The following publication Q. Xian, Z. Qiu, S. Kala, K. F. Wong, J. Guo and L. Sun, "Behavioral and Functional Assessment of Ultrasound Neuromodulation on Caenorhabditis Elegans," in IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control, vol. 68, no. 6, pp. 2150-2154, June 2021 is available at https://doi.org/10.1109/TUFFC.2021.3057873.

Behavioral and functional assessment of ultrasound neuromodulation on *Caenorhabditis elegans*

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***Abstract*—Ultrasound brain stimulation is a promising modality for probing brain function and treating brain diseases. However, its mechanism is as yet unclear and in vivo effects are not well-understood. Here we present a top-down strategy for assessing ultrasound bio-effects in vivo, using *Caenorhabditis elegans*. Behavioral and functional changes of single worms and of large populations upon ultrasound stimulation were studied. Worms were observed to significantly increase their average speed upon ultrasound stimulation, adapting to it upon continued treatment. Worms also generated more reversal turns when ultrasound was on, and within a minute post-stimulation they performed significantly more reversal and omega turns than prior to ultrasound. In addition, *in vivo* calcium imaging showed that the neural activity in the worms' heads and tails was increased significantly by ultrasound stimulation. In all, we conclude that ultrasound can directly activate the neurons of worms *in vivo*, in both of their major neuronal ganglia, and modify their behavior.**

***Index Terms*—Ultrasound neural stimulation, *C. elegans*, Calcium imaging.**

I. Introduction

Noninvasive neuromodulation of deep brain structures provides a powerful way to understand brain function and treat brain disorders. Some existing technologies include transcranial magnetic stimulation (TMS), transcranial electric stimulation (iACS and tDCS), ultrasound brain stimulation and optogenetics [1]. Among these technologies, ultrasound neuromodulation is considered one of the more promising modalities for eventual clinical translation, as low-frequency ultrasound can penetrate the intact skull and be targeted to small regions [1-3]. Indeed ultrasound alone has been shown to activate or inhibit neurons both *in vitro* [4] and *in vivo* [5]. However, the mechanism underlying this neuromodulatory ability is still unclear. One major hypothesis of low-intensity low-frequency ultrasound stimulation (LILFUS) in the brain is that ultrasonic waves activate mechanosensitive ion channels located on the cell membrane [6]. We have previously shown that LILFUS treatment could activate the Piezo1 channel *in vitro* [7]. However, further study is required to elucidate the underlying mechanisms in order to improve its effectiveness.

The nematode worm, *Caenorhabditis elegans* is a good model for investigating this hypothesis due to its unique advantages such as easy genetic modifications and transparency for optical imaging. It has a small nervous system consisting of just 302 neurons and well-characterized robust behaviors. *C. elegans* worms have therefore been widely used as a model animal in neuroscience studies, including some studying the effects of ultrasound. Worms have been treated using higher-frequency ultrasound (10, 45 MHz) to test whether ultrasound could activate mechanosensitive ion channels such as MEC-4 [8]. Another study used lower-frequency ultrasound (2 MHz), combined with microbubbles, to enable activation of the TRP-4 channel in worms [8-11]. Similarly, Zhou et al. demonstrated the activation of ASH neurons in *C. elegans* by a surface acoustic wave device at 28 MHz [12]. These studies provide evidence of ultrasound's ability to stimulate neuronal ion channels *in vivo*, but the protocols used lack of the advantages of transferring to clinic because higher frequency ultrasound is unable to access to deep brain regions or can be entangled with different physical mechanisms. Secondly, administering microbubbles into the brain would be difficult and risky in higher mammals, including for future clinical translation. Thus, the clinically-relevant questions remains whether low-frequency ultrasound (1 MHz or below), without the presence of enhancers like microbubbles, can activate neurons *in vivo*.

