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Multiplexing Focused Ultrasound Stimulation with Fluorescence Microscopy

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TITLE:**Multiplexing Focused Ultrasound Stimulation with Fluorescence Microscopy****AUTHORS & AFFILIATION:**

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SUMMARY:

Low-Intensity Pulsed Ultrasound Stimulation (LIPUS) is a modality for non-invasive mechanical stimulation of endogenous or engineered cells with high spatial and temporal resolution. This article describes how to implement LIPUS to an epi-fluorescence microscope and how to minimize acoustic impedance mismatch along the ultrasound path to prevent unwanted mechanical artefacts.

ABSTRACT:

By focusing low-intensity ultrasound pulses that penetrate soft tissues, LIPUS represents a promising biomedical technology to remotely and safely manipulate neural firing, hormonal secretion and genetically-reprogrammed cells. However, the translation of this technology for medical applications is currently hampered by a lack of biophysical mechanisms by which targeted tissues sense and respond to LIPUS. A suitable approach to identify these mechanisms would be to use optical biosensors in combination with LIPUS to determine underlying signaling pathways. However, implementing LIPUS to a fluorescence microscope may introduce undesired mechanical artefacts due to the presence of physical interfaces that reflect, absorb and refract acoustic waves. This article presents a step-by-step procedure to incorporate LIPUS to commercially-available upright epi-fluorescence microscopes while minimizing the influence of physical interfaces along the acoustic path. A simple procedure is described to operate a single-element ultrasound transducer and to bring the focal zone of the transducer into the objective focal point. The use of LIPUS is illustrated with an example of LIPUS-induced calcium transients in cultured human glioblastoma cells measured using calcium imaging.

INTRODUCTION:

Many diseases require some form of invasive medical intervention. These procedures are often expensive, risky, require recovery periods and thus add a burden to health care systems. Non-invasive therapeutic modalities have the potential to provide safer and cheaper alternatives to conventional surgical procedures. However, current non-invasive approaches such as pharmacotherapy or transcranial magnetic stimulation are often limited by trade-offs between tissue penetration, spatiotemporal resolution and unwanted off-target effects. In this context, a focused ultrasound constitutes a promising non-invasive technology with the potential to manipulate biological functions deep inside tissues with high spatiotemporal accuracy and limited off-target effects.

Focused ultrasound stimulation consists of delivering acoustic energy at precise locations deep inside living organisms. Depending on acoustic pulse parameters, this energy can have a variety of medical uses. For instance, the Food and Drug Administration has approved the use of High-intensity Focused Ultrasound (HiFU) for thermal ablation of prostate tumors, tremor-causing brain regions, uterine fibroids and pain-causing nerve endings in bone metastases¹. HiFu-mediated microbubble cavitation is also used to transiently open the blood-brain barrier for the targeted delivery of systemically-administered therapeutics². The spatial-peak pulse-average intensity (I_{sppa}) and spatial-peak temporal-average intensity (I_{spta}) used for HiFU applications are typically above several kW cm^{-2} and produce pulse pressure of several tens of MPa. These intensity values are far above the FDA-approved I_{sppa} and I_{spta} limits for diagnostic ultrasound, 190 W cm^{-2} and 720 mW cm^{-2} , respectively³. In contrast, recent studies have shown that non-destructive pulsed ultrasound stimulation that are within or near the range of diagnostic ultrasound intensity limits (LIPUS) can be effective to remotely and safely manipulate neural firing⁴⁻⁸, hormonal secretion^{9,10} and bioengineered cells¹¹. Yet, the cellular and molecular mechanisms by which cells sense and respond to ultrasound remain unclear, precluding clinical translation of LIPUS. Hence, in the past few years, studies of artificial membranes, cultured cells and animals stimulated with ultrasound have gained momentum to reveal biophysical and physiological processes modulated by LIPUS¹²⁻¹⁵.

Sound consists of a vibration propagating through a physical medium. An ultrasound is a sound with a frequency above the human audible range (*i.e.*, above 20 kHz). In a laboratory setting, ultrasound waves are generally produced by piezoelectric transducers that contain a material that vibrates in response to an electrical field oscillating in a specific high-frequency bandwidth. Two types of transducers exist: single element transducers and transducer arrays. Single element piezoelectric transducers possess a curved surface which acts as a focusing lens and hence concentrates acoustic energy into a defined region called the focal zone. Single element transducers are much cheaper and easier to operate than transducer arrays. This article will focus on single element transducers.

The size of the focal zone of a focused single element transducer depends on the geometric properties of the acoustic lens and on its acoustic frequency. To achieve a millimeter-size focal zone with a single element transducer, ultrasound frequencies in the MHz range are generally required. Unfortunately, acoustic waves at such frequency are very rapidly attenuated when propagated in a tenuous medium such as air. Thus, MHz ultrasound waves need to be

generated and propagated to the sample in a denser material such as water. This constitutes the first challenge in integrating LIPUS modality to a microscope.

A second challenge is to minimize physical interfaces between materials with different acoustic impedances (which is a product of material density and the acoustic velocity) along the acoustic path. These interfaces can reflect, refract, scatter and absorb acoustic waves, making it difficult to quantify the amount of acoustic energy effectively delivered to a sample. They may also create unwanted mechanical artefacts. For instance, reflections produced perpendicular to acoustic mismatch impedance interfaces create backpropagating waves that interfere with forward-propagating ones. Along the interference path, the waves cancel each other at fixed regions of spaces called nodes and sum up at alternating regions called anti-nodes, creating so-called standing waves (**Figure 1**). It is important for the experimentalist to be able to control or eliminate these experimental interfaces *in vitro* as they may not exist *in vivo*.

Fluorescence measurement of optical reporters is a well-known method to interrogate transparent biological samples in real-time and with no physical disturbance. This approach is thus ideal for LIPUS studies as any physical probes present in the sonicated area will introduce mechanical artefacts. This protocol describes the implementation and operation of LIPUS to a commercial epi-fluorescence microscope.

PROTOCOL:

1. Growing Cells on Acoustically-Transparent Polyester Film

1.1. Drill a 12 mm hole size at the bottom of a standard 35 mm culture dish using a vertical press-drill. Move the drill slowly and wear eye protection. Remove pieces of plastic attached to the bottom of the dish using a blade to create a smooth surface on the external side (**Figure 2**).

1.2. Apply a thin layer of marine-grade epoxy or glue at the external bottom surface of the dish.

1.3. Place a film of polyester (2.5 μm thickness) against the external bottom surface of the dish and press firmly to make sure the epoxy/glue spreads evenly between the film and the thick plastic surface. Gently pull the film in a centrifugal manner with fingers to create a flat surface (**Figure 2**).

1.4. When the epoxy/glue has dried, briefly rinse-dry the polyester-bottom dish with 95% ethanol and sterilize by placing the dish and the inside surface of its lid under a strong 254 nm UV excitation source. Adjust duration and intensity to deliver a UV dose of approximately 330 mJ cm^{-2} for complete destruction of most types of micro-organisms. This energy approximately corresponds to a duration of 5 min using a 1000 $\mu\text{W cm}^{-2}$ UV illumination.

1.5. Aliquot commercially available extracellular matrix protein mixtures (EMPM) in small tubes (50-100 μL) and store them at $-20\text{ }^{\circ}\text{C}$ or less in sterile conditions.

