

# Unmyelinated Peripheral Nerves can be Stimulated *In Vitro* Using Pulsed Ultrasound

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## Abstract

Appreciation for the medical and research potential of ultrasound neuromodulation is growing rapidly, with potential applications in non-invasive treatment of neuro-degenerative disease and functional brain mapping spurring recent progress. However, little progress has been made in our understanding of the ultrasound-tissue interaction. The current study tackles this issue by measuring compound action potentials (CAPs) from an *ex vivo* crab walking leg nerve bundle and analysing the acoustic nature of successful stimuli using a Passive Cavitation Detector (PCD). An unimpeded ultrasound path, new acoustic analysis techniques and simple biological targets are used to detect different modes of cavitation and narrow down the candidate biological effectors with high sensitivity. In the present case, the constituents of unmyelinated axonal tissue alone are found to be sufficient to generate *de novo* action potentials under ultrasound, the stimulation of which is significantly correlated to the presence of inertial cavitation and is never observed in its absence.

**Keywords:** neurostimulation, neuromodulation, *in Vitro*, peripheral nerves, therapeutic ultrasound, cavitation, Axons

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## 1 Introduction

2 Diseases and dysfunction of the nervous system, both central and peripheral,  
3 are common causes of morbidity and mortality around the world. Despite huge in-  
4 vestment into pharmaceutical solutions for some of the more prevalent problems,  
5 progress has been slow. For a few of these diseases, successful new treatments have  
6 been found in neurostimulatory medical devices. Examples include Deep Brain Stim-  
7 ulation (DBS) for Parkinson’s disease (Bronstein et al., 2011), Vagus Nerve Stimu-  
8 lation (VNS) for epilepsy and depression (Groves and Brown, 2005) as well as Sacral  
9 neuromodulation for incontinence (Thaha et al., 2015). The gold standard for all of  
10 these are implantable electrodes, which themselves are associated with much mor-  
11 bidity from the need for highly invasive surgery, regular battery replacements and  
12 immunosuppression.

13 Though implants are improving, techniques that allow non-invasive neurostim-  
14 ulation such as Transcranial Magnetic Stimulation (TMS) (Lee et al., 2012) and  
15 Direct Current Stimulation (DCS) (Nitsche et al., 2009) are gaining favour since  
16 they avoid the complications mentioned above. However, neither of these techniques  
17 can replicate the location specificity, or stimulation of deep structures that implants  
18 can achieve.

19 Ultrasound (US), through the development of High Intensity Focused Ultrasound  
20 (HIFU) for ablative surgery and blood brain barrier disruption, has demonstrated its  
21 ability to overcome both of these targeting issues, reaching anywhere in the brain and  
22 other body areas with millimetre precision. Its application to elicit neuromodulation  
23 at lower intensities is still relatively new but is rapidly gaining momentum.

24 Examples of the neuromodulatory effect of US were first reported as early as  
25 1929 (Harvey, 1929), but surfaced only occasionally until the last decade. These

26 early, pre-2008 exploratory studies almost all focused on examining effects on pe-  
 27 ripheral nerves (Fry, 1968; Younan et al., 2013; Sheltawy and Dawson, 1966; Lele,  
 28 1963; Gavrilov et al., 1977; Wright et al., 2015; Mihran et al., 1990; Dalecki et al.,  
 29 1995; Wright et al., 2002; Tsui et al., 2005; Foley et al., 2008) with a few targeting  
 30 central nervous structures (Tsirulnikov et al., 1988; Wall et al., 1953). This pref-  
 31 erence shifted dramatically towards central nervous targets after 2008 when Tyler’s  
 32 group demonstrated that hippocampal slices could be stimulated at much lower in-  
 33 tensities than those used on peripheral nerves (Tyler et al., 2008). Furthermore,  
 34 a comparison of threshold neuromodulation intensities in studies on peripheral or  
 35 central nervous tissue shows the same large difference (Peripheral Nervous System  
 36 (PNS) mean threshold =  $59 \text{ W/cm}^2$   $\sigma = 68$  (Fry et al., 1950; Lele, 1963; Gavrilov  
 37 et al., 1977; Wright and Davies, 1989; Dalecki et al., 1995; Tsui et al., 2005; Fo-  
 38 ley et al., 2008; Colucci, 2009; Kim et al., 2012; Legon et al., 2012; Dickey et al.,  
 39 2011; Tych et al., 2013; Lee et al., 2014; Hu et al., 2014), CNS mean threshold =  
 40  $3 \text{ W/cm}^2$   $\sigma = 3$  (Tyler et al., 2008; Tufail et al., 2010; Min et al., 2011b,a,b; Yoo  
 41 et al., 2011; Moore et al., 2015; Kim et al., 2014a; King et al., 2014; Kim et al., 2015,  
 42 2014b; Legon et al., 2014; Lee et al., 2015; Deffieux et al., 2013; Hameroff et al., 2012;  
 43 Younan et al., 2013; Yang et al., 2012)). Subsequent to 2008, studies on the effects  
 44 of low intensity US in the living brain have yielded a range of exciting results, such  
 45 as stimulating motor activity (Tufail et al., 2010), affecting GABA release (Yang  
 46 et al., 2012), reversibly inhibiting epileptic activity (Min et al., 2011a) and eliciting  
 47 somatosensory sensations (Lee et al., 2015).

48 Despite recent progress in the application of the technique, still very little is  
 49 known about the mechanism at work behind the observations. Understanding in  
 50 this regard has been hampered by poor characterisation of the ultrasound field,  
 51 especially in small animal models where small cranial volumes make reflections and

standing waves a significant problem (Young and Henneman, 1961). Combined with the biological complexity of brain tissue and the variety of models used, very little consensus has been achieved on successful US parameters, exemplified by occasional directly conflicting or negative findings (Colucci, 2009; Gavrilov and Tsirulnikov, 2012).

There is at least consensus that ultrasound stimulates nervous tissue through a mechanical effect, not a thermal one. The field is far from united on the nature of this mechanical interaction, but the leading two theories for the key mechanism involve either acoustic radiation force or cavitation.

Cavitation is most often brushed aside as a potential mechanism in the CNS stimulation literature due to the low intensities used to elicit neurostimulation (Tufail et al., 2010; Deffieux et al., 2013; Yoo et al., 2011; Lee et al., 2015), below the FDA recommended Mechanical Index (MI) limits for soft tissue ultrasound (Duck, 2007). The limitations with this claim however are that the MI limit was formulated from observations of bubbles in free water, is concerned only with preventing inertial cavitation of sufficiently large bubbles to cause significant damage, and that MI is only a guide and cannot be used to truly predict the occurrence of cavitation as this will depend on the tissue type, bubble nuclei, dissolved gas content and other factors. Though some studies have reported very high pressure thresholds for *in vivo* cavitation in the brain (Gateau et al., 2011), others have found significant non-inertial cavitation at much lower intensities (240 mW/cm<sup>2</sup>) (ter Haar et al., 1982; Ter Harr et al., 1986). Though these two studies had much longer duration exposures of over a minute, the finding does indicate that bubble nuclei can be affected in some way by low intensities over much shorter durations.

