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TITLE:
Studying Cavitation Enhanced Therapy

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KEYWORDS: Ultrasound, Microbubbles, Drug Delivery, Bioeffects, Cavitation, Sonoporation, Therapy Monitoring, Passive Acoustic Mapping.

SUMMARY: The presented experimental protocol can be used to perform real time measurements of cavitation activity in a cell culture device with the aim of enabling investigation of the conditions required for successful drug delivery and/or other bioeffects.

ABSTRACT: Interest in the therapeutic applications of ultrasound is significant and growing, with potential clinical targets ranging from cancer to Alzheimer's disease. Cavitation - the formation and subsequent motion of bubbles within an ultrasound field - represents a key phenomenon underpinning many of these treatments. There remains, however, considerable uncertainty regarding the detailed mechanisms of action by which cavitation promotes therapeutic effects and a need to develop reliable monitoring techniques that can be implemented clinically. In particular, there is significant variation between studies in the exposure parameters reported as successfully delivering therapeutic effects and the corresponding acoustic emissions. The aim of this paper is to provide an experimental protocol using widely available components for performing studies of cavitation mediated bio-effects that include real time acoustic monitoring. It is hoped that the protocol will enable more widespread incorporation of acoustic monitoring into therapeutic ultrasound experiments and facilitate easier comparison across studies of exposure conditions and their correlation to relevant bio-effects.

INTRODUCTION:

Ultrasound (US) has been used widely as a diagnostic imaging technique because of its safe and non-invasive nature, its ease of implementation at a patient's bedside, and its cost effectiveness[1]. Next to its diagnostic and monitoring capabilities, US has considerable potential for therapeutic applications. Early work explored its use in thrombolysis, DNA transfection, and drug delivery[2–4] and therapeutic US now represents a very active area of research, with applications including tumor treatment[5–7], immunotherapy[8,9], blood-brain barrier (BBB) disruption[10–12], thrombolysis[13–15], and bacterial infection treatment[16,17]. A key phenomenon underpinning these applications is cavitation: the nucleation, growth, and oscillation of gaseous cavities due to changes in fluid pressure[18,19].

There is a range of mechanisms by which cavitation produces biological effects. For example, the highly nonlinear nature of bubble oscillations under the influence of an applied ultrasound field can generate microstreaming in the surrounding liquid that can both enhance drug convection[20] and exert shear stresses on the tissue in the vicinity of the bubbles. This is particularly prevalent when bubbles are in the vicinity of a boundary, causing bubbles to oscillate non-spherically, and may potentially promote drug uptake through shear-induced permeabilization[21–24]. At higher pressures, larger amplitude oscillations and rapid bubble collapse are observed, imparting direct mechanical stress[25] and frequently generating shock waves, and consequent large pressure gradients, that can disrupt and permeabilize tissues[26,27]. The collapse of bubbles near a surface can also result in the formation of high-velocity liquid microjets[28–30]. These microjets can penetrate tissue, potentially creating pores or inducing secondary stress waves[31,32]. The permeabilization of biological membranes at both the tissue and cellular levels is variously referred to sonophoresis, used primarily in the context of US-induced enhancement in skin permeability[33,34], and sonoporation, used mainly to describe the reversible permeabilization of the cellular membrane due to formation of membrane pores[35,36].

Viscous absorption in the liquid immediately surrounding the oscillating bubble can produce a substantial heating effect[37]. Moreover, the highly non-linear oscillations produce acoustic radiation at frequencies higher than the driving ultrasound field. This leads to increased absorption in the surrounding tissue and further heating[38]. Bubble collapse may also be accompanied by chemical effects due to the transient high temperatures and pressures in the bubble core, such as generation of highly reactive species and electromagnetic radiation, known as sonoluminescence[32]. These effects have been investigated to assess potential damage and/or activation of relevant cellular pathways for delivery[39] and exploited in local activation of light-sensitive drugs in an approach known as sonodynamic therapy[40–43].

Many US mediated bioeffects may be initiated solely through control of US field parameters (pressure amplitude, frequency, pulse length and repetition frequency, and duration of exposure), but to reliably generate cavitation in biological tissue often requires high input energies and hence carries an elevated risk of damage. Introduction of exogenous or artificial cavitation nuclei may substantially reduce the input energy required to produce the broad range of effects discussed above and further introduces additional effects that may not be possible with US alone. Cavitation nuclei include gas bubbles[26,44], liquid droplets[45–47] and solid particles[48–50],

with nanoscale cavitation nuclei being an emergent area of investigation for their benefits in terms of prolonged circulation time, improved extravasation and prolonged cavitation activity[49,51–53].

The most commonly used nuclei are gas microbubbles (MBs), originally used as contrast agents in diagnostic imaging. They are typically 1-2 micrometers in diameter and contain a core of a high-molecular-weight gas with low aqueous solubility in the surrounding medium. The core is surrounded by a protective lipid, protein, or polymer shell most commonly consisting of bioinspired phospholipids[54]. When exposed to a US field, the compressibility of the MBs causes them to undergo volumetric oscillations, consequently producing a strong acoustic scattering, which is responsible for the success of MBs as a contrast agent. As mentioned, these oscillations also lead to the aforementioned mechanical, thermal, and chemical effects that can be harnessed in therapeutic applications. The MB coating process also offers a mechanism for encapsulating drugs within the MB structure and for attaching drugs and/or targeting species to the MB surface. This technique facilitates the triggered release of drugs to reduce systemic toxicity[55]. It has also recently been shown that material from the microbubble surface may be transferred to biological structures, enhancing drug delivery through so called "sonoprinting"[56–58].

Monitoring of US-mediated cavitation activity can provide insights into the resultant biological effects both in vitro and in vivo and potentially allows for the tuning and optimization of these effects. The two most widely applied methods for monitoring cavitation activity are i) optical, which use ultra high speed video microscopy and is generally not feasible in vivo*;* and ii) acoustic, which records the re-radiated sound fields produced by oscillating and/or collapsing bubbles. Both the amplitude and frequency components of the acoustic signal contain information on bubble behavior. Low concentrations of bubbles at low incident US amplitudes have been shown to produce predominantly harmonic emissions (integer multiples of the driving frequency)[59]. As driving pressures increase, the bubble emission spectrum may also contain fractional components known as subharmonics and ultraharmonics[60] that indicate stronger nonlinear behavior, as well as broadband noise indicative of inertial cavitation. Integer harmonics are a primary indicators of bubble oscillation but can also be caused by non-linearities anywhere in an experimental system, e.g., due to non-linear propagation. By contrast, fractional harmonics and broadband noise are very strongly correlated with bubble dynamics.

The relationship between bubble behavior and the detected acoustic emissions may be complicated by factors including the incident US field, the nucleation environment, and the characteristics of the detection pathway[60]. Nevertheless, important information about bubble behavior and their interactions with cells can be gained by discerning trends in frequency and energy in the acoustic spectrum. These data can also provide valuable information that can be used to form the basis for clinical treatment monitoring techniques. To fully exploit this information, the development of robust, translatable, and reproducible experimental methods is required.

Currently there is substantial variation in reported protocols. In terms of the apparatus, a range of design approaches has been undertaken. Several groups have made use of parallel-plate

chambers[56,61–63], either custom built or commercially available (e.g., OptiCell, ThermoFisher Scientific). Hu et al.'s (2013) developed a cell chamber coupled with an US sonication module and real-time confocal imaging[64], Carugo et al. (2015) used a system comprising a commercially available cell culture dish with a custom-made PDMS lid to allow for submersion in a water bath during US exposure[65], and Pereno et al. (2018) used a device consisting of layered acoustofluidic resonators that allow for simultaneous optical and acoustic characterization of bubble dynamics and bubble-cell interactions[66]. The use of custom-fabricated and application-specific designs complicates characterization of the US field and other environmental exposure conditions, making cross study comparisons challenging. For example, there is considerable variation in the US parameters identified for achieving successful sonoporation, which include center frequencies ranging from 0.02 to 15 MHz, duty cycles varying from 1% to continuous wave, and rarefactional pressures ranging from 0.1 to 20 MPa[23,64,67–70] (**Table 1**). There is similarly considerable variation in the spectral components (harmonics, sub-harmonics etc.) that have been identified as being associated with particular bioeffects.

The aim of this work, therefore, is to provide an easily reproducible system design and implementation framework for the in vitro study of cavitation-induced cellular bioeffects with the specific inclusion of a cavitation monitoring capability.

PROTOCOL

1. System Design Principles

NOTE: This section presents the design principles used to create systems for ultrasound exposure and cavitation monitoring. These principles are illustrated with two existing systems for acoustic transfection (SAT) (shown in **Figure 1**). Each system consists of a cell exposure compartment, an ultrasound source, and a single element transducer functioning as a passive cavitation detector (PCD), all of which are integrated into a benchtop test chamber. These designs build upon the prior system development described in Carugo et al. (2015)[65].

1.1. Maximize ease of use.

1.1.1. Make the cell exposure compartment compatible with existing culture techniques and imaging systems by using existing commercial cell culture devices as seeding/growth substrates.

1.1.1.1. For SAT2, use a culture dish (35 mm diameter, of which a 21 mm diameter area is observable, see **Table of Materials**).

1.1.1.2. For SAT3, use a Transwell insert (6.5 mm diameter, see **Table of Materials**). The Transwells have a permeable membrane and hence need to be placed in cell media rather than water.

1.2. Enable rapid loading and sealing of the cell exposure compartment.

1.2.1. Form the SAT2 cell exposure compartment by press fitting a flexible polymer lid over the culture dish (Carugo et al. 2015[65]). As seen in **Figure 1C**, the lid has a pair of 1.2 mm diameter holes that allow filling the compartment with an 18 G blunt needle syringe. After filling, seal these filling ports with short plastic rods (**Table of Materials**).

1.2.2. Fill the SAT3 compartment by syringe or pipette and seal by press fitting a rubber stopper/bung.

1.2.3. Enable rapid loading of the sealed cell exposure compartment into the test chamber. Holders for the cell exposure compartments were built into the chamber lids, where a light press-fit is sufficient to ensure proper alignment. With the systems shown in **Figure 1**, the time for sample changes can be as short as 20 seconds when multiple cell exposure compartments have been prepared in advance.

1.2.4. Minimize the chamber internal volume so that the system is portable and the amount of required water/media can be minimized. Doing so also accelerates recovery from accidental spills or leaks of cavitation agents out of the cell exposure compartment.

NOTE: The SAT2 internal volume is approximately 0.8 L. The SAT3 internal volume is approximately 7.6 L – made larger to accommodate easy loading and change of source transducer or its configuration. An internal chamber of 0.3 L was added to minimize the disposable volume and to allow biologically relevant fluids other than the tank fill water (e.g., cell culture media) to be used. The internal chamber bottom is made from 30 μm thick mylar sheet to allow maximum acoustic transmission.

1.2.5. Make the chamber and the internal components out of optically clear materials when possible, so that any problems (e.g., leaks, entrapped macrobubbles) can be quickly observed and remedied.