1.6. In a sterile environment (*e.g.*, inside a biosafety cabinet), dilute a frozen stock of EMPM with a desired culture medium to 1:100. Work on ice to prevent EMPM polymerization at room temperature. Quickly apply 100 μ L of the medium mixture onto the polyester film. Place the lid back on the dish to maintain sterility.

1.7. Incubate EMPM-coated polyester bottom dishes in a cell culture CO₂ incubator at 37 °C for 6-12 h.

1.8. After incubation, aspirate the excess medium and directly seed the surface with cells at the desired density. Work under sterile condition to maintain sterility.

2. LIPUS Implementation

2.1. Place a water tank underneath the objective of an upright microscope with large working volume and without illumination hardware in the transmission path.

2.2. Using commercially-available optomechanical components, place a sample holder below the objective and a transducer holder underneath the sample holder. For subsequent sample search and ultrasound alignment, mount these two holders on translation stages.

2.2.1. Place the moving parts and actuators of translation stages either outside the tank or above the water line to avoid water damage. Only use non-corrosive materials such as anodized aluminum or stainless steel for immersed optomechanical components.

2.3. Fill the tank with deionized and degassed water before utilizing the immersion transducer. The water line should coincide with the horizontal plane of the sample holder (Figure 3).

NOTE: Deionized water prevents electrical coupling in presence of high electric fields. Degassing will also prevent scattering and alterations of acoustic waves. Drain water after each experiment using a pump or valve so that the water line falls below the position of the transducer. Also, replace or filter water frequently and clean-up the water tank as needed to avoid growth of microorganisms.

3. Oblique Acoustic Excitation

3.1. Using commercially available optomechanical components, orient the transducer in an oblique position with respect to the optical path. This will ensure that any reflected waves will be directed away from the sample (Figure 3 and 4).

4. Driving the Transducer

NOTE: Ultrasound transducers convert oscillating electrical energy into mechanical expansion/contraction of a piezoelectric material. This conversion produces energy loss in the form of heat energy. Hence, while transducers do possess a peak input voltage limit, they also possess an electrical power limit to avoid thermal damage to the piezoelectric element:

$$P = \text{duty cycle} \times \frac{V_{rms}^2}{Z} \text{ (Equation 1)}$$

with the duty cycle the relative fraction of time of electrical stimulation, P the electrical power (in Watts), V_{rms} the input root-mean-square voltage (in Volts) of the alternative voltage source and Z the electrical impedance (in Ohms).

$$V_{rms} = \frac{V_{pp}}{2\sqrt{2}} \text{ (Equation 2)}$$

with V_{pp} the peak-to-peak input voltage applied to the transducer.

4.1. Create a sinusoidal wave form containing the desired frequency, number of cycles per pulse, and pulse repetition frequency using a commercial function generator. However, the relatively high V_{pp} needed to effectively drive standard ultrasound transducers often requires the addition of a power amplifier to amplify the output (*i.e.*, increase the amplitude of V_{pp}) of the function generator.

NOTE: For example, a transducer's manufacturer indicates the power limit for a given transducer is 35 W. Will a sinusoidal peak-to-peak input voltage (V_{in}) of 500 mV at a duty cycle of 50% and amplified through a 50 dB/100 W amplifier be within the power limit of this transducer?

4.1.1. To answer this question, calculate the voltage after amplification. For a radio-frequency (RF) power amplifier, the amplification factor (dB) is defined by:

$$dB = 20 \log \frac{V_{out}}{V_{in}} \text{ (Equation 3)}$$

Thus, the amplified voltage has an amplitude output V_{pp} ($V_{pp} = V_{out}$) of:

$$V_{pp} = 0.5 \times 10^{\frac{dB}{20}} = 158 \text{ V}$$

Using Equations 1 and 2, and using 50 Ω as electrical impedance, the corresponding power generated by this voltage is:

$$P = 0.5 \times \frac{[0.5 \times 0.707 \times 158]^2}{50} \approx 31 \text{ W}$$

This stimulation is therefore within the power limit of the transducer.

4.1.2. Using the example above, calculate the waveform parameters (V_{pp} , frequency, pulse duration and pulse repetition frequency) that correspond to the power and voltage limits provided by the transducer's manufacturer. Make sure to respect these limits to avoid damaging the transducer and other connected instruments.

213 4.2. Choose a function generator that operates within a frequency range compatible with
214 the ultrasound transducer. Adjust the frequency of the function generator to the nominal peak
215 frequency of the transducer.

216
217 4.3. Create a sinusoidal voltage pulse of the desired duration and repetition frequency using
218 the burst mode of the function generator. Adjust peak-to-peak voltage to a desired value. Make
219 sure that the pulse duration is shorter than the elapsed time between two consecutive pulses.

220
221 4.4. Check that the waveform corresponds to the desired signal by connecting the output of
222 the function generator to the input of an oscilloscope.

223
224 4.5. Connect the output of the function generator to the input of a power RF amplifier
225 (Figure 4). Make sure that the stimulation parameters are within the limits of the transducer's
226 manufacturer.

227 228 5. Beam Alignment

229
230 5.1. Choose a hydrophone that operates with a frequency range and acoustic intensity
231 compatible with the frequency and intensity of the ultrasound transducer.

232
233 5.2. Carefully bring the tip of a hydrophone probe into focus within the objective field of
234 view at the position corresponding to the position of the sample (Figure 4).

235
236 5.3. Make sure that both probe and transducer are immersed in deionized and degassed
237 water. Do not bump the tip of the hydrophone with any physical object other than water as this
238 will alter its coating and affect the measurement.

239
240 5.4. Perform a gross pre-alignment of the transducer by visually positioning its acoustic axis
241 toward the hydrophone probe. Make sure that the distance between the transducer's surface
242 and the hydrophone tip correspond approximately to the transducer's focal length.

243
244 5.5. Connect the hydrophone output to one of the oscilloscope's signal input. Connect the
245 synchronization trigger from the function generator to another oscilloscope input. Visualize
246 both signals simultaneously on the oscilloscope.

247
248 5.6. Drive the transducer with few ultrasound cycles at a low duty cycle and low amplitude
249 to avoid damaging the probe. Check with the hydrophone's manufacturer safe operation
250 conditions to avoid damaging the hydrophone tip.

251
252 5.7. Adjust the s/division knob according to the travel time of ultrasound from the
253 transducer's surface to the hydrophone. Look for a hydrophone signal on the oscilloscope after
254 the synchronization trigger.

5.8. Slowly actuate the transducer using a motorized or manual XYZ stage. Leave the transducer into the position that correlates with the maximal hydrophone signal (**Figure 4**).

Note: If no signal is detected it is possible that the intensity of the acoustic pulses is too low or that the beam is mis-aligned or scattered by an object. Check regularly that the hydrophone and transducer are visually pre-aligned and that no bubbles or physical object are present in the path except the polyester film. If no signal is still detected, increase the input voltage by a small amount to increase the amplitude of hydrophone signal.

6. Determination of Ultrasound Pulse Pressure and Intensity

6.1. With the beam aligned, measure the peak-to-peak amplitude of the hydrophone output at the oscilloscope for various voltages driving the transducer. Make sure not to exceed the pressure limit recommended by the hydrophone's manufacturer.

6.2. Convert these measurements into pressure and/or acoustic intensity values using the calibration method provided by the hydrophone's manufacturer.

