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A multimodal instrument for real-time *in situ* study of ultrasound and cavitation mediated drug delivery

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The development of a multimodal instrument capable of real-time *in situ* measurements of cavitation activity and effect in tissue mimicking phantoms during ultrasound and cavitation mediated drug delivery experiments is described here. The instrument features an acoustic arm that can expose phantoms to high-intensity focused-ultrasound while measuring cavitation activity and an optical arm that monitors cavitation effect using confocal microscopy. This combination of modalities allows real-time *in situ* characterisation of drug delivery in tissue and tissue mimicking phantoms during ultrasound and cavitation mediated drug delivery experiments. A representative result, obtained with a tissue mimicking phantom and acoustically activated droplets, is presented here as a demonstration of the instrument's capabilities and potential applications. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>). [<http://dx.doi.org/10.1063/1.4978811>]

I. INTRODUCTION

In the treatment of solid tumours, achieving delivery of systematically administered chemotherapy agents to cancer cells which may be 100 μm or more from a blood vessel presents a significant challenge.^{1–5} For example, the concentration of the drug doxorubicin drops to half its perivascular concentration at a distance of 40 to 50 μm from blood vessels, while the mean distance to hypoxic cells is between 90 and 140 μm .⁴ Furthermore, delivery becomes still more limited the larger the drug used. Antibody therapies such as trastuzumab have been shown to reach less than 30% of the tumour mass.⁶

The application of ultrasound and cavitation is a promising method for improving treatment of solid tumours. For example, Carlisle *et al.* have shown that tumour infection increased over 30-fold when an adenovirus was co-delivered with gas microbubbles and exposed to focused ultrasound.⁷ Increased treatment efficacy will not only improve patient outcomes but will also increase the efficiency of treatment, with fewer treatment cycles needed overall. This should, in turn, lead to a reduction in the severity and duration of side-effects while also lowering the cost of treatment.

In addition to its applications in solid tumour therapy, ultrasound and cavitation can also be used to enhance drug delivery in a range of other diseases, e.g., thrombolysis, gene delivery. Furthermore, it provides new ways of achieving targeted release or activation of therapeutic agents.^{8,9}

Due to its potential, the study of the interactions between ultrasound, cavitation, and the surrounding tissue and how they affect drug delivery is an important and growing field of research. A particular difficulty is that cavitation interactions occur at the micro to millisecond time scale while producing effects on the order of hundreds of microns to millimetres. This combination of time and length scales necessitates

trade-offs between temporal resolution, spatial resolution, and spatial field-of-view. Typically the effects of ultrasound and cavitation on drug delivery are characterised only at the end of an experiment, and only after extensive physical post-processing of samples, e.g., sectioning and immunohistochemistry. As a result, the time between the end of ultrasound exposure to the start of characterisation can range from hours to days. A system that can provide *in situ* measurements of ultrasound and cavitation mediated drug delivery on relevant length scales would be highly desirable, as it would provide data at intermediate stages and at end points, while being free from post-processing distortions and artefacts.

We have designed and implemented a multimodal instrument that is capable of exposing tissue mimicking phantoms to high-intensity focused-ultrasound (HIFU), and at the same time, monitor in real-time and *in situ*, using acoustic and optical modalities, the degree of cavitation activity at the ultrasound focus as well as the effects induced by cavitation. The instrument consists primarily of an HIFU transducer used to deliver ultrasound energies to tissue mimicking phantoms, a passive cavitation detector (PCD) used to detect cavitation noise, and a long working distance confocal laser scanning microscope for optical measurements of cavitation induced effects.

