Cellular Localization and Dosage Regulation of Neural Stimulation Enabled by 1.05 GHz Ultrasonics

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***Abstract*— A novel neural stimulation system is demonstrated that is capable of delivering tunable ultrasonic stimulus at the cellular level and additionally providing feedback information towards dosage regulation. GHz aluminum nitride ultrasound transducers provide continuous wave stimulation to *in vitro*, retinoic acid differentiated SH-SY5Y neuroblastoma cells at 1.05 GHz. Results show stimulation of optically recorded calcium ion transients in SH-SY5Y cells, with during stimulation increases in F/F_0 of 1.5 ± 0.328, differing from pre-stimulation with $p < 0.01$. The ultrasonic pulse-echo return signal of the ultrasound transducer is used to estimate and tune the ultrasound dosage using transducer design, input stimulus, and interface medium. Results and theory suggest that higher acoustic impedance cell culture medium and scaffolds will allow for delivery of higher ultrasonic intensity to neural cells. Differences in echo return amplitudes for various interface medium and input stimulus amplitudes are reported at $\alpha = 0.01$.**

Keywords*— *ultrasonic neural stimulation, GHz ultrasound, SH-SY5Y, in vitro, neural cell culture, MEMS

I. Introduction

Neural stimulation and modulation with ultrasonic energy has great potential for impact in the neurosciences as it is essentially label-free excitation of cells. Ultrasonic neuromodulation suffers from lack of energy localization within a single cell, informed dosimetry, and mechanism validation [1]. The role of ultrasound in neuromodulation has a rich history, while specific mechanisms are lacking. Ultrasound energy delivered to tissue with controlled localization is especially important for cellular and tissue engineering and clinical applications. Being able to focus and limit the energy delivery within the cellular level holds the potential to study sonic neuromodulation at the single cell level. At GHz frequencies, the wavelength in tissue can be sub-micron, and the loss depth can be also within a few microns to provide a focused does of ultrasonic energy [2]. While high frequency ultrasonics presents clear challenges due to steep signal attenuation, this attenuation profile can be utilized to further localize the stimulus in the axial direction, providing potential sub-cellular cellular localization of ultrasound stimulus, as shown in Figure 1. Here phased arrays of transducers can be used to focus an ultrasonic beam with a sub-wavelength focus to scan individual neurons.

The pulse-echo operation of this transducer consists of driving thin film piezoelectric transducers with short pulses that propagate through the silicon chip, and are incident on the opposing interface and travel back to the transducer. The

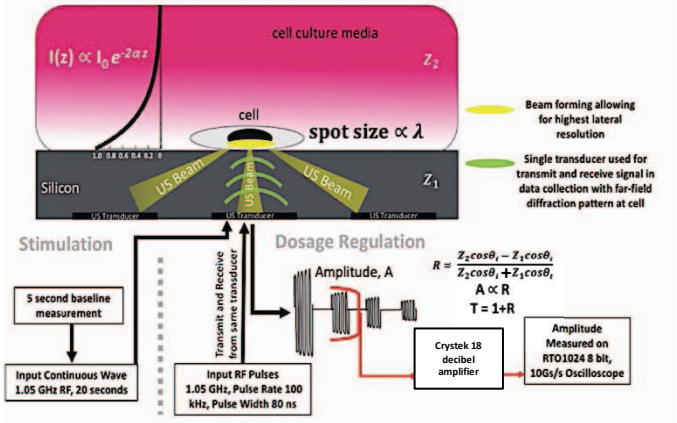

Fig. 1. GHz attenuation profile localizes signal axially, wavelength localized signal laterally to the level of one cell. Pulse echo return allows for dosage regulation following the Fresnel transmission-reflection interphase physics of acoustics.

multiple echoes of the incident ultrasonic pulse contain the information of the ultrasonic impedance mismatch between silicon and the tissue. As shown in Figure 1, the return echo of the incident pulse can be used to estimate the delivered ultrasonic intensity, making this a plausible method towards feedback regulation and ultrasonic dosimetry. Time varying reflected signals can provide information on how a different or time evolving interface medium or stimulus intensity is influencing the dosage. This has significance for both research and clinical applications. Implantation of devices often elicits an immune response, inflammation, and scar tissue formation. The acoustic impedance of the tissue is strongly influenced by these phenomena. This is also important for growth inducing conditions of tissue engineering in the *in vitro* research setting. This paper demonstrates one of the first studies in which this high frequency, localizable GHz signal stimulates *in vitro* neurons, to demonstrate neural stimulation and senses impedance of the neuron-transducer interface.