NOTE: The acoustic intensity can be determined from the pressure and vice versa using the formula:

$$I = \frac{P^2}{\rho c} \quad (\text{W m}^{-2})$$

with I the acoustic pressure (in W m^{-2}), P the acoustic pressure (in Pa), ρ the density of propagating material (1000 kg m^{-3} for water) and c the speed of sound in propagating medium (for water, $c = 1500 \text{ m s}^{-1}$).

6.3. Create calibration curves using these measurements.

NOTE: The pressure vs. voltage and intensity vs. voltage curves have a linear and parabolic shape, respectively.

6.4. Determine the pressure and/or intensity value of a desired driving voltage by using the corresponding calibration curve.

7. Calcium-Sensitive/LIPUS Live-Cell Fluorescence Imaging

7.1. Replace the cell's culture medium with a desired imaging buffer containing $5 \mu\text{M}$ of a cell-permeant calcium-sensitive dye (e.g., Fluo-4 AM). Incubate the culture dish in a CO_2 incubator at 37°C for 1 h.

7.2. Carefully wash cells with the same buffer free of dye.

7.3. Place the dish in the sample holder. Excite the cells using blue light illumination (490 nm) and adjust excitation intensity and camera exposure to avoid excessive bleaching or pixel saturation.

7.4. Perform time-lapse imaging using desired image acquisition settings. Use an immersion objective for better image quality and with long working distance to reduce undesired reflections (see **Figure 4**).

REPRESENTATIVE RESULTS:

Figure 5 is an example of LIPUS experiment multiplexed with calcium imaging. Glioblastoma cells (A-172) were grown on EMPM coated polyester film in standard culture medium (supplemented with 10% serum and 1% antibiotics) and incubated with the calcium-sensitive fluorescent reporter Fluo-4 AM. Cells were imaged using a 10X immersion lens and illuminated with a white LED light source and fluorescence light was collected using a standard GFP filter set. LIPUS was applied by manually by driving a 4 MHz transducer with a pulse waveform of 158 V peak-to-peak amplitude, 0.1 ms pulse duration and 10 ms pulse repetition frequency (*i.e.*, 1% duty cycle). These parameters correspond to $I_{\text{sppa}} = 88 \text{ W cm}^{-2}$ (well below the diagnostic limit of 190 W cm^{-2}) and $I_{\text{spta}} = 877 \text{ mW cm}^{-2}$ (slightly above the diagnostic limit of 720 mW cm^{-2}), respectively. The results show this stimulation produced robust calcium elevations (**Figure 5B, 5C, 5D**).

For short pulse durations (*i.e.*, with no significant heat dissipation during the pulse) and assuming the heat capacity of the sample (*i.e.*, cells grown on polyester film and immersed in aqueous solution) is similar to that of water, the change of temperature (ΔT_{max}) produced during each pulse can be estimated by¹⁶:

$$\Delta T_{\text{max}} = 0.12 \text{ J kg}^{-1} \text{ K}^{-1} \text{ s}^{-1} \text{ cm}^{-2} \text{ (?????????)}$$

with a pulse duration of 0.1 ms and pulse intensity of 88 W cm^{-2} :

$$\Delta T_{\text{max}} = 0.12 \times 0.0001 \times 88 \approx 1 \text{ m}^\circ\text{C}$$

with 1% duty cycle, the small amount of heat deposited at the focal zone during each 0.1 ms pulse is likely removed by thermal conduction during the 9.9 ms spanned between two consecutive pulses. Hence, the robust calcium signals seen in **Figure 5B, 5C, 5D** are most likely induced by non-thermal mechanism(s).

Figure and Table legends

Figure 1: Standing wave formation at a reflecting interface. The presence of an interface between materials with different acoustic impedance reflects an incoming pressure wave (blue) with a wavelength λ . Since both waves travel in opposite directions, an oscillating phase shift is established. *Top*: at this time, the phase shift is 180° , producing destructive interference of the standing wave (green wave). *Middle*: After the waves have moved a distance corresponding to $\lambda/4$ with respect to the top panel, the phase shift is null and both waves amplify via

constructive interferences, producing a standing wave of higher amplitude. *Bottom*: After the waves have moved an additional distance of $\lambda/2$ (hence a total of $\lambda/4 + \lambda/2 = 3/4 \lambda$ from the top reference), the phase shift becomes null again, producing a standing wave of high amplitude but with inverse polarity. Note that some positions within the path have a constant null pressure (node, black circles) while other positions constantly oscillate between minimum and maximum pressures (antinodes, green circles).

Figure 2: Growing cells on acoustically-transparent polyester film. The figure shows the bottom part of a 35 mm dish with a large 12 mm hole in its center. The hole is subsequently covered with a thin polyester film. The film is firmly glued to the external bottom of the dish using marine grade epoxy.

Figure 3: Implementation of an ultrasound set-up to an upright fluorescence microscope. A custom-made water tank is placed under an upright fluorescence microscope without transmitted illumination hardware. A motorized sample holder positioned under the objective is attached to optomechanical components fixed to the vibration table outside the tank. The transducer is positioned underneath the sample and attached to a translation stage affixed to optomechanical components inside the tank.

Figure 4: Schematic diagram of the entire set-up. The set-up includes an epi-fluorescence upright fluorescence microscope to enable fluorescence imaging of cultured cells. The transducer is shown with an oblique orientation with respect to the optical path. This configuration avoids backward reflection of acoustic waves along the acoustic path, thus preventing standing wave formation and/or repetitive stimulation of the sample with multiple ultrasound echoes. A desired waveform is produced by a function generator which can be manually switched on or electronically triggered by a computer interface (Transistor-Transistor-Logic or Universal Serial Bus). The amplitude and delay of the signal measured by the hydrophone needle (red) can be analyzed using an oscilloscope.

Figure 5: Example of LIPUS-induced calcium signals in human glioblastoma cells A-172. (A) Raw fluorescence image of A-172 cells grown on a polyester-bottom 35 mm culture dish and loaded with a cell-permeant version of the calcium-indicator Fluo-4. The red traces represent cell boundaries automatically identified by a computer program and labeled as regions of interest (ROI). (B) Time course of relative fluorescence change ($F_t - F_0 / F_0$, or $\Delta F / F_0$) for each ROI during a LIPUS experiment. Images were acquired at a speed of 1 frame per second by a standard CCD camera and LIPUS was applied for 10 s between frames 20 and 30. The LIPUS waveform consists of 100 μ sec pulses containing 400 cycles at 4 MHz and repeated every 10 ms for 10 s. (C) Plot showing the time course of the mean $\Delta F / F_0$ calculated for all ROIs (error bars not shown). (D) Plot showing the percentile of ROI exhibiting $\Delta F / F_0$ above a user-defined activation threshold.

DISCUSSION:

A main advantage of focused ultrasound is its ability to non-invasively deliver mechanical and/or thermal energy to biological samples with high spatio-temporal precision. Other

techniques intended to mechanically stimulate cells usually employ invasive physical probes (e.g., cell-poking) or requires the interaction of high energy laser beams with foreign objects (e.g., optical tweezers). Magnetic heating can heat specific spatial locations inside biological samples but requires the presence of foreign magnetic nanoparticles. On the other hand, precise non-invasive heating of small sample (e.g., cell cultures) in aqueous media is possible using infrared or microwave excitation¹⁷⁻¹⁹.

