Visualization of intracellular calcium transport between cells using high frequency ultrasound and FRET live-cell imaging

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***Abstract*— The live visualization with fast kinetics of the interaction between cells has been long term challenges because of the lack of efficient stimulation methods. We propose an approach to achieve single cell resolution stimulation and FRET-base calcium live cell imaging to visualize fast kinetics of calcium transport between physically connect neighboring cells. Chemical stimulation stimulates cells within a dish at the same time and is not suitable for the study of cell-cell interaction. We replaced chemical stimulation with ultrasound-based mechanical stimulation approach to provide precise spatiotemporal resolution. To achieve this, we integrated 3D translation stages and epi-fluorescence microscope and a developed 150 MHz high frequency ultrasound with f_{number} of 1 and aperture size of 1 mm. The 150 MHz transducer can focus within 10 micrometers in diameter and directly stimulate cells by disturbing cell plasma membranes without microbubbles. High frequency stimulation was used to introduce calcium ions into cytoplasm of cells. Results demonstrate calcium transport between cells, visualized by FRET calcium biosensor after only one cell was stimulated by the developed high frequency ultrasonic transducer.**

Keywords — Calcium transport, high frequency ultrasound, single cell stimulation, FRET biosensor

I. INTRODUCTION

Intracellular and intercellular molecular events of cells are closely related to disease progress and provide understanding of fundamental mechanism how external stimulations are transduced into certain genetic expression in cells[1-3]. Fluorescence resonance energy transfer (FRET) based imaging has revolutionized biology and biochemistry by providing live cell monitoring of molecular signals with precise spatiotemporal resolutions [4]. Calcium ions are strictly regulated in cells to maintain consistency and important molecules to identify phenotype changes of cells that predicts cell differentiation and death[5]. In FRET-based calcium biosensor (FRET-CA), FRET changes between enhanced cyan fluorescent proteins (ECFP)[6] and enhanced version of yellow fluorescent proteins (YPet)[7] fluorophores indicate the changes of the intracellular concentration of calcium. This FRET-CA has swift FRET kinetics and high efficiency, making this biosensor suitable to monitor fast and small changes of intracellular calcium concentration [8]. One way of observing cell response using FRET is a chemical stimulation by using binding molecules to the receptor of a specific signaling pathway. Chemical stimulation is suitable for high throughput screening and generally useful to test the performance of FRET biosensors. However, the chemical stimulation is not suitable to study cell-cell interactions because chemical in a dish stimulates all cells at the same time. We have developed an innovative single cell stimulation technique using high frequency ultrasound to study cell-cell interaction at single cell resolution [9, 10]. A ultrasonic transducer with the center frequency of 150 MHz has the focal area of 10 μm, which is smaller than normal cells. The acoustic energy at the focal area of 150 MHz transducer is enough to directly disrupt cell plasma member to induce calcium influx of cell cytoplasm without any help of microbubbles [3, 9, 10].

In this study, HFUS stimulation system was developed by combining epi-fluorescence microscope and 3D translation stage (Fig. 1). We hypothesize that calcium may be transported from one cell to another through intracellular communication, which can be visualized by FRET-CA and single cell stimulation using a high frequency ultrasound (HFUS). We stimulate one cell with HFUS and monitor FRET-CA changes to observe calcium transport between cell, physically connected with each other.

II. MATERIALS AND METHODS

A. Cell Culture and reagents

Hela cells from ATCC were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine, serum (FBS). A humidified 95% air, 5% CO2 incubator at 37°C was used for cell incubation. The transfection of FRET-CA was performed with the lipofectamine 3000 (Thermo Fisher Scientific). Transfection was processed according to the manufacturer's instructions. The medium was refreshed 18 hours after transfection. Then, cells were seeded into fibronectin coated glass-bottom dishes for live cell imaging. Cells were cultured for another 18 hours after seeding on the fibronectin-coated dishes to provide time to fully express FRET-CA.