In the present study we examine the bio-effects of LILFUS neuromodulation using a top-down strategy with transgenic *C. elegans* [13], expressing a neurally-integrated calcium indicator, GCaMP6s. Their whole-body neuron activity during free-roaming behaviors under ultrasound stimulation was monitored by calcium imaging. We found that low-frequency ultrasound stimulation alone triggered worms to alter the rate and the type of their movements. Neurons in the worms' heads were seen to activate significantly upon ultrasound stimulation. Our results thus confirm that LILFUS can activate the neurons *in vivo* directly, without microbubbles. In addition, stronger

Manuscript submitted March 24, 2020. This work was financially supported by the General Research Fund (15102417 and 15326416), Innovation Technology Fund (MRP/018/18X), and Science and Technology Planning Project of Guangdong Province (2018B030331001).

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fluorescent responses to ultrasound were observed in the worms' guts compared to their heads, suggesting that ultrasound can also be utilized to stimulate nerve terminals of intestinal muscles.

II. Materials and Methods

A. Ultrasound system

The acoustic components were composed of a function generator, a power amplifier, and ultrasound transducers with a center frequency of 1 MHz, which could generate ultrasound with different stimulation protocols. The generated ultrasonic waves were coupled into a dish and delivered to the *C. elegans* moving freely on the gel surface. The acoustic field was characterized with a hydrophone to ensure the acoustic beam covered the optical imaging field of view. The optical components of this customized system were composed of a widefield upright fluorescence microscope. A schematic illustration of such an ultrasound stimulation device consists of ultrasound transducer, NGM with *C. elegans* and camera for recording, shown in Fig, 1a and 3a. We used low frequency, low intensity ultrasound stimulus on the worms, and recorded video of their movements using the microscope (20X). The parameters of ultrasound used were: 200 bursts of central frequency 1 MHz, 200 μs pulse width, 1 kHz PRF, spatial peak-temporal peak pressure 0.37 MPa.

B. Worm strains and maintenance

Wild-type stains were grown on Nematode Growth Medium (NGM) agar plates seeded with OP50 Escherichia coli bacteria at 20℃ using standard methods [14]. We used genotype wtfls5 transgene worms (rab-3p::NLS::GCaMP6s+rab-3p::NLS::tag RFP) which integrated calcium indicator GCaMP6s and calcium-insensitive fluorescent protein RFP in the nuclei of all neurons [13].

C. Worm average speed recording and calculation

Worms were grown at 20℃ for 72 hours to reach the young adult stage for the average speed quantification test. About 20 adult worms were synchronized on to a 5 cm NGM plate seeded with OP50 for the average speed assay. Approximately 50 worms were tested for 22 min. A temperature of 20℃ was maintained throughout the experiment. The worms were allowed to move freely for 5 minutes and they were recorded after showing stable movement for 2 min. They were then stimulated with ultrasound for 20 minutes. The average speed was quantified and analyzed every 5 min using ImageJ.

D. Calcium imaging

An experimental system was set for real-time recording of worms' behavior and neural activity during ultrasound stimulation. A small NGM pad was prepared for reducing the locomotion of C. elegans. Molten NGM (100 μl) was dropped on a larger glass coverslip and a smaller glass coverslip was pressed on the NGM drop after 5 s. After the molten NGM solidified, it was moistened with M9 buffer. Then, adult worms were picked from a cultured NGM plate and placed in the gap between the NGM agar and larger coverslip. Redundant M9 buffer was removed with a Kimwipe. Ultrasound gel was put on the small coverslip and an ultrasound transducer was mounted above it. The worms were allowed to move freely 10 min for recovering followed by calcium imaging and behavior recordings as outlined previously [15, 16]. The individual worms' behaviors were manually tracked.

E. Statistical analysis

Data were analyzed using GraphPad Prism5 and ImageJ. The observer was not blind to the genotype of the group being tested. The populations used for imaging data were analyzed by a student's t-test.

