

Development of a confocal ultrasound device using an inertial cavitation control for transfection *in-vitro*.

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Abstract. Sonoporation using low-frequency high-pressure ultrasound (US) is a non-viral approach for *in vitro* and *in vivo* gene delivery. We developed a new sonoporation device designed for spatial and temporal control of ultrasound cavitation. This device was evaluated for the *in vitro* transfection efficiency of a plasmid coding for Green Fluorescent Protein (peGFP-C1) in adherent and non-adherent cell lines. The frequency spectrum of the signal received by a hydrophone is used to compute a cavitation index (CI) representative of the inertial cavitation activity. The influence of the CI on transfection efficiency, as well as reproducibility were determined. A real-time feedback loop control on CI was integrated in the process to regulate the cavitation level during sonoporation. In both adherent and non-adherent cell lines, the sonoporation device produced a highly efficient transfection of peGFP-C1 (40-80%), as determined by flow cytometry analysis of GFP expression, along with a low rate of mortality assessed by propidium iodide staining. Moreover, the sonoporation of non-adherent cell lines Jurkat and K562 was found to be equivalent to nucleofection in terms of efficiency and toxicity while these two cell lines were resistant to transfection with lipofection.

Introduction

Transfection is a widely used tool for the analysis of the role of a target gene, determination of its function, identification of regulatory sequences controlling its expression as well as for the demonstration of biological processes involved in molecular interactions causing diseases. Depending on the cell type, different techniques of nucleic acid transfection into eukaryotic cells can be used. Viral vectors provide the most efficient means for *in vivo* and *in vitro* gene delivery. However, the use of viral vectors carries significant biological risks and difficulty of vector production [1]. Chemical methods using natural or synthetic carriers to deliver genes into cells are safe, effective for transfection of adherent cell lines but suffer from their low efficiency in the case of non-adherent cell lines as well as *in vivo* [2]. Physical methods for gene delivery such as micro-injection, electroporation, gene gun and ultrasound-mediated methods have been the object of considerable research during the last decade. Physical methods involve the use of physical force to increase the permeability of the cell membrane, allowing genetic materials and therapeutic molecules to enter into the cell [3]. Among them ultrasound-

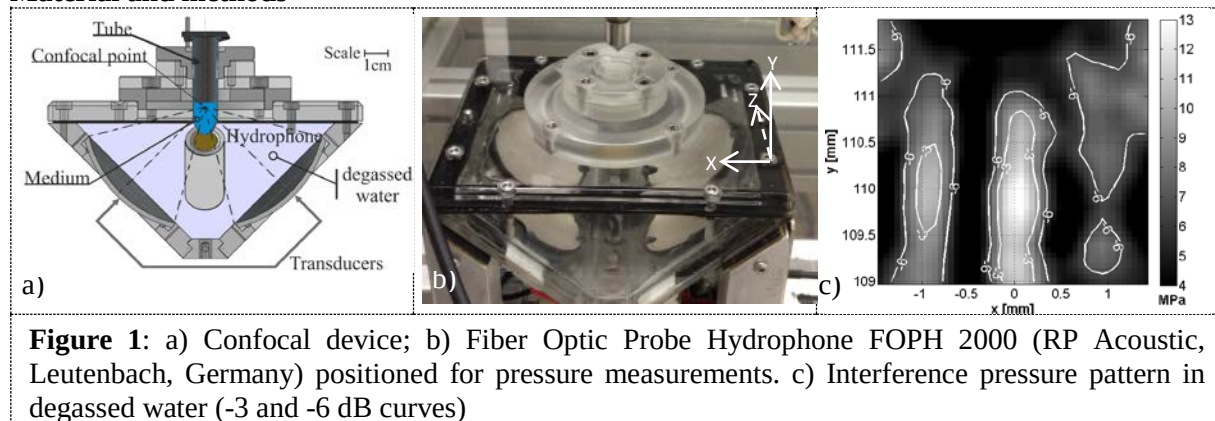
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induced biological effects are commonly assumed to be caused by acoustic cavitation which results in the creation of transient holes in the cell membrane (sonoporation).