II. INSTRUMENT DESIGN

A. Acoustic arm

The acoustic arm of the instrument consists of an HIFU transducer with a fundamental frequency of 1.067 MHz and a focal length of 62.64 mm. The HIFU transducer is used to deliver ultrasonic pulses with peak-negative pressures of up to 4 MPa. Cavitation induced by these pulses is detected

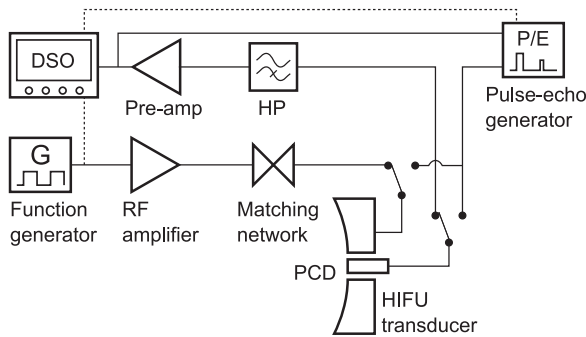


FIG. 1. Schematic of the acoustic arm of the instrument. Pre-amp: 25x (SR445A; Stanford Research Systems, US); HP: 5 MHz high-pass filter (F5081-5P00-B, Allen Avionics, Inc., US); pulse-echo generator: DPR 300, JSR Ultrasonics, US; RF amplifier: 55 dB radio-frequency amplifier (1140LA, Electronics & Innovation, Ltd., US); matching network: H102-28D, Sonic Concepts, US; HIFU transducer: H102-28D, Sonic Concepts; PCD: V319, Panametrics-NDT, US; dashed lines indicate trigger signals.

acoustically using a focused PCD with a centre frequency of 15 MHz and a focal length of 76.20 mm. The HIFU transducer and the PCD are arranged coaxially, with the PCD nesting within a central circular cutout within the HIFU transducer.

The setup shown in Figure 1 is responsible for driving the HIFU transducer and measuring the PCD output. This setup also facilitates alignment of the HIFU transducer and PCD foci to each other and to the optical focus of the instrument.

B. Optical arm

The optical arm of the instrument consists of a custom built long work distance confocal laser scanning microscope, shown schematically in Figure 2. Excitation light from the 15 mW 516 nm laser diode is collimated by L1 after the reflection off the dichroic mirror, then focused into phantoms by L2. Fluorescence generated in phantoms is then collected by L2 and focused onto the pinhole by L1 after transmission through the dichroic mirror and a 550 nm long-pass filter. The pinhole allows fluorescence originating from the focal plane of L2 to pass through and be detected by the photomultiplier tube (PMT).

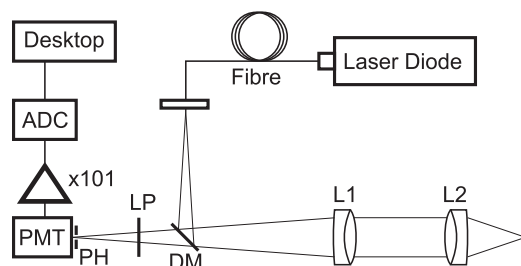


FIG. 2. Schematic of the optical arm of the instrument, a confocal laser scanning microscope. Laser diode: 516 nm (LP520-SF15; Thorlabs, US); DM: dichroic mirror (DMLP505; Thorlabs); L1: collimating lens $f = 125$ mm, $\varnothing 25$ mm (AC254-125-A-ML; Thorlabs); L2: multi-element objective, $f = 51$ mm, 15 mm clear aperture (CA) (AMP-75.0-15.0-425-675-PM with companion lens; Acal BFi, UK); LP: 550 nm long-pass filter; PH: pinhole, $\varnothing 15$ μ m; PMT: photomultiplier tube (H9306-04; Hamamatsu Photonics, JP).

The microscope's 51 mm working distance is key to enabling *in situ* measurements during ultrasound and cavitation mediated drug delivery experiments, where phantoms are typically submerged within a water tank. Due to the combined thickness of the water tank wall (5 mm) and the intervening volume of water (10 to 40 mm), a long working distance is necessary in order to image phantoms *in situ*.

A pinhole diameter of 15 μ m was chosen to accommodate the imaged size of L2's Airy disk.¹⁰ At a wavelength of 550 nm, L2's Airy disk has a radius of 2.28 μ m, calculated using $x = 1.22\lambda(F/D)$. After magnification by the lenses, the imaged Airy disk has a radius of 5.59 μ m, and thus a diameter of 11.18 μ m.

