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Acoustic mechanogenetics

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Abstract

From basic studies in understanding the role of signaling pathways to therapeutic applications in engineering new cellular functions, efficient and safe techniques to monitor and modulate molecular targets from cells to organs have been extensively developed. The developmental advancement of engineering devices such as microscope and ultrasonic transducers allows us to investigate biological processes at different scales. Synthetic biology has further emerged recently as a powerful platform for the development of new diagnostic and therapeutic molecular tools. The synergetic amalgamation between engineering tools and synthetic biology has rapidly become a new front in the field of bioengineering and biotechnology. In this review, ultrasound and its generated mechanical perturbation are introduced to serve as a non-invasive engineering approach and, integrated with synthetic biology, to remotely control signaling and genetic activities for the guidance of cellular functions deep inside tissue with high spatiotemporal resolutions. This ultrasound-based approach together with synthetic biology has been applied in immunotherapy, neuroscience, and gene delivery, paving the way for the development of next-generation therapeutic tools.

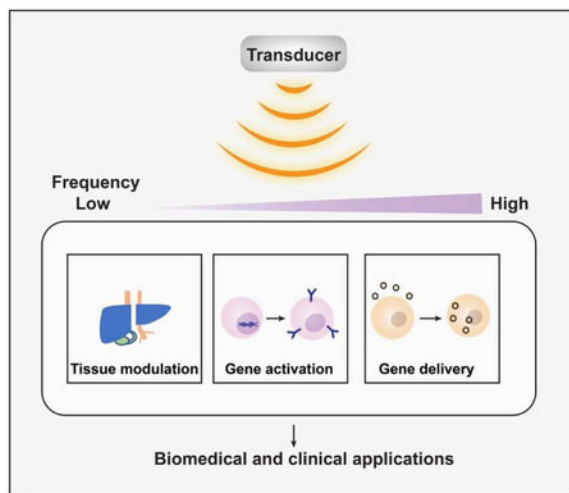
Graphic abstract

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Competing Financial Interests

The authors declare no competing financial interests.

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Keywords

Ultrasound; mechanogenetics; synthetic biology; bioengineering; biotechnology

Introduction

Ultrasound has been used for more than 50 years as a diagnosis and therapeutic tool. Ultrasound imaging using a single element, or an array transducer has long history and is widely used at research laboratories and clinics [1, 2]. An effort to use ultrasound as therapeutic tools has been surged due to non-invasive, remote, and safe characteristics that can be used to target biological objects ranging from cells to tissues [3-5].

Current methods to control genetic activities rely mainly on chemicals, radio waves, magnetics, or light [6, 7]. Chemical inducers, radio waves, and magnetic field typically lack spatial resolution, with radio and magnetic waves requiring additional signal amplification and transmission to activate genetically engineered cells. Although light or optogenetics can control genetic regulations with high precision in space and time, it cannot reach deep tissues [8]. In contrast, ultrasound waves can be safely and non-invasively focused into small volumes of tissue deep inside the body [9].

In this review, we first introduce basic definitions and general concepts as well as the mechanical effects of ultrasound. We then introduce techniques that modulate functions of biological targets from cells to organs. Applications to immunotherapy and the intracellular delivery of macromolecules using ultrasound are also presented. Lastly, future perspectives are discussed.

General principles of ultrasound and interaction with biological target

History

In the early 1940s, Karl Theodore Dussik first developed an idea to visualize cerebral ventricle using transmission ultrasound [10] while ultrasonic surgery was proposed after

recognizing the benefits of noninvasiveness using thermal ablation [11, 12]. Ultrasound hence showed a potential as a versatile source for both diagnosis and therapy. In the 1950s, Edler and Hertz developed echocardiography, with the invention of array transducers further advancing the ultrasound medical imaging field. On the therapeutic direction, thermal effect by ultrasound was the main mechanism before the cavitation effect was observed. Indeed, the cavitation was studied intensively in the 1980s with cavitation of microbubble proposed for diagnostic and therapeutic purposes [13, 14]. Since then, studies on the effects of ultrasound to biological targets from cells to animals have been conducted and have shown that ultrasound has the capability to modulate function and fate of biological targets. Low frequency ultrasound conjugated with microbubbles as an amplifier and high frequency ultrasound directly have demonstrated that noninvasive and specific control of biological targets can advance in the field of immunotherapy and drug delivery. The scale of biological targets and the types of applications can be broadly extended by tuning the properties of the ultrasound.