1.3. Maximize acoustic transmissibility of the exposure compartment.

1.3.1. Maximize transmissibility through the choices of compartment wall materials and thicknesses. Under the assumption that the liquids on either side of a wall are essentially the same (e.g., water), the magnitude of the normal incidence pressure transmission coefficient[71] is: $|T| = \left(1 + \left(\gamma \sin(\frac{2\pi L}{\lambda})\right)^2\right)^{-\frac{1}{2}}$, where λ is the wavelength in the wall of thickness L, $\gamma = 0.5 \cdot |z_L/z_o - z_o/z_L|$, and z_L and z_o are the characteristic impedances (products of density and sound speed) for the wall material and liquid, respectively. T=1 indicates perfect transmission.

1.3.2. For broad spectrum monitoring of cavitation (e.g., 1-8 MHz), most laboratory polymers (e.g., PDMS, PTFE, polystyrene) will alter the transmitted pressure by no more than 10% if the thickness of material is less than 1/10th of a wavelength in the material. This condition can be difficult to meet with standard supplies at high frequencies (e.g., #1.5 coverslip at 8 MHz), so it is

good practice to predict or directly calibrate the transmission frequency response.

1.3.3. For narrow band transmission of the ultrasound source signal into the cell exposure compartment, allow a thicker layer if it is approximately an integer multiple of a half wavelength in the layer material. For example, the PDMS lid in SAT2 is used at a thickness of 2.0 mm (~1 wavelength at 1MHz, c_{PDMS} ~ 1000 m/s).

1.4. Maximize the exposure region through selection of source and cell environment.

1.4.1. To maximize the number of exposed cells, make the area of cell attachment as broad as possible while maintaining compatibility with available culturing and imaging equipment.

1.4.2. Use an ultrasound source with a field that spans the cell attachment area with minimal spatial variability by using the pre-focal region of a large focused source (SAT2) or a focused or lensed source with a main lobe width that matches the diameter of the cell attachment area (SAT3). See the **Table of Materials** for specific sources.

1.4.3. Minimize the field complexity introduced by the cell compartment holder by mechanically supporting the cell compartment well away from the strongest part of the incident field, minimizing the scattering cross section of the holder or placing absorbing material on the holder. Examples are shown in **Figure 1A** and **1D**.

1.5. Ensure repeatable exposure conditions.

1.5.1. Terminate the acoustic field in a fixed boundary to eliminate variability that can arise from air-water interfaces in partially filled chambers. In SAT2 and 3, this is accomplished by installing an acoustic absorber (see **Table of Materials**) on the chamber lid with a further benefit of reducing the field complexity that may arise from boundary reflections.

1.5.2. Monitor and record the source drive voltage at the amplifier output/source input so that minor variability or major malfunction can be detected quickly. Use a voltage probe or other device that is safe to use over the drive voltage range of interest. Periodically check the calibration of the voltage probe using a well-known source such as a waveform generator.

1.5.3. Control, monitor, and record the temperature of the chamber and its contents. Responses of cells, transducers, and propagation medium may all be temperature sensitive. In SAT2, monitoring and control are accomplished with a pair of circulation ports connected to a water conditioning system, whilst SAT3 employs an aquarium heater (not shown). Set the water temperature as needed to mimic the relevant physiological conditions for the therapeutic application.

1.5.4. Carefully degas the chamber liquid(s) to minimize the likelihood of unintended cavitation and/or scattering from pre-existing bubbles in the propagation path.

1.6. Calibrate the fully assembled system.

1.6.1. Include a means of measuring the pressure field incident upon the exposed cells when all system components are in place, including the cell exposure compartment. In SAT2 and 3, this is accomplished with an opening in the chamber lid through which a needle or fiber optic hydrophone could be inserted without disturbing the field to be measured. Make the measurements as close as possible to where the cells are located.

1.6.2. Choose a hydrophone with a sensitive radius (a_{rcv}) is small enough that it will not misreport the pressure field being measured. The acceptable size is a function of source frequency (f) and radius (a_{src}) as well as the distance between source and the field scan (z_{rcv}). A general criterion for hydrophone size selection is: $\frac{4\pi f}{c}\frac{a_{src}\,a_{rcv}}{z_{rcv}} < 1$, leading to: $a_{rcv} < \frac{c}{4\pi f}\frac{z_{rcv}}{a_{src}}$, where c is the sound speed[72].

1.6.3. Ensure that the hydrophone is calibrated under the conditions used in system characterization, including the temperature. Specifically, if the hydrophone is held at an angle with respect to the scan plane, the hydrophone must be calibrated at that angle, as directivity effects may differ significantly from those expected based purely on geometry. The change in hydrophone sensitivity with respect to temperature should be available from the manufacturer.

1.6.4. Scan the entire region in which cells may be exposed. To capture an appropriate level of field detail, use a scan spacing no coarser than 1/5th of a wavelength at the highest frequency of interest. If unexpected field complexity is observed, consider using short burst signals (e.g., 1-3 cycles) to allow identification and quantification of direct and scattered field contributions.

1.7. Incorporate a cavitation monitoring capability.

1.7.1. Determine the monitoring transducer type and placement as part of the design of the overall system, rather than as a retrofit. In practice, this leads to a system that is maximally compact without sacrificing the ability to reliably align the critical system components.

1.7.2. Place a cavitation monitoring device in the system such that it can be repeatably positioned with minimal added setup time or disturbance to workflow. In SAT2, this is accomplished with a single element piezoelectric transducer acting as a PCD fitted into the chamber lid, whilst SAT3 integrates the PCD into the source base using a 90° reflector.

1.7.3. Select the PCD shape according to the objectives of the experiments. In **Figure 2**, calculations of the half-amplitude contours of unfocused (left) and focused (right) devices show the profound differences in spatial sensitivity with respect to frequency. The unfocused device is better suited for large volume monitoring with modest spatial variation with respect to frequency, while the focused device is better suited for more radially compact measurements at the frequencies of interest.

1.7.4. Select the PCD center frequency and bandwidth to fit the needs of the experiment. The center frequency is typically chosen to be at least five times that of the ultrasound source in order to minimize sensitivity to direct source emissions. Bandwidth is typically maximized in order to observe a broad range of bubble behaviors (harmonic and broadband noise).

1.7.5. Select conditioning, recording, and processing methods to allow analysis of cavitation data, as described in the next section.

2. Instrumentation and Processing for Cavitation Monitoring

NOTE: This section presents the signal flow components and functions recommended for collection of cavitation monitoring data, and the data processing that leads to qualitative and quantitative assessments of cavitation activity.

2.1. Instrumentation (See also **Figure 3**).

2.1.1. Unless the application calls for a customized device, select a PCD from the broad range of commercially available single element transducers, typically marketed for non-destructive testing of submerged targets. These suppliers also have cabling and accessories (e.g., the mirror reflector in SAT3).

2.1.2. Minimize PCD response to the ultrasound source. This may be done both through selection of the PCD (center frequency and bandwidth) by using a notch or high pass filter. The latter is implementable either as a standalone module or as part of a signal conditioning device.

2.1.3. Optimize the use of digitizer dynamic range. PCD signals may cover several orders of magnitude, both over a long exposure (as bubbles are eliminated) or if running experiments at varying ultrasound drive levels. It is therefore necessary to check that the signal conditioning chain scales all signals so that they can be properly recorded.

2.1.4. Use a digitizer with a large dynamic range (at least 12 bits) to capture as much data as possible with minimal likelihood of high signal clipping and maximal signal to noise ratio (SNR) of the smallest signals. When comparing devices, review the specifications for signal to noise and distortion and/or effective number of bits, as these are more complete descriptions of attainable dynamic range. Also consider whether the size of the memory buffer is sufficient for the desired rate of data capture.

2.1.5. Include a preamplifier in the signal chain, so that the smallest expected signals can be adequately captured. In our experience, PCD self-noise is well below that of most digitizers, so a modest degree of preamplification (e.g., <100x) can still improve the SNR of the final result.

2.1.6. Filter the ultrasound source frequency prior to preamplification to avoid saturating the amplifier.

2.1.7. If an ultrasound pulser/receiver is used to provide gain and/or filtering capabilities, use it in pulser mode to confirm path length/alignment or check for unexpected scatterers in the propagation path between the PCD and the cell exposure compartment.

2.1.8. Enable real time streaming of data to storage. The SAT2 and SAT3 systems both employ 12-bit streaming USB oscilloscopes, which have the conveniences of portability and well-built user interfaces.

2.1.9. Confirm proper impedance matching in the signal chain to avoid gain or bandwidth errors. PCD devices typically have output impedances near 50 ohms, so a suitable process is to replace the PCD with a known signal from a waveform generator (with 50 ohm output impedance) and confirm that the signal size appearing on the digitizer matches expectations, scales linearly when the injected signal is changed, and no clipping is observed for the largest signal of interest.

2.2. Processing

2.2.1. Correct the raw voltage signals for all known gains and sensitivities in the signal path that relate to processing of data in the frequency range of interest.

2.2.2. If the data were recorded with DC input coupling or otherwise show a DC-offset, remove this offset by direct subtraction or with a high pass filter.

2.3. Compute the power spectrum P of each recorded signal.

2.3.1. Set the Fourier transform length N_{ft} so that the fundamental frequency of the ultrasound source f_0 is a large integer multiple of the transform bin width: $N_{ft} = nf_s/f_0$, where n is an integer and f_s is the sample frequency of the digitized data. Set $n \geq 50$ for clear capture of spectral features. For the example of a 1 MHz fundamental sampled at 50 MHz, N_{ft} is 2500 and the bin width is 0.02 MHz.

2.3.2. As the transform length N_{ft} is usually smaller than the duration of recorded signal, use a power spectral density (P) estimator such as Welch's method[73]. Power in a frequency band spanning f_1-f_2 is $P = \int_{f_1}^{f_2} PSD\ dF$, where dF is the transform bin width.

2.3.3. To characterize bubble activity during each exposure, estimate the contributions to the power spectrum from integer multiples of f_0 (harmonics), odd integer multiples of $f_0/2$ (ultraharmonics), and broadband noise (inertial cavitation).

2.3.4. Harmonic and ultraharmonic content may be most simply estimated by selecting the power spectrum values at specific frequencies, although large amplitude tonal responses may spread into a smaller number of adjoining frequency bins.

2.3.5. Estimate broad band cavitation power by subtraction of the harmonic and ultraharmonic

contributions from the total power spectrum. Alternately, these contributions may be estimated using more sophisticated pre-processing[74].

2.3.6. Estimate the cumulative cavitation signal energy over the duration of the exposure, preferably according to spectrum feature / bubble activity type.

2.3.7. Assuming all data records had equal duration T_r, the cumulative energy in the recorded data is $E = T_r \sum_{m=1}^{M} P_m$, where M indicates the number or records.

2.3.8. If there are gaps between recordings, as may happen when the exposure is continuous, and the saved files capture a fraction of the total exposure duration T_e, the cumulative energy may be estimated as $E = (T_e/M) \sum_{m=1}^{M} P_m$

2.3.9. Estimate the measurement SNR by comparison of spectrum levels with those of background noise recorded in absence of ultrasound.

3. Experimental Protocol

3.1. SAT Preparation

3.1.1. Minimize the likelihood of cavitation in the propagation path by degassing the fill liquid (typically filtered water) under a pressure of -10^5 Pa for at least two hours. Confirmation with a dissolved oxygen probe that the partial pressure of oxygen is below 10 kPa is recommended.