The output of the PMT is amplified by a single-supply amplifier with a gain of 101, then sampled by a 16 bit 40 kHz custom-built ADC. The ADC consists of an ATmega2560 (Atmel Corporation, US) and an ADS7825 IC (Texas Instruments, US). The ATmega2560, running custom firmware written in C, interfaces between the ADS7825 and the Python desktop software. In addition to command-and-control, the ATmega2560 firmware also provides data buffering functionality.

When triggered by the Z stage's quadrature position encoder, the ADC samples the amplified PMT output multiple times, where the number of samples per trigger is programmable. The samples are then summed together and stored as a single unsigned 16-bit integer, effectively performing an averaging operation without the normalisation step. It is possible to store the result in an unsigned integer because the PMT output is always positive and the amplifier is of a single-supply design. When unsigned integer overflow is detected, 0xFFFF is stored to signal saturation.

The multi-sampling capability increases the sensitivity of the microscope while reducing the signal-to-noise ratio. Together with the PMT control voltage, the multi-sampling mechanism determines the sensitivity of the microscope.

Optical sections of the phantoms are generated by scanning the objective focus along the X, Y, and Z axis using linear stages (Figure 3). Motion along the Z axis, carrying the objective, is driven by a linear motor (LCS25-025-51; SMAC Corporation, US) with a maximum in-use speed of 100 mm s⁻¹ and 25 mm of travel. Motion along the X and Y axes is driven by

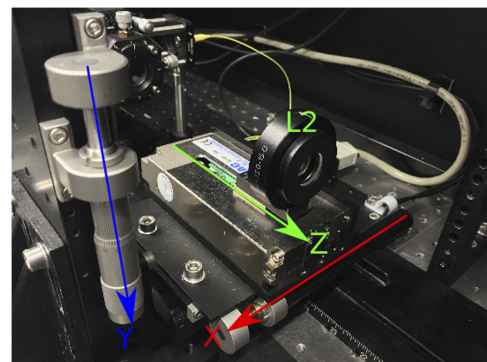


FIG. 3. Linear stages for raster scanning. The stages are connected in Z-X-Y arrangement, with the Y stage bearing the vertical load of both the X and Z stages.

screw-drive systems (X-LSM025A-E03; Zaber Technologies, Inc., Vancouver, British Columbia, Canada and TRB25CC; Newport Corporation, US) with a maximum speed of 2 mm s^{-1} . This scanning mechanism is possible because the diameter of the collimated beam produced by L1 overfills the objective, L2. Raster scanning of the phantoms by moving L2 is preferred over moving the phantoms due to the desire to keep the ultrasound exposure site stationary, and ease of movement afforded by air compared to water.

Control software for the microscope was written in Python 2.7 and interfaces with motion stages and the ADC over serial connections. A graphical user interface based on wxPython is also provided for the ease of use. To obtain an optical section, the operator specifies the desired scanning range and resolution along X, Y, and Z, as well as the number of samples per trigger. The control voltage of the PMT and the forward current of the laser diode are set manually by the operator.

III. INSTRUMENT CHARACTERISATION

Whereas the acoustic components of the instrument are commercial-off-the-shelf parts with well defined characteristics, the optical arm of the instrument was custom built and in need of characterisation prior to deployment.

A. Optical axial resolution

The confocal microscope operates in air and images water-submerged phantoms through the side of a glass water tank. When used in this manner, the instrument's point spread function (PSF) has a full-width half-maximum of $63.84 \mu\text{m}$, measured by scanning against a submerged mirror target through 12.5 mm of de-ionised water (Figure 4). This is in reasonable agreement with the theoretical value of $57.14 \mu\text{m}$ calculated using Equation (1)¹¹ with $\lambda = 516 \text{ nm}$, $n = 1$ and after account for axial scaling due to refractive index mismatch between air and water.^{12,13} The discrepancy between the measured and theoretical values may be explained by the fact that the pinhole used was larger than the 1 Airy unit assumed by Equation (1).