Sound waves

Sound waves can cause a disturbance of molecules in medium that propagates from one point to another in finite time without requiring gross movement of medium. The spectrum of sound can vary from several hertz (Hz) to several hundred mega hertz (MHz). The audible sound spectrum for human beings is generally from 20 to 20,000 Hz. Ultrasound is usually considered as the frequency ranges over 20 kHz. Medical ultrasound usually uses frequency ranges between 1 and 20 MHz for imaging purposes and below 2 MHz for therapeutic purposes as shown in Figure 1A. In research laboratories, frequency ranges over 50 MHz are often used to investigate cellular response and develop high resolution imaging device because of technical advancement of fabrication process for high frequency ultrasonic transducers [15, 16].

Ultrasound design parameters

Depending on the size of biological targets and engineering purposes, users must select the appropriate center frequency of ultrasound waves, bandwidth, intensity (power), spatial resolution. The center frequency of ultrasonic waves and bandwidth are first determined depending on engineering purposes. If the biological target is made of very lossy (high attenuation) material, low frequency ultrasound is preferred. An appropriate center frequency is required to resonate the specific size of the microbubbles. High center frequency and large bandwidth are required to obtain fine images with improved lateral and axial resolutions, respectively. Narrow bandwidth is desired to interrogate specific target regions within biological tissues. Ultrasound medical imaging does not need high intensity but needs frequent samplings with high pulse repetition frequency (PRF). Therapeutic ultrasound requires high intensity at the focal region to ablate abnormalities or break kidney stones which can be achieved by increasing the duty factor or peak-to-peak voltages (V_{pp}). Most properties are easily adjusted by using a waveform generator or a function generator (Figure 1B).

Thermal interaction

The amplitude of ultrasound waves decreases when the sound waves propagate in a lossy medium such as biological tissue. Attenuation is the main cause of the dissipation and it can be categorized by absorption and scattering. Naturally, the energy of ultrasound waves may turn into heat as the waves propagate, and ultrasound waves may change its propagation direction after the interaction with structures in a medium. As long as the rate at which heat is converted is greater than the rate at which the heat is leaving, temperature may increase due to absorption. Studies have established mathematical equations to explain thermal interactions between ultrasound waves and biological tissues, and the temperature increase can be predicted by simple equations to provide a rough estimation within a short duration of time [17, 18]. Photoacoustics can be explained by thermal and stress confinements, which are mainly due to the interaction of light with biological tissues. After thermal expansion, ultrasound waves with wide frequency spectrum propagate, which are then used to reconstruct images for photoacoustic imaging modality [19].

Non-thermal interactions

The most representative non-thermal interaction between ultrasound waves and biological tissue is the cavitation or acoustically generated cavitation [20]. US Food and Drug Administration (FDA) regulates the exposure of ultrasound energy within certain levels depending on organs. One of the regulations is Mechanical Index (MI), which explains the mechanical impact of ultrasound energy to biological tissue. The relationship between acoustic pressure and the generation of cavitation is presented previously [21]. Besides serving as ultrasound contrast agents to improve echogenicity and image contrast, microbubbles are routinely applied herein to amplify ultrasound energy for therapeutic purposes. Oscillatory motion of microbubbles under sonication may break blood-brain-barrier (BBB) or loosen connective tissues for easier drug delivery [22]. By conjugating moieties with microbubbles, targeted delivery to specific sites such as tumors and safe activation by ultrasound sonication remotely can be achieved [23]. Another therapeutic application using microbubbles and low frequency ultrasound is to enhance cell permeability. Upon exposure to ultrasound, stable or unstable inertial cavitation affects the permeability of cells, named sonoporation. This membrane alteration can be transient or sustained to cause permanent membrane disruption [24]. Many studies have shown the success of sonoporation-related intracellular delivery, although quite some of these methods suffer from high cell cytotoxicity and unreliable control of microbubbles [25, 26].