In this study, a controlled *in vitro* environment is used, simplifying both the biological and the acoustic environment so that insight can be gained into the mech-

anism by which mechanical forces are transduced into propagating electrical activity in axons. Given this goal, it was decided that the best first course of action was to isolate and understand the direct stimulation phenomena observed previously by the authors in the crab walking leg nerve axon (Wright et al., 2015). To this end, a test setup was designed with several key capabilities:

- Ultrasonic stimulation of a nerve bundle with known exposure parameters.
- Electrical stimulation of the bundle, providing saturated control measurements of the CAP before each US stimulus.
- Measurement of cavitation activity at the US stimulus site.
- Measurement of electrical CAPs at a distal site, resulting from either stimulus modality.

Using this experimental approach combined with modelling of ultrasonic radiation forces at various stimulus parameters, the likely stimulus mechanism was determined by calculating the correlation of radiation force or cavitation activity with successful stimulation. Other features of the successful US stimuli, such as response latency and response reliability, were also investigated to determine the responsible force mechanism.

## Materials and Methods

### *Experimental Setup*

The equipment used in the current setup shown in figure 1 is detailed here. US stimulus waveform was produced by two function generators (Agilent 33220A, Agilent, Santa Clara, CA, USA), one gated by the other to produce the pulsed

100 protocol which was then amplified by a class AB linear power amplifier with 55  
101 dBm gain (E&I 1020L 200 W, E&I , Rochester, NY, USA). The three US stimulus  
102 transducers, and the transducer used as a PCD are detailed in Table 1. The signal  
103 of the PCD was amplified by a voltage amplifier (SRS inc. Model 445A, Sunnyvale,  
104 CA, USA) providing a 5 times gain.

105 Electrical nerve stimulus was produced using a constant current isolated stim-  
106 ulator (Digitimer DS3, Digitimer, Hertfordshire, UK). Electrical recordings from  
107 the nerve were taken using a differential amplifier (WPI DAM50, World Precision  
108 Instruments, Sarasota, FL, USA) at  $100\times$  DC gain. Electrical and acoustic data  
109 was acquired by an oscilloscope (Lecroy HDO6054, 12.5 MHz sampling frequency,  
110 Teledyne LeCroy, Chestnut Ridge, NY, USA). Synchronisation of US and electrical  
111 stimulation, and signal acquisition was performed using a 4 channel I/O module and  
112 DAQ chassis (NI 9402 and NI 9171 cDAQ, National Instruments, Austin, TX, USA).

113 The nerve bath was separated into three electrically isolated sections. The two  
114 ends of the bath performing the electrical stimulation and recording were filled with  
115 mineral oil (figure 2) and the middle chamber with a crab ringers solution (525  
116 mmol/L NaCl, 13.3 mmol/L KCl, 12.4 mmol/L  $\text{CaCl}_2$ , 24.8 mmol/L  $\text{MgCl}_2$  and 5  
117 mmol/L dextrose). All electrodes used for stimulation and recording from the nerve  
118 bundle (shown in figure 2) were made from silver chloride coated silver electrodes. To  
119 reduce atmospheric electrical noise, the entire setup was contained within a copper  
120 mesh Faraday cage.

121 A deep water bath (20 cm) with an acoustic absorbing layer (figure 1) was used  
122 to prevent ultrasound reflection interfering with the US field at the focal point.  
123 Reflections within the water bath were measured to affect the peak focal pressure by  
124 less than 5% at any of the amplitudes used in this study. The water bath was cooled  
125 with ice and monitored to ensure that it stayed between 1-4 °C. The cold slows down

126 the nerve's rate of conduction which serves to separate its response from stimulation  
127 artefacts and keeps the nerve viable for longer. The focused PCD was fixed in place  
128 within the water bath at an angle and distance such that its focal zone overlapped  
129 the focus of the stimulus transducer on the nerve bundle and such that it avoided  
130 receiving the direct field of the stimulus transducer (figure 1).

### 131 *Nerve Preparation Procedure*

132 All nerves were taken from live crabs (*Cancer pagurus*) sourced on the day of  
133 use from London markets. As invertebrates, crabs are not subject to regulatory  
134 requirements on animal testing in the UK. Nerves were extracted from the crab leg  
135 by stripping away each joint section, removing the shell and muscle from around  
136 the nerve bundle, leaving as much as possible of the nerve intact. During extraction  
137 the nerve was regularly sprayed with chilled (4-10 °C) crab ringer's solution. The  
138 nerve was then ligated at both the proximal and distal ends with red cotton thread.  
139 Cutting above the distal ligation, the nerve was detached from the claw, transferred  
140 into the nerve bath and wetted with chilled saline. The nerve was handled by the  
141 string attachments and passed through the two blocking gates (figure 2), then pulled  
142 straight between them. This ensured that the nerve was located directly under the  
143 ultrasonic focus (within  $\pm 0.1$  mm). Oil was then added to the two side channels  
144 and saline to the centre. Surface tension in the small aperture of the blocking gates  
145 (most of which was occluded by the nerve diameter) prevented the oil and saline  
146 from mixing between the chambers.

147 Once loaded, the chamber was transferred to a holder on the surface of the water  
148 bath and an US coupling cone fitted on top, ensuring that no bubbles were trapped  
149 in the US propagation path using a small endoscopic camera viewing from below.  
150 The nerve bath and all implements were cleaned and sterilised with ethanol before

151 use.

### 152 *Nerve Bundle Characterisation*

153 Five extracted nerve bundles were fixed in 3% glutaraldehyde (0.05 mol/L sodium  
154 cacodylate buffer pH 7.2-7.4) directly after extraction. The bundle was then sectioned  
155 and fixed in araldite resin using a methylene blue/azure II/ basic fuchsin stain for  
156 light microscopy examination. Axon fibre density was estimated using a digitally  
157 applied, randomly positioned 50  $\mu\text{m}$  square, counting only the axonal cells with  
158 more than 50% of their volume within the square. This was repeated three times  
159 for each of the 5 nerve bundles. Neuron density was calculated to be 136/100  $\mu\text{m}$   
160 ( $\sigma = 27$ ), combined with the mean cross-sectional area of a nerve bundle the total  
161 number of nerve axons in an extracted bundle was found to be 1017 ( $\sigma = 202$ ).