$$FWHM_z = \frac{0.88\lambda}{n - \sqrt{n^2 - NA^2}}. \quad (1)$$

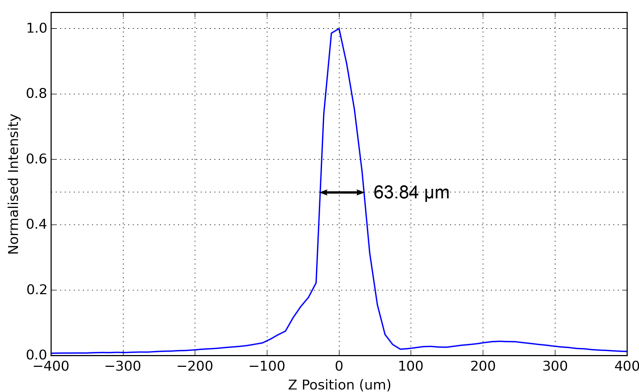


FIG. 4. Point spread function of the instrument, measured by scanning against a mirror target submerged in water through 12.5 mm of water.

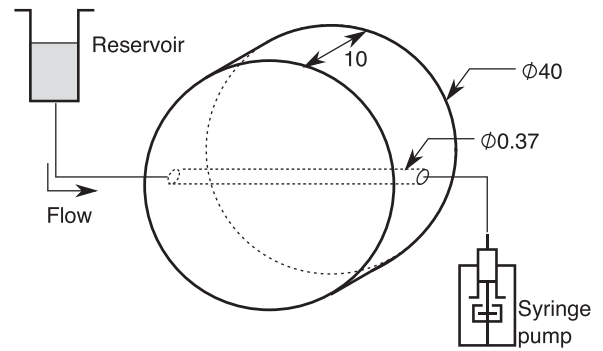


FIG. 5. Schematic of agarose phantom and flow loop. Phantoms are enclosed in water-tight containers with optically and acoustically transparent faces. Measurements are in millimetres.

B. Scanning speed

An XZ plane scan of $5 \text{ mm} \times 5 \text{ mm}$ at $50 \mu\text{m}$ lateral resolution (determined by stage step size) and with 8 samples per trigger takes 30 s to complete. The scanning speed is primarily limited by the acceleration of stages, and secondarily by the number of samples required per trigger and the sampling speed of the ADC. If the time required for the ADC to perform the necessary number of samples per trigger is longer than the time between triggers, then some triggers will be missed, and the total number of readings will be fewer than expected. When this occurs, the operator is notified via the desktop control software.

IV. IMAGING OF ULTRASOUND AND CAVITATION INDUCED CHANGES

A. Materials and methods

To demonstrate the capabilities of the instrument, it was used to track in real-time and *in situ* the distribution of a fluorescent drug analogue within an agarose phantom when exposed to ultrasound and in the presence of a cavitation agent. The phantom used is a 2% w/v agarose cylinder with a $370 \mu\text{m}$ diameter transverse flow channel. A syringe pump and reservoir connected to the flow channel via Luer connectors allowed

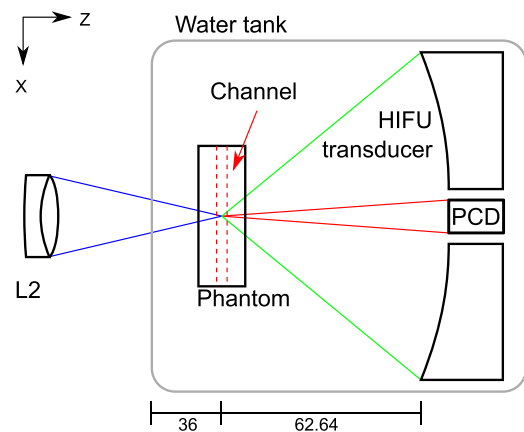


FIG. 6. Alignment of the objective (L2), the HIFU transducer, the PCD, and the flow channel. The optical focus is indicated in blue, while the HIFU and PCD foci are indicated in green and red, respectively. Measurements are in millimetres.