Overview of acoustic and mechanical effects on the biological system and medical applications

Tissue ablation

Tissue ablation is broadly and frequently used in clinical surgeries, especially in cancer therapy. Traditional tumor ablations, including laser ablation, radiofrequency ablation and ultrasound ablation, have been widely used in clinical treatments [27]. Among them, ultrasound ablation can achieve non-invasiveness with high spatial precision. High-intensity focused ultrasound (HIFU) can be focused at the region of interest and the absorption of the

ultrasound energy by the tissue can rapidly increase the tissue temperature and cause cell necrosis. Starting from 1990s, the thermal effect of ultrasound has been used in the clinical treatment of prostate tumor, breast tumor, uterine fibroids and many other tumors [28]. However, thermal effects usually have limited spatial resolution due to tissue inhomogeneity. Considering this, more effort has been focused on the pure mechanical ablation of tissues. In the last two decades, two approaches that use shock waves to provide non-thermal tissue ablation (called histotripsy) have been proposed [29, 30]. The first approach uses short (10-20 μ s) and intense ultrasound pulses to rapidly generate the ‘cloud bubbles’ only at the local focus in the tissue and the implosion of the bubbles can disintegrate the cells. The second approach is called the boiling histotripsy. Relatively longer and intense acoustic pulses are used to initiate ‘boiling bubbles’ within milliseconds, and the interaction of the boiling bubbles with the tissue can fragment cells into debris. Although these two methods use different principles to generate the pure mechanical effects in tissue, both methods produce lesions in the tissue[31, 32].

Ultrasound stimulation of brain activities

Ultrasound has also been applied to stimulate brain activities. Non-thermal stimulation of neuron cells in a brain circuit was first demonstrated using low intensity ultrasound by activating voltage-gated ion channels [33]. Since then, pulsed low frequency and low intensity ultrasound has been applied to modulate deep-brain function and human brain activity by stimulating brain and peripheral nerve circuits, verifying the mechanical effect of pulsed ultrasound beam on activation of neuronal membranes and mechanotransduction channels [5]. Non-invasive stimulation of cortical and hippocampal circuits, as well as the peripheral nerve circuits in human subjects, were also achieved without undesired heating effects [34-36].

Ultrasound application at molecular/cellular levels

Cells are constantly exposed to physical cues in the forms of shear stress, compression, and stretching [37]. Early applications of acoustic mechanical stimulations were mainly applied at the tissue level with innate mechanosensitivity. With the development of synthetic biology and cellular/molecular imaging tools, recent studies applied ultrasound to induce mechanical perturbation for the control of molecular signaling pathways. For example, ultrasound was used to actuate functionalized lipid microbubbles covalently attached to single mechanosensitive live cells to study cytoskeleton and RohA/ROCK signaling [38] (Figure 1C). In another study, by exogenously expressing an *E. coli*-derived mechanosensitive ion channel MscL in mammalian cells coupled with microbubbles, low frequency ultrasound was demonstrated to induce the opening of engineered mechanochannels for the calcium influx *in vitro* [39] (Figure 1C). In worm *Caenorhabditis elegans*, the low-pressure ultrasound was coupled with microbubbles to sensitize specific neurons that endogenously express the mechanosensitive channel TRP-4 and induce neuronal behavior changes [4].