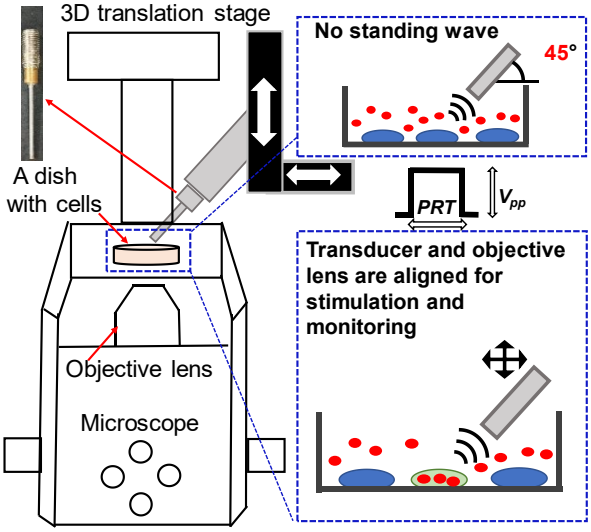

Figure 1. Epi-fluorescence microscope and 150 MHz ultrasonic transducer were integrated to stimulate single cells and monitor FRET changes. The high frequency ultrasound transducer has the center frequency of 150 MHz and f_{number} of 1 with an aperture of 1mm. To avoid standing waves between the transducer and the bottom of a cell culture dish, the transducer was fixed to the 3D translation stage with 45°. V_{pp} and PRT are peak-to-peak voltage and pulse repetition time to stimulate a single cell.

B. High Frequency Ultrasound Transducer

A 150 MHz press focused lithium niobite ($LiNbO_3$) transducer with a f_{number} of 1 and an aperture size of 1 mm was fabricated (Fig. 1). The transducer consists of 10 µm $LiNbO_3$ layer with 2 µm of parylene coating. Electrode was sputtered on $LiNbO_3$ with chrome and gold (50 nm and 100 nm respectively). Pulse/echo and axial and lateral resolution of the developed transducer was measured (Fig. 2). As theoretical calculation predicts, lateral resolution is 10 µm and axial resolution is 8.5 µm. Point spread function of the transducer is symmetric and well-shaped as shown in Figure 2(b).

C. Imaging acquistion and stimulation system

We used integrated system between microscope and high frequency ultrasonic transducer as shown in Fig. 1. The transducer was attached and controlled by 3D translation stages. To prevent standing waves, the transducer was positioned at an angle of 45° to the glass bottom dish. Prior to the experiment, the focus of the microscope and the transducer were aligned. The transducer emitted ultrasound pulses for 1 minute with a pulse repetition time (PRT) of 1 millisecond with 5% duty factor, and peak-to-peak voltage (V_{pp}) of 21 V for stimulation. Images were collected with the Ti2 Nikon fluorescence microscope and a cooled charge-coupled device (CCD) camera using the Nikon NIS-Elements AR software. Nikon NIS-Elements AR was used to calculate the pixel-by-pixel ratio images of FRET-YPet over ECFP after subtracting basal fluorescence level. Statistical analyses of FRET change was calculated. The filter setting CY for FRET-CA consisted of CFP EX 440/20 nm and EM 480/40 nm, and FRET-YPet EX 440/20 nm and EM 535/30 nm combined with dichroic mirror 455 nm. FRET-YPet/CFP emission ratio were acquired to take the ratio between FRET-YPet and ECFP emission.

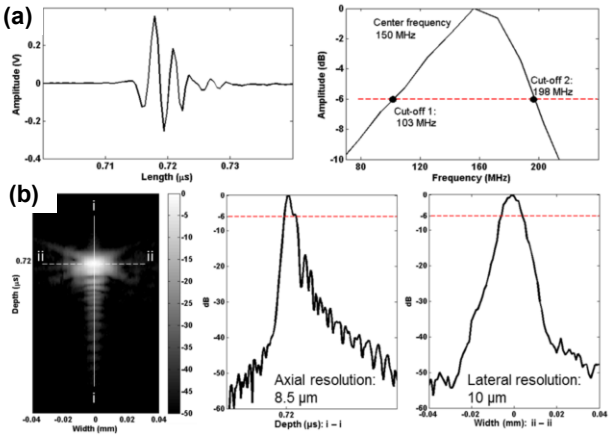

Figure 2. Pulse/echo and resolution measurements of 150 MHz ultrasonic transducer used in this study. (a) Time domain and frequency response of echo. (b) axial and lateral resolution of the transducer by scamming 4 µm tungsten wire.