3.1.2. Fill the test chamber slowly to minimize re-introduction of air into the degassed liquid. Continue degassing the filled chamber if necessary.

3.1.3. Clear all residual bubbles from the transducer and media container surfaces immediately after filling and again just before initiating exposure experiments.

3.1.4. Make sure the temperature of the chamber and its contents have stabilized before commencing exposure experiments.

3.1.5. Allow the ultrasound source power amplifier to warm up (per manufacturer recommendation) so that the gain and output are stable with respect to time.

3.2. Exposure Compartment Preparation

3.2.1. Cavitation agent suspension

3.2.1.1. When diluting the cavitation agent, gently and continuously stir to make a uniform suspension without entrapping macrobubbles or destroying the agent (especially if they are shelled bubbles).

3.2.1.2. When working with microbubbles, withdraw and dispense slowly using the largest gauge needle available to minimize destruction during the loading process[75]. An 18 G blunt fill needle has been used regularly with the SAT systems.

3.3. SAT2 Preparation

3.3.1. Sterilize the PDMS lid before use in experiments with live cells.

3.3.2. Form the cell exposure compartment by press fitting the PDMS lid to the culture dish.

3.3.3. Prepare a syringe with an 18 G blunt needle and fill with approximately 10 mL of liquid (e.g., microbubble suspension or water control).

3.3.4. Insert the needle through one of the PDMS fill holes and slowly fill the chamber, tilting so that macrobubbles can escape through the open fill hole. For best results, tilt the chamber so that the open hole is vertically above the fill hole.

3.3.5. When filled, close the open hole by inserting a short (4-5 mm) polymer rod. Set the assembly so both holes are horizontal.

3.3.6. Remove the blunt filling needle while injecting extra fluid so that air is not drawn in. Close the hole with another polymer rod. This process completes the sealing of the cell exposure compartment.

3.3.7. Visually check the compartment for evidence of entrapped macrobubbles, and if any are found, repeat 3.3.3.-3.3.6.

3.3.8. Press fit the cell exposure compartment in the compartment holder. Install the chamber lid in place atop the chamber. Lowering the lid with an angle to horizontal discourages macrobubbles from resting on the submerged parts (absorber, holder).

3.3.9. Consider the buoyancy of the particles in suspension when deciding the orientation of the cell exposure compartment (e.g., floating bubbles or sinking nanoparticles) and how this will affect their contact with cells.

3.3.10. In all operations, use as little force as possible to minimize the flexure of the cell growth surface and detachment of cells.

3.4. SAT3 Preparation

3.4.1. Fill the Transwell with approximately 150 μL of liquid (e.g., microbubble suspension or water control).

3.4.2. Form the cell exposure compartment by carefully sealing the transwell with a rubber plug,

removing any overflow liquid with a clean paper towel or wipe. Before use in experiments with live cells, sterilize the rubber plug.

3.4.3. Visually check the compartment for evidence of entrapped macrobubbles, and if any are found, remove the plug, remove the macrobubbles and repeat 3.4.2.

3.4.4. Press fit the cell exposure compartment in the compartment holder. Install the chamber lid in place atop the chamber. Lowering the lid with an angle to horizontal discourages macrobubbles from resting on the submerged parts (absorber, holder).

NOTE: As noted above, macrobubbles may cause a variety of non-repeatable and potentially detrimental effects on US exposure experiments. Most critically, macrobubbles trapped in the cell exposure compartment may cause PCD responses and local cellular bioeffects that are not representative of the intended treatment. Always visually inspect all system components to find and remove macrobubbles before initiating US experiments.

4. Data Collection

4.1. Establish background PCD response levels by conducting initial experiments with a cell exposure compartment filled with control liquid (e.g., degassed water or cell media).

4.2. Record PCD data without driving the ultrasound source to establish background electronic noise levels.

4.3. Record PCD while driving the ultrasound source at the full range of planned drive levels. This data will indicate what parts of the acoustic response are unrelated to the cavitation agents to be tested subsequently.

NOTE*:* Common laboratory liquids (e.g., PBS or cell media) will exhibit cavitation if not degassed.

4.4. Before commencing measurements, give time for the suspension to thermally equilibrate with the chamber temperature. A fine needle thermocouple may be useful for this purpose.

4.5. Monitor the experiments in real time in both the time and frequency domains.

4.5.1. Time domain monitoring of the PCD reveals whether signals are sized appropriately for the current instrumentation settings. Specifically, signal clipping is to be avoided, since it will appear in the frequency domain as multi-tone harmonic response.

4.5.2. Time domain monitoring of the PCD also reveals if cavitation signals are seen earlier than expected based on propagation time from ultrasound source to exposure compartment to PCD. If such signals are seen, this may indicate leakage of cavitation agent into the test chamber.

4.5.3. Frequency domain monitoring of the PCD indicates the type of bubble behavior and can

be used to adjust to drive levels as needed to achieve the desired cell stimulus (e.g., lower drive levels for harmonic excitation).

4.6. To ensure that the first exposures are not missed, start the data collection process prior to turning on the ultrasound source drive signal.

4.7. Monitor the amplifier output signal that drives the ultrasound source (as opposed to the waveform generator output) throughout the experiment to ensure that the exposure is proceeding as expected. Use a high voltage probe for this measurement and make sure that the oscilloscope is set to compensate for probe attenuation.

4.8. After exposing a sample, carefully remove it from the test chamber.

4.8.1. Remove and clean the PDMS lid (SAT2)/rubber plug (SAT3) in preparation for any subsequent use.

4.8.2. Transfer the culture dish (SAT2)/Transwell (SAT3) as needed for subsequent analysis (e.g., microscopy, fluorescence imaging).

4.8.3. After a small number of exposures (e.g., 3-5), it is good practice to re-acquire baseline cavitation signals (in the absence of cavitation agent) and compare with the original data set to make sure the chamber media have not been contaminated.

REPRESENTATIVE RESULTS:

Figure 4 shows examples of time and frequency domain PCD responses, illustrating three distinct cavitation behaviors. All data were collected on SAT3 using SonoVue microbubbles diluted 5x in PBS. The temperature for all examples in this section was 19 ± 1 °C. The ultrasound source was driven with a 2.0 ms pulse at 0.5 MHz to achieve incident pressures of 0.20 (**Figures 4A and 4B**), 0.30 (**Figure 4C and 4D**), and 0.70 MPa (**Figures 4E and 4F**). The signal recordings began 1.4 ms before the t=0 start of the ultrasound pulse. The inset traces show the signal as recorded (red) and with a 2 MHz high pass filter (blue) for a time window centered at the time of flight from source to cell exposure compartment to PCD. The low-level response before this time is due to directly received radiation from the source, which is common in configurations where the PCD is behind the ultrasound source.

At the lowest incident pressure, the PCD response consists entirely of integer harmonics of the 0.5 MHz fundamental ultrasound frequency. Increasing from 0.20 to 0.30 MPa results in pronounced ultraharmonics in the spectrum in addition to further elevated integer harmonics. The time domain waveforms at these two pressures look similar, although the 0.30 MPa results show more variability over the pulse duration. At the highest pressure, the time domain waveform amplitude has grown nonlinearly relative to the lower pressures as a result of clearly elevated broadband noise visible in the spectrum. This noise is commonly considered to be a result of inertial cavitation and in this example, corresponds to destruction of microbubbles.

To see this more clearly, PCD responses as a function of time are shown in **Figure 5**. In the left panel (**Figure 5A**), full spectra are shown over a 50 second exposure time, during which the source emitted 2.0 ms pulses every 0.20 seconds. Corresponding total, harmonic and broadband powers are shown in the right panel (**Figure 5B**). The ultrasound was turned on at t=3.0s, at which time large-amplitude broadband responses were seen. The initial spike is thought to correspond to the destruction of the largest bubbles in the suspension (SonoVue is polydisperse) and is a common observation in cavitation experiments with shelled bubbles and even with non-degassed media (e.g., PBS).

After a few seconds, the broadband response rapidly diminished, apparently due to bubble destruction, and the signal is predominantly comprised of harmonics. This suggests that the freed gas and remaining microbubbles are vibrating stably and non-inertially. At t~50s, the broadband component has fallen to the level of the original background noise. Exposure tests like this are therefore important when trying to understand the timescales during which different bubble effects may be acting upon the cells in the chamber.

Bubbles are likely to translate in response to radiation forces generated during US exposure and movement of microbubbles in and out of the PCD field of view can lead to increased variability in the monitored cavitation signal, especially when dealing with dilute suspensions. The sensitive region of the PCD should therefore span as much of the cell exposure surface as possible. A response comparison of focused and unfocused PCDs with identical center frequencies (see **Figure 2**) is shown in **Figure 6**, using a 20:1 dilution of microbubbles in normal PBS. The time and sample-averaged spectra in panel (**Figure 6A**) show that the unfocused PCD contains a stronger broadband response, accompanied by reduced sample-to-sample variability in both harmonic (**Figure 6B**) and ultraharmonic powers (**Figure 6C**).

It is important to recognize that media used for in vitro cell work are not degassed and may present an enhanced background level of bubble activity. **Figure 7** shows the response of PBS used in its supplier-provided form and after two hours of degassing under vacuum, after which the air saturation was reduced from 92% to 46% as determined with an optical sensor (PreSens, Germany). The spectra in **Figure 7A** were averaged over exposure time and repeats with five independent samples show clearly elevated ultraharmonics in normal PBS. Powers summed over three harmonics (**Figure 7B**) are well within the standard deviation of each experimental output. By contrast, the ultraharmonic sums in **Figure 7C** show that normal PBS has nearly an order of magnitude higher level and substantially higher variability between samples. These examples indicate that a common cell-compatible medium may exhibit behaviors that could be (incorrectly) attributed to the presence of microbubbles. Since it is usually impractical to degas culture medium due to the negative impact upon cells and/or cavitation agent stability, it is critical to perform suitable controls in any cavitation-related study.

FIGURE AND TABLE LEGENDS:

Figure 1. Illustrations of two ultrasound exposure system designs incorporating cavitation monitoring: SAT2 (A-C), SAT3 (D-F). (A) SAT2 annotated assembly with side wall removed for clarity. (B) SAT2 with side wall intact. (C) SAT2 cell exposure compartment, disassembled. (D)

SAT3 annotated assembly. (E) SAT3 in normal (left) and lensed (right) configurations for beam width matching at different frequencies. (F) SAT3 cell exposure compartment, disassembled.

Figure 2: Calculations of half amplitude pressure field contours for 12.7 mm diameter unfocused (left) and spherically focused (right) transducers. Frequencies of 2, 4 and 8 MHz are shown as red, blue, and green contours, respectively, for a PCD element at the coordinate origin (0,0). The outermost contours of the unfocused device are relatively insensitive to frequency, but the interior structure is frequency dependent. The spherically focused field contracts as frequency increases, but inside the contours, the fields vary smoothly.

Figure 3. Instrumentation for cavitation signal conditioning and recording (blue arrows), US source excitation (red lines), and data acquisition triggering.