The application of ultrasound to cellular engineering

Ultrasound application for mechanogenetics

One promising therapeutic application of acoustic mechanogenetics is immunotherapy. With the use of central memory T cells capable of suppressing cancer relapse for several years [40]. CAR T cell immunotherapy is transforming cancer therapy. However, major challenges remain for CAR-based immunotherapy against solid tumors. For instance, the on-target, off-tumor toxicity due to non-specific targeting of the CAR-T cells against normal/non-malignant tissues can be life-threatening [41]. There is hence an urgent need for the control of CAR-T cell activation with high spatiotemporal precision. Ultrasound could serve as a promising inducer for gene activation in clinical settings because it can be safely and non-invasively delivered into small volumes of tissue deep inside the body with high spatiotemporal resolutions [9]. Accordingly, A modular method has been developed to engineer cells with synthetic genetic circuits that can sense ultrasound stimulation. Ultrasound can hence be applied remotely and non-invasively to these engineered cells to control genetics and CAR protein expression for recognizing antigens to kill the target tumor cells [23]. This method utilizes Piezo1 ion channel as a mechanical sensor (Figure 2A), which has been identified as a component of mechanically activated (MA) cation channels [42]. With microbubbles conjugated on HEK293T cells or primary T cells, the ultrasound stimulation of Piezo1 and the consequent calcium influx can activate calcium-sensitive pathways to drive the expression of designed target genes. While continuous evolution and optimization will be required for *in vivo* and clinical applications, this study laid the basis for developing remote and non-invasive modular system for controllable cancer immunotherapy.

Intracellular delivery of macromolecules in vitro

We have developed an ultrasound technique to deliver macromolecules into target cells *in vitro* [43, 44]. Ultra-high frequency ultrasound beam can mechanically disrupt cell membrane to increase permeability of cells. Direct membrane disruption can only be achieved by ultra-high frequency ultrasound because it has a very confined focal area of smaller than the size of a single cell and enough focusing gain (Figure 2B). 150 MHz ultra-high frequency ultrasonic transducer was specifically developed for this application with a lithium niobate (LiNbO₃) single crystal. For the purposes of diagnosis and therapeutics, the function of cells can be modulated by the introduction of such exogenous molecules as DNA plasmids and recombinant proteins. Direct intracellular delivery of macromolecules using 150 MHz high frequency ultrasound was named as acoustic-transfection. Acoustic-transfection has three prominent benefits: 1) simultaneous intracellular delivery of various types of molecules ranging from nucleic acids to recombinant proteins, 2) the accumulation of molecules at target cells, and 3) single cell level selectivity (Figure 2B). These studies lay the groundwork for developing future devices for therapies involving patient-specific induced pluripotent stem cells and for deciphering intratumor heterogeneity and tumor evolution through CTC analysis.

Future Perspectives

Fluorescent proteins (FPs) and their derived biosensors, such as those based on fluorescence resonance energy transfer (FRET), have allowed the visualization of dynamic molecular activities at subcellular levels in live cells [45]. Optogenetics integrating optical and genetic methods has enabled the control of specific molecular events in living systems with high spatiotemporal resolutions [46]. The ultrasound- controllable mechanogenetics can non-invasively manipulate cells deep in the body for the control of physiological and therapeutic outcomes, hence bringing the full power of remote control of gene and cell activation to the general scientific and clinical community, similar to how fluorescent proteins and optogenetics have revolutionized live biological sensing and actuating.

By combining the orthogonal mechanogenetic and optogenetic controls, fully controllable live cells can be developed, activated or deactivated at any given time for cellular studies and therapeutic approaches. In addition, the combination of ultrasound-controllable mechanogenetics with genetic regulation tools, e.g. CRISPR and Cas9, can allow dynamic genome editing *in vivo* at any location deep in living organisms[47]. Integrated with epigenetic modulators and nuclease-deficient Cas9 (dCas9) targeting specific genome locus, this ultrasound-controllable mechano-genetics can be applied to remotely and non-invasively control the locus-specific epigenetic landscapes and endogenous gene expression profiles (Figure 3A) [48]. It is expected that each component of this ultrasound-controllable mechano-genetics, i.e. ultrasound controllers and transducers, genetic/epigenetic transducing modules, and mechano-sensors, will continue to be evolved for greater precision. The leverage of technological advancements of these different fields into this ultrasound-controllable mechano-genetics should in turn drive the development of these individual fields to open up new avenues.

We envision that wearable patches carrying ultrasound transducers will be developed in the near future to be controllable by mobile phones and/or control devices via wireless and Internet connection. In fact, stretchable electronic circuits have been developed to produce wearable patches and ultrasound transducer arrays [49]. As such, this ultrasound-controllable mechano-genetics technology will lead to a paradigm shift in translational medicine and pave the way for future telemedicine approaches, providing programmable health care at a remote distance for individual patients transplanted with ultrasound-controllable cells (Figure 3B). In summary, the ultrasound-controllable cell activation will usher in a new era in life science and technology, and highlight the translational power by integrating fundamental science and engineering principles for biomedical and clinical applications.