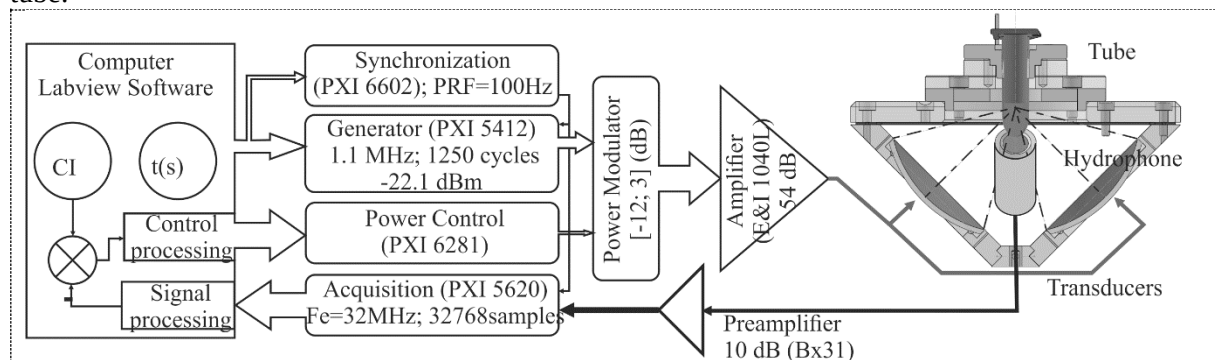
The objective of this work is to develop a device to transfect with increased efficiency and acceptable viability in a reproducible manner. The device should work without adjuvant or contrast agent and should be adapted to routine use in research laboratories.

Material and methods



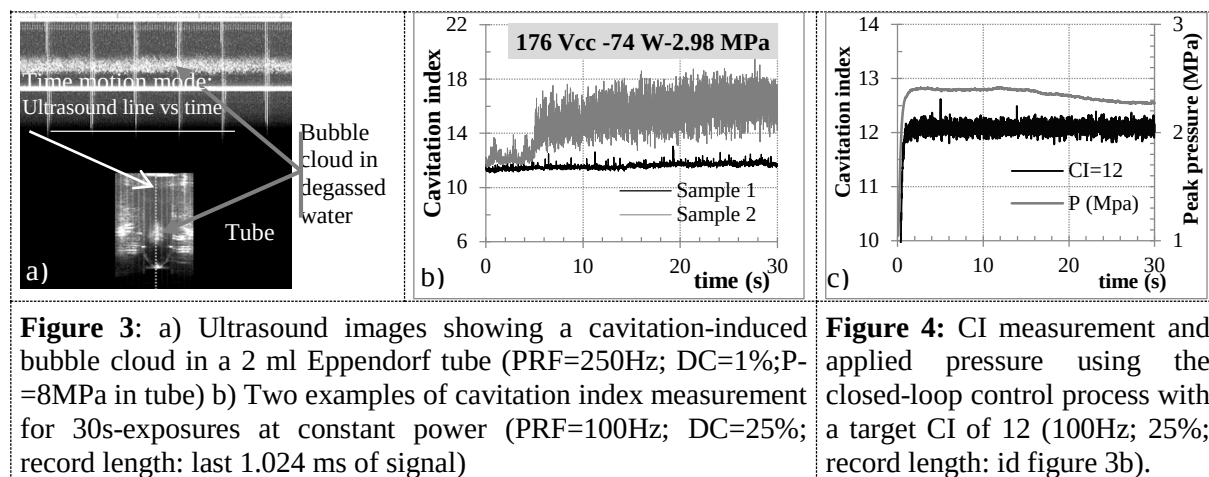
The ultrasonic set-up is based on two focused transducers PZ28 (Ferroperm, Kvistgaard, Denmark), operated at the frequency of 1.1 MHz. The diameter and the radius of curvature of these transducers were 50 mm. The transducers were positioned in a confocal manner with a 90° angle between the acoustic axes (figure 1(a)). Due to the interference between the two ultrasound beams, the pressure field presents a pattern around focus that traps the bubbles of various size on the pressure nodes and antinodes (figure 1(b-c)). The bubble cloud in a 2 ml Eppendorf tube filled of water is therefore precisely localized as presented by ultrasound imaging (figure 3(a)); Pulse Repetition Frequency PRF = 250Hz, Duty cycle DC = 1%, Peak negative pressure $P_- = 8\text{MPa}$).