III. Result and Discussion

We first tested the effects of LILFUS on free-moving *C. elegans* (wild type, Him-5) by monitoring their behavior of a large population by using a customized system. Our ultrasound setup consisted of a transducer generating the ultrasound beam coupled to a waveguide at the bottom of a petri dish of agar, which contained the worms (Fig. 1a). We stimulated the whole body of worms and observed their responses in a wide range. A camera placed above the plate was used to record the worms' movement trajectories, and the recordings were used to quantify their average speeds. During the first 5 minutes of a 20-minute LILFUS stimulation (200 bursts of central frequency 1 MHz, 200 μs pulse width, 1 kHz PRF, spatial peak-temporal peak pressure 0.37 MPa and 5 s interval.), the worms' average speed increased from a baseline of 100 pixels/frame to 130 pixels/frame (Fig. 1b). After 5 minutes, the worms gradually reduced their average speed to near the baseline level. Thus, we found that our LILFUS setup could stimulate could trigger a reaction in the worms, as shown by them speeding up their movement, and that the worms adapt to the stimulation after

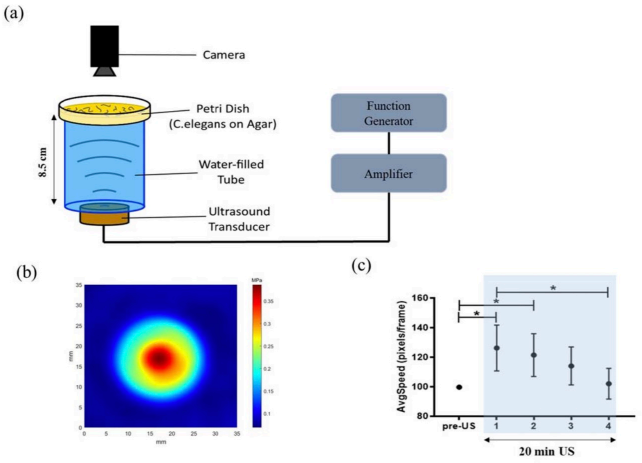


Fig. 1. *C. elegans* worms respond to LILFUS stimulation by altering their speeds. (a) A schematic diagram of our ultrasound system for recording the average speed of worms. (b) The adaptation probability of the worms after repeated US stimuli at constant intervals for 20 minutes, divided into 4 sections of 5 minutes each. The average speed of worms during each section was then calculated. (n = 4 trials, with each trial consisting of ~50 worms. *P < 0.05, unpaired two- tail t-test.

approximately 5 minutes.

We further investigated detailed behavioral changes of single worms during LILFUS stimulation. The worms' movements were tracked and classified as reversals or Omega turns [16] (Fig. 2a). We found that 84.38% worms exhibited immediate "reversal" responses upon LILFUS stimulation (Fig. 2b). The behavior of the worms both prior to and 30 seconds post-LILFUS stimulation were also analyzed. We found that the worms' rate of both reversals and omega turns after LILFUS stimulation were approximately 4 and 5 times respectively what they were before the stimulation (Fig. 2c). Before ultrasound stimulation, worms generated 0.5 ± 0.1387 reversals, but the number increased to 4 times that (2 ± 0.2774) after LILFUS. Similarly, worms not stimulated with LILFUS produced 0.2143 ± 0.1138 omega turns and this increased to 5 times after stimulation (1.071 ± 0.2864). Taken together, these data demonstrate that LILFUS can induce obvious behavior changes in *C. elegans*.

We then used calcium imaging to study more deeply how LILFUS could affect functional changes in neural stimulation in vivo. Intracellular Ca^{2+} imaging is a widely-used marker of neuronal activity [17, 18], and allows greater temporal resolution in observing the effects of ultrasound. The fluorescence of GCaMP6s in worms' heads was monitored to assess neural activities during LILFUS stimulation using a fluorescence microscope, with the ultrasound being administered from above and using an ultrasound coupling gel (Fig. 3a). The total fluorescence intensity of GCaMP6s in the heads of worms was monitored for response to ultrasound. Worms that showed reversal responses had their head GCaMP6S fluorescence significantly increased LILFUS stimulation, whereas in the groups without LILFUS and the worms which did not show reversal responses upon LILFUS, the fluorescence intensity in the head remained almost unchanged (Fig. 3c). Compared to control group and with (responding) behavior group, GCaMP6S fluorescence increased by 49.24 ± 11.4 (n = 7 worms, P = 0.001, unpaired two tail T-test). Also, on average, 39.08 ± 8.514 of GCaMP6S fluorescence increased in (responding) behaviour group compare with the group of (no response) without behaviour response (n = 7 worms in the group of (responding) with behaviour response, n = 3 in the group of (no response) without behaviour response, P = 0.001, unpaired two tail T-test). Thus among the worms that were able to respond to ultrasound, LILFUS alone could stimulate neuronal activation in their heads.