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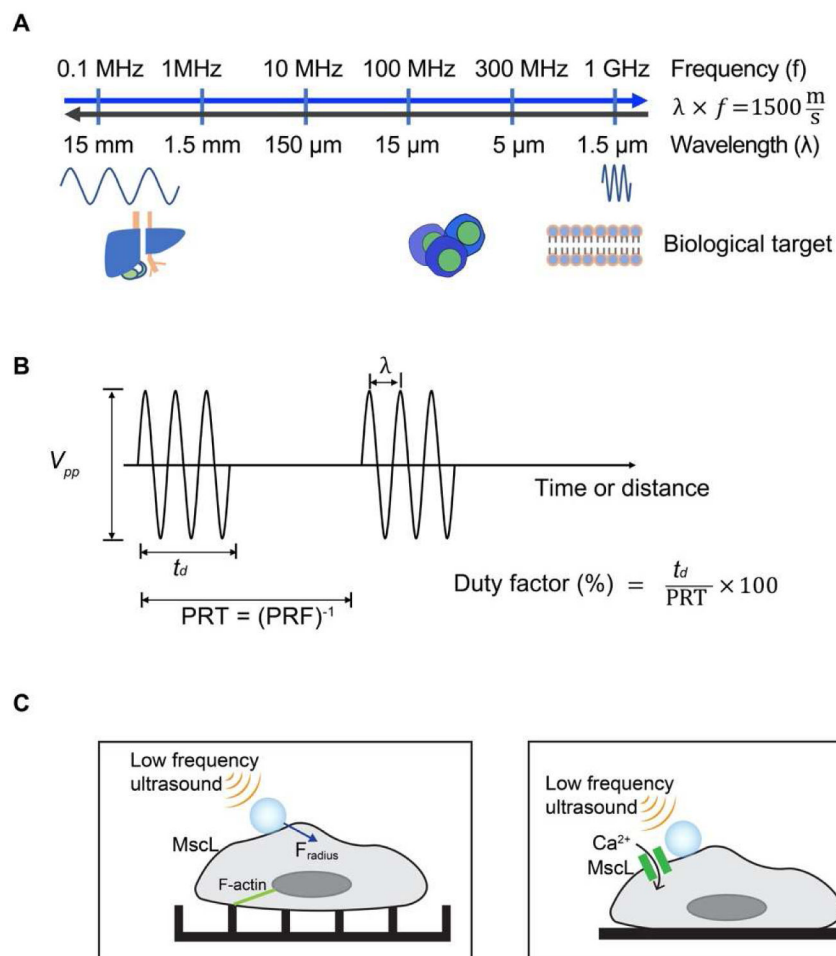


Figure 1. Sound waves and ultrasound parameters.

(A) In ultrasound field, frequency and wavelength are related to speed of sound of water or biological targets. Therapeutic ultrasound usually use frequency below 1 MHz and medical imaging applications uses 1 to 20 MHz frequency ranges. Recent development of ultra-high frequency ultrasonic transducers opens a new area for high frequency ultrasound research applications such as cellular manipulation using ultrasound. (B) Depending on applications, appropriate pulse parameters are required. PRT (PRF) represents pulse repetition time (frequency). (C) Schematics showing low frequency ultrasound excitation on microbubbles (MB) attached via RGD-integrin binding to the cell membrane to study actin cytoskeleton signaling (left); or to activate exogenously expressed mechanosensitive channel MscL opening (right).