For transfection, longer pulse are applied to the culture medium (650 μl ; PRF = 100Hz; DC = 25%). The placement of the 2-mL tube and the volume of fluid are fixed so that the focal point is situated on the symmetrical axis of the tube 2 mm below the air medium interface (figures 1(a), 2). The coupling media between the transducers and the tube is degassed water (O_2 concentration <3 mg/L). This setup is appropriate for easily initiating cavitation with no more than 4 MPa peak negative pressure inside the tube.



To characterize the inertial cavitation phenomenon, we have chosen to study the evolution of the broadband noise induced by the bubble collapse. A hydrophone is used to record the signal back-scattered by the cavitating bubbles. A cavitation index (CI) is determined by computing the mean of this signal frequency spectrum in dB in the frequency range 0.1 to 7.1 MHz [4]. To this value a reference

value representative of the electronic noise (~ 93 dB measured when the ultrasound power is switched off) is removed.



The signal applied to the transducers is first generated numerically before being amplified by a voltage-gain controlled pre-amplifier. This pre-amplifier has a short response time and allows a feedback on its amplification gain at each burst (up to 250 Hz). Since the inertial cavitation activity can evolved differently for the same excitation parameters (figure 3(b)) a LabVIEW-based program computes after each burst a new gain using the past CIs and the target CI and the pre-amplifier gain is updated. The pre-amplified signal is then fed to a RF power amplifier (E&I 1040L Electronics & innovation Ltd, 400W, 54dB) and applied to the transducers. This closed control loop provides a tighter control of the inertial cavitation activity (figure 4). The electrical peak power ranges from 3 to 100 W corresponding to a peak pressure up to 8 MPa in water and from 0.4 to 3.5 MPa inside the tube.

To demonstrate that the set-up is able to maintain a constant inertial cavitation activity experiments were performed with a chemical dosimeter of inertial cavitation [5]. Acoustic cavitation generates free radicals from the breakdown of water molecules. The initial step of water decomposition is the production of hydroxyl and hydrogen radicals. It has been shown that terephthalic acid (TA) [benzene-1, 4-dicarboxylic acid] is suitable for detecting and quantifying free hydroxyl radicals ($\text{OH}\bullet$) generated by ultrasound-induced inertial cavitation. During this process, the TA solution (20 mM of TA in PBS) reacts with $\text{OH}\bullet$ generated through water sonolysis to produce 2-hydroxyterephthalic acid (HTA that can be detected using spectroscopy with excitation and emission wavelengths at 318 and 426 nm, respectively).

As the non-adherent cell lines are considered hard to transfect we proposed to restrict the study presentation to only 3 non-adherent cell lines: K562 (human chronic myelogenous leukemia), Jurkat (T-lymphoblastoid), RL (human follicular lymphoma) and 1 adherent cell line BT474 (human breast tumour). Prior to treatment, non-adherent or trypsinized adherent cell lines were harvested and prepared for sonoporation (650 μl in 2 mL tube containing $10 \cdot 10^6$ cells/ml in Opti-MEM® medium with 10 μg of peGFP-C1 plasmid) or for nucleofection using a procedure according to the manufacturer's recommendations (Nucleofector® Lonza, Basel, Switzerland; $10 \cdot 10^6$ cells/ml in 100 μl solution with 2 μg of peGFP-C1 plasmid). 24 hours post-treatment the transfection efficiency and cell viability were evaluated by flow cytometry after propidium iodide (Invitrogen Carlsbad, CA, USA) staining. For sonoporation 2 or 3 experiments in triplicate were analysed.