162 Investigation into the cause of the increased likelihood of initial response success  
163 found in a previous study by the authors (Wright et al., 2015), led to the observation  
164 of microbubbles on the surface of the nerve bundle by light microscopy. These bubbles  
165 are introduced by the extraction process as the bundle is submerged into the saline  
166 bath. In the 0-5 minute period post nerve submersion, a mean of 11 bubbles ( $\sigma = 8.8$ ,  
167  $n = 10$ ) with a mean diameter of 78  $\mu\text{m}$  ( $\sigma = 54$ ) were seen over 2 cm of nerve. The  
168 microbubbles were not observed past the first two US stimuli of an experiment as  
169 larger bubbles were observed to rise to the surface after US exposure and smaller  
170 ones dissolved rapidly into the surrounding saline. As only the first couple of stimuli  
171 are affected by these bubbles, it was decided not to degas the nerve ringers solution,  
172 as this would cause the axons to die faster.

### 173 *Ultrasound Setup*

174 To produce a highly predictable experimental US field a good understanding  
175 of the field and focus produced by each ultrasound transducer was required. The



176 free field spatial pressure distribution of the three HIFU transducers used in the  
177 experiments (Table 1) were measured using fibre-optic hydrophones (plane tipped,  
178 10  $\mu\text{m}$  diameter, calibrated frequency range of 500 kHz to 50 MHz) in a degassed  
179 water tank.

180 The spatial, temporal peak pressures for each transducer were located in 3 di-  
181 mensions and measured at relatively small peak negative pressure amplitudes (0 to -2  
182 MPa) by three fibre-optic hydrophones, taking the mean positive and negative pres-  
183 sures for each transducer at three different input powers. Mean measurements from  
184 multiple hydrophones were used to minimise inaccuracy from sensitivity variation be-  
185 tween different probes. Larger pressure amplitudes were not directly measured due  
186 to the risk of damage to the hydrophones and therefore inaccurate measurements, as  
187 per the manufacturer’s recommendations (limited to <3 MPa at 1 MHz). Instead,  
188 the measured pressure values were used to parametrise a Khokhlov-Zabolotskaya-  
189 Kuznetsov (KZK) based model of acoustic fields for each transducer, changing the  
190 output efficiency parameter to match the measured outputs. This model takes into  
191 account non-linear effects by modelling the propagation of the first 50 harmonics  
192 around the fundamental frequency. The model was then used to predict peak nega-  
193 tive pressures of exposures below -2 MPa used in the current study (Table 2). The  
194 KZK model has been validated using low f-number transducers similar to the ones  
195 used in this study (Canney et al., 2008). Furthermore, radial peak positive pressure  
196 at the focal point of each transducer was obtained by solving the calibrated KZK,  
197 and was confirmed to be within a 10% tolerance of hydrophone measured profiles.

198 Rigid ultrasound coupling cones were machined from perspex for each transducer  
199 that both sealed in degassed water for near field transmission and mechanically locked  
200 onto the nerve bath. These fixed the focal point along the central axis, 5 mm beyond  
201 the end of the cone. The apertures of the cones were set at 20 mm, much larger

202 than the diameter of the 1st side lobe (measured using a fibre-optic hydrophone in  
203 degassed water to be 15.2 mm in diameter to the 2nd nul point for the 0.67 MHz  
204 transducer 5 mm before the focal point). The truncated end of these cones was sealed  
205 with thin mylar film ( $12\ \mu\text{m}$ ), providing an acoustically transparent window into the  
206 nerve bath. The wide top of the cones fitted each transducer tightly, preventing  
207 leakage of water and lateral targeting errors. The presence of the cone, affixed to  
208 each transducer was found to have no measurable effect on the dimensions or peak  
209 positive pressure of the focal points, measured in a degassed water tank.

210 Thin mylar film was also used as an acoustic window in the nerve bath, separating  
211 the water in the cone from the nerve bath, and the saline in the nerve bath from the  
212 water bath underneath. The width of the acoustic window in the nerve bath was 10  
213 mm.

214 Ultrasound targeting error was analysed by producing visible heating spots in  
215 thermo-chromatic gels with each transducer (figure 3). The centre point of the colour  
216 change and its lateral deviation from the centre line of the chamber were measured  
217 three times for each transducer, dismantling and re-constructing the apparatus each  
218 time. Deviation was found to be a maximum of 160 ( $\sigma = 67$ ), 84 ( $\sigma = 11$ ) and 89  $\mu\text{m}$   
219 ( $\sigma = 40\ \mu\text{m}$ ) for 0.67, 1.1 and 2 MHz respectively. As the errors are much smaller  
220 than the width of the nerve (1-2 mm), a portion of the nerve bundle will always be  
221 exposed to the focal maximum.

## 222 *Ultrasound and Electrical Stimulation Protocols*

223 Electrical stimulation of the nerve bundle was performed to provided a measure-  
224 ment of maximum CAP amplitude and conduction speed, monitoring the health of  
225 the bundle and allowing the proportion of the bundle stimulated by US to be de-  
226 termined. Electrical and US stimulation were paired in these experiments so that

every US stimulus was preceded by an electrical stimulus, 3 seconds apart (a CAP in this chilled and unmyelinated model lasts 100 ms). The timing between each electrical and US stimulus pair was alternately varied between 30 and 90 seconds, causing the whole pattern to repeat every 120 seconds with an average of 1 stimulus pair per minute (figure 4a). Each nerve bundle was exposed to 22 of these stimulus pairs resulting in a total experimental time of 22 minutes. The paired pulse protocol was designed to investigate if recovery times had an effect on either the cavitation environment (i.e. on the presence of cavitation nuclei) or biological environment.

Electrical stimulation was applied via the stimulation electrode (figure 2a) using a 0.2 ms constant current pulse. Stimulation amplitude was adjusted before each experiment to achieve saturation. Full saturation may not have been achieved every time due to varying levels of saline short between the stimulation electrodes and the earthed central bath. Larger crabs with generally thicker nerve bundles were preferentially selected to reduce this effect, as their nerve bundles better occluded the holes in the blocking gates.

Ultrasound parameters were initially chosen based on precedence in the literature for successful neurostimulation protocols (King et al., 2013; Kim et al., 2014a; Tufail et al., 2010). Variation and optimisation of these stimulus parameters in preliminary experiments (data not shown) led to a novel protocol described below.

