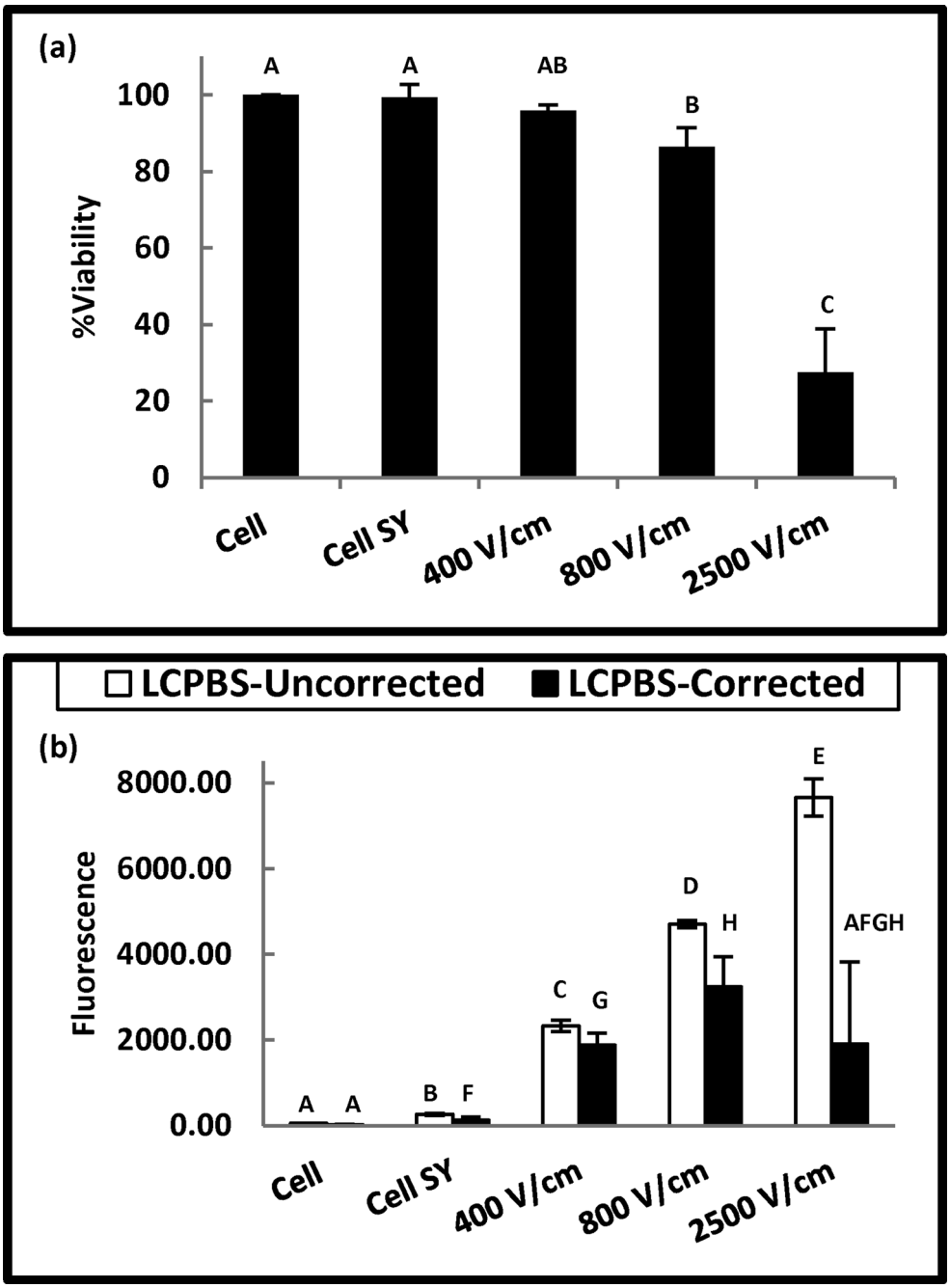


Fig. 4 **a** Viability of cells pulsed in LC-PBS at low, medium, and high field strengths. **b** Uncorrected and corrected fluorescence of cells pulsed in LC-PBS at low, medium, and high field strength (Sy = SYTOX®-green). Letters shared in common indicate no significant difference. Means with different letters are significantly different. For example, two groups that both possess the letter A indicate a p-value>0.05 between those two groups and for those that possess different letters such as A and B denote a p-value<0.05 between those two groups

repectively. The largest decrease in cell viability (76 %) was observed at the highest field strength (2500 V/cm). The data presented in Fig. 4a indicates that the use of low conductivity media resulted in better viability for cells pulsed at low and medium field strengths.

Figure 4b also illustrates the uncorrected and corrected fluorescence data associated with cells pulsed in LC-PBS. The unpulsed cells showed little to no fluorescence for both uncorrected (open bars) and corrected (filled bars) data, before and after SYTOX®-green addition. The open bars demonstrate a fluorescence magnitude increase by 9, 18, and 29-fold after field strengths of 400, 800, and 2500 V/cm were applied, respectively. This trend was identical to the one shown in Fig. 3b for cells pulsed in PBS. From the uncorrected plot it could be erroneously concluded that increase in the field strength leads always to increase in the dye delivery across the cell membrane. When analyzing the corrected data it was observed that this trend was only partially true. The filled bars for 400 (15-fold increase) and 800 V/cm (26-fold increase) show that there was an increase in fluorescence magnitude, but at 2500 V/cm

the increase was the same as that at the lower field strengths. This finding demonstrated that beyond a threshold, higher field strengths resulted in a level of cell death too high to effectively deliver the dye. Figure 4b also illustrates the effect of changing media on the magnitude of fluorescence. When comparing the corrected fluorescence values in LC-PBS (Fig. 4b) to PBS (Fig. 3b) the magnitudes in the low conductivity media were amplified by 22, 34, and 3-fold at 400, 800, and 2500 V/cm, respectively. From these results, it can be concluded that the dye delivery by electroporation in LC-PBS was greater than in PBS.

Conclusion

The technique demonstrated in this study, unlike others in published literature, is a simple non-counting method that relies on a single dye and a single instrument. This assay improves data collection and interpretation for optimizing electrical as well as other parameters for effective electroporation/molecular delivery. Data are adjusted using the fluorescence from non-viable cells that were lysed in the presence of the dye. Using murine melanoma (B16-F10) cells, it was demonstrated that the fluorescent data reflects inaccurate trends when the fluorescence from non-viable cells was not taken into account. Applying the compensation assay to ascertain the effects of media conductivity on SYTOX®-green delivery allowed a more accurate interpretation of the media properties. Corrected data showed that by using a low conductivity medium 10 to 30-fold dye delivery increase was possible at all field strengths tested. This trend was more difficult to identify in the uncorrected data and underscores the value of the fluorometric technique reported in this paper.

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