Since commercial systems able to perform ultrasound excitation in conjunction with fluorescence microscopy are not available, many biophysicists have created customized systems tailored to their specific applications^{8,12,13,20}. The implementation of such systems can, however, be difficult for non-experts. This article described the basic operation to drive a focused ultrasound beam toward the focal plane of an upright epi-fluorescence microscope objective while limiting acoustic reflections and standing waves artefacts that are produced at mismatch acoustic impedance interfaces. These acoustic artifacts are not usually explicitly taken into consideration in the published literature.

If using an acoustic beam perpendicular to the optical path, the beam will ultimately encounter the liquid-solid interface formed by the front lens of an immersed objective or, if an air objective is being used, the water-air interface above the sample. These interfaces will reflect the acoustic waves back to the sample, producing standing waves (see **Figure 1**). To mitigate or avoid these reflections, it is recommended to position the transducer with an oblique angle with respect to the optical path.

The use of polyester-bottom culture dishes not only minimize acoustic reflections produced by the thick plastic culture dishes bottom, they also enable the experimentalist to stimulate the sample from underneath with an upright microscope. This is a preferable configuration as compared to an inverted microscope to position an immersed transducer with an oblique orientation with respect to the optical path.

This protocol is designed for use with focused ultrasound transducers, but experimentalist may also use non-focused (planar) ultrasound transducers as well. Note that, since LIPUS is intended for precise stimulation of desired areas within tissue, focused ultrasound transducers are generally preferred for both *in vitro* and *in vivo* applications. Acoustic beams produced by planar transducers are also broader, making it more difficult to reduce reflections and other mechanical artefacts.

The focal zone of a single element focused ultrasound transducer depends on many parameters such as the element diameter, the acoustic frequency and the sound velocity of the propagating material. For a standard MHz transducer, the focal area is typically confined in a millimetric or sub-millimetric region. A trade-off exists between the size of the focal zone and the ability of the acoustic beam to penetrate inside tissue without too much loss due to attenuation: the higher the frequency, the smaller the focal zone but the weaker the penetration.

To effectively stimulate cells visualized under a microscope, the acoustic focal zone of the transducer must overlap with the optical focal plane of the objective. To this aim, it is necessary to precisely align the acoustic beam using a commercially available hydrophone. There are two main types of hydrophones: hydrophone needles and fiber optic systems. Both types can be used. During alignment, it is important to make sure that the acoustic intensity is within the pressure limits of the hydrophone and that the frequency of the transducer is within the frequency range of the hydrophone's sensitivity.

Use the calibration provided by the manufacturer (if available) to convert the voltage amplitude output of the hydrophone into actual acoustic pressure (hydrophones are usually calibrated with a number in V Pa^{-1} or similar units). Hydrophones also usually have a preferential orientation (directionality) with respect to the acoustic beam, hence it is preferred to position the hydrophone in the same direction of the acoustic axis. If not possible, hydrophones may have a chart of attenuation vs. directional angle, allowing directional post-correction after the measurements have been taken.

Beam alignment can be a frustrating task, especially when operating a transducer with a narrow focal zone. The hydrophone signal should appear with a delay with respect to the pulse driving the transducer. This delay corresponds to the time taken by the ultrasound to travel from the transducer's surface to the hydrophone probe (in water, sound waves travel at a speed of approximately $1,500 \text{ m s}^{-1}$) (see **Figure 4**). Note that the delay of RF signals traveling through electrical cables is small, only about 3 ns m^{-1} , which, in the present case, can be safely ignored. A way to test if the beam is well aligned is to calculate the distance between transducer and hydrophone using the delay measured at the oscilloscope and the known sound velocity in water. For instance, a delay of approximately $17 \mu\text{s}$ is expected for a transducer with a focal length of 25 mm.

If the use of commercial hydrophone is not possible (*e.g.*, unusual transducer frequency with not matching hydrophones or limited space for placing the hydrophone), the acoustic beam can be aligned with the pulse-echo method²⁰. This is usually done by first placing a small reflecting object of a size similar or smaller than the beam diameter of the transducer into the focus within the microscope field of view. The transducer is then used both as acoustic emitter (to send ultrasound pulses) and receiver (to detect the echo from the reflecting object). Note that in this configuration, a dedicated amplifier is necessary to amplify the rather small echo signal coming out of the transducer.

For smooth operation of RF instruments, it is important that all the cables and connections to instruments have matching electrical impedance, otherwise undesired electrical reflections will occur and alter the electrical waveform. This is usually the case with most Bayonet Neill-Concelman (BNC) cables and RF equipment having 50Ω impedance. Some oscilloscopes, however, have a high input impedance of $1 \text{ M}\Omega$ and thus will reflect a RF signal fed through a 50Ω BNC cable. In this case, a simple 50Ω feed-through terminator should be inserted between the end of the BNC cable and the oscilloscope's input to properly terminate the signal without loss.

This method is technically limited by the instrumentation used for delivering ultrasound pulses and to perform fluorescence imaging. For example, a standard single-photon fluorescence microscope would only enable imaging of two-dimensional samples. However, it can perform more complex LIPUS imaging of three-dimensional samples like brain slices or small organs using multi-photon excitation.

Another challenge with LIPUS experiments is to distinguish mechanical vs. thermal effects imparted by acoustic beams. A mechanical displacement oblique to the optical path can be detected if it displaces the image in the plane axis (x,y) or defocuses the sample in the z-axis. These displacements depend on the combined optical resolution of the microscope camera and objective. In addition, as these motions would only occur during sonication, one would need to use a frame rate high enough to synchronize the temporal overlap between camera exposure and pulse duration.

To investigate ultrasound-induced thermal effects, the use conventional physical probes to measure temperature is not recommended because of unavoidable vibrations of the probe. However, the technique described here is well suited to measure temperature changes using genetically-encoded thermosensitive fluorescence reporters or thermosensitive dyes^{21,22}. In the future, as more biocompatible fluorescent reporters become available, this technique will enable the study of ultrasound effects on many other biophysical parameters.