The primary stimulus protocol used in this study was, 80 pulses of 0.67 MHz driving frequency at 10 kHz Pulse Repetition Frequency (PRF), over an 8 ms Total Stimulus Duration (TSD) (50% duty cycle). Short duration stimuli (8 ms) were chosen to ensure temporal separation of the electrical noise artefact from the received electrical nerve signal. Intensity was varied between the values shown in table 2. Orders of intensities being tested on a single nerve were randomised to prevent systematic error from nerve inhibition or other effects. The 1.1 and 2 MHz exposure

parameters shown in table 2 were calculated to match the radiation forces produced by the 0.67 MHz exposures.

To test the effect of longer exposure durations on the nerve response dynamics at 0.67 MHz, a second set of stimulus experiments, with the same parameters as above, were performed using 100 ms instead of 8 ms stimulus durations (1000 pulses).

To test the effect of different pulsing protocols on stimulation success without exploring the entire parameter space, the parameters found in a recent successful *in vivo* US neurostimulation study by Lee et al. were tested (250 kHz fundamental, 500 Hz PRF, 50% duty cycle for 300 ms, with 3 s between each stimulus (Lee et al., 2015)) at the higher frequency of 0.67 MHz in our current setup. These parameters were used initially at 0.7 W/cm<sup>2</sup>, shown to be effective in Lee’s study, and then incrementally increased in the same steps seen in table 2 until a response threshold was found. As with all exposure protocols, each stimulus intensity was repeated 22 times on a new nerve bundle.

KZK modelling determined that there was significant non-linear propagation of ultrasound at the power levels used in the current study, increasing at higher amplitudes at all frequencies (Table 2). This results in higher positive pressures compared to the peak negative values. To facilitate easy comparison with other papers in the literature, Spatial Peak Pulse Average Intensity (I<sub>SPPA</sub>) will be used throughout the rest of the paper but it should be noted that these are linear approximations and the positive and negative pressure peaks will be the most accurate metrics, especially at higher amplitudes. These peak pressures are displayed along side peak and pulse average intensity values at all frequencies and driving powers (Table 2).

277 To detect CAPs, the electrical signal was split into 10 ms windows with no overlap  
 278 and an FFT performed on each. As the extracellular population recording of a CAP is  
 279 a summation of many cells' ionic fluxes in the environment around the nerve bundle,  
 280 the low frequency component (0-5 MHz) was used for detection. An amplitude  
 281 threshold for CAP detection was set at 5 times the standard deviation ( $\sigma$ ) of the  
 282 low frequency background activity or  $5\sigma$  of the total background noise in the time  
 283 domain. The integrated area under the curve of a CAP, 5 ms each side of the peak  
 284 voltage amplitude was used to measure the response amplitudes for both electrical  
 285 and US stimulated CAPs.

286 The, electrically stimulated CAP response was used as a reference point to nor-  
 287 malise the absolute amplitudes recorded from US stimulation.

288 CAP response latency was measured from the onset of the ultrasound or electrical  
 289 stimulus to the peak of the resultant CAP. This was the median latency of all the  
 290 fibres in the bundle which includes US travel, nerve response and CAP transmission  
 291 time. The first of these was constant and calculated to be  $47 \mu\text{s}$  and  $40 \mu\text{s}$  for the 0.67  
 292 and 1.1/2 MHz transducers respectively. The CAP transmission time along the nerve  
 293 was estimated on a nerve by nerve basis using the preceding CAP transmission time  
 294 from the interleaved electrical stimuli and the known relative distances between the  
 295 electrodes. Subtracting these from the total lag time, an estimate for the US response  
 296 latency was calculated for each. This method assumes that the stimulation occurs  
 297 in the centre of the US focal point every time, uniform conduction velocity along the  
 298 length of the nerve and that the relative position of the CAP peak amplitude does  
 299 not change with time or different stimulation modalities.

300 The US transducers may induce noise in the recording electrodes (Francis et al.,  
 301 2003). Though this source of noise was greatly reduced by using an earthed saline

302 bath, a temporal and spectral filtering algorithm was designed to prevent such noise  
303 being mistaken for CAP responses.

### 304 *Analysis of PCD Signals*

305 PCD recordings were analysed to determine the presence of inertial cavitation,  
306 which is characterised by a high energy, short duration, broadband signal. Analysis  
307 of time domain signal spikiness (kurtosis) and energy (variance) in multiple frequency  
308 bands was therefore used to detect inertial cavitation events.

309 In cases where multiple cavitation events are occurring simultaneously, as was  
310 usually the case in this study, smaller amplitude events can be difficult to detect  
311 and quantify using standard methods (Chen et al., 2003; Tu et al., 2006). A multi-  
312 resolution signal processing method is used here which demonstrates a promising  
313 performance for this application (Haqshenas and Saffari, 2015). The technique uses  
314 the wavelet transformation to decompose the signal into several components across  
315 the following frequency ranges:  $\frac{f_N}{2^{n+1}} - \frac{f_N}{2^n}$ ,  $n = 0, \dots, M - 2$ , where  $f_N$  is the Nyquist  
316 frequency (6.25 MHz) and  $M$  is the levels of decomposition (5 levels).

317 After performing the discrete wavelet transformation, short Fourier transform  
318 (STFT) and statistical analysis (i.e. variance and kurtosis) of each component of  
319 the signal are carried out to identify and characterise different cavitation regimes  
320 (Haqshenas and Saffari, 2015). Inertial cavitation is indicated by a high value of  
321 time domain kurtosis. The kurtosis threshold was set using the standard deviation  
322 of kurtosis in the lowest amplitude exposures as a baseline noise measurement, as  
323 no inertial cavitation was observed in standard spectrographic analysis. A kurtosis  
324 threshold for cavitation detection was therefore set at 6.

325 In the case of the 100 and 300 ms exposures, the key 10 ms section of the US  
326 stimulus likely to have caused any resultant CAP, was determined by subtracting

327 the expected CAP transmission lag time from the point when the CAP peak was  
 328 received. A 10 ms section of PCD signal data was analysed around the resulting  
 329 time point, illustrated by the vertical red lines in (figure 4c). In cases where no  
 330 US stimulated CAP was detected, a random 10 ms time section of PCD data was  
 331 analysed for comparison.