Figure 4. Time (left) and frequency (right) domain PCD responses recorded with microbubbles diluted 5x in PBS. Incident peak pressures were (A, B) 0.2 MPa, (C, D) 0.4 MPa, (E, F) 0.7 MPa, all at 0.5 MHz. Signal recordings begin 1.4 ms before the t=0 start of the 2.0 ms duration ultrasound pulse. (A, C, E) Time domain signals (red) are shown on a fixed vertical scale, indicating how the response level changes with incident pressure. The inset traces show the signal as recorded (red) and with a 2 MHz high pass filter (blue) for a time window centered at the time of flight from source to cell exposure compartment to PCD. (B, D, F) Noise and signal power spectral densities are calculated for t<0 and t>0, respectively.

Figure 5. Spectrum histories over a 50 second exposure of a suspension of microbubbles in diluted 5x in PBS. (A) Full spectra and (B) total, harmonic and broadband signal powers, all as a function of time. Drive conditions were 0.5 MHz, 0.7 MPa peak pressure, 2.0 ms pulse duration, 200 ms pulse repetition period.

Figure 6. Effect of PCD focusing geometry recorded with PBS. Drive conditions were: 1.0 MHz, 0.50 MPa peak pressure, 3.0 ms pulse duration, 10 ms pulse repetition period. (A) Full spectra averaged over exposure time and three independent sample repeats. (B) Power in 3, 4 and 5 MHz harmonics, and (C) Power in 2.5, 3.5 and 4.5 MHz ultraharmonics. Thick lines are sample means, shaded areas indicate +/- 1 standard deviation.

Figure 7. Effect of degassed media recorded with PBS. (A) Full spectra averaged over exposure time and five independent sample repeats. (B) Power in 3, 4 and 5 MHz harmonics, and (C) Power in 2.5, 3.5 and 4.5 MHz ultraharmonics. Thick lines are sample means, shaded areas indicate +/- 1 standard deviation. Drive conditions were 1.0 MHz, 0.50 MPa peak pressure, 1.0 ms pulse duration, 200 ms pulse repetition period.

Table 1. Summary of the range of reported parameters facilitating sonoporation *in vitro*.

DISCUSSION:

The critical steps for any acoustic measurement were encapsulated by Apfel in 1981[76] as "know thy liquid, know thy sound field, know when something happens." In the context of this protocol, these encompass the transducer calibration and alignment and the water preparation and bubble handling steps. First, it is essential that the hydrophone used to calibrate the driving transducer and/or the PCD is itself accurately calibrated through regular external servicing or in-house comparison to a reference standard. Similarly, the response of both the driving transducer and PCD need to be regularly characterized to check for any change in output and/or loss of sensitivity. If the driving conditions and receive sensitivity of the system are unknown, then it will be impossible to infer any meaningful relationship between exposure conditions, bioeffects and acoustic emissions. Directly related to this, the alignment of the transducers to each other and the sample chamber need to be carefully checked to ensure that the exposure conditions within the chamber are as expected and the sampling volume for the PCD corresponds to the region of interest. As indicated, the temperature and gas content of the suspending medium can affect the final results significantly and consistency is extremely important in this respect[77,78]. Similarly, the preparation, characterization, and handling of the cavitation agent suspension require very close attention to ensure that the expected size distribution and concentration of particles is present within the sample. For example, if the concentration of bubbles is too high, there will be effective shielding of the sample volume from the incident ultrasound field. Microbubble agents are particularly susceptible to destruction and coalescence and further guidance on their handling may be found in[79].

A very common problem with detection of cavitation signals is achieving an adequate SNR. This is due partly to the nature of the signal itself, as described, but may also be due to sources of electrical noise within the experimental set up. Checking the connections between system components, in particular those involving co-axial cables, may help to eliminate some of these. Replacing or repairing co-axial cables may be necessary. Identifying and removing or deactivating other equipment in the laboratory such as pumps that may cause electrical noise can also help. Poor electrical impedance matching between system components can be a further cause of poor signal to noise ratio and also potentially of damage to equipment and should be carefully checked. The triggering settings on the signal generator and oscilloscope should similarly be checked to confirm that they are configured appropriately for the experiment and have not reverted to the manufacturer default settings. If there is significant destruction of bubbles during handling, it may be helpful to attach a second syringe to the outlet port and use this to gently extract fluid from the chamber, thereby drawing in the suspension. This can also help in eliminating macro bubbles or enabling flow during US exposure, if desired.

It is not possible to completely eliminate acoustic reflections within the sample chamber and hence the incident field will not be completely uniform over the whole the sample volume. As mentioned in steps 1.3.2 and 1.3.3, the transmissibility of acoustic windows will be frequency dependent and thus the desired bandwidth for acoustic emission measurements should be carefully considered. In particular there may be significant multiple reflections of higher frequency components. This is another reason why calibration of the field within the fully assembled system is so important for minimizing the uncertainty in incident pressure.

Appropriate gating of the recorded signals should also be considered to minimize the effects of multiple reflections. The use of commercial devices for convenience and the need for acoustic transparency means that some optical transparency must be sacrificed. This may impact the quality of subsequent imaging, e.g., to assess cell viability or drug uptake. Some of the membranes used in commercial devices are also porous and, thus, imperfect isolation occurs between the sample chamber and the surrounding water bath. As above, the corresponding risk of contamination can be mitigated by using a smaller sub-chamber, the contents of which can be regularly replaced. The cell culture devices indicated in the **Table of Materials** are suitable primarily for cell monolayers that may not be representative of tissues in terms of all ultrasound/cavitation mediated bioeffects. The proximity of the cells to a solid surface will also affect microbubble dynamics in a way that may not be reflective of conditions in vivo, e.g., promoting microstreaming and microjetting as described in the introduction. These limitations can be addressed, however, through a simple substitution of alternative tissue models.

The aim in proposing the SATs is to provide a means of improving the reproducibility of acoustic exposure conditions and acoustic emissions between studies of ultrasound mediated bio-effects, thus hopefully facilitating better understanding of the underlying mechanisms and the development of treatment monitoring techniques to improve safety and efficacy. The systems are designed to be compatible with commercially available cell culture devices, enabling a wide range of biological assays to be performed according to the application of interest and enabling the performance of high throughput experiments, removing the need for time consuming alignment procedures between runs. By standardizing protocols for the characterization of exposure conditions and the capture of acoustic emissions, the system dependent variability can hopefully be reduced. The range of parameters that should be explored for a particular experiment will depend upon the application (desired bio-effect, cell type, depth of target tissue if in vivo etc.) and the nature of any cavitation agent being used. Given the large number of variables (US frequency, pressure amplitude, pulse length, pulse repetition frequency etc.) fully exploring the whole parameter space is unlikely to be practicable. An advantage of the proposed protocol is that it enables some bounds on this parameter space to be quickly established. For example, it enables determination of the minimum pressure at which a cavitation signal is generated, the maximum pressure or pulse length that can be used before cell detachment/death occurs, and the pressure at which fractional harmonics or broadband noise are produced. It is recommended that such a set of scoping measurements be carried out as a first step in any study.

As presented, the SATs are designed for real-time monitoring of acoustic emissions, with biological assays being performed outside of the experiment. It would be relatively simple, however, to modify the SAT to enable direct optical observation of the sample chamber via a microscope objective. This could in turn be coupled to a fluorescence and/or high-speed microscopy system to enable observation of drug uptake and bubble dynamics, for example. The PCD output as currently presented in terms of voltage indicates: i) the types of cavitation behavior and their relative proportions; ii) how long these cavitation behaviors persist; iii) whether the observed time-cumulative exposure characteristics are correlated to a particular bioeffect; and iv) whether the relative levels and time-dependent behaviors are consistent with

previous experiments in the exposure system. Whilst the receive sensitivity of the PCD can be quantified, in order to reliably characterize the acoustic emissions in terms of absolute energy, additional spatial information is required. This could be achieved by replacing the PCD with an array probe to implement passive acoustic mapping (PAM)[80]. This would however increase the complexity of signal processing and the computational time and power required.

Other instrumentation for measurement of membrane electrical resistance or application of physical targeting methods, for example magnetic fields, could also be incorporated. It would also be possible to use three-dimensional tissue structures such as tumor spheroids, organoids, or even ex vivo tissue samples on acoustically "soft" gel substrates in place of the cell monolayers to study ultrasound and cavitation mediated effects in more realistic tissue environments.

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DISCLOSURES:

The authors have nothing to disclose.

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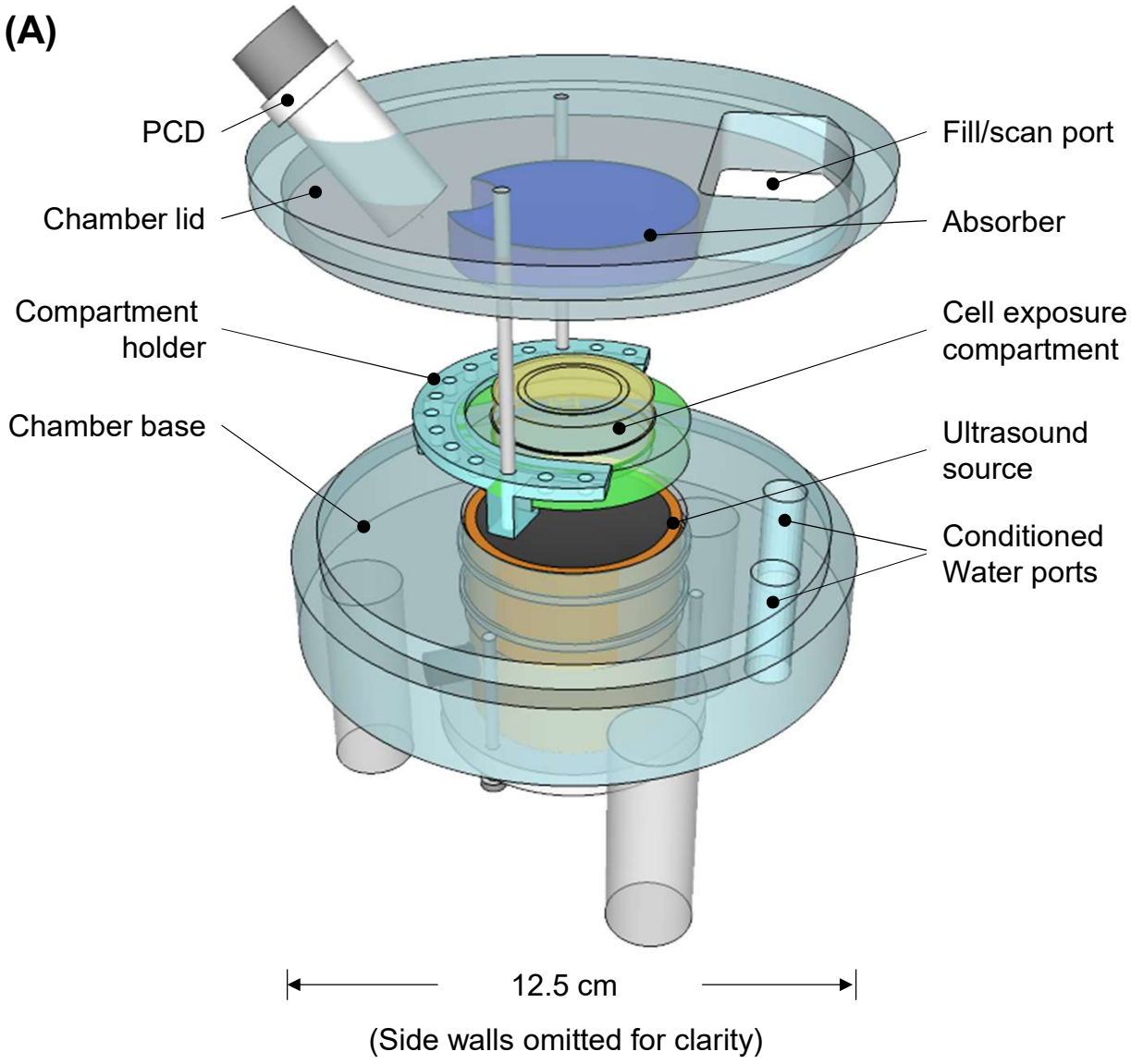