Results and discussion

Figure 5 shows that the cavitation index is positively correlated with the chemical dosimeter and is representative of the inertial cavitation activity. The saturation observed for CI upper than 14 is due to a strong mechanical agitation of the liquid medium. In this case the liquid is projected by the ultrasound radiation forces and the $\text{OH}\bullet$ production by inertial cavitation seems to be altered. This mechanical

agitation was also observed for the open loop in figure 3b. To complete these observations cells did not supported this regimen and were all destroyed after 30s treatment. Figures 6 and 7 show that the device is able to transfect adherent and non-adherent cells with an acceptable level of efficiency. They also show a correlation between the CI, the transfection efficiency, and the viability. Finally, Figure 8 shows that the device efficiency is comparable to what is attainable using nucleofection on two cell lines. Notice that nucleofection is known as one of the most effective non-viral methods for *in vitro* gene delivery [6, 7].

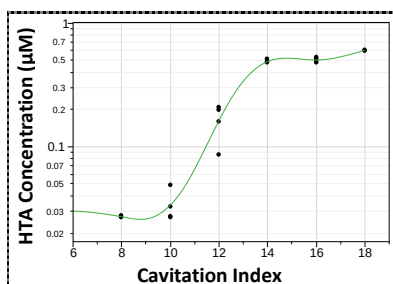


Figure 5: HTA concentration vs CI on 650μl of 20mM TA solution sonicated during 60s

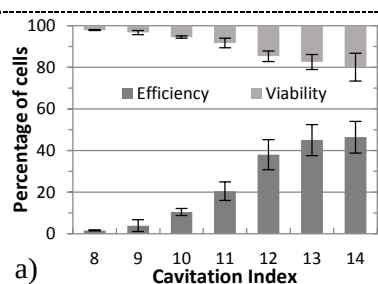


Figure 6: Transfection efficiency and viability vs CI of (a) Jurkat and (b) K562 cell lines 24h after ultrasound treatment (30s). The error bars correspond to standard deviations

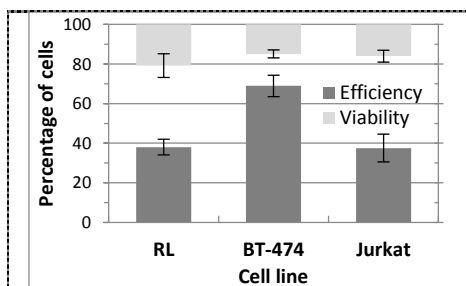
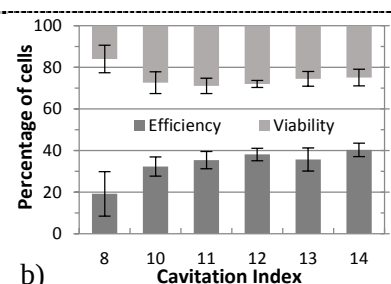


Figure 7: Transfection efficiency and viability for CI=12 during 30s for 3 cell lines

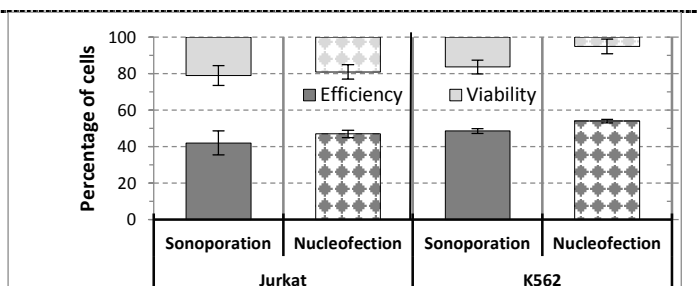


Figure 8: Comparison of sonoporation method with nucleofection on 2 non-adherent cell lines hard to transfect

Conclusion

We developed a user-friendly and cost-effective ultrasound device, well adapted for routine *in vitro* high-yield transfection experiments and which does not require the use of any transfection reagent or gas micro-bubbles. It provides a well-adapted method for low cost routine pDNA *in vitro* delivery for both adherent and non-adherent cell lines. Our results confirm ultrasound as an alternative non-viral technology for the efficient transient transfection of a wide range of different cells including non-adherent cells or fresh human cells, and the preparation of stably transfected cells.

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