### 332 *Calculation of the Acoustic Radiation Force*

333 Radiation forces produced by the 0.67 MHz stimulation protocol shown in table  
 334 2 were calculated by summing the force caused by acoustic absorption within the  
 335 nerve, with the force caused by acoustic reflection from the surface of the nerve. The  
 336 former is calculated as follows (Leighton, 1994):

$$I_{\text{SPPA}} = \frac{P_{ac}^2}{\sqrt{2}Z}, \quad (1) \quad F_{abs} = \frac{2\alpha I_{\text{SPPA}}}{c}, \quad (2)$$

337 where  $P_{ac}$  is the peak pressure,  $Z$  is the characteristic acoustic impedance of  
 338 brain tissue (1.6 MRayls),  $F_{abs}$  is the radiation force due to the absorption of acoustic  
 339 energy,  $\alpha$  is the absorption coefficient of neural tissue in neppers per meter, calculated  
 340 with the equation:

$$\alpha = \alpha_0 f^y, \quad (3)$$

341 where  $f$  is frequency,  $y$  is the frequency dependence exponent (an exponent of 1.3  
 342 and  $\alpha_0$  of 8.6 for brain tissue was used (Duck, 1990)) and  $c$  is the speed of sound in  
 343 soft tissue (1562 m/s) (Roy, 1991). These equations assume plane wave and linear  
 344 propagation.

345 Radiation forces due to the reflection at the saline-tissue interface were then  
 346 calculated (Leighton, 1994):

$$R = \frac{Z_2 - Z_1}{Z_2 + Z_1}, \quad (4) \quad F_{ref} = \frac{2I_{SPPA}R}{c}, \quad (5)$$

347 where  $R$  is the pressure reflection coefficient,  $Z_1$  and  $Z_2$  are the specific acoustic  
 348 impedances for saline and tissue respectively and  $F_{ref}$  is the radiation force acting  
 349 on the boundary due to reflection assuming a linear plane wave, perpendicular angle  
 350 of incidence and a reflecting surface area much larger than the wavelength. For the  
 351 purposes of this summation, the difference between the planes upon which the force  
 352 was acting was assumed to be negligible due to the small dimensions of the nerve.

353 These equations were used to calculate the radiation forces produced by the 0.67  
 354 MHz exposures and find the focal intensities required at 1.1 and 2 MHz to produce  
 355 identical forces. This resulted in a range of 17-475 W/cm<sup>2</sup> at 1.1 MHz and 12-343  
 356 W/cm<sup>2</sup> at 2 MHz (Table 2).

### 357 *Damage Detection*

358 Damage to the nerve bundle was detected by two means. The primary method  
 359 was to measure proportional reductions in electrical stimulation amplitude from one  
 360 stimulation to the next. A significant damage event detected by these means was  
 361 defined as more than a 20% reduction caused by a single US stimulus. This threshold  
 362 was defined by 1.5 times the maximum point to point decline detected in nerves not  
 363 exposed to US. Three such control nerves were recorded over 22 minutes using the  
 364 same experimental protocol but without power to the US transducer.



365 Damage that may have been caused by US stimulation over a longer period was  
366 tested for by determining the correlation coefficient of the decline in CAP amplitude  
367 with acoustic kurtosis and signal energy at all frequency bands in each nerve experi-  
368 ment. Correlation was also tested for between the same variables, irrespective of US  
369 frequency or individual nerve experiments, across each stimulation protocol.

370 The second method used to detect significant damage events was through iden-  
371 tification of after-discharge (repetitive nerve activation) after a successful US stimu-  
372 lation event. After-discharge is known as a sign of poration in the nerve membrane  
373 as the charged ions equilibrate causing the membrane to regularly depolarise (Lee  
374 et al., 1995). After-discharge was identified when the standard deviation of the raw  
375 electrical signal (100-150 ms after the CAP peak) was more than 1.5 times greater  
376 than the background  $\sigma$  measured before CAP initiation.

## 377 Results

378 142 nerves responsive to electrical stimulation were exposed to a range of ultra-  
379 sound parameters which were shown to be capable of eliciting large, synchronous  
380 CAP events from the unmyelinated crab leg nerve bundle (figure 4b). Responsive  
381 nerve bundles could be stimulated multiple times in the same location, with stimulus  
382 reliability varying between 5 and 80%, strongly depending on fundamental frequency  
383 and pulse average intensity of stimulation. Nerve responses occurred unpredictably  
384 at different US exposures throughout the 22 minutes of an experiment, however,  
385 there was an increased response probability for the first stimulus (15% of all exper-  
386 iments responded on the first attempt compared to a mean of 7% success for any  
387 other of the 22 stimuli).

388 The lowest intensity at which stimulation was observed was at  $100 \text{ W/cm}^2 \text{ I}_{\text{SPPA}}$   
389 for the 8 ms, 0.67 MHz stimulus. Inertial cavitation signals were detected in all

390 successful stimuli and found to be significantly correlated with nerve responses in  
391 the 100 ms, 0.67 MHz stimulus experiments in all frequency bands ( $P < 0.05$ ). The  
392 results from each stimulus protocol variant are presented in this section.

393 Direct stimulation of the nerve via the electric field was ruled out by a sham  
394 experiment where the US cone was raised, creating a reflecting air gap between the  
395 cone and the saline bath and the primary US stimulation protocol repeated at high  
396 intensity. No direct stimulation was observed in this manner across 3 electrically  
397 responsive nerves and 66 individual stimuli (562 W/cm<sup>2</sup>, 8 ms TSD, 0.67 MHz, 10  
398 kHz PRF, 50% duty cycle, 30/90 s repetition period).

#### 399 *8 ms 0.67 MHz Stimuli*

400 61 electrically responsive nerves across 26 crabs were tested using a range of  
401 11 different US stimulation intensities (Table 2). Nerve response reliability and  
402 amplitude for each intensity stimulus are shown in figure 5. signal energy and kurtosis  
403 of the PCD data are shown in figure 6. The overall response reliability was less than  
404 25% at all intensities (figure 5a).

405 The lowest intensity where neurostimulation was observed was 100 W/cm<sup>2</sup>. Cor-  
406 relation coefficients between the amplitude of the CAP response and both PCD signal  
407 energy and kurtosis, find significant ( $P < 0.05$ ,  $n = 22$ ) correlation in two nerve experi-  
408 ments (out of 61) at 485 and 562 W/cm<sup>2</sup> across all frequency bands. Mean response  
409 latency was 3.16 ms ( $n = 106$ ), measured from stimulation onset and excluding the  
410 time taken for the CAP to reach the recording electrodes.

411 Inertial cavitation was found to be ubiquitous at pulse average intensities past  
412 100 W/cm<sup>2</sup>, with broad band (1.56-6.25 MHz) inertial events (kurtosis > 6) occurring  
413 in more than 70% of US stimuli (figure 7). This matches with the threshold for  
414 successful US stimulation also seen at 100 W/cm<sup>2</sup>. The majority of these cavitation

415 events are not associated with any resultant nerve activity.

