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Localized Sonoporation: In-Situ Disruption of Actin Cytoskeleton at Single-Cell Level

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1. Introduction

As a new way of perforating the cell membrane, sonoporation (i.e. generation of membrane pores using ultrasonic means) has attracted a lot of enthusiasm among researchers in the drug and gene delivery field. This approach generally works by exploiting the acoustic cavitation mechanism in which microbubbles are first introduced nearby cells to serve as cavitation nuclei, and then the cavitational forces needed for membrane puncturing are generated by sonicating the microbubbles to induce cavitation. Sonoporation is fundamentally different from other methods like electroporation. Its uniqueness lies in the ability for ultrasound to be delivered remotely to the site of interest using mature beam focusing principles in acoustics.

Despite its technical advantages, sonoporation is not yet mature for routine application in drug and gene delivery. What is critically missing is not a demonstration of whether sonoporation can directly facilitate internalization of exogenous substances (numerous reports are already available on this aspect), but specialized investigations that focus on unraveling the biophysical dynamics pertinent to this poration method, particularly at a single-cell level. Unless these biophysical details are revealed, considering the diligent use of sonoporation in biomedicine is candidly out of the question.

2. Statement of Contribution

Our team has made new scientific discoveries on the cytomechanical impact of sonoporation by investigating the dynamics of actin cytoskeleton: a subcellular filamentary net physically connected to the plasma membrane[1]. The following questions, unanswered hitherto, have been addressed:

1) What is the structural impact of sonoporation on the actin cytoskeleton?
2) If actin structure is indeed perturbed by sonoporation, is the impact local or global?

All our findings were synchronized with the onset of localized sonoporation at a single-cell level. This ensures that our findings are directly linked to the time course of the sonoporation process.

3. Materials and Methods

The experimental protocol used in this study is illustrated in the timeline shown in **Fig. 1**. Each of the stages will be briefly described as follows.

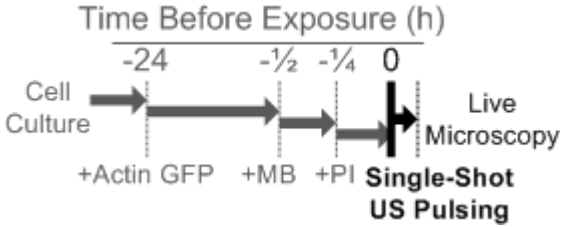

Fig. 1 Timeline of experimental protocol on live imaging of sonoporation-induced actin disruption.

Cell Culture: Using an incubator with 37°C operating temperature and 5% carbon dioxide, we first cultured the cells needed for our research. The cell line model that we used was ZR-75-30 human breast carcinoma cells (ATCC). Their membrane deformability is higher than normal cells, so they tend to favor swift recovery whenever plasma membrane disruption occurs. This is a suitable characteristic for our investigation, as we wish to avoid intrinsic factors that impede cells from recovery after a sonoporation episode. The culture medium that we used was RPMI 1640 (Sigma-Aldrich); 10% FBS (ATCC) was added as supplements during culturing.

Transfer to Cell Chamber: Once cells reached exponential growth phase, they were transferred to a custom-made cartridge-shaped cell chamber that resembled a cartridge structure with a glass cover slip as the bottom surface. There were 10,000 cells seeded onto the cover slip in each experiment. 24 h period was given to allow the transferred cells to form a monolayer on the cover slip.

Labeling of Actin Cytoskeleton: The cells on the cover slip were transfected with 2 μl of CellLight actin-GFP (Invitrogen). Transfection time was 24 h, and it took place in a dark environment. Afterward, the cells were washed with PBS once.

Use of Targeted Microbubbles: To achieve site-specific sonoporation on a single-cell level, we

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fabricated targeted microbubbles (1-4 μm in diameter) and used them in this work. Binding preference was tagged to VEGF receptors on the membrane of the ZR-75-30 cells. Shell material was a lipid composite with DSPC, PEG stearate, and DSPE; gas core was C_3F_8 gas. Microbubbles were added in suspension form, and 5 min time was given to allow them to attach to the membrane. Unattached ones were washed away afterward. With this procedure, we achieved single-bubble binding per cell.