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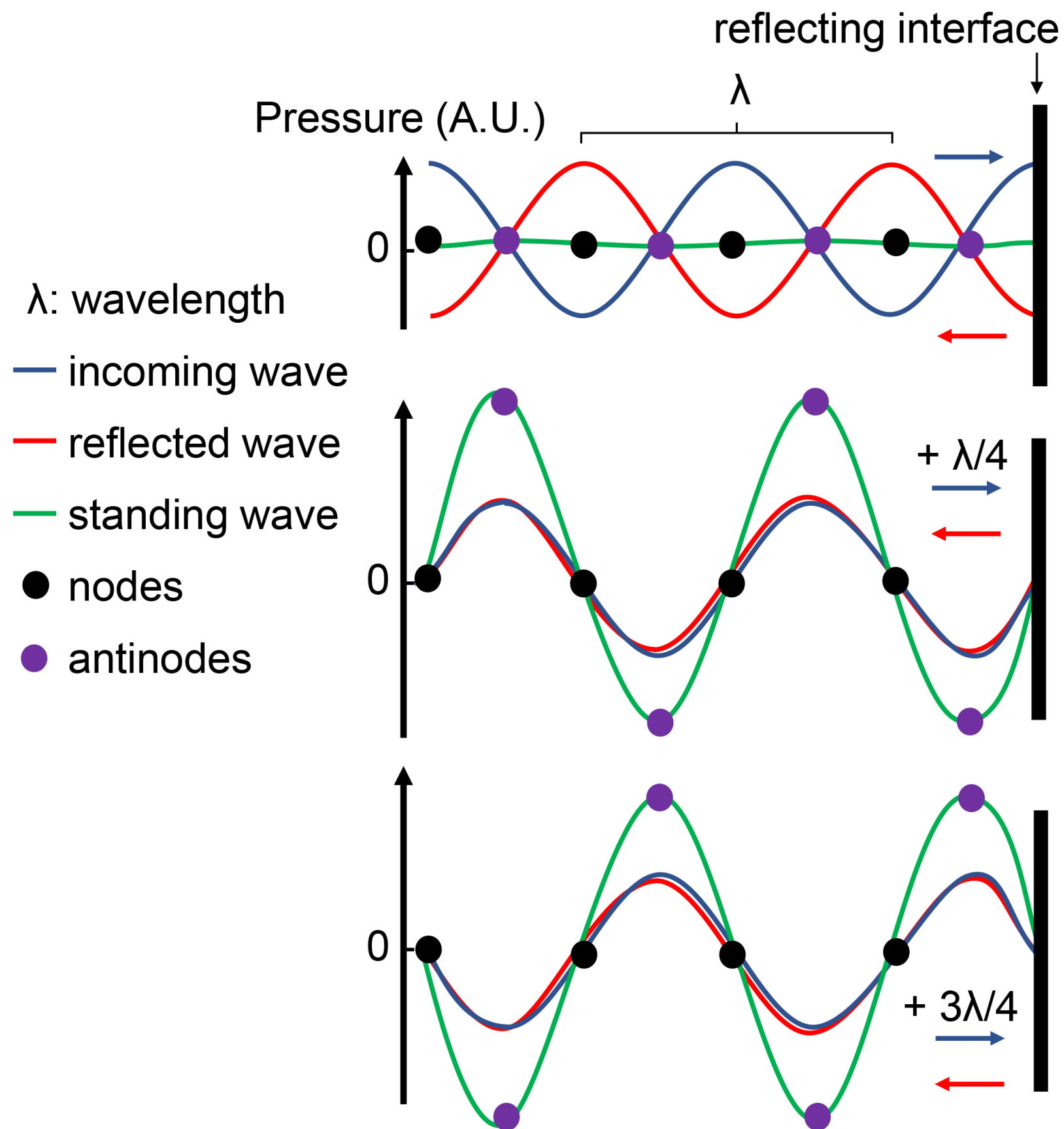
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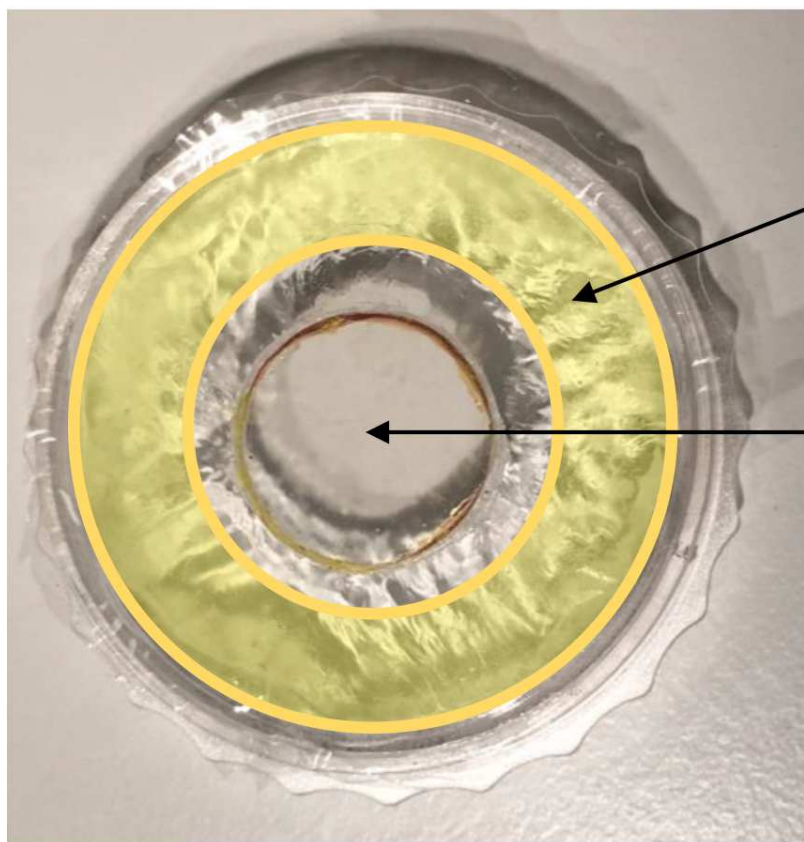
The authors have nothing to disclose.

