New sonotransfer evaluation method using fluorescent dye ethidium bromide and image processing program ImageJ

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Introduction. Ultrasound (US) application, in the presence of microbubbles (MB) in extracellular medium enables various exogenous molecules transfer into the cell [1, 2]. The leading hypothesis states that this is achieved when temporal pores are formed in the cell membrane [3]. However, sonotransfer mechanisms are not yet fully understood. Therefore sonoporation requires large sample volumes (1 - 2 ml of cell suspension) and thus higher amounts of poration-reporter molecules (fluorescent dyes or plasmids). This makes sonoporation experiments expensive and therefore requires development of new, more practical methods for evaluation of sonoporation efficiency [4].

In this paper we adapted low cost fluorescent dye ethidium bromide (EB) to the sonoporation experiments. Fluorescent microscopy images were processed with open source program ImageJ to enhance fluorescent cell visualisation. Moreover, ImageJ allowed us to measure the relative amount of EB present inside of the cell. Obtained results show 22% successful EB reversible sonotransfer to cells with particular US and microbubble concentration.

Methods. Chinese Hamster ovary cells (CHO) were used for experiments. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich). Experiments were made also in DMEM medium supplemented with 10% FBS and 1% antibiotics. Final cell concentration during the experiment was 330000 cells per millilitre.

Microbubbles (MB) used in experiments were made in our laboratory: 2 mg/ml polyethyleneglycol-40 stearate (Sigma-Aldrich) and 2 mg/ml DL-alpha-phosphatidylcholine (Sigma-Aldrich) were dissolved in 1 ml sterile 0.9 % NaCl solution, which was saturated by 5 ml sulphur hexafluoride gas (Linde Gas AGA, Sundbyberg, Sweden), mixed and sonicated with 20 kHz power output 150 W for 30 s and 300 W for 30 s. MB concentration at the beginning of experiment was 2×10^7 MB/mL. The cell and MB ratio used in experiments was 1:60. Fluorescent dye EB (final concentration 250 μM) was used for sonotransfer.

Pulsed wave US was achieved with 1 MHz unfocused 19 mm diameter transducer (UAB Medelkom, Lithuania). Signal was made using computerised US pulser-receiver USBox SX (Lecoeur Electronique, Chuelles, France). Pulse delivered 300 kPa peak negative pressures at 1% duty cycle for 1 s.

After US application cells were incubated for 15 minutes at room temperature. Afterwards cells were centrifuged and supernatant was removed. Cells were suspended in 40 µl DMEM medium supplemented with 10% of FBS and 1% of penicillin-streptomycin. Images were taken with fluorescent microscope Motic AE31. Texas Red® / Cy3.5™ filters for fluorescence imaging were used.

Used software. Cell images were processed with ImageJ software. It is open source program created by National Institute of Health by an employee of the Federal Government of United States. One of the functions, which program can perform, is background subtractions, which is based on "rolling ball" algorithm [5]. It removes smooth continuous background from the image. In this way the ability to detect fluorescent cells is greatly enhanced (see Fig. 1).

ImageJ also allows calculating mean grey value (the mean of all pixel values) in outlined areas. Consequently, selected fluorescent cells integrated density (IntDen) can be obtained by multiplying measured mean grey value (MGV) to outlined cell area. Knowing background mean grey value (BMGV) a Correlated Total Cell Fluorescence (CTCF) can be found by using formula (1):

$$CTCF = IntDen - (Area\ of\ selected\ cell \times BMGV). \quad (1)$$

CTCF corresponds to a relative unit that can be used to quantify cell fluorescence. With more EB delivery through cell membrane, fluorescence intensity inside the cell is increased allowing accurate quantification of EB sonotransfer.

Results. In order to enhance fluorescence visibility, initial experiments were made to obtain good resolution visible and fluorescent light cell images and to process fluorescent images with ImageJ software. To the best of our knowledge, there are no clear protocols using EB as a fluorescent dye in sonoporation experiments.

Obtained example from sonotransfer results using EB is represented in Fig. 1. When clear fluorescence image was obtained using ImageJ program (see Fig. 1 C), CTCF measurements divided all cells in two populations. One was high fluorescence intensity group (HFG), other - low fluorescence intensity group (LFG). Example of each group is shown in Fig. 1 C. Analysing images taken with visible light also revealed two groups. First group was killed cells (see Fig. 1 A, filled arrow). Second group was healthy appearing cells (see Fig. 1 A, open arrow). Surprisingly, all killed cells fell into HFG group. Correspondingly healthy appearing cells fell into LFG group. This match can give the assumption that HFG corresponds to killed cells and LFG corresponds to viable, EB positive (sonopotransferred) cells. Without fluorescent image being processed with ImageJ software, LFG can hardly be seen and mostly would not be counted (compare Fig. 1 B and C images). This is why LFG, which are the cells wanted to be measured, mainly disappears from results.

