**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

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Mylar, Matrigel, etc.

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: Isppa = 190 W/cm2 and Ispta = 720 mW/cm2) 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 (Isppa = 88 W/cm2 and Ispta = 877 mW/cm2). 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 Vrms instead of Vrms^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 he 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 Vpp 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!