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epoxy/glue

12 mm hole

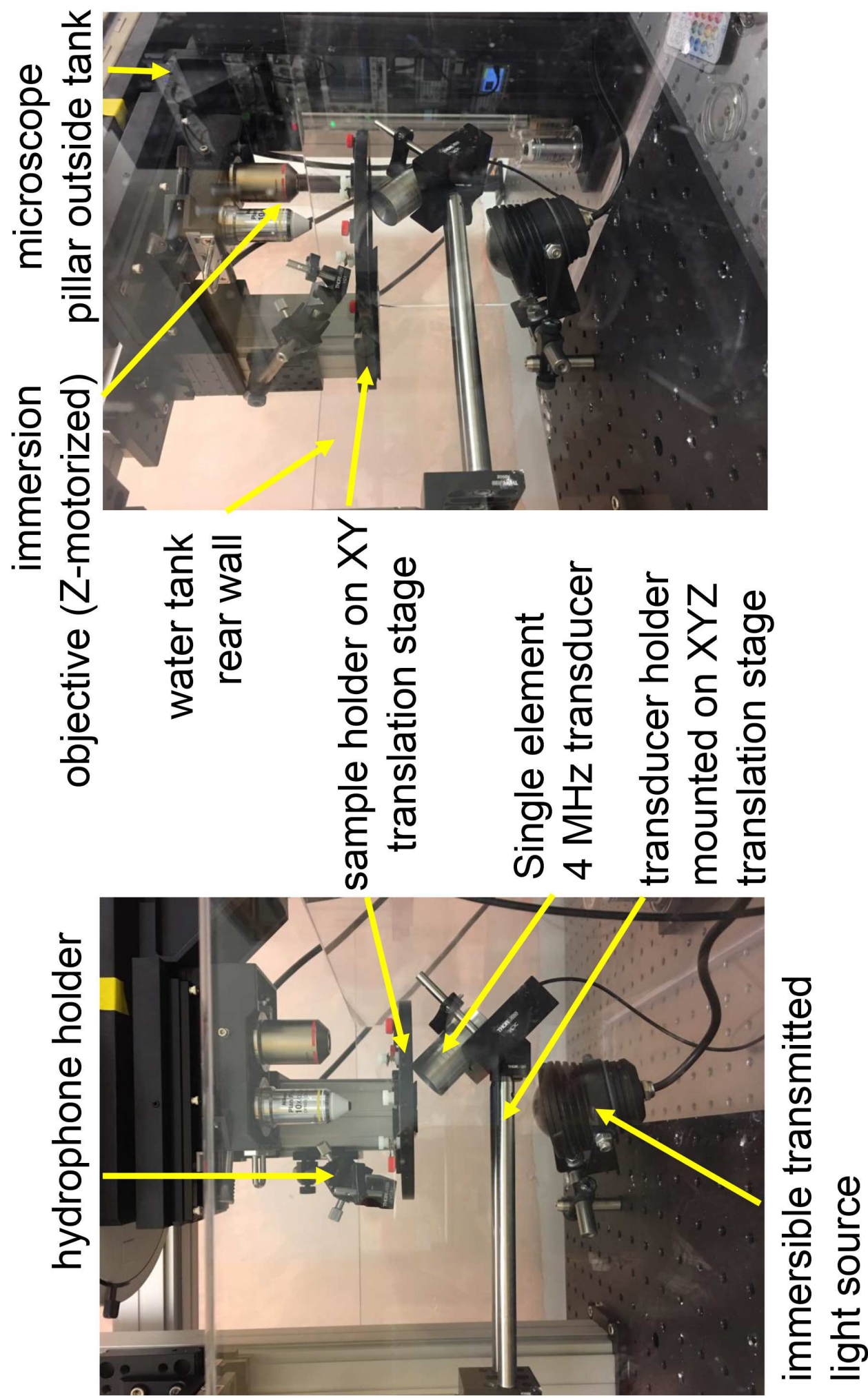
bottom view



polyester film

top view

Figure3



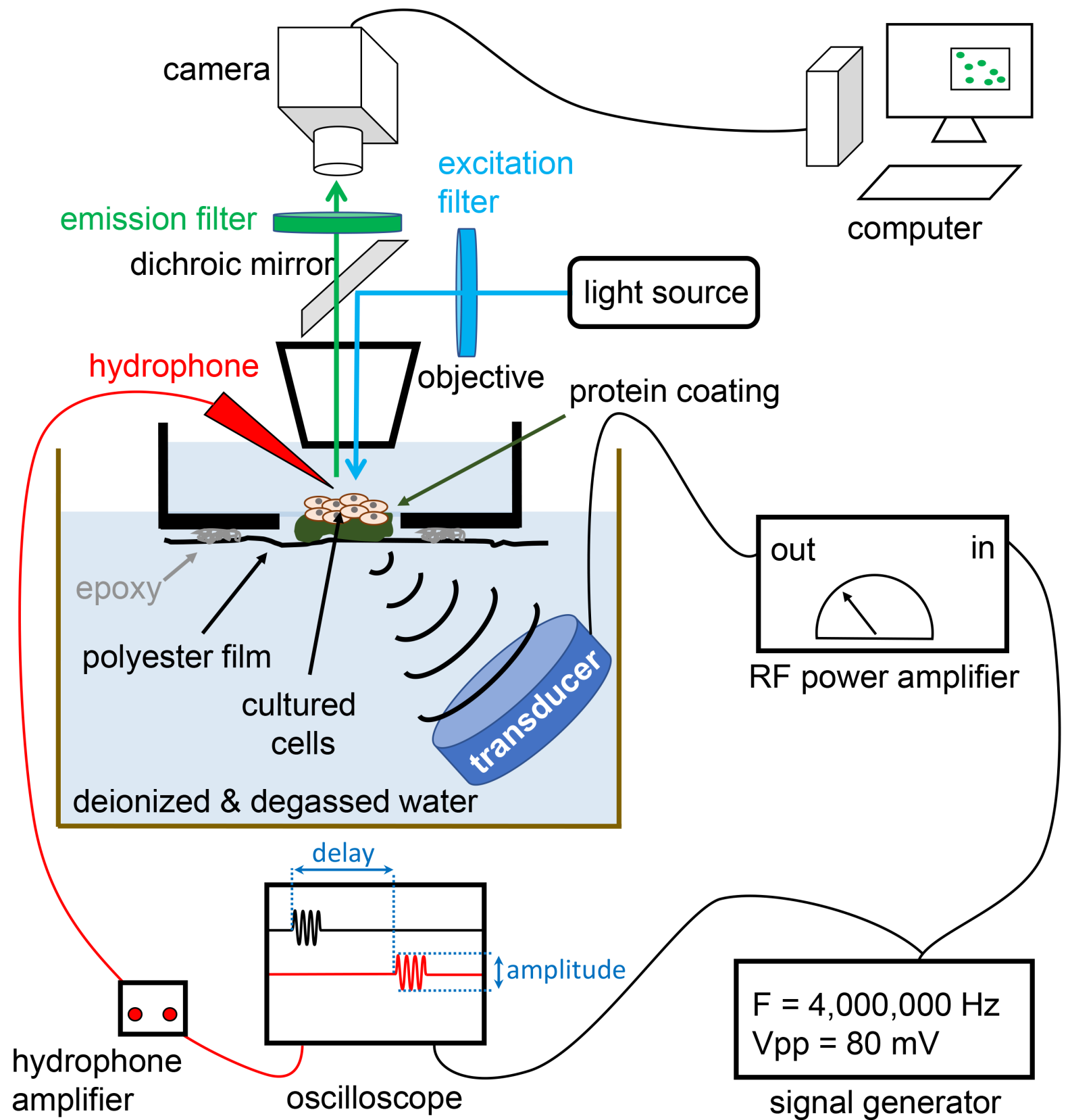
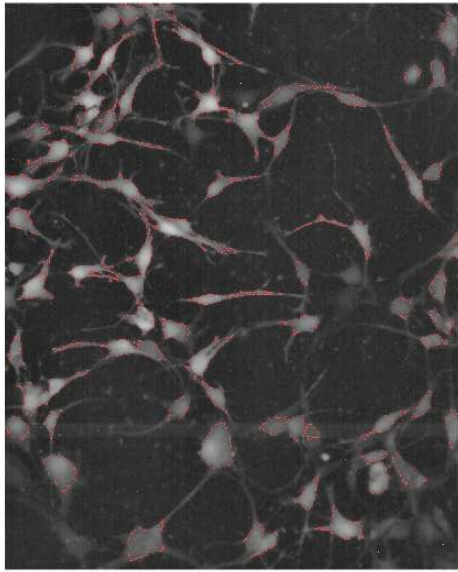
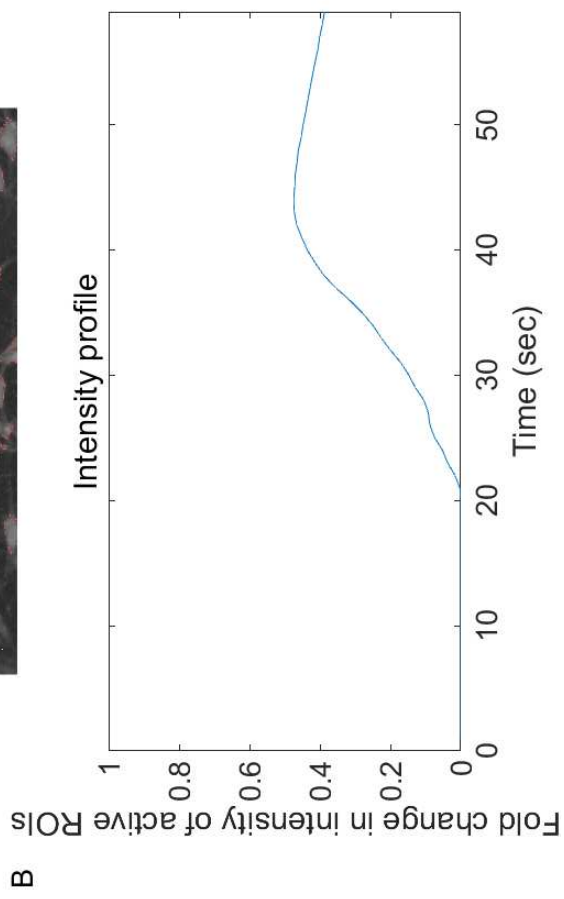