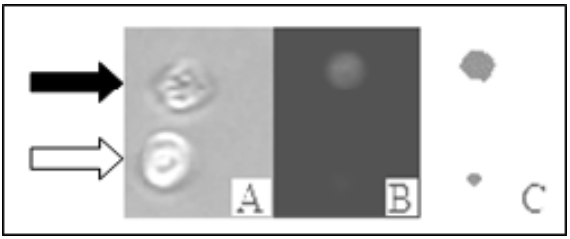

Fig. 1. Killed and viable cell visualisation with light and fluorescent microscopy after cell sonoporation. Panel **A** shows cell visualisation under light microscope. Filled arrow indicates dead cell, open arrow indicates reversibly sonoporated cells. Panel **B** represents fluorescent microscopy image, demonstrating EB sonotransfer into the cell. Panel **C** is represent image shown in panel **B** after processing with ImageJ software. EB was used as a fluorescent dye. Sonoporation was induced with US at 1 MHz, 1% duty cycle, 300 kPa acoustic pressures for 1 s. Measurements were made at least 20 minutes after sonoporation.

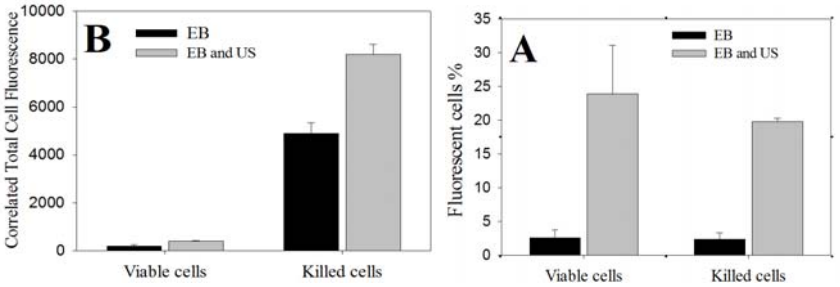

Fig. 2. Percentage of EB positive (fluorescent) cell A) and CTCF in viable and killed cell populations before and after US application. EB at 250 µM final concentration was used as fluorescent dye. Sonoporation was induced with US at 1 MHz, 1% duty cycle, 300 kPa acoustic pressures for 1 s. Measurements were made at least 20 minutes after US application. Each experiment point was repeated at least 3 times. Errors bars represent standard error of mean.

Further experiments were performed to evaluate whether a new method can be applied to find out the percentage of killed and sonoporated cells. To find out percentage of reversibly sonoporated cells, we subtracted viable fluorescent cells before US application from those after US application. This resulted reversible sonoporation at used condition reached about 22% (Fig. 2A). Similarly to evaluate a percentage of killed cells due to US delivery (irreversible sonoporation) we subtracted a low percentage of dead cells in control from those obtained after US. Thereby US application resulted in about 17% irreversibly sonoporated cells. (Fig. 2 A).US application resulted in twice higher CTCF in comparison to untreated by US. CTCF corresponds to EB relative quantity inside the cell after background subtraction (Fig. 2 B).

Conclusions. Fluorescent dye EB as a low cost fluorescent dye and an open source program ImageJ properties were successfully adapted to evaluate

sonoporation efficiency. ImageJ properties allowed to enhance fluorescent microscopy images enabling to visualise cells with low fluorescence intensity. Therefore, accuracy of evaluated EB sonotransfer effectiveness can be greatly increased.

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Ultrasound (US) application to cells in the presence of microbubbles can induce cell sonoporation. During this process exogenous molecules can enter (be sonotransfered) into permeabilised cells. Molecule sonotransfer experiments require considerable amounts of fluorescent dye that greatly increases experiment cost. This shows that new and more practical methods for evaluation of sonoporation efficiency are needed. In this study we adapted low cost fluorescent dye ethidium bromide (EB) and an open source ImageJ software to quantify sonoporation efficiency ImageJ properties allowed enhancing fluorescent microscopy images enabling to visualise cells with low fluorescence intensity. Therefore, accuracy of evaluated EB sonotransfer effectiveness can be greatly increased.