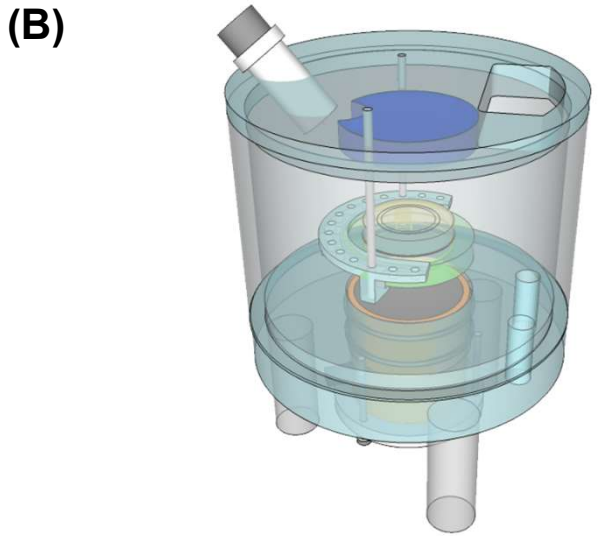

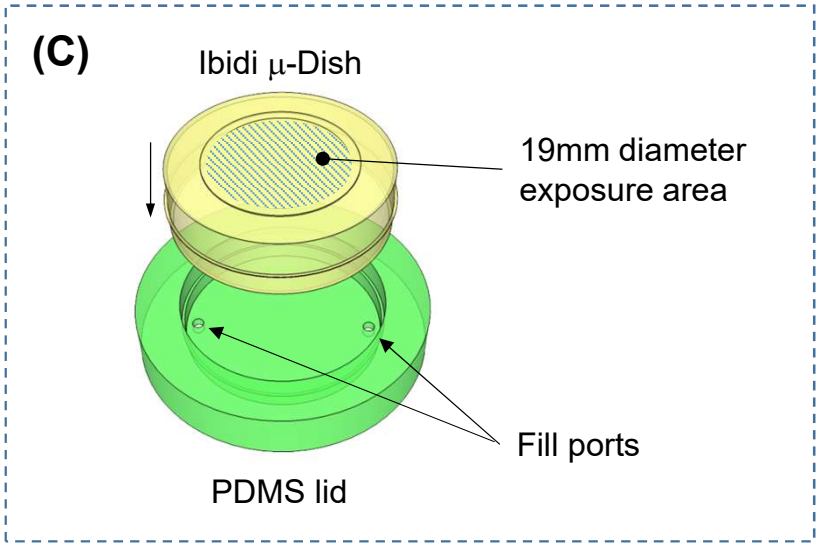

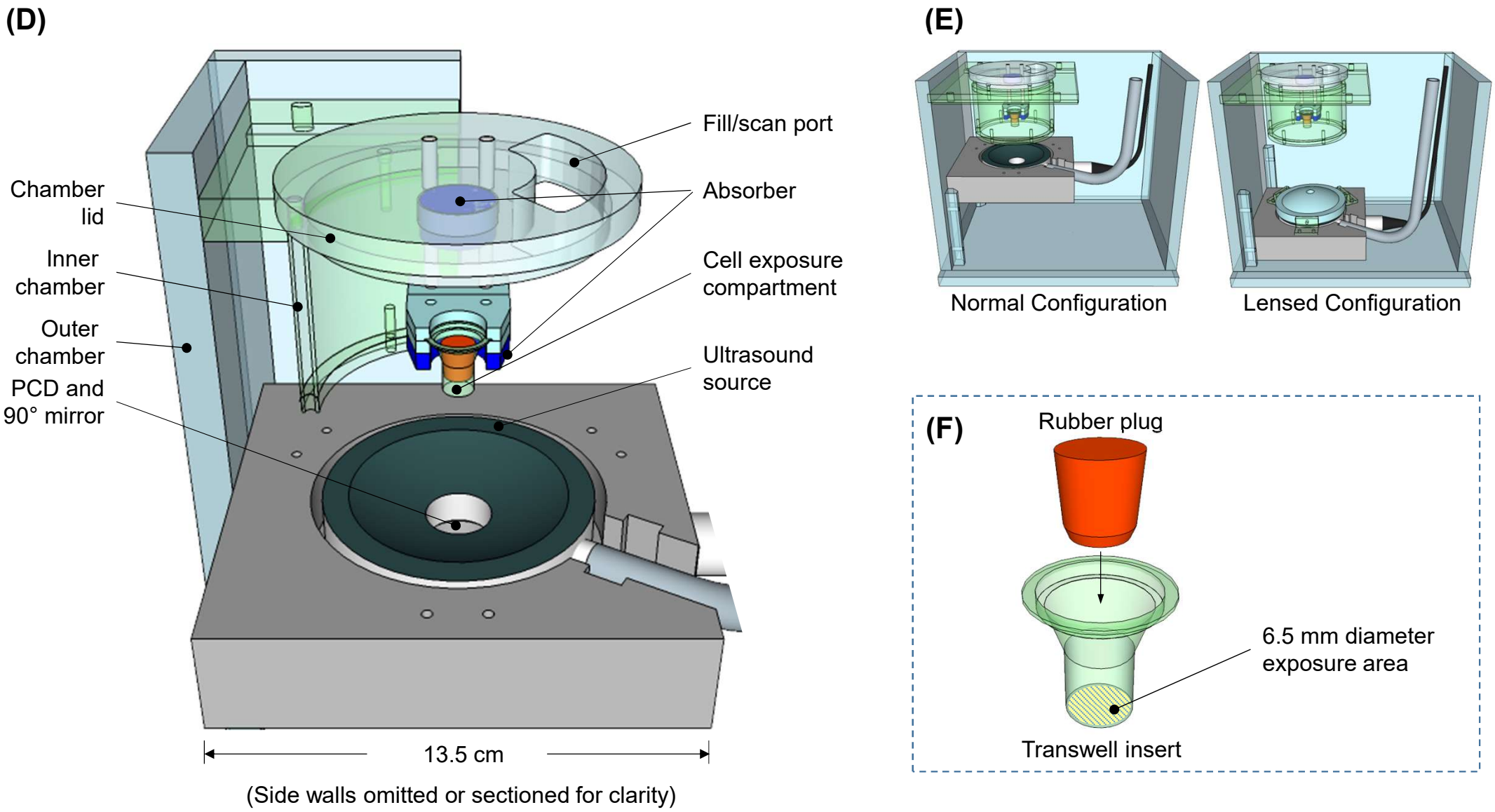

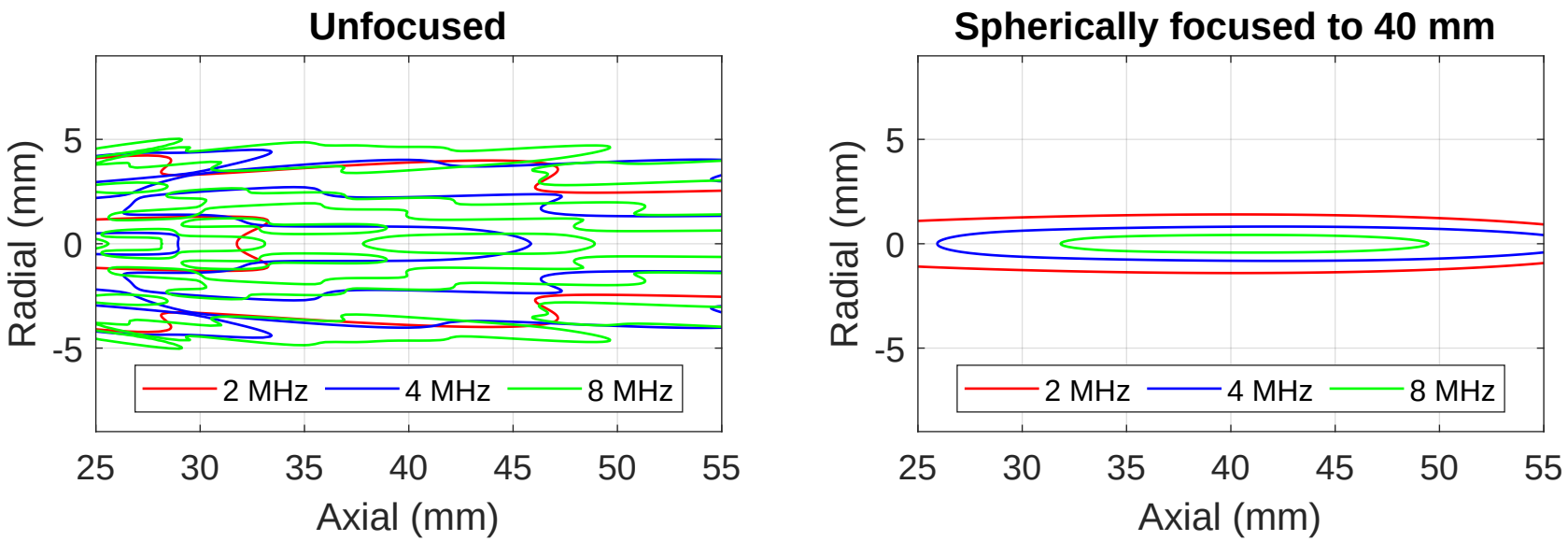

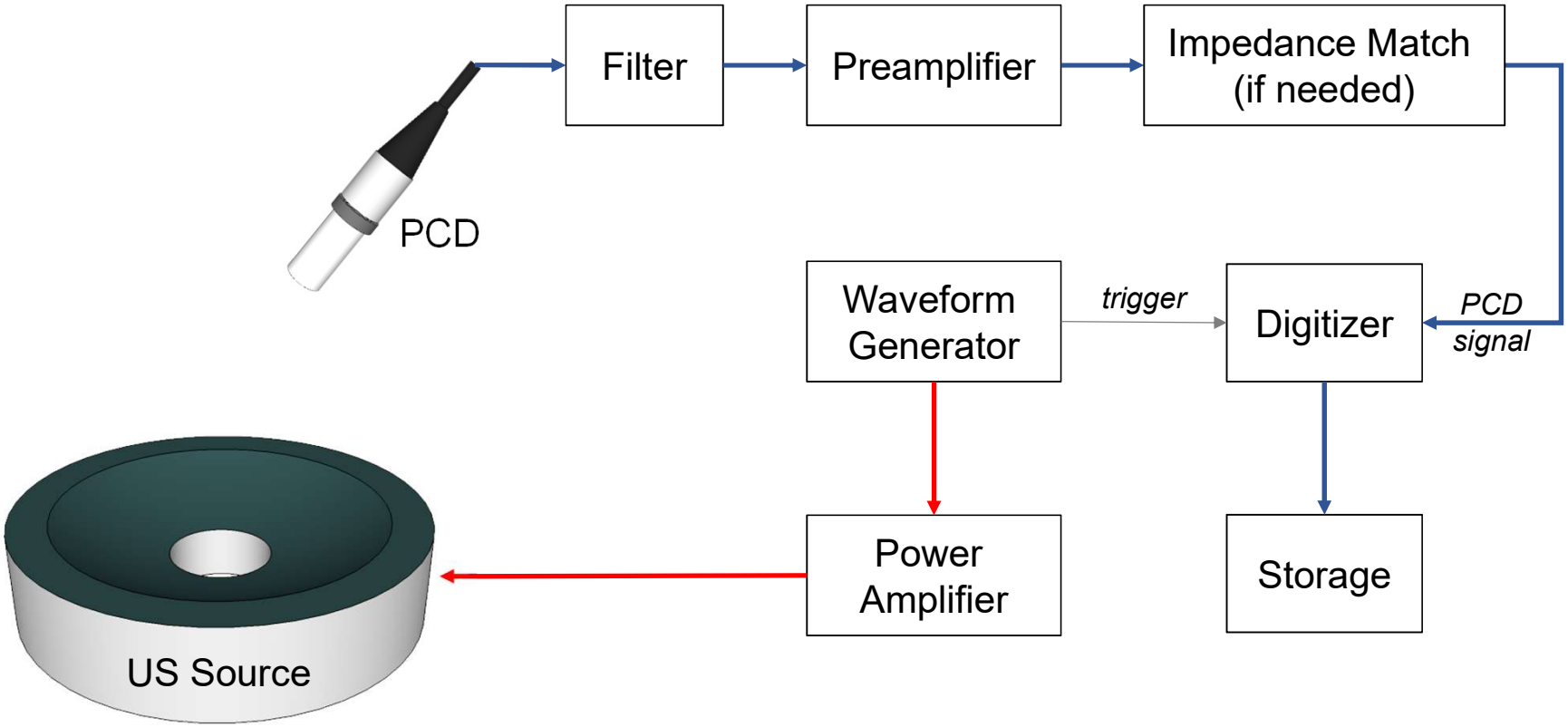

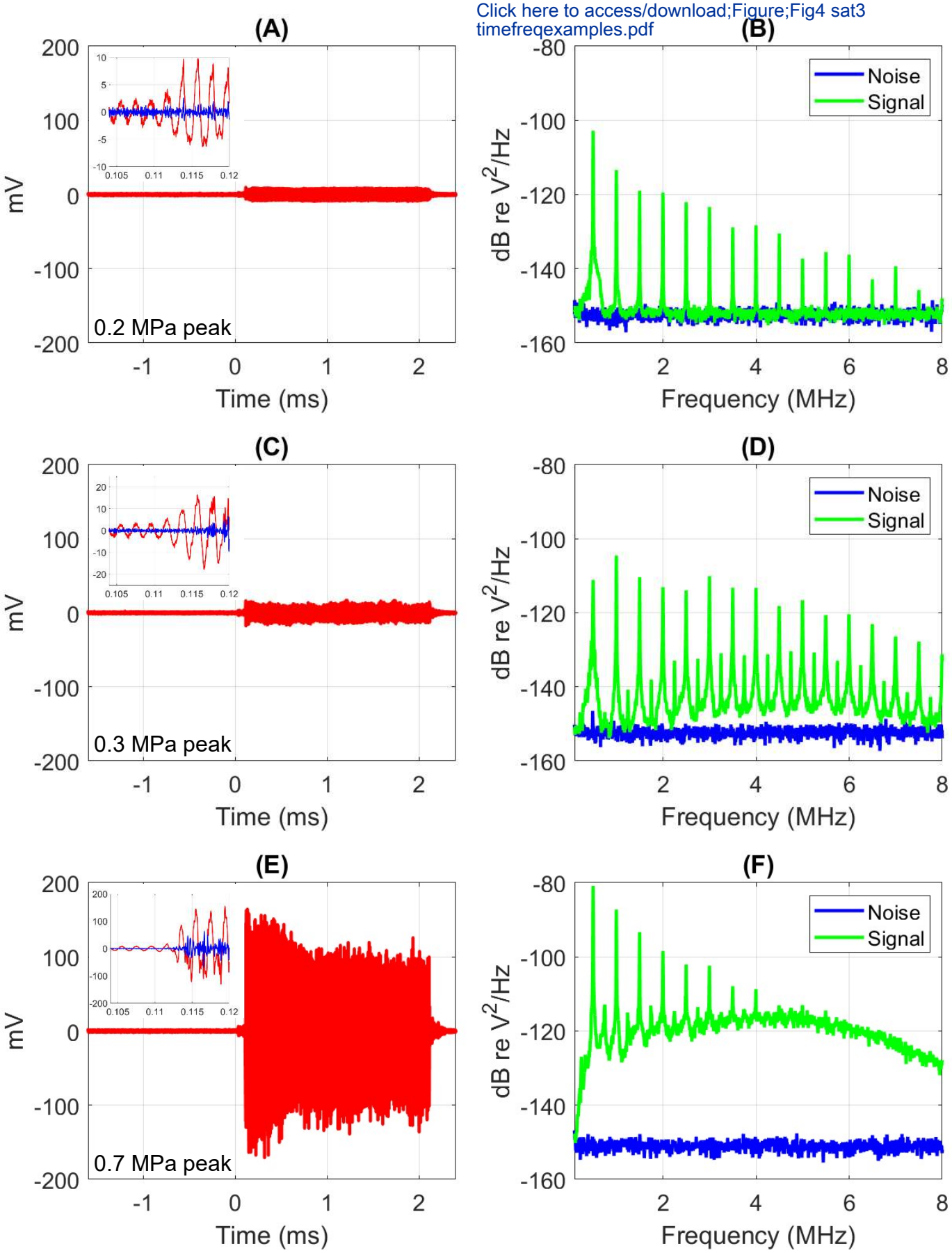

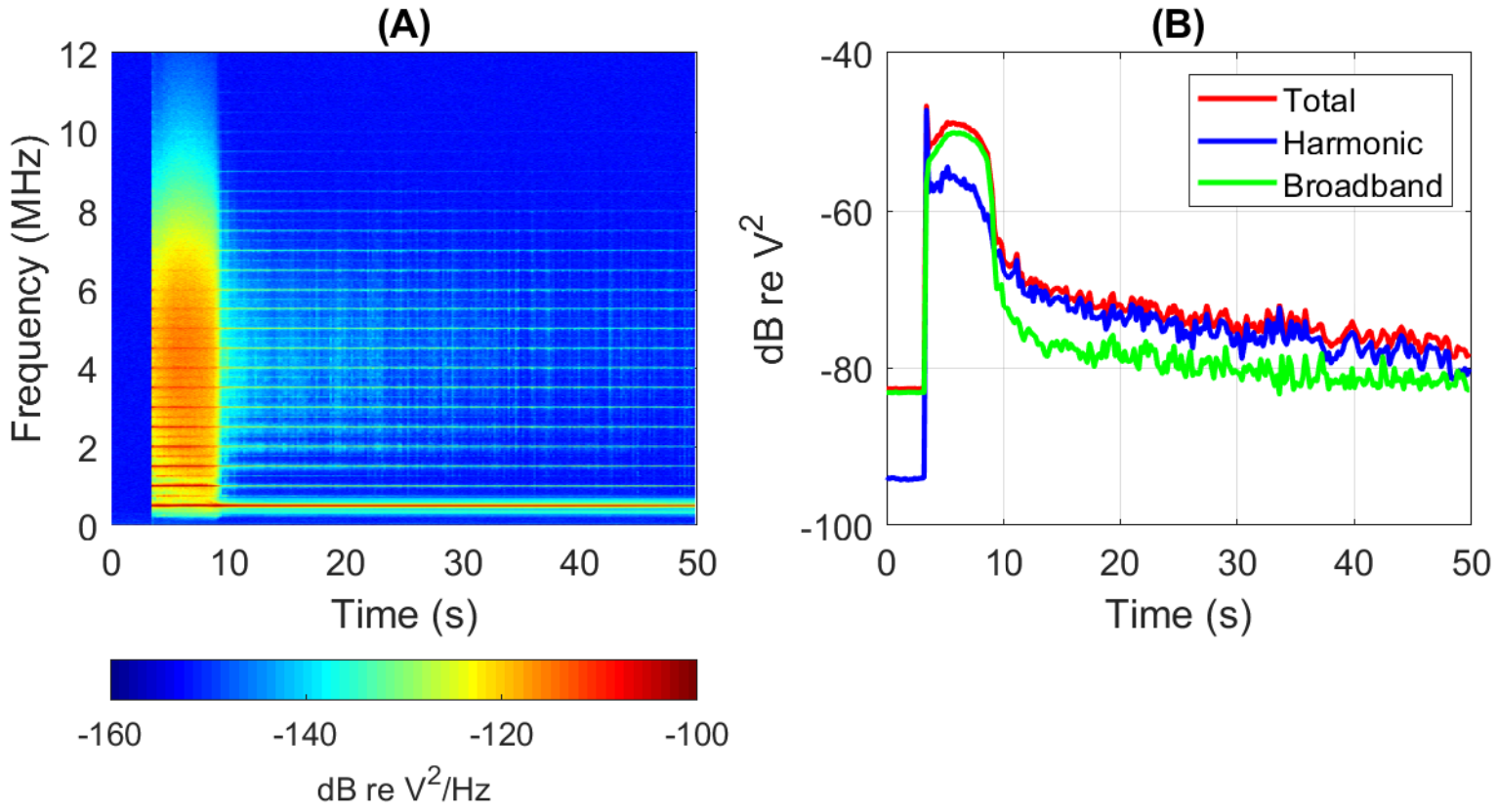

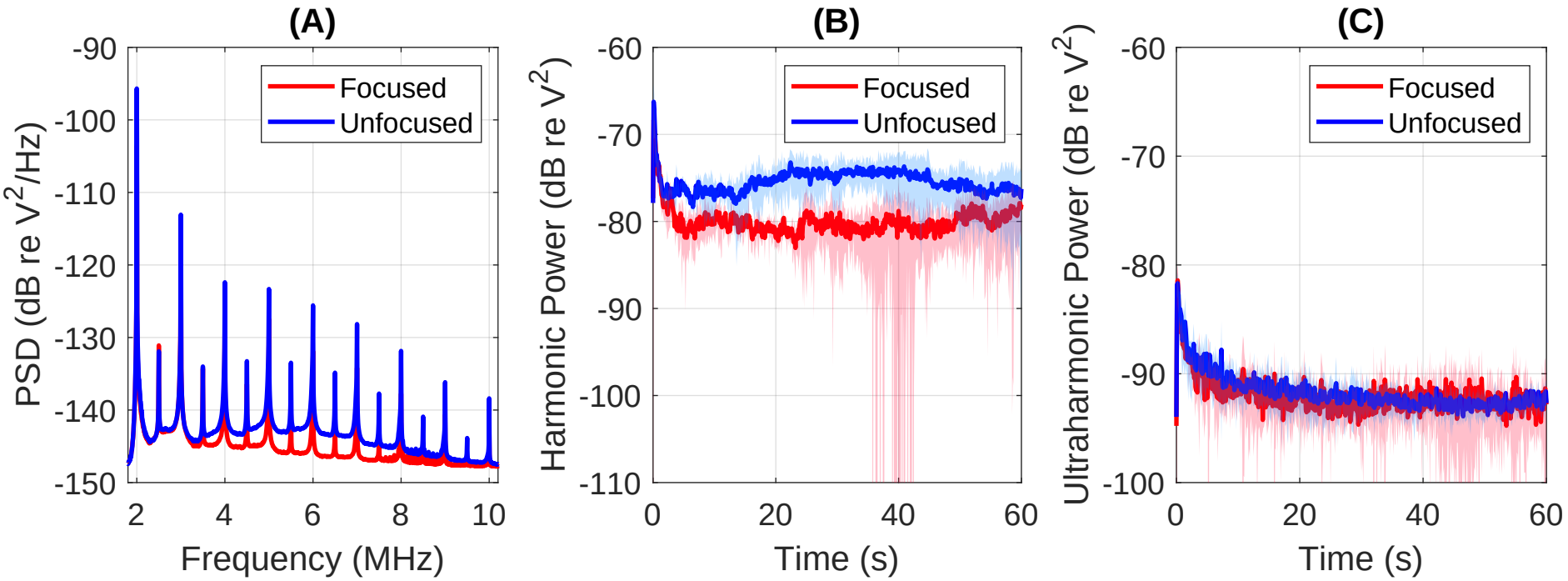

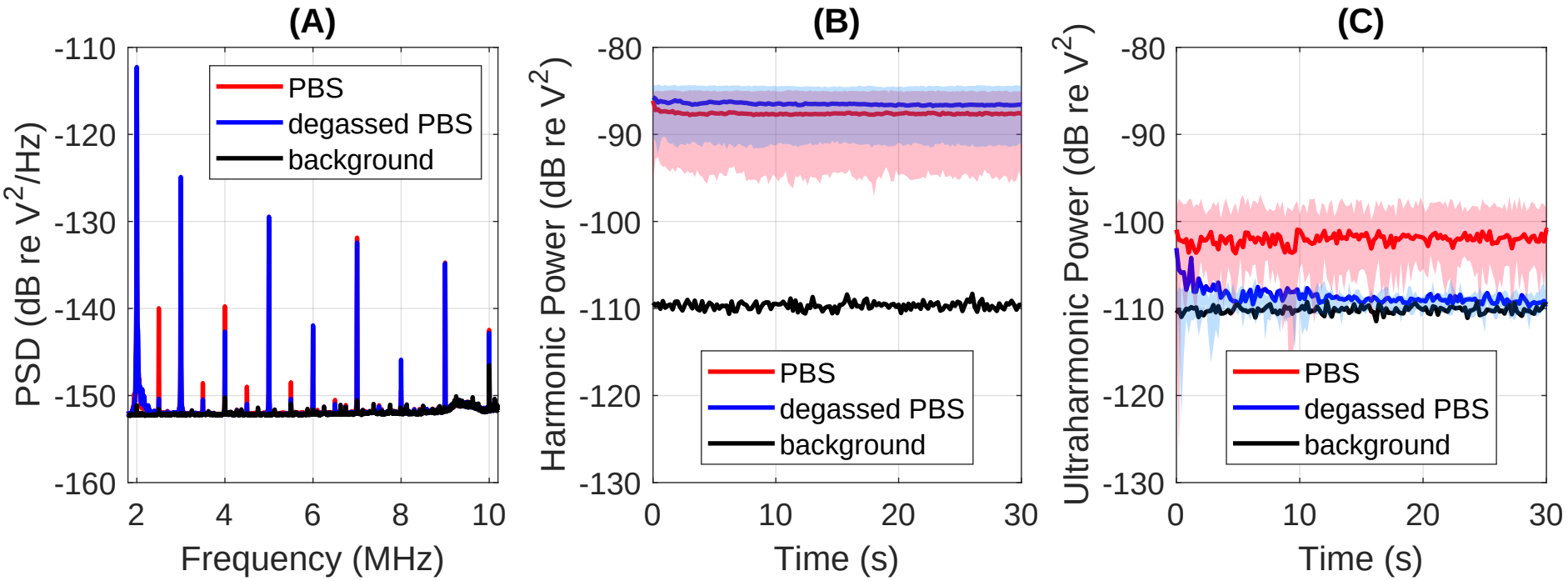

Parameter	Unit	Minimum	Maximum
frequency	MHz	0.02	15
pressure (peak negative)	MPa	0.1	20
pulse length	cycles	1	CW
duty cycle	%	1	CW
exposure time	s	10	1000

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Absorber	Precision Acoustics	APTFlex F28 panel	1.0 cm standard thickness
Amplifier (power)	E&I Ltd.	1040L	400W power amplifier to drive ultrasound source
Amplifier (pre)	Stanford Research Systems	SR445A	Fixed gain multi-stage preamplifier for PCD signals
Aquarium heater	Aquael	Ultra 50W	Different models for different tank sizes.
Digitizer	TiePie Engineering	HS5-110-XM	Extended memory option: 32M points per channel
Hydrophone	Precision Acoustics	FOH	0.01 mm diameter sensitive area minimises directivity effects
Microbubbles	Bracco	SonoVue	FDA approved microbubbles
PCD mirror (SAT3)	Olympus NDT	F-102	90 degree beam reflection
PCD transducer	Olympus NDT	V320-SU	Immersion transducer, 7.5MHz
PCD waterproof cable	Olympus NDT	BCU-58-1 W	
PDMS (SAT2 compartment lid)	Corning	Sylgard 184	See Carugo et al. (2015) for preparation guidelines
Polymer rod (SAT2 seal)	Zeus	PTFE monofilament	
Rubber plug (SAT3 lid/seal)	VWR	391-2101	6mm bottom dia., 8mm top dia., red
Signal generator	Agilent	33250	Waveform generator for ultrasound source
Substrate for cell exposure compartment, SAT2	Ibidi	μ-Dish 35mm	
Substrate for cell exposure compartment, SAT3	Corning	Transwell 6.5mm	
Ultrasound source (SAT3)	Sonic Concepts	H107 with central hole	Use of a HIFU-capable source allows pressures >1MPa to be generated both at the focus and pre-focally for expanded spatial coverage

Editorial comments:
Changes to be made by the Author(s):

Comment	Response
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please use American English.	Thank you, we have checked the manuscript as requested and revised as needed.
2. Please revise and shorten the Introduction to remove some of the background, but include all of the following: a) A clear statement of the overall goal of this method b) The rationale behind the development and/or use of this technique c) The advantages over alternative techniques with applicable references to previous studies d) A description of the context of the technique in the wider body of literature e) Information to help readers to determine whether the method is appropriate for their application	We have refined the introduction as requested.
3. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. Please ensure you provide sufficient detail to facilitate the filming of the video.	The spaces have been added and the selected text indicated.
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.	The text has been revised as requested.