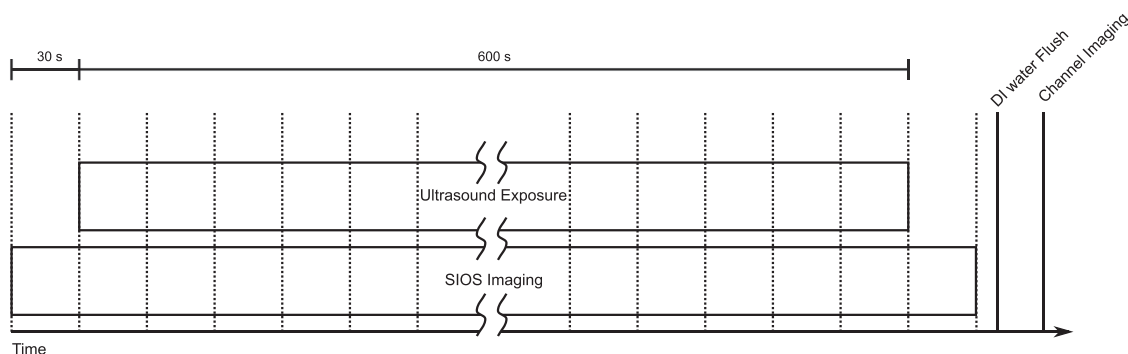


FIG. 7. Time-line of experiment. Dashed lines represent the start of confocal imaging of the flow channel.

for the controlled flow of liquids (Figure 5). The phantom was housed in a water-tight container with acoustically and optically transparent windows, which isolated the phantom from the environment and allowed it to be mounted to positioning systems.

In order to expose the phantom to ultrasound, it was attached to a gantry positioning system and submerged in deionised and degassed water at a temperature of 20 °C. The position of the phantom must be such that the channel is simultaneously at the focus of both the acoustic and optical arms of the instrument. To achieve this, the foci of the acoustic and optical arms of the instrument were made to coincide using a reflective metallic sphere 3 mm in diameter. The phantom was then positioned such that the flow channel is at the optical focus, which, owing to the previous alignment of optical and acoustic foci, also places it within the ultrasound focus. The final alignment is shown in Figure 6.

A suspension containing a fluorescent dye (4.4 mg of 40 kDa TRITC-Dextran (42874; Sigma-Aldrich, US) dissolved in 24 ml of deionised water) and a cavitation agent in the form of acoustically activated droplets was used in the experiment. The droplets are amphiphilic polymer coated droplets containing iron oxide particles with a nominal size of 800 μm and a concentration of 1×10^8 droplets per ml. They contain a liquid core of perfluorohexane, which when exposed to ultrasound rapidly boils and expands, forming micrometre-sized

bubbles which then cavitate in response to ultrasound. The volume flow rate of the suspension was 27 $\mu\text{l min}^{-1}$.

When aligned and under flow, the channel was exposed to ultrasound at 1.067 MHz, 1.8 MPa peak negative pressure, 5 Hz pulse repetition frequency (PRF), and 1.17% duty cycle for 10 min. Confocal imaging of the flow channel occurs immediately before, during, and after ultrasound exposure (Figure 7). For every ultrasound pulse, induced cavitation activity is measured for 1 ms at 50 MHz sampling frequency.

Following ultrasound exposure, the channel was flushed with deionised water and then excised from the phantom (Figure 8) for imaging with an inverted microscope (Eclipse Ti; Nikon, Inc., US).

B. Results

Confocal images of the channel, centred on the ultrasound exposure site, are shown in Figure 9. After approximately $t = 240$ s of ultrasound exposure tunnel formation in the direction of ultrasound propagation can be observed in image 8. Over the next 120 s, in images 9 to 12, the tunnel grows to a length of approximately 2266 μm . Thereafter tunnel growth in the direction of ultrasound propagation slowed considerably. Using image 21, the final tunnel length is estimated to be 2370 μm .