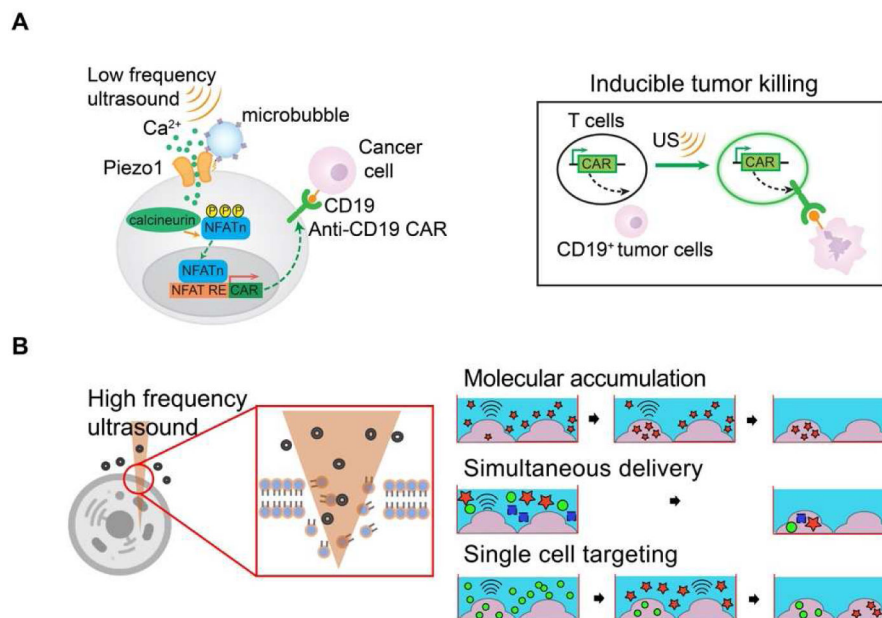


Figure 2. Cell engineering applications.

(A) Schematic showing low frequency ultrasound excitation on microbubbles (MB) attached to the mechanosensitive ion channel Piezo1 expressing cell membrane to activate anti-CD19 CAR gene expression. The subsequent calcium entry triggers the downstream pathways, including calcineurin activation, NFAT dephosphorylation and translocation into the nucleus. The nucleus-translocated NFAT can bind to upstream response elements to initiate gene expression (left). Ultrasound induced T cells can specifically kill antigen expressing tumor cells (right). (B) 150 MHz high frequency ultrasound beam directly disturbs cell membrane for intracellular delivery of macromolecules, known as acoustic-transfection technique. Three prominent capabilities of acoustic-transfection are molecular accumulation by repeated acoustic-transfection, simultaneous delivery of multiple molecules, and single cell level targeting to deliver multiple molecules into target cells.

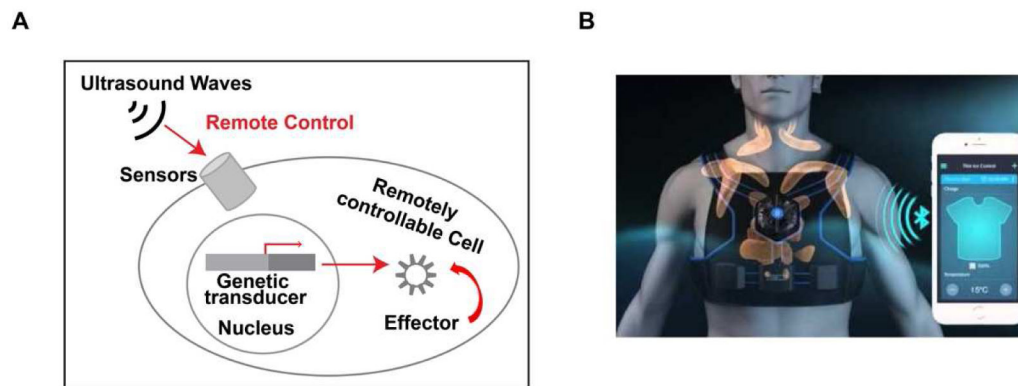


Figure 3. A diagram depicting the future application of ultrasound-controllable mechanogenetics.

(A) Ultrasound can be applied to remotely activate molecular sensors and genetic transducer for the production of effector in controlling the target cell. (B) A cartoon depicting the remotely controlled telemedicine. Wearable patches containing ultrasound transducers can be distally activated by mobile phones via internet and wireless network to control engineered cells for therapeutics.