5. 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: ibidi, Corning	The text has been checked and revised as requested. References to the table of materials have been added in place of the commercial language.
6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps.	The text has been checked and revised as requested.

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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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. For the entire protocol, please include enough specifics (with notes indicating what can and was modified and why) and highlight these specifics to allow filming.	
7. For example, in 1.3, please provide details that will allow replication and filming of the video: which ultrasound source do you recommend, which focused source should be used, how do you support the cell compartment well away from the strongest part of the incident field etc.	The text has been checked and revised as requested (but avoiding use of commercial information as above). The reader is now referred to the table of materials for information on the ultrasound source, and is referred to Figs 1(A) and 1(D) for examples of how to hold the cell exposure compartment.
8. 1.4: which acoustic absorber do you use, what should the temperature of the chamber and aquarium heater be?	The absorber and heater information has been added to the table of materials.
9. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.	The table has been removed and uploaded as a separate file with its title and description.
10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal names.	The references have been checked and corrected where necessary.
11. Please sort the Materials Table alphabetically by the name of the material.	The table has been revised as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors identified the gap that there is considerable variation in the experimental protocol for generating cavitation and monitoring it. This variation can come from cavitation agents used, incident field , apparatus used, and type of bubble behavior observed. The authors have discussed a generalized protocol which can be tuned to suit specific needs of the reader. Experimental practices to increase reproducibility and ease of use have been outlined. Details on how to choose monitoring equipment and data processing parameters for an intended application are given. A specific discussion on the protocol to use author's customized equipment for cavitating microbubbles in cell cultures is also included. The results shown give a fair idea of the equipment, the various types of bubble behavior, and some critical components that play a role in cavitation analysis. The authors have discussed critical steps, limitations, and future applications of this protocol in the end.

Comment	Response