#### 416 *100 ms, 0.67 MHz Stimuli*

417 19 electrically responsive nerves across 6 crabs were tested using the same range  
418 of US stimulation intensities (Table 2) as used in the 8 ms protocol. Nerve response  
419 reliability and amplitude for each intensity stimulus are shown in figure 8a and b  
420 respectively with the PCD signal kurtosis shown in c. The lowest intensity where  
421 neurostimulation was observed was 169 W/cm<sup>2</sup>.

422 Significant positive correlation ( $P < 0.05$ ) between nerve response amplitude and  
423 cavitation measures (kurtosis and signal energy of key PCD time sections) was found  
424 in 5 individual nerve experiments (56% of US responsive nerve experiments) across  
425 all frequency bands.

426 All cavitation and nerve response data in the 100 ms exposures was aggregated  
427 to determine the correlation, irrespective of US driving intensity and separate nerve  
428 experiments. Significant positive correlation was found between kurtosis of acoustic  
429 signals and nerve response amplitude across all frequency bands (0.39-0.78 MHz:  
430  $r = 0.25$   $P < 0.005$ , 0.78-1.56 MHz:  $r = 0.23$   $P < 0.005$ , 1.56-3.13 MHz:  $r = 0.18$   
431  $P < 0.05$ , 3.13-6.25 MHz:  $r = 0.2$   $P < 0.005$ ,  $n=304$ ). This strongly implicates  
432 the involvement of inertial cavitation. In individual STFT and wavelet analysis of  
433 the PCD data from every successful US stimulation, inertial cavitation signals were  
434 found in the expected time section without exception.

435 Similar to the 8 ms exposures, ubiquitous cavitation activity detectable in all  
436 frequency bands was found over 169 W/cm<sup>2</sup> shown in figure 8c. However, Analysis  
437 of the kurtosis of separate time sections showed that the majority of the inertial  
438 activity was restricted to the first 10 ms time bin (figure 9). Some events did occur  
439 after the initial burst of cavitation such as shown in figure 4c, at a much lower event

440 frequency, demonstrated by the much lower mean kurtosis values seen in figure 8c  
441 compared to figure 6b.

#### 442 *300 ms 0.67 MHz Stimuli*

443 Reproductions of the intensities and pulse parameters found in CNS stimulation  
444 literature (Lee et al., 2015) at 0.67 MHz were unable to generate CAP responses in  
445 the crab nerve bundle. Incrementally increasing the intensity of stimulation resulted  
446 in a threshold for CAP generation at 169 W/cm<sup>2</sup>.

#### 447 *1.1 and 2 MHz Stimuli*

448 58 electrically responsive nerves across 11 crabs were tested using a range of 5  
449 different US stimulation intensities that equalled the radiation forces produced in the  
450 0.67 MHz exposures (Table 2). At these intensity values, no nerve responses were  
451 observed.

452 Occasional high kurtosis events were seen with 1.1 and 2 MHz exposures, though  
453 the acoustic signal energy in frequency bands other than driving was near zero  
454 (<0.1% of total signal energy at all points) with very low standard deviation between  
455 experiments. Therefore the cavitation activity present was considered negligible.

#### 456 *Damage*

457 US was found to damage the exposed nerve bundles in some cases. The lowest  
458 intensity example of after-discharge in the 0.67 MHz 8 ms protocol, at 230 W/cm<sup>2</sup>, is  
459 shown in figure 10c. Two other after-discharge events in separate nerve experiments  
460 were observed above this intensity threshold (at 485 and 562 W/cm<sup>2</sup>), all three were  
461 concurrent with reduced electrically stimulated CAP amplitude.

Each of the damage events causing after-discharge were examined spectrographically. Large broadband noise signatures marking inertial cavitation events (figure 10d) were seen preceding all these instances of significant damage. The cause was therefore deemed likely to be inertial cavitation induced membrane rupture.

Significant positive correlation ( $P < 0.05$ ,  $n = 22$ ) between decline of the electrically stimulated CAP and acoustic kurtosis & signal energy was seen in two of the three after-discharge occurrences mentioned above and in three more nerve experiments at 419 W/cm<sup>2</sup>. Positive correlation is also found in 3/19 nerve experiments in the 100 ms exposures. In total, damage was observed in 4% of all nerve experiments at any intensity or frequency. No significant damage as a result of US exposure occurred at either 1.1 or 2 MHz.

Sham experiments were performed on three nerves from one crab where no US was used. Degradation of the electrically stimulated CAPs across 22 minutes (Mean normalised decline per minute = 0.009,  $\sigma = 0.013$ ) was not significantly different from mean decline of US exposed nerve bundles, where signs of major damage events as above were not seen at any intensity. The rate of decline in CAP amplitude over the 22 minutes also did not significantly change between exposure intensities (figure 10b). No significant correlation between damage and either signal energy or kurtosis was found when measured across all data for each stimulation protocol, irrespective of US frequency or individual nerve experiments.

## Discussion

Our results demonstrate that unmyelinated axonal tissue alone is sufficient to generate *de novo* action potentials in response to ultrasound stimulation. Examining the nature of this response allows several insights into the underlying mechanisms, which, in the present case, the authors demonstrate to be cavitation.

487 The lowest threshold at which responses were seen in any of the experiments  
488 conducted here, was an order of magnitude greater than pulse average intensities  
489 used in some studies achieving successful stimulation in rat brain tissue (Kim et al.,  
490 2012, 2014b,a; Tufail et al., 2010; Yoo et al., 2011). These studies use lower frequency  
491 ultrasound (250-350 kHz) which has indeed been shown to be a critical factor by  
492 the current study and others (King et al., 2013; Kim et al., 2014a; Gavrilov et al.,  
493 1977; Muratore et al., 2009; Lee et al., 2014) which may account even for this large  
494 discrepancy in the pulse average intensity threshold. From investigations repeating  
495 the pulsing parameters of an applied study in the human brain (Lee et al., 2015),  
496 pulse protocol does not appear to play a role. The mechanism observed here and in  
497 many *in vivo* brain studies may be the same but given the very different cavitation  
498 environments, until further research can be performed the mechanisms should be  
499 treated as distinct. Subsequent discussion will therefore focus on the characterisation  
500 of the currently observed stimulation phenomenon.

501 The intensity thresholds found in this study are much closer to those reported by  
502 Gavrilov’s group and others targeting peripheral nerve structures (Gavrilov et al.,  
503 1996; Wright and Davies, 1989; Mihran et al., 1990; Legon et al., 2012; Tych et al.,  
504 2013), re-enforcing the apparent divide in threshold amplitude between neuromodu-  
505 lation of the CNS and the PNS. The extent of the separation in required intensities  
506 between these two paradigms demonstrate the importance of identifying in different  
507 tissue types, the specific US effects and their thresholds. This could then be used to  
508 develop a fuller understanding of the US-tissue interaction and targeted ultrasound  
509 therapies, including but not limited to neurostimulation.