Widefield images of the tunnel, taken after ultrasound exposure and channel extraction, are shown in Figure 10. The

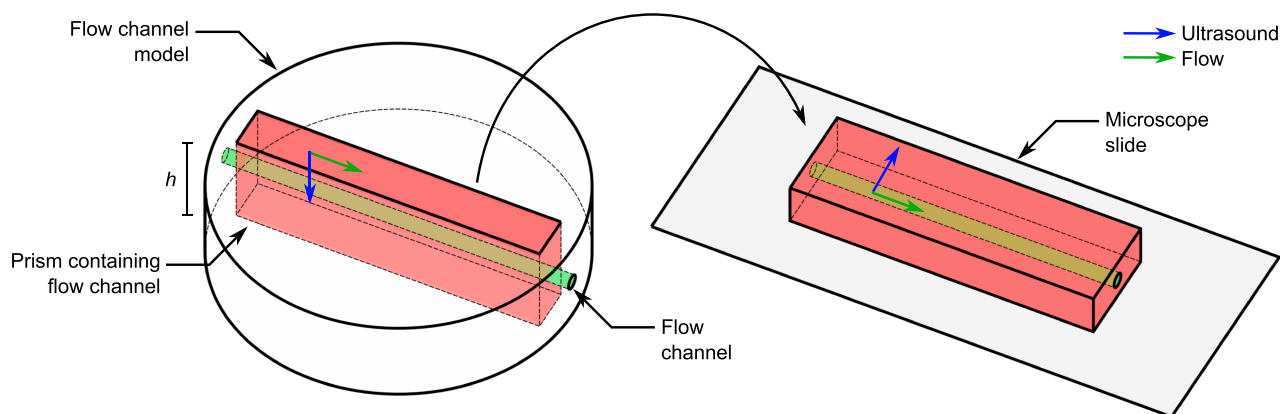


FIG. 8. Excision of a flow channel for end-point microscopy. The rectangular prism containing the flow channel, shown in red, was approximately 30 mm long, 10 mm height, and 4 mm deep. Once excised from the flow channel model, the prism was then rotated and placed on a microscope slide for imaging.