III. RESULTS

The stimulation using HFUS was performed to examine cellular interaction between connected cells at single cell resolution. The FRET ratio of FRET-CA biosensor was taken under the filter setting CY. The targeted cell by HFUS before the stimulation (t= 0 sec) (white arrow) and regions of interest (areas 1-3) are shown in Figure 3(a). The calcium transport from the stimulated cell to the adjacent cell is demonstrated in Figs. 3(b-d). Figure 3(e) shows cells after stimulation (t= 4 min). The FRET ratio images in Figures 3(b)-(d) and the time courses of FRET-YPet / ECFP ratio are plotted in Figs. 3(f). These results demonstrate that HFUS firstly stimulated the targeted cell in area 1 with single cell resolution. The increase of FRET ratio is observed between the cells in area 2 as shown in Figure 3(c). Finally, calcium transport to the neighboring cell was observed due to FRET increase in area 3 of the

neighboring cells as shown in Figure 3(d). Time course plot in Fig. 3(f) shows FRET changes of areas 1-3. FRET peak of area 3 is followed by the FRET peak of areas 1 and 2, which indicates calcium transport between two neighboring cells.

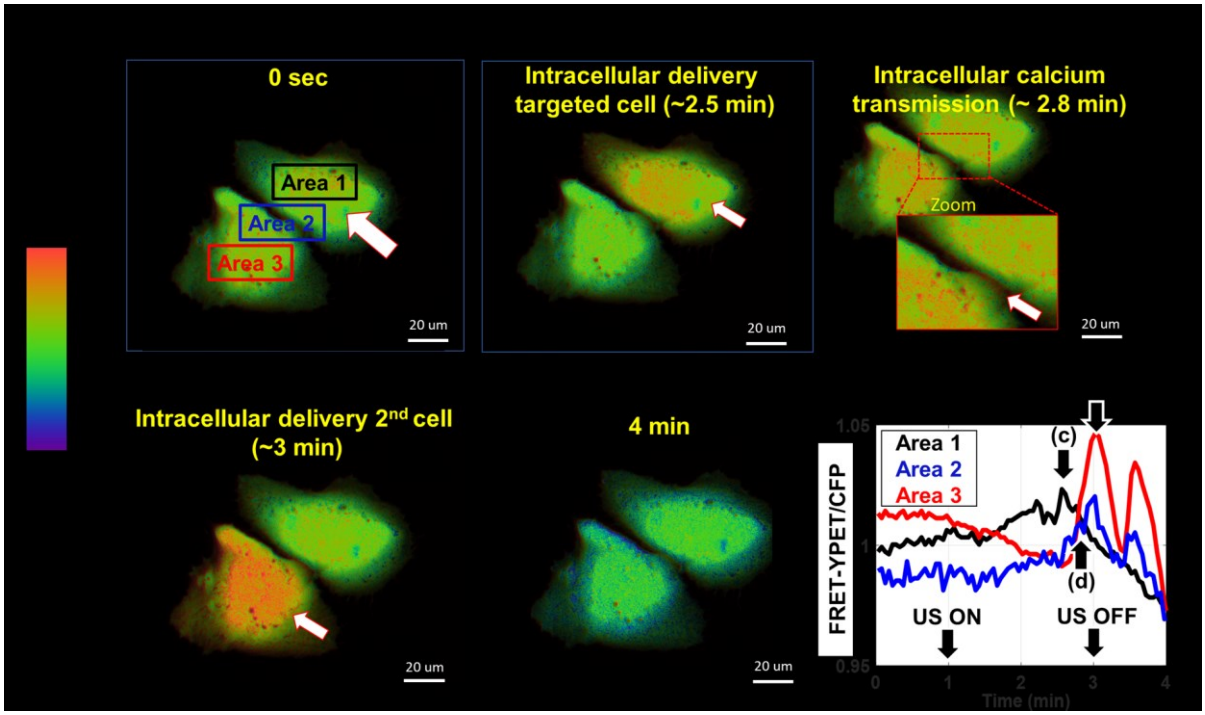

Figure 3. Calcium transmission between cells under HFUS stimulation. FRET-CA was used to visualize calcium transport (a) before and (b-d) during HFUS stimulation. The white arrow in (a) represents the targeted cell for the HFUS stimulation. (e) FRET image after the HFUS stimulation shows two cells are still in tact. (f) Time course of FRET-YPet to ECFP ratio plot represents that areas 1, 2, and 3 shows peak FRET sequentially, which indicates calcium transport from areas 1 to 3. US ON / OFF means when the HFUS was turned on and off. FRET images of (b), (c), and (d) correspond to time point at 2.5 min, 2.8 min, and 3 min in plot (f). Color bar on the left indicates low FRET as purple and high FRET as red.

IV. Conclusions

The results establish the new approach for single cell resolution intracellular stimulation system using high frequency ultrasound and FRET biosensor. This approach shows the calcium transport between neighboring cells, which may be used for cell-cell interaction study with single cell resolution.

Acknowledgment

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