II. Materials and Methods

A. Transducer Design and Setup Criteria

70 μm wide square aluminum nitride (AlN) piezoelectric films between molybdenum (Mo) electrodes with passivating and insulating silicon-dioxide layers form the on silicon (Si) chip transducers. The transducers have a nominal thickness mode resonance at ~1.05 GHz, with higher order modes that were not

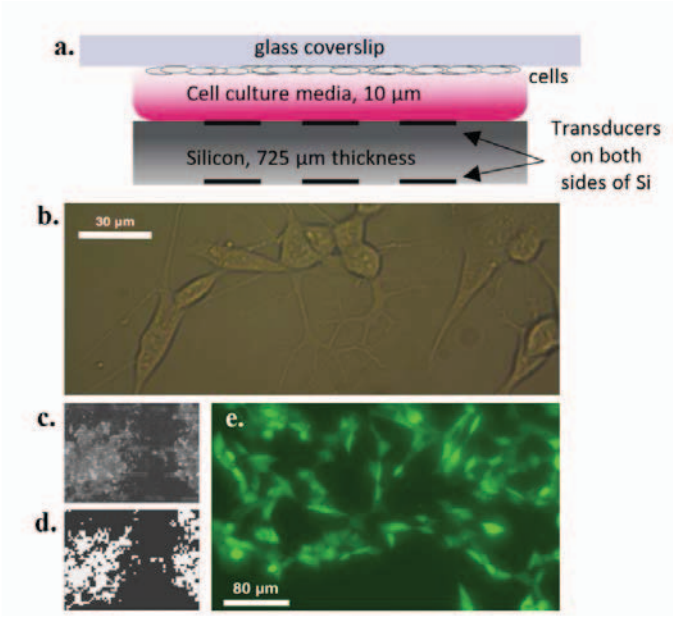

Fig. 2. a. Setup for stimulation experiments with layer thicknesses depicted. Transducers are wire-bonded to printed circuit board (not shown). b. RA differentiated cells with visible neurites, 48 hours 40X magnification. c. Raw input data for processing. d. Image processing cell classification mask. e. Fluo-8 loaded SH-SY5Y cells, 20X magnification

explored here. The operating frequency was chosen for a maximum echo signal for resonance operation. Neural stimulation data in this paper is collected using two simultaneously activated 70 µm wide transducers spaced by one dormant 70 µm transducer, stimulated at an input voltage of 2.5 V_{pp} sinusoid at 1.05 GHz. Maximum displacement values from similar transducers recorded from the Polytec UHF-120 Vibrometer show displacements of u_o= ~20 pm for 5 V_{pp} at resonance. The displacement in liquid at the interface would be $u_t = \frac{2Z_T}{Z_T+Z_I}u_i$ for pressure waves. The displacement is approximately u_t= ~1.35pm for the cell culture media interface at 2.5 V_{pp} corresponding to ~6 mW/cm^2 at the interface. The intensity decays as $I = I_0 e^{-\alpha x}$ where $\alpha = 0.134\frac{dB}{\mu m\ GHz^2}$. With the cells being 10 μm away from the interface, the order of magnitude intensity at the cells should be ~1-2 mW/cm^2.

The transducers are activated at the backside of an identically fabricated, two-sided transducer array, separated by a 725 µm thick layer of Si. The excitation from the back side allows for the far field diffraction pattern to form at the interface with the cells, with both main and side lobes present in the intensity profile [3].

The experimental setup is designed to stimulate neural cells and obtain proof of concept that GHz ultrasonics can stimulate the ion channels of neural cells, with the realization that phased array, beam-forming, and transducer design can be used to address sub-cellular volumes. Excitation of one neuron can spread to connected neurons in the in-vitro preparation. This setup is preferred as it provides us statistical certainty due to more stimulated cells per experiment. Although single neuron localization is possible, *in vitro* connectivity is crucial to differentiation of neurons and sufficient membrane ion channel densities. When cells are in a network, proper differentiation occurs, but it is hard to distinguish stimulus from in-network signal propagations. Proof of single neuron signal isolation could be achieved using several neurons that lack connectivity being selectively targeted by GHz ultrasonics with mechanistic proof of signal isolation at that volume. In cell culture medium, acoustic streaming forces can influence neurons past the focal spot of the beam. Thus even high speed imaging and electrode array outputs that can track signal propagation in a network will still require mechanism evaluation. This will be detailed in future research.

In order to study neuromodulation, continuous wave ultrasonic stimulus for 20 seconds was applied, and was recorded for an additional 5 seconds before stimulus to obtain the baseline measurement. Action potential activity was optically measured, during the stimulus duration and baseline period is reported. The effects of stimulation after administration and intensity response will be detailed in future studies.