510 *Ultrasound Force Mechanism*

511 *Response Dynamics*

512 The first thing that was noted about the nerve responses was the stochastic  
513 success rate, where large events that involved many axons in the bundle occur infre-  
514 quently. This points to a correspondingly probabilistic cause that occurs on a scale  
515 affecting a large proportion of the fibres in the bundle or not at all, consistent with  
516 cavitating bubbles occurring outside of nerve fibres. If the mechanism of stimulation  
517 was on a small scale such as the bilayer sonophore model (Krasovitski et al., 2011),  
518 the many isolated events that act at the individual cell level would be expected to  
519 produce a reliable response when aggregated to the level of the entire bundle.

520 The same argument against cellular scale probabilistic effects can be applied to  
521 discount a radiation force mechanism. In a system where nothing is being changed  
522 between US exposures, radiation force as a result of tissue absorption and reflection  
523 should remain constant as well as any effects on the nerve it elicits, but this is not  
524 what was observed. Second to this, the radiation forces produced in the 0.67 MHz  
525 exposures were calculated and reproduced at 1.1 and 2 MHz (Table 2) and found  
526 to be ineffective at generating responses from the nerve. Indeed these modelled  
527 forces are lower than compressional experiments in the literature shown to generate  
528 mechanical stimulation of axons (Rivera et al., 2000), though conductance changes  
529 from weak compression may contribute to the overall effect (Julian and Goldman,  
530 1962; Olesen et al., 1988).

531 The response latency dynamics of the current observed phenomena also does not  
532 match with studies that find only the onset or offset of US stimuli to be effective  
533 (Menz et al., 2013; King et al., 2013; Gavrilov et al., 1977; Dalecki et al., 1995;  
534 Krasovitski et al., 2011; Plaksin et al., 2014) which would be consistent with radiation

535 force or bilayer sonophore mechanisms. In the current findings, stimuli occurrences  
536 are distributed throughout the 8 and 100 ms exposures, with each part of the pulse  
537 train having a similar chance of stimulating the nerve.

#### 538 *PCD Data*

539 In both the 8 ms and 100 ms datasets a plateauing of kurtosis and signal energy  
540 are seen after 230 W/cm<sup>2</sup> (figure 6). This is likely to be caused both by the non-  
541 linear scaling of peak negative pressures with intensity (Table 2), and saturation in  
542 the occurrence (but not amplitude) of cavitation. Concurrently with this observa-  
543 tion, response reliability also saturates around this intensity (figure 5 and figure 8).  
544 Therefore increasing US intensity past a point will not increase the likelihood of  
545 stimulation and may increase the violence of events and likelihood of damage.

546 Across all US stimuli, inertial cavitation was most often observed with no resul-  
547 tant nerve response. The probable reason for this is that the focal area ( $6 \times 3.5$   
548 mm FWHM) and potential volume in which cavitation is likely to occur, was much  
549 larger than the volume occupied by the nerve bundle (1-2 mm diameter). Cavitation  
550 therefore may not be occurring in close proximity to the nerve.

551 This affected the analysis of the 8 ms much more than the 100 ms exposures as  
552 the non-proximal cavitation activity is found throughout the 8ms exposure and only  
553 in the first 10 ms of longer stimuli. This lower average background activity over the  
554 longer exposure period led to significant correlations between US stimulated CAP  
555 amplitude and PCD signal kurtosis in individual nerve experiments and across the  
556 whole dataset.

557 Correlation coefficients can mask infrequent stimulation events that may occur  
558 without any sign of cavitation. A key finding of this paper therefore, is that, through  
559 detailed individual PCD signal analysis, broad frequency band inertial cavitation



560 events were detected in the expected time section preceding 100% of successful US  
561 neurostimulation events in both the 8 and 100 ms datasets.

## 562 *Damage*

563 Damage was found to occur as a result of US exposure in several cases, strongly  
564 correlated to inertial cavitation at all intensities. The lowest instance of damage  
565 occurring close to the threshold ( $230 \text{ W/cm}^2$ ) for stimulation raises concerns as to  
566 the safety of US stimulation at these intensities. However, given the present scope  
567 for optimisation and refinement of the stimulation parameters, it is hoped that the  
568 risk of damage can be eliminated in future. It is also as yet unclear how a full, *in*  
569 *vivo* situation will affect both the success of the stimulation effect and the occurrence  
570 of damage but the latter should be examined in more depth *in vitro* before applying  
571 the current technique to animal models.

## 572 *Biological Mechanism*

573 Given the nature of the causative US forces discussed above and the presence  
574 of axonal tissue alone, the authors suggest that the mechanism of membrane de-  
575 polarisation has been narrowed down to two options. The first option involves the  
576 opening of ion channels by membrane stretch induced by cavitation forces such as  
577 microstreaming drag, direct jetting, or radiation forces on bubbles. The second is  
578 general ionic flux and resultant depolarisation through a sonoporation effect caused  
579 by the same cavitation mechanisms (Wan et al., 2015).

580 Responses that were followed by after-discharge (figure 10c) from identified dam-  
581 age events were likely due to large scale membrane perforation or tearing that re-  
582 sulted from inertial cavitation forces. It is hoped that through planned future work  
583 using high speed imaging and computational bubble modelling, the critical biological  
584 interaction can be determined and a force threshold identified.

## 585 Conclusions

586     Reported here are successful parameters for ultrasonic neurostimulation in the  
587 peripheral crab leg nerve bundle, demonstrating that the constituents of unmyeli-  
588 nated axonal tissue are sufficient to generate *de novo* action potentials in response  
589 to US stimulus, in the majority of cases without lasting damage. The threshold  
590 for this stimulation was much higher than similar procedures performed on CNS  
591 models but in good agreement with other PNS focused studies. Low intensity stim-  
592 ulation parameters shown to be successful *in vivo* in the literature were unsuccessful  
593 at generating any response from the nerve bundles. Given the difference in thresh-  
594 old intensities, the current observed stimulation phenomenon is assumed to have a  
595 distinct US force mechanism.

596     In characterising the observed stimulation phenomena, inertial cavitation activity  
597 was found to be highly correlated to successful US stimulation, with its acoustic  
598 signature present in every example. With further work into protocol refinement  
599 and control of cavitation nuclei, this US stimulation mechanism will have incredible  
600 potential for both clinical and research applications. Future work by the group will  
601 aim to determine the exact cellular level forces required to generate stimulation in  
602 this and other models.