Sonoporation Tracer: PI (Sigma-Aldrich) was used as the sonoporation tracer. It was added to the cell chamber before exposure. Note that PI is a suitable indicator for sonoporation[2] because it is naturally impermeant through the membrane unless there is a disruption of membrane integrity. As long as viability is maintained, sonoporated cells would exhibit strong PI fluorescence, while unsonoporated ones would be PI negative.

Live Microscopy: To monitor sonoporation dynamics in real-time and at single-cell level, the dye-loaded and microbubble-bound cells within the cell chamber were placed onto the scan stage of a confocal microscopy system. Microbubble binding to cells can be readily observed in bright-field mode. The fluorescence levels of actin-GFP and PI were traced using wavelength settings suggested by the dye vendor, and the monitoring was conducted in-situ before and after instigation of sonoporation that was initiated by single-shot ultrasound pulsing.

Ultrasound Exposure: Ultrasound was delivered to the cell chamber via a single-element transducer with 1 MHz frequency and 1" diameter. The probe was mounted onto a special waveguide that was at 40° mounting angle and 70 mm away from the cell chamber. Hydrophone calibrations showed that the in-situ peak negative pressure level was 0.45 MPa (i.e. the pressure magnitude over the microscope's field of view). A single shot of ultrasound pulse with 30 cycles was applied to trigger microbubble collapse and in turn instigate sonoporation.

4. Experimental Results

Using our experimental protocol, we achieved local instigation of sonoporation on a single-site basis. This was induced by the collapse of a single microbubble, whose pre-exposure position is shown in the merged fluorescence image (bright field + PI + actin-GFP) given in **Fig. 2a**. Note that, before exposure, no PI was within the cell as expected. Direct indication of sonoporation can be deduced from the PI entry into the cell after the application of ultrasound pulse (see **Fig. 2c**). More strikingly, at the same time, the actin cytoskeleton underwent structural snapping. Comparing between **Fig. 2b** and **Fig. 2d**, the main cables of the actin network adjacent to the sonoporation site (dashed circle) were destroyed after sonoporation. In addition, disassembly of actin cables away from the site was found to take place in tandem.

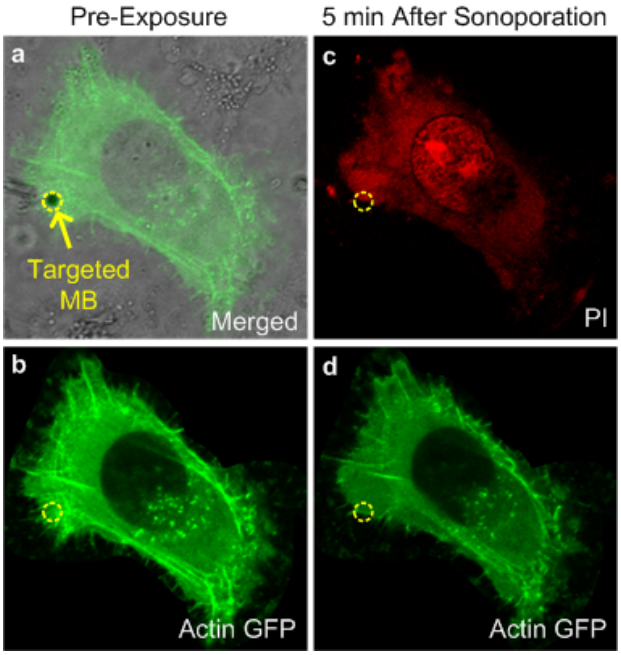

Fig. 2 Example of sonoporation-induced actin network disruption at single cell level. Pre-exposure images shown for: (a) Merged (bright field + actin GFP + PI), with position of the targeted MB highlighted; (b) actin-GFP. After sonoporation is induced for 5 min: (c) PI influx is evident; (d) loss of actin-GFP cables can be observed.

5. Discussion

These findings are of scientific importance in that they provide direct evidence to show that the biophysical impact of sonoporation is not limited to membrane-level perforation. Indeed, they point to the fact that mechanical disruption of subcellular structures, such as the actin cytoskeleton, would take place as well. Further findings and statistical insight will be presented at the meeting.

Acknowledgement

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References

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