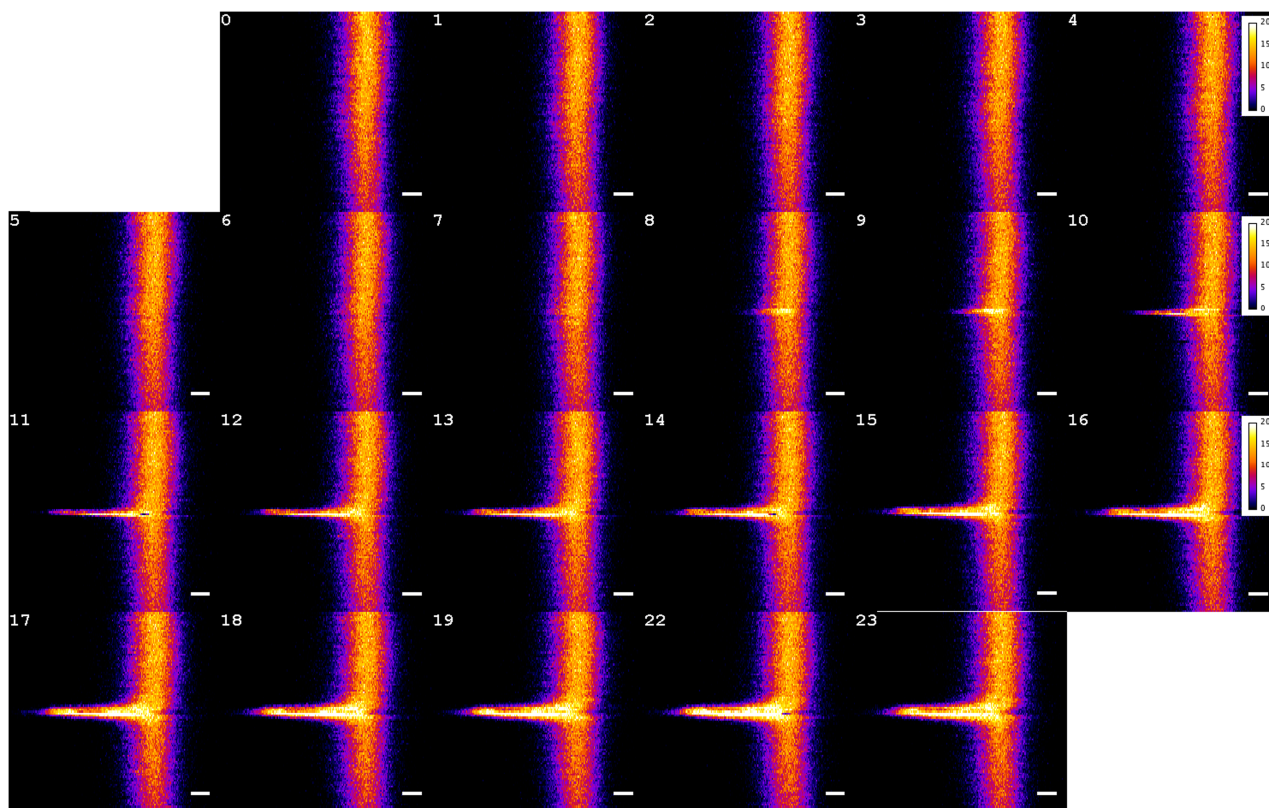


FIG. 9. Confocal images of TRITC-Dextran distribution in an agarose phantom exposed to ultrasound and cavitation in the presence of acoustically activated droplets. Images were acquired *in situ* immediately before (0), during (1-20), and immediately after (21) ultrasound exposure. Lateral scan resolution was $50\ \mu\text{m}$, and image acquisition time was 30 s for all images. Colour bars have units of fluorescence. Scale bars are $500\ \mu\text{m}$.

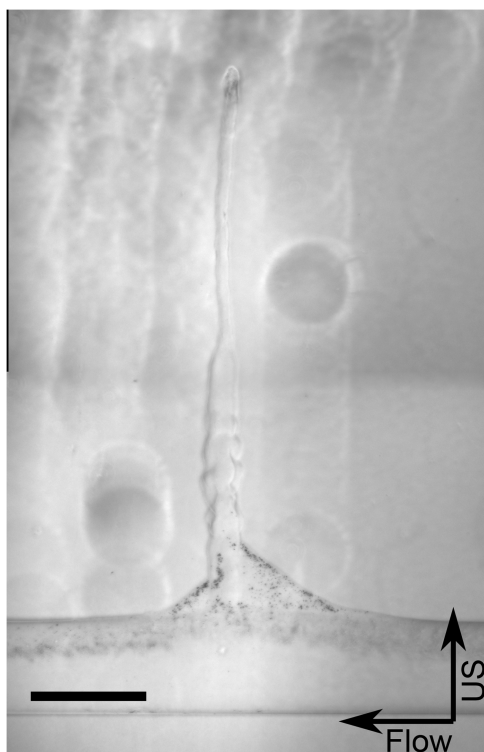


FIG. 10. Widefield microscopy of the tunnel produced by cavitation at the ultrasound focus. Scale bar is $500\ \mu\text{m}$.

length of the tunnel, measured from Figure 8, is $2411\ \mu\text{m}$, which is in good agreement with the estimated tunnel length from confocal images taken *in situ*.

Concurrent with confocal images, cavitation noise from cavitating droplets was also measured and shown in Figure 11. The level of cavitation noise increased linearly over the duration of the experiments, with a transient increase at $t = 270\ \text{s}$, which correlates well with the start of tunnel emergence in images 8 and 9. This increase may be the result of a vaporisation cascade, where the vaporisation of one droplet triggers the vaporisation of others in the vicinity.

The emergence and growth of the tunnel between $t = 240\ \text{s}$ and $t = 360\ \text{s}$ were not reflected in PCD measurements beyond the transient increase in cavitation power at $t = 270\ \text{s}$, with PCD signal power increasing linearly before, during, and after tunnel formation. This is contrary to the expectation that cavitation noise can be used to monitor cavitation effect as both are the result of cavitation activity. However, given the non-linear behaviour of cavitation effects, i.e., tunnel formation, and the linear increase in detected cavitation noise power, this is not the case.