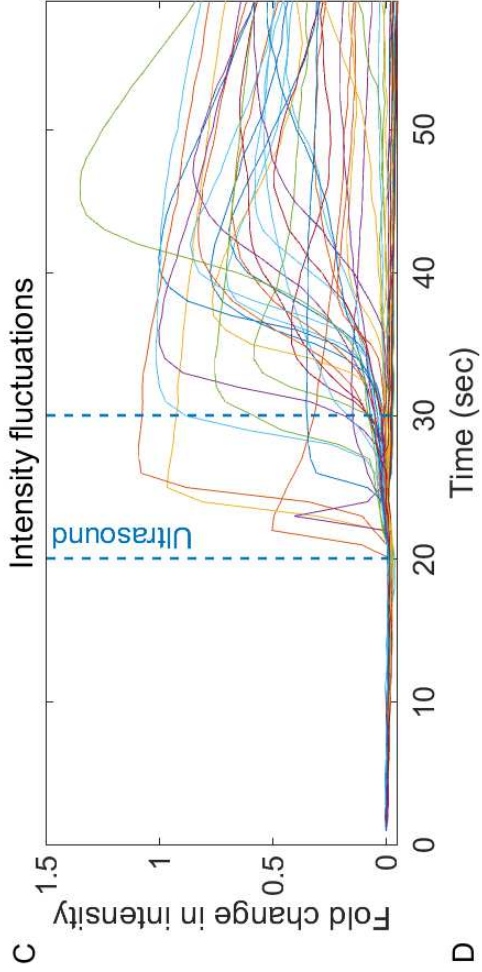
Figure5



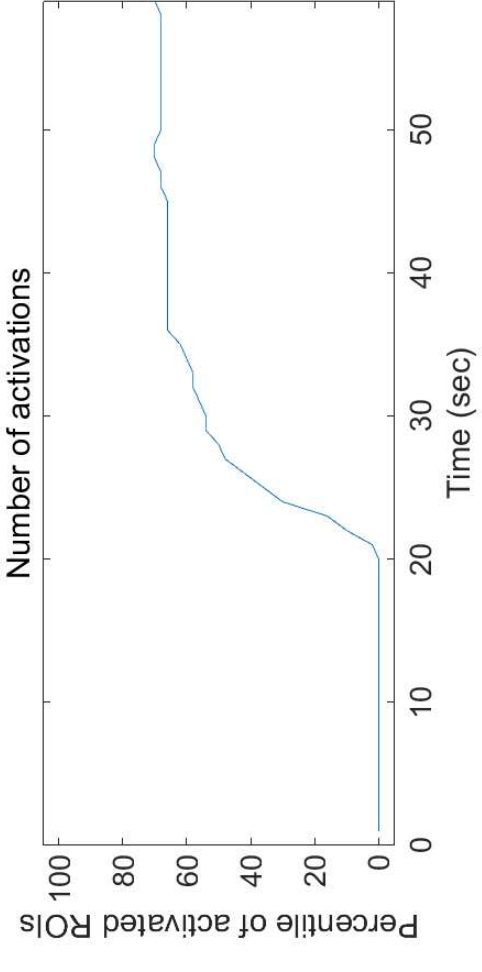
A



B



C



D

Name of Equipment /	Company	Suggested Catalog number / Models
upright microscope with large working volume	Thorlabs	CERNA
	Scientifica	SliceScope
optomechanical	Thorlabs	n/a
needle hydrophone	ONDA Corporation	HNP/C/R/A/T series + AH/G pre-amplifier
	Precision Acoustics	n/a
fiber optic hydrophone	ONDA Corporation	HFO series
	Precision Acoustics	n/a
oscilloscope	Keysight Technology	DSOX2004A (4-channels 70MHz)
function generator	Keysight Technology	33500B (20MHz single-channel)
RF power amplifier	Electronic Navigation Industries (ENI)	325LA, 525LA, 240L, 350L, A075, 2100L, 3100LA
	Electronics & Innovation (E&I)	
immersion ultrasound transducer	Olympus	focused immersion transducers
	Benthowave Instrument	HiFu transducer BII-76 series
	Precision Acoustics	Piezo-ceramic or HiFu transducers
	Ultrasonic-S-lab	HiFu transducers made to order
high-density Matrigel	Corning	VWR 80094-330
Mylar film 2.5 microns	Chemplex	CAT.NO:107



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Author(s):

Jerome J LACROIX, Alper D OZKAN

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Article Title:

An experimental set-up for multiplexing Focused Ultrasound Stimulation with

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Responses to editors and reviewers

Our responses are highlighted in blue after each comment.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully read the manuscript and corrected as many typos as we could.

2. Please provide an email address for each author.

An email is now provided for each author

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We have made sure that no commercial names are present in the main text and figures. Commercial names are however listed in the table for equipment/materials.

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have eliminated all personal pronouns.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have edited our protocol accordingly.

6. Lines 94-98, 123-128, 144-150, 152-179, 190-197, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

We have edited our protocol accordingly.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

1.1: Please specify the size of the hole drilled and the culture dish used.

These are now specified.

1.4: Please specify the intensity and time used in this step.

These parameters are now specified.

4.2: Please describe how this is done.

We have added few steps to explain how to create a signal waveform.

6: Please describe how to perform fluorescence imaging.

We have added few steps to explain how to perform fluorescence imaging.

8. 4.1: Please note that calculations are not appropriate for filming. Please un-highlight this step.

Done!

9. Figure 3: Please include a space between the number and its unit (i.e., 4 MHz).

Done!

10. Discussion: Please also discuss any limitations of the technique, the significance with respect to existing methods, and any future applications of the technique.

We have extended the discussion and added few paragraphs to discuss the limitations and future applications of the techniques (L390-407) and how it compares with others (L310-317).

11. References: Please do not abbreviate journal titles.

Corrected.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a protocol to build a setup that combines ultrasound probing and a regular fluorescence microscope. The experimental setup allows the cell response to the ultrasound waves to be observed with fluorescence contrast. The manuscript adequately describes the general procedures to construct the setup. The response of biological cells to ultrasound has not been fully understood in the field, and the ability to observe the response at a cellular structure level would be useful to help understand the mechanisms.

Major Concerns:

The main concern I have is that the protocol seems very straight forward. The procedures require only basic knowledge in ultrasound, optics, mechanics, and biology. Researchers who would like to conduct a similar experiment may easily come up with a similar setup without references.

To make this work more valuable, it would be great to include some detailed and quantitative system characterization, e.g. characterizing ultrasound pressure, attenuation, resolution, and reflectance. Also, the motion artifact induced by the ultrasound should also be characterized. For example, what is the portion of the signals shown in Figure 5 purely due to the displacement caused by the ultrasound? In addition, it would be helpful to provide a discussion on the alternative solutions, e.g. alternative geometry and using ultrasound absorbers to avoid reflection, using a pulse-echo transceiver to help alignment, etc.