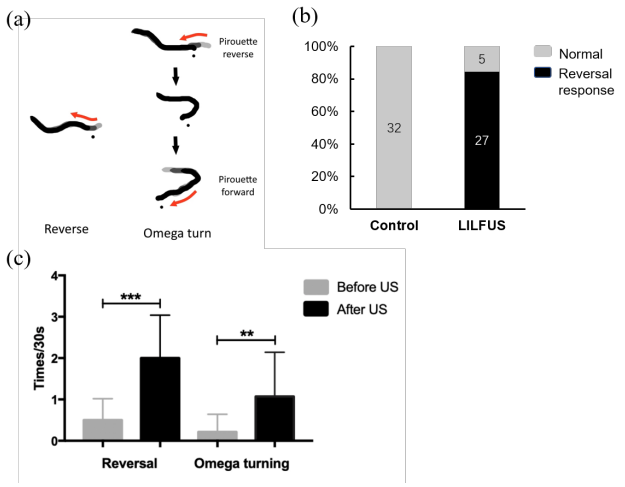


Fig. 2. LILFUS alone induces worms to modify their behaviors. (a) Schematic showing the criteria for worm behavior evaluation; (b) The proportions of the reversal behavior to an ultrasound stimulus; (c) Quantified responses of worms' behaviors before and after LILFUS stimulation. Bars represent the mean ± SEM of multiple experiments n = 14 (activated C. elegans), *** P <0.0001, ** P < 0.01, unpaired two tailed t-test.

In addition to activity in the head, significant changes in GCaMP6s fluorescence were observed in the intestine of *C. elegans*. GCaMP6s fluorescence increased significantly in the intestine after 6-8 s of LILFUS compared to before the stimulation (Fig. 4a). Of 29 worms studied, we observed no changes in intestinal GCaMP6S fluorescence in 28 worms in the control group, whereas in LILFUS-stimulated worms 19 out of 29 worms (65.52%) showed increased intestinal fluorescence (Fig. 4c). Thus, LILFUS stimulation could significantly increase activity in the intestinal neurons of worms in addition

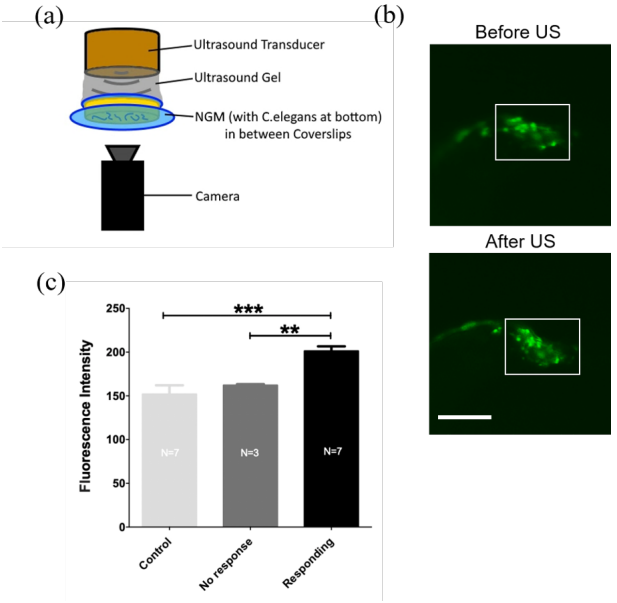
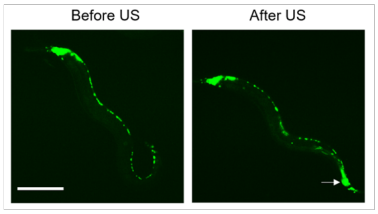