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793 **Figure Captions**

794 **Figure 1:** Schematic diagram of the experimental setup used to generate alternate  
795 electrical and US stimuli recordings.

796 **Figure 2:** Schematic diagram of the nerve bath with electrodes and important  
797 features labelled.

798 **Figure 3:** Visualisation of the size and position of the focal points produced by  
799 each ultrasound transducer within the nerve bath (a) 0.67 MHz (b) 1.1 MHz  
800 (c) 2 MHz. Temperature induced colour changes were produced in an polyacry-  
801 lamide gel. 10s continuous wave exposures with different focal intensities were  
802 used with each transducer to achieve a good visualisation of the focal area.

803 **Figure 4:** (a) Timeline of the interleaved US and electrical stimulation protocol.  
804 Electrical stimulation is marked by vertical black lines, US stimulation by ver-  
805 tical blue lines. Each US stimulus is comprised of a pulse train of 0.5 ms pulses  
806 at 50% DC. The entire stimulus protocol is repeated every 2 minutes 11 times  
807 for every nerve experiment. (b&c) Example of a CAP (electrode voltage data  
808 - red, left axis. Shown by a small deviation from the mean) which was stimu-  
809 lated by an ultrasound pulse ( b) 0.67 MHz, 8 ms, 562 W/cm<sup>2</sup> c) 0.67 MHz,  
810 100 ms, 562 W/cm<sup>2</sup>). PCD data showing the US stimulus is included above  
811 the electrode voltage data (acoustic amplitude - blue, right axis). High ampli-  
812 tude acoustic signal containing cavitation signatures are detected at the start  
813 of both stimuli. (c) Vertical red lines show the period of the acoustic pulse  
814 train within which the stimulus event is expected to have occurred given esti-  
815 mated CAP conduction times. Increased acoustic signal amplitude containing  
816 cavitation signatures is seen within this period.

817 **Figure 5:** Response success statistics for the 8 ms, 0.67 MHz stimulation protocol.  
818 Nerves that did not respond to electrical stimuli were excluded. Numbers of  
819 successful stimuli at each intensity are displayed above each bar. (a) Total  
820 response reliability for all nerves tested at each intensity level. Two nerve  
821 experiments with over 50% reliability were excluded as outliers. (b) Mean  
822 amplitude of US induced CAPs as a proportion of the electrically induced  
823 CAP amplitude.

824 **Figure 6:** Mean and standard deviation error bars of (a) signal energy and (b) time  
825 domain kurtosis in four frequency bands decomposed from PCD recordings of  
826 the 8 ms, 0.67 MHz US stimulation protocols. The frequency band containing  
827 the US driving frequency is highlighted in red.

828 **Figure 7:** Percentage of US stimuli (8 ms, 0.67 MHz protocol) showing above thresh-  
829 old kurtosis ( $>6$ ) in four frequency bands at each stimulus intensity. In each  
830 frequency band, 11 columns are present representing the different stimulus in-  
831 tensities. The colourmap on the right is in units of  $W/cm^2$ .

832 **Figure 8:** ((a) and (b)) Response success statistics for the 100 ms, 0.67 MHz stimu-  
833 lation protocol. Nerves that did not respond to electrical stimuli were excluded.  
834 Numbers of successful stimuli at each intensity are displayed above each bar  
835 (a) Mean response reliability for all nerves tested at each intensity level. (b)  
836 Mean Amplitude of US induced CAPs as a proportion of saturated electrical  
837 stimulus recording taken before each US stimuli. (c) Mean acoustic signal kur-  
838 tosis of four frequency bands across all intensities. Error bars show standard  
839 deviation.

840 **Figure 9:** Mean values of PCD signal kurtosis of the lowest frequency band over

841 100 ms, split into 10 ms divisions and a range of stimulation intensities ( $I_{SPPA}$ ).

842 **Figure 10:** (a) Example of electrically stimulated CAP amplitude (line) and US  
843 stimuli (+) over 22 stimuli on a single nerve. This example was exposed using  
844 the 0.67 MHz, 8 ms stimulation protocol at 562 W/cm<sup>2</sup>. (b) Mean decline over  
845 time of the electrically stimulated CAP amplitude of the 0.67 MHz, 8ms stim-  
846 ulated nerves, normalised to the amplitude of the first stimulus with standard  
847 deviation error bars. (c) Example of after-discharge due to nerve damage using  
848 the 0.67 MHz, 8 ms stimulation protocol at 230 W/cm<sup>2</sup>. (d) spectrographic  
849 analysis of the PCD signal of the first damage causing ultrasound event in (a).

Table 1: My caption

Model	Manufacturer	CF (MHz)	Focal Length (cm)	Aperture (cm)	LFA (cm)	WFA (cm)
PA409	Precision Acoustics	0.67	7.2	6.0	4	0.5
H-101-MR	Sonic Concepts	1.1	6.3	6.4	1	0.14
H-106	Sonic Concepts	2	6.3	6.4	0.6	0.08
XL50PCD	Ultran	5.8	7.7	1.3	-	-

## 850 Tables

851 **Table 1:** HIFU transducer reference table. CF = Centre Frequency, LFA = Length  
852 of Focal Area, WFA = Width of Focal Area. Focal area dimensions are given  
853 according to the FWHM. The sensitivity bandwidth of the XL50 PCD was 4.8  
854 MHz at -6 dB with a bandwidth centre frequency of 6.8 MHz.

855 **Table 2:** Intensities, negative and positive peak pressures and radiation forces at  
856 three frequencies. Intensities and pressures were chosen to create equal radia-  
857 tion forces across the frequencies used.

858

	0.67 MHz			1.1 MHz			2 MHz		
Radiation	Pressure			Pressure			Pressure		
force (mN/cm <sup>2</sup> )	(MPa)		I <sub>SPPA</sub> (W/cm <sup>2</sup> )	(MPa)		I <sub>SPPA</sub> (W/cm <sup>2</sup> )	(MPa)		I <sub>SPPA</sub> (W/cm <sup>2</sup> )
1	-0.8	0.8	20	-0.7	0.7	17	-0.7	0.5	12
5	-1.4	1.6	76						
6	-1.6	1.8	100	-1.5	1.7	84	-1.4	1.3	61
9	-1.9	2.2	140						
11	-2.1	2.4	169	-1.9	2.2	143	-1.7	1.8	103
15	-2.4	2.9	230						
18	-2.6	3.2	274	-2.4	2.8	232	-2.1	2.4	167
23	-2.8	3.6	352						
27	-3.1	4.0	419	-2.9	3.6	353	-2.5	3.0	255
31	-3.3	4.3	485						
36	-3.5	4.7	562	-3.3	4.2	475	-2.8	3.6	343