These results demonstrate the optical and acoustic capabilities of the instrument as well as its utility in the study of ultrasound and cavitation mediated drug delivery, revealing relationships between cavitation activity and cavitation effect that are otherwise hidden with end-point only imaging.

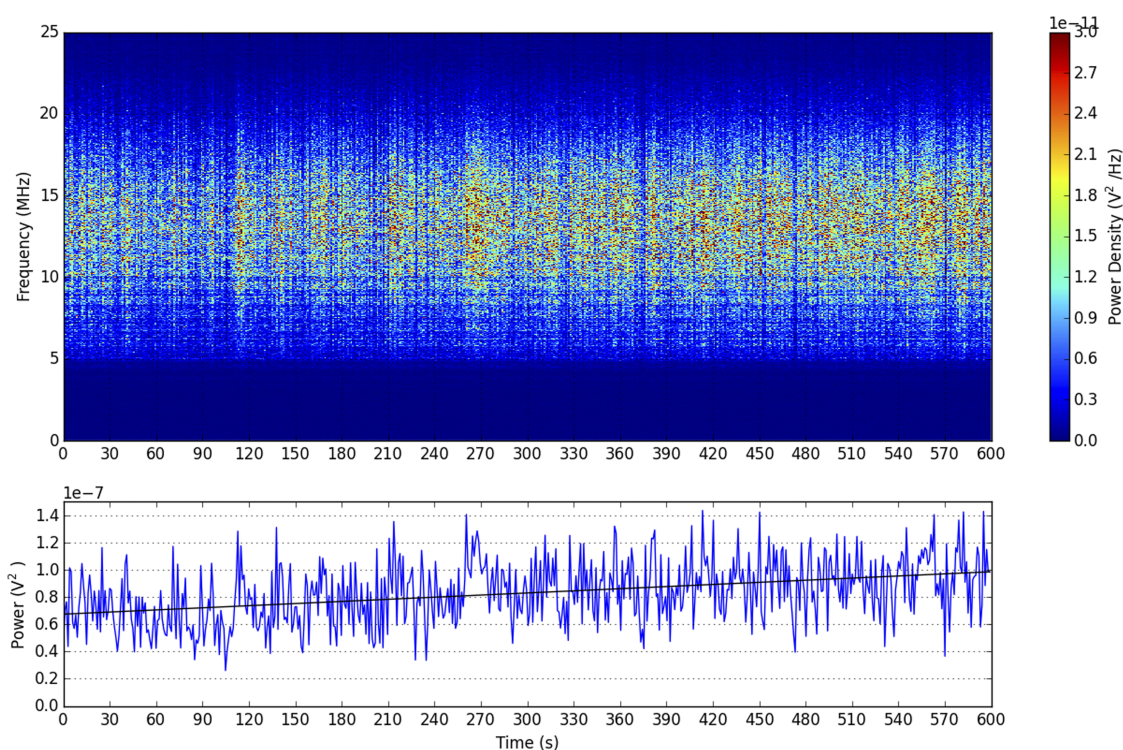


FIG. 11. PCD spectrogram and power curve recorded during ultrasound exposure. Power plot has been fitted with a 1st degree polynomial.

V. CONCLUSIONS AND FUTURE WORK

A multimodal instrument has been developed in which cavitation activity and effect within tissue mimicking phantoms can be monitored concurrently during ultrasound and cavitation mediated drug delivery experiments. The results presented here demonstrated the capabilities and utility of the instrument, where measurements were taken in real-time and *in situ* without the need for post-processing. Compared with the widefield microscopy images taken after post-processing, good agreement with *in situ* measurements was found.

Future work planned for the instrument includes optimising the optical path such that the beam incident on the dichroic mirror is collimated, replacing the 15 μm pinhole with a 12.5 μm pinhole to increase the optical axial resolution, and software upgrades to enable 3D imaging and dynamic lateral scan resolution. The latter involves increasing the lateral scan resolution within the region of interest while decreasing it elsewhere, keeping the overall scan time constant while providing higher resolution images of cavitation effects.

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