Fig. 3. LILFUS stimulation activates neurons in the heads of worms. (a) Schematic of the fluorescence imaging and LILFUS system. (b) GCaMP6s fluorescence in the whole body and magnified picture of the head. Wtfls5 transgenic *C. elegans* were used, which possess the fluorescent calcium indicator GCaMP6s in all neurons. The scale bar in this panel represents 100 µm. (c) Average GCaMP6s fluorescence intensity of the neuron in the head of non-treatment group (control), US group without behavior responses, and US group with behavior responses. Bars represent the mean ± SEM of multiple experiments. n for control and responding groups = 7 worms, n for no response group = 3 worms, unpaired two tailed t-test.

to stimulating neurons in the head.

(a)



(b)

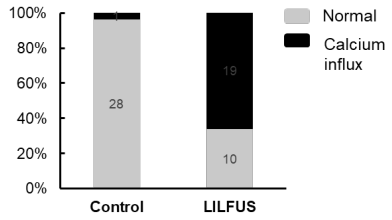


Fig. 4. Intestinal neurons of *C. elegans* worms respond to LILFUS stimulation. (a) Representative images are shown of increased calcium and neural activity in the worms' intestines before and after stimulation with US The scale bar in this panel represents 200 μm. (b) The number of worms whose intestinal neurons responded to LILFUS stimulation (t=6-12s interval after LILFUS stimulation). The numbers are from 29 worms observed in 6 independent experiments.

IV. Discussion

Ultrasound brain stimulation has garnered lot of attention in recent years, but with unsatisfactory elucidation of its workings and some doubt about its effects [19, 20]. Previous studies have shown that ultrasound as a mechanical pressure wave could activate mechanosensitive ion channels to give rise to the neural activity at low frequencies [21, 22], in the presence of microbubbles [9], or at higher frequencies in *C. elegans* [9]. However, these conditions are not clinically relevant as microbubbles cannot pass through blood vessels and are extremely unstable, and high-frequency ultrasound cannot penetrate through the rodent skull [23]. This significantly limits the areas that may be targeted with such treatment. Furthermore, the physical mechanism of ultrasound acting on biological samples vary depending on the frequency and parameters used [24], which could make applying their conclusions to low-frequency ultrasound problematic. Our results show for the first time that LILFUS alone can activate neurons in vivo, in the heads of worms, making it more directly relevant whether ultrasound could successfully stimulate neuronal mechanosensitive ion channels in the brains of mammals.

There are other possible mechanisms proposed to explain ultrasound's bio-effects in addition to the radiation force hypothesis [25]. Here we utilized an unbiased, whole-body observation strategy to assay the possible effects of the stimulation. We showed that while neurons in the head and body could be activated by LILFUS, there was also a significant effect on neurons observed in the intestinal terminus. As there are known to be mechanosensing neurons in the intestine [26], our results suggest that LILFUS was activating these very mechanosensitive neurons. This suggests further novel applications of LILFUS stimulation on the neural activity in important enteric circuits, like the human intestine-brain axis [26].

Taken together, our findings demonstrate that LILFUS can activate neurons directly in the absence of microbubble to initiate behavior in vivo in worms, with worms adapting to the stimulus after 20 minutes' treatment. Our top-down strategy also allowed us to observe the new phenomenon of intestinal neuronal responses to LILFUS.

V. Conclusion

We found that *C. elegans* responded and adapted to the LILFUS stimulation in a matter of minutes. We also found that LILFUS could activate neurons in the worms' heads and intestines by using a transgenic *C. elegans* line expressing GCaMP6s in each neuron. Thus, we show that LILFUS could activate neurons and initiate behavioral changes in *C. elegans*. The response dynamics presented here are shared by other rapidly adapting mechanosensory neurons, including *C. elegans* [27] and Drosophila [28]. This phenomenon is akin to the well-known mechanical stimulation adaptation mechanism when ultrasound is used to stimulate cells *in vitro* [29].

Acknowledgment

This study was financially supported by the General Research Fund (15102417 and 15326416), Innovation Technology Fund (MRP/018/18X), and Science and Technology Planning Project of Guangdong Province (2018B030331001).

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