We thank the reviewer for these suggestions. The shape of acoustic beam (beam size, focal length, etc...) is typically available from the transducer's manufacturer. In-house characterization of the beam shape can be done using an automated XYZ scanning of the hydrophone position relative to the transducer. Characterization of the transducer pressure (and acoustic intensity) can be done with a calibrated hydrophone. We have added the new protocol part 6 to explain how to proceed. Technical considerations for beam alignment and pressure characterization are also discussed in the revised discussion and can be obtained from the hydrophone's manufacturer.

It is inherently difficult to quantify the motion artefact for relatively short pulses of low amplitudes. Reducing camera exposure to temporally match ultrasound pulse length may produce images with visible XYZ displacements with respect to images taken outside sonication period. This point is discussed L395-401. There is also a new comment describing a quick calculation method to determine the generation of thermal effects L254-264.

We have not personally experimented with sound absorbing materials. The alternative of using pulse-echo method for beam alignment is now discussed L374-381.

Minor Concerns:

The authors proposed to use LIPUS instead of HiFU, but the calculation and component list are based on the HiFU transducers, which is confusing.

This is correct, and we agree that this can be confusing. The list of materials contains both non-HiFU (e.g. Olympus transducers) and HiFU transducers. The distinction between LIPUS and HiFU is mainly based on whether the transducer can sustain a high driving voltage and power. “LIPUS-only transducers” are easily damaged or destroyed by accidental voltage/power overdrives while HiFU transducers are much more resilient. Hence it may be preferable to use a HiFU transducer even when only doing LIPUS experiments.

Also, it would be great to provide a general pressure range for these two regimes.

Thank you for the suggestion, we agree that is something that was needed, and we have added a paragraph in the introduction to discuss the intensity regime used in both situations (L46-63). The intensity upper limit for diagnostic ultrasound (with some tissue exceptions: $I_{sppa} = 190 \text{ W/cm}^2$ and $I_{spta} = 720 \text{ mW/cm}^2$) are usually accepted as a good estimate for what is considered “low-intensity” stimulation. The range for clinical HiFU intensity and/or pressure (for thermal ablation or microbubble cavitation) is one or several orders of magnitude higher.

What is the ultrasound pressure for the experiment results shown in Figure 5?

We have added the acoustic intensity values for the experiment based on our hydrophone measurements L243-253 ($I_{sppa} = 88 \text{ W/cm}^2$ and $I_{spta} = 877 \text{ mW/cm}^2$). These values correspond to a pulse peak pressure of about 1.18 MPa. These values are near the upper limit for diagnostic ultrasound.

I think the left side of Equation 2 should be V_{rms} instead of V_{rms}^2 .
That is correct, thank you for spotting that one.

Reviewer #2:

Manuscript Summary:

The authors objective for this manuscript is to outline how to use an existing, semi-custom, fluorescence imaging setup for in-vitro pulsed ultrasound activated calcium imaging in live cells. This type of experimental approach is greatly benefited by visual examples and demonstrations, as is reported in this manuscript.

Major Concerns:

In order to be most effective, while considering the large instrumentation and parameter space for LIPUS, specific examples and utility of immersion transducers should be carefully outlined. Additionally, specific recommendations should be given for hydrophone selection based on frequency ranges intended to be used.

Thank you for this comment, the technical literature regarding the utility and applications of transducers and hydrophones is huge. This article only deals with focused ultrasound MHz transducers that enables millimetric size focal zone because those are most useful for both in vitro and in vivo application (e.g. targeting a small area inside a tissue). A comment to discuss the trade-off between penetration and focal zone has been added L343-349.

The choice of hydrophone based on frequency and acoustic intensity range is now discussed L187-188.

Depending on the parameters used to drive the transducer of choice, it is imperative to demonstrate that the energy used to stimulate the biological sample is due to mechanical energy rather than thermal energy (unless thermal energy is the goal for the experiment). In order to do so, the authors should describe a procedure, with appropriate instrumentation, on how to measure small changes in temperature within the experimental setup.

Thank you for raising this important point. We have added a rapid calculation method that enable determination of temperature change for short ultrasound pulses L254-264. We also propose to use genetically-encoded thermometers (e.g. FRET-based or polarization anisotropy-based) or other thermosensitive dyes to measure actual temperature changes (L402-407) as the presence of any physical temperature probe may produce additional heat by mechanical frictions/vibrations. The details to conduct those experiments are beyond the scope of this article but can be found in the cited literature.

One other technical note that should be considered is in regards to aligning the acoustic beam to the sample. It should be highlighted that the hydrophone measurements in the setup should be taken on the axial plane of the transducer, and at the Z-position of

where the sample will be placed. A clear visual demonstration of this, along with supported text, should be included. Also, careful handling of the hydrophone should be stated. Including the water level height within the water tank should be included.

Thank you for noticing this important point. We have added a paragraph L357-363 to explain the directionality issue of hydrophone measurement. Careful handling of the hydrophone is also stated L189-193. The level of water in the tank is purely arbitrary as long as the transducer is immersed and there is continuity of water with no air gaps between the transducer and the sample.

Minor Concerns:

Line 122: Details on microscope, objectives (air/immersion), camera, translation path needed (x,y,z) etc.

Details regarding instrumentation in the main text are limited due to the non-commercial language policy of JOVE. Commercial information can be found in the material Table. Objective/camera information can be found L246 and in Fig5 legend. The main requirement of the microscope is to be upright with a large working volume (stated L122-123).

Line 143: How will aligning focused vs unfocused transducer compare? Is this protocol not suitable for linear (non-focused) transducers?

That is a very good question. Technically, this protocol also works with planar transducers but in this case, it may be difficult to control standing waves and other mechanical artefacts due to their broader beam size as compared to focused transducers. This is now discussed L337-342.

Line 56: Please include units for each variable

The units have been added, thank you.

Line 162: Include notes on limitation of driving frequencies and prf with some function generators

Thank you, we have edited paragraphs 4.1 and 4.2 accordingly.

Line 168: Definition/classification of high-intensity transducers? References? How can this be translated to other transducers?

Thank you for pointing out this confusion, this example was purely hypothetical. We have edited it to avoid generalization of power limit for a HiFU transducers. The

distinction between HiFU and non-HiFU transducers is a grey area. The term HiFU is given to the therapeutic procedure capable of heating (and even boiling) tissue. For this purpose, transducers must sustain considerable amount of electrical driving power. The driving power limits of transducer depend on the manufacturer's design and piezoelectric material. As such, transducer power limits may continuously vary from very low power limit (0.1 Watts or lower) to very high limits (e.g. hundreds of Watts).

Line 180: Temperature measurements should also be taken

A method to determine temperature changes for pulsed stimulation is now given L254-264. Alternative methods to measure temperature without producing heating artifacts are suggested L402-407.

Line 189: Mention limitations with unfocused transducers

A discussion regarding planar vs focused transducers is now mentioned L337-342.

Line 198: A figure would help visualize this as it can be cumbersome, depending on the objective lens you are using

We agree, this has been highlighted in Figure 4

Line 200: Mention significance of degassing water

This is now mentioned in a Note L133-137

Line 205: Be sure to describe that this position is where the sample needs to be placed.

Thank you, this is now specified L 189-190.

Line 210: Explain what aspects of the setup to troubleshoot if V_{pp} measurement and subsequent solving of power do not meet expected results.

Thank you, we have added a Note L 208-212 to explain the troubleshooting procedure.

Figure 5: x-axis should be converted into seconds

Done!