1. The authors pointed out that there is a wide variation of exposure parameters for achieving successful cavitation and this protocol aims to address that. However, in the protocol there is no discussion on what the best acoustic parameters are for achieving successful cavitation nor a discussion on how to find them. They may also vary with respect to different cavitation agents. A discussion on these aspects may further aid the usability of this protocol.	Thank you, this is a very good point. The aim of the paper is to present a protocol that is suitable for a wide range of therapeutic ultrasound studies and we have therefore deliberately avoided prescribing a specific set of exposure parameters. The methodology for establishing that set of parameters, however, should be mentioned and we have revised the discussion to address this point as follows: “The range of parameters that should be explored for a particular experiment will depend upon the application (desired bio-effect, cell type, depth of target tissue if in vivo etc.) and the nature of any cavitation agent being used. Given the large number of variables (US frequency, pressure amplitude, pulse length, pulse repetition frequency etc.) fully exploring the whole parameter space is unlikely to be practicable. An advantage of the proposed protocol is that it enables some bounds on this parameter space to be quickly established. For example, it enables determination of the minimum pressure at which a cavitation signal is generated; the maximum pressure or pulse length that can be used before cell detachment/death occurs; the pressure at which fractional harmonics or broadband noise are produced. It is recommended that such a set of scoping measurements be carried out as a first step in any study.”

1. In line 167, perhaps the author is talking about Fig. 1(C) rather than 1(D) as it is difficult to visualize any holes on the lid in Fig. 1(D). Labelling these holes in the corresponding figure may assist in better visualization.	Thank you, we have corrected the text to reference "Fig. 1(C)". That sub-figure already identifies the filling ports. The revised section is: 1.1.2.1. The SAT2 cell exposure compartment is formed by press fitting a flexible polymer lid over the μ-dish (Carugo *et al.* 2015). As seen in Figure 1(~~D~~C), the lid has a pair of 1.2 mm diameter holes that allow filling the compartment with an 18g blunt needle syringe. After filling, these filling ports~~holes~~ are sealed with short plastic rods.
2. In line 244, a reference for the formulation would further aid the readers to know the details of the derivation of the formulation	Thank you, this has been added
3. In Line 327, a reference for providing the details of the welch method would be helpful for the readers.	Thank you, this has been added
4. For point 2.2.4.1 and 2.2.4.2, a detail that can further aid data processing can be to mention an optimum size of frequency bins that one should use for ultraharmonic and harmonic frequencies.	Thank you, we have added a clarifying example to 2.2.3.1. We do also already provide a recommendation for how to set the transform length for resolution of spectral features. "For the example of a 1 MHz fundamental sampled at 50 MHz, Nft = 2500 and the bin width is 0.02 MHz."
5. For point 3.2.2, how large is the largest gauge needle. It may be helpful to provide a maximum or a minimum limit.	Thank you, this has been added together with references to previous studies on microbubble destruction and the role of needle gauge.

Reviewer #2:
REVIEWER COMMENTS
Manuscript Number: JoVE61989
Title: Experimental protocol for studying cavitation enhanced therapy.
Authors: Michael Gray, Alexandra Vasilyeva, Veerle Brans, Eleanor Stride.

Comment	Response
This is an excellent contribution to the technical field, very well written and appropriately detailed. It addresses an important topic; namely, the specification of a system or systems(s) allowing the controlled exposure of microbubbles and other targets such as cells to an acoustic field. This should enhance the reproducibility of such studies, promoting an improved understanding of the bio-effects of ultrasound and confidence in its controlled application. I have only a limited number of comments for the authors to consider.	We are very grateful for the reviewer's kind comments.
1. Page 4, line 186: it is a small point, but maybe refer to "optically clear materials".	Thank you, this is a very good point and has been added. We have modified 1.1.5 as follows: "1.1.5. Make the chamber and the internal components out of optically clear materials when possible, so that any problems (e.g. leaks, entrapped macrobubbles) can be quickly observed and remedied."
2. Page 4, lines 190-206: Later in the manuscript (Limitations of method on pages 14 &15), the issues of multiple reflections are mentioned. It should also be mentioned here i.e. it is not just the transmissibility of the acoustic window that is key, but the fact that the reflections may return to the chamber. In this case, you may be over-correcting for membrane transmission, particularly at higher frequencies where the amplitude reflection is significant even for thin membranes.	We completely agree with the reviewer and have modified the paragraph to make this point more explicit and also added a cross reference back to sections 1.2.2 and 1.2.3 to highlight the issue of variation in transmissibility with frequency. "It is not possible to completely eliminate acoustic reflections within the sample chamber and hence the incident field will not be completely uniform over the whole the sample volume. As mentioned in 1.2.2 and 1.2.3 the transmissibility of acoustic windows will be frequency dependent and thus the desired bandwidth for acoustic emission

	measurements should be carefully considered. In particular there may be significant multiple reflections of higher frequency components. This is another reason why calibration of the field within the fully assembled system is so important to minimize the uncertainty in incident pressure. Appropriate gating of the recorded signals should also be considered to minimize the effects of multiple reflections."
3. Page 5, Section 1.4.2, lines 225-227: the authors raise the issue of using a voltage probe directly measuring the input drive voltage to the transducer. This is an important measurement as it defines the applied acoustic stimulus but I wondered whether the authors could state that it is important that the voltage probe is calibrated (you might be surprised how poor probes are in terms of this aspect) and specify additionally the voltage measurement uncertainty. This will allow readers to identify the transducer input voltage should they choose identical commercially available transducers.	We have added the following to 1.4.2: "1.4.2. Monitor and record the source drive voltage at the amplifier output / source input, so that minor variability or major malfunction can be detected quickly. Use a voltage probe or other device that is safe to use over the drive voltage range of interest. Periodically check the calibration of the voltage probe using a well-known source such as a waveform generator."
4. Page 5, Section 1.5, lines; 235-251: I was a little confused regarding how this would be done especially regarding the comment "…..all system components are in place". To confirm, is the "Cell exposure compartment" still in place for the scan? If so, is the aim to get as close to the compartment as possible and scan over its rear (exit) surface. Or, is the compartment not in place, in which case the exposure conditions may not be representative due to multiple reflections. It may be worth mentioning that whilst needing to characterise the exposure under the actual conditions employed (long millisecond bursts), reducing the burst length might enable the existence of other reflections to be identified and their contribution estimated.	Yes, this includes the cell exposure compartment, with the intention of making the measurements as close as possible to where the cells are located. We have modified section 1.5.1 accordingly: "1.5.1. Include a means of measuring the pressure field incident upon the exposed cells when all system components are in place, including the cell exposure compartment. In SAT2 and 3, this is accomplished with an opening in the chamber lid through which a needle or fibre optic hydrophone could be inserted without disturbing the field to be measured. Make the measurements as close as possible to where the cells are located." We have added a comment about pulse length to 1.5.4. for diagnostic purposes: "1.5.4. Scan the entire region where cells may be exposed. In order to capture an appropriate level of field detail, use a scan spacing no coarser

	than 1/5th of a wavelength at the highest frequency of interest. If unexpected field complexity is observed, consider using short burst signals (e.g 1-3 cycles) to allow identification and quantification of direct and scattered field contributions."
5. Page 5, section 1.5.2, lines 245-246: worth adding temperature here, as the sensitivity of the hydrophone will be temperature dependent. Exposures at 35°C may be difficult the quantify without some form of model as hydrophone calibrations are commonly carried out around ambient.	We have modified the text in 1.5.3 as follows: "1.5.3. Ensure that the hydrophone is calibrated under the conditions used in system characterisation, including the temperature. Specifically, if the hydrophone is held at an angle with respect to the scan plane, the phone must be calibrated at that angle, as directivity effects may differ significantly from those expected based purely on geometry. Note: the change in hydrophone sensitivity with respect to temperature should be available from the manufacturer."
6. Page 6, Section 2, line 58: Could the authors comment on whether there is any benefit in the PCD being calibrated in terms of its V Pa-1 response? If not, why not? This could be a separate test of the relative response of the PCD at a single frequency against the hydrophone used for the exposure test. It could be a useful cross-check and might put spectra such as Figure 5B on an absolute basis, which would be a useful parameter in terms of study reproducibility.	This is an interesting question. The PCD signal collected as described in the protocol represents a spatial integration of acoustic emissions whose strength and distribution depend on the low frequency source field, the geometry of the exposure compartment, and the cavitation agent therein (its distribution, concentration, response properties etc.). A calibration performed with a point-like source or reciprocity calibration with a hydrophone would reveal sensitivity at one location in the PCD beam pattern. This would need to be integrated over the exposure compartment volume with assumptions made about cavitation spatial coherence and distribution, both of which are generally not known (arguably they are unknowable due to the stochastic nature of cavitation) and could change with low frequency source drive level and the cavitation types (harmonic vs. broad band) that result. This process would still yield a PCD signal that represents a system-specific spatially integrated output. Unfortunately, therefore, given the

	assumptions and uncertainties in the process, it is not clear that the calibration is at all absolute or able to provide additional physical insight. The PCD output as currently presented in voltage units indicates: 1) the types of cavitation that are present and in what proportion, 2) how long these cavitation behaviors were present, 3) whether the observed time-cumulative exposure characteristics were correlated to a particular bioeffect, and 4) whether the relative levels and time-dependent behaviors were consistent with previous experiments in the exposure system. Measurement scenarios where absolute energy distributions are desired really require array-based cavitation mapping techniques. However, their implementation cost is orders of magnitude more expensive to implement than a single-channel PCD. Therefore, for compact, portable, and high throughput systems for in vitro assessments, we still believe that PCDs are useful despite their limitations. We have summarised the above in the discussion as follows: "The PCD output as currently presented in terms of voltage indicates: 1) the types of cavitation behavior and their relative proportions, 2) how long these cavitation behaviors persist, 3) whether the observed time-cumulative exposure characteristics are correlated to a particular bioeffect, and 4) whether the relative levels and time-dependent behaviors are consistent with previous experiments in the exposure system. Whilst the receive sensitivity of the PCD can be quantified, in order to reliably characterize the acoustic emissions in terms of absolute energy additional spatial information is required. This could be achieved by replacing the PCD with an array probe in order to implement Passive acoustic mapping (PAM)80. This would however increase the complexity of signal processing and the computational time and power required."

7. Page 7, Section 2.1.5, line 309: Direct signal inject is also a useful test for system linearity and detection of saturation limits.	Thank you, we have added these features of injection testing to the text: "2.1.5. Confirm proper impedance matching in the signal chain in order to avoid gain or bandwidth errors. PCD devices typically have output impedances near 50 Ohms, so a suitable check would be to replace the PCD with a known signal from a waveform generator (with 50 Ohm output impedance) and confirm that the signal size appearing on the digitiser matches expectations, scales linearly when the injected signal is changed, and no clipping is observed for the largest signal of interest."
8. Page 8, Section 2.26, line 2.2.6: This is a useful measurement. Can I just clarify whether or not the authors use it at all i.e. in terms of subtracting from the "cell exposure" spectra?	The background is used as a reference so that the confidence in a particular response metric (e.g. size of 3rd ultraharmonic or total broadband power) is meaningful but is also helpful as a diagnostic for electronics problems. This measurement made is in addition to controls with ultrasound but without cavitation agents. We typically display the background level in figures to indicate its level relative to the signal(s) of interest rather than subtracting it during processing.
9. Page 8, Section 3.2.2.6: "while inject" should be "while injecting".	This has been corrected, thank you.
10. Page 10, Section 3.3.5: I believe it is better to measure the direct drive voltage being applied to the transducer using a high impedance scope or voltage probe.	Section 3.3.3.5 makes this recommendation, but we have modified the wording to improve its clarity: "3.3.3.5. Monitor the amplifier output signal that drives the ultrasound source (as opposed to the waveform generator output) throughout the experiment in order to ensure that the exposure is proceeding as expected. Use a high voltage probe for this measurement and make sure that the oscilloscope is set to compensate for probe attenuation."
11. Page 11, line 500: How is the standard deviation calculated? Given that this is a time-varying system and spectra are being acquired continually? Or does this refer to what you could call "true repeats" i.e. with newly	The standard deviations are across 'true repeats' at each time point, since cavitation behaviors are generally dependent on exposure history.

replenished chambers with identical constituents and nominally identical exposure conditions?	
12. Page 11, Table of Materials: given the aim is to establish free-field condition in the exposure chamber it seems a little strange to exclude the absorber details. If it is commercially available, then a specification would be available to the interested researcher; if it is ''homemade'', what is its specification in terms of echo-reduction?	Details have been added to the Table of Materials. All acoustic absorbers were made from APTFlex F28 precast panels with 1.0 cm thickness, Precision Acoustics Ltd., Dorset, UK.
13. Page 11, Table of Materials: similarly, given the importance of measuring exposure to reproducibility, information on the characterisation system should be stated in terms of hydrophone used (manufacturer/type), angle of tilt and uncertainty at the particular frequencies of interest. This uncertainty is not just the uncertainty on the calibration certificate by may involve variations over the volume on interest - see the prior comment 4.	Hydrophone details have been added to the Table of Materials and revisions made to the manuscript as above in respect of comments 4 and 5.
14. Page 13: I may have missed this but what temperature was used for these exposures? What was the uncertainty in temperature?	This information has been added to the first paragraph of this section: “Figure 4 shows examples of time and frequency domain PCD responses, illustrating three distinct cavitation behaviors. All data were collected on SAT3 using SonoVue microbubbles diluted 5x in PBS. The temperatures for all examples in this section were was 19 ± 1 °C.”

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