B. Cell Culture Methods and Protocol

In order to realize a neural sample, differentiation protocols supported by researched SH-SY5Y neuroblastoma differentiation methodologies are adapted [5]. These cells are an immortalized cancer cell line that possess both epithelial and neuronal phenotypic characteristics. They are popularly used in neuroscience research, and known to have ionic transients when differentiated towards neural phenotype. In order to preserve high cell density, neural connectivity, and optimal cell adhesion, cells are differentiated to the point of prominent neurite structures, which is at the 48-hour time point after Retinoic Acid (RA) supplementation and serum deprivation of media. It was observed that the further stages of differentiation requiring additional neurotrophic factors render the cells to be more neuronal, but also require surface coatings and diminish cell density and neural connectivity. In addition to the neurite outgrowth, ionic transients are elicited with ultrasonic stimulation at the 48-hour differentiation time point, providing validity to the use of this differentiation stage to obtain data for our proof of concept study of *in vitro* neural effects of 1.05 GHz ultrasonics. Extensive control experiments were performed, to be detailed in future publications, and will make note of the use of Gentamicin as a Ca^{2+} ion channel blocker in this paper. The interface is constructed using a ~10 µm cell culture media contact layer, between the silicon transducer chip and the glass slide with neural cells. The undifferentiated SH-SY5Y neural cells are seeded at a density of 15,000 cells/cm^2 on a 22mm x 22mm glass slide in 0.25 mL of media and grown to 60-70% confluency for 48 hours. After this time point, RA differentiation media is introduced to obtain neurite like processes after 48 hours as shown in Figure 2b. The glass slide with the neural cells post-Ca^{2+} imaging agent loading is then inverted and placed onto 10 µL of Hank's Balanced Saline Solution with calcium and magnesium (HBSS) atop the silicon wafer housing the transducers. This creates a ~10 µm cell culture media layer, given volume and layer thickness calibration results. The interface is on the side of the silicon opposite and

exactly mirroring the transducers that are wire-bonded to a circuit board providing the coaxial input RF supply.

C. Optical Action Potential Detection

The Ca^{2+} transients, that occur on a much longer time scale than other action potential associated ionic transients, were optically imaged. This improves the signal to noise ratio (SNR) and simplifies the acquisition process. CalciFluor™ Fluo-8, AM is used with a standard, adapted protocol for loading conditions, as shown in Figure 2e [4,6]. Image acquisition time is kept under 120 seconds as to minimize the effects of photobleaching. A standard FITC filter set is used for excitation and emission filtering.

The OMAX A35140U camera is used for video acquisition, with a lower frame rate of 1 fps and 1 sec exposure time given observed SNR and optimal performance. The calcium transient induced by plausible action potentials are > 2 sec, and closer to 5 sec for differentiated SH-SY5Y cells. In order to confirm our results, similar data was acquired at ~50 fps with a Thorlabs 340M-USB fast frame rate camera. The data shown in the paper is from the OMAX A35140U. Brightfield images are obtained using AmScope MA-1000.

D. Ultrasonic Impedance Change Sensing

As shown in Figure 1, the theory of acoustic impedance influence on the transmission-reflection coefficient to ascertain the approximate acoustic power delivery to the neural cells. A pulsed RF signal at 1.03 GHz carrier frequency is used, with an amplitude from 3.1 to 7.5 V_{pp}, at a pulse repetition rate of 100 kHz, and a pulse width of 80 ns. The return signal from the transducer is amplified by a CRYSTEK 18 decibel small-signal amplifier and sampled at 10 GigaSamples/s by an 8-bit oscilloscope (Rohde and Schwarz RTO 1024). The amplitude of the first return is obtained through the oscilloscope measure function, and statistics are collected for > 5000 pulses. Liquids and gels with a thickness > 1mm are interfaced with the surface (contained in a temporarily adhered PDMS well) opposite the wire bonded transducer. Solids are pressurized against surface with ~15 kPa.

E. Data Processing

An image processing algorithm was implemented on MathWorks, MATLAB R2016a that allows for classification of regions of interest (ROI) pertaining to cells, background subtraction, quantification of transient activity, and graphical outputs of regional activation patterns in time scale data. The algorithm thresholds each frame of the video of data using a high and low threshold marker to prevent obtaining data from oversaturated regions or regions with no cells present. Thresholding is compared at regions in the beginning and the end of the time series, and background subtracted regions that retain a fluorescence ratio of 1 are classified as cells. The transducer and pad areas that are also imaged are excluded using this step. The threshold window size is ~20 x 20 μm^2, corresponding to a region slightly smaller than the cell to compensate for movement due to stimulus. Background subtraction is performed by taking a region that has both cells and background and this area is averaged and subtracted from both the initial baseline (no ultrasound stimulation) data and the during stimulation data. All data is obtained continuously through the camera. Each ROI is normalized to the 5 sec before stimulation time average of the respective ROI. The final result of $\frac{F-b}{F_0-b}$, where F is the fluorescence at any time point, F_0 is the fluorescence average at baseline, and b is the background average value is plotted. Figure 2c shows one frame of the raw data, and figure 2d shows the identified ROIs, with regional averages of activated regions mapped in figure 3a. Scale bars are excluded from Figure 2c,d as they are extracted directly from the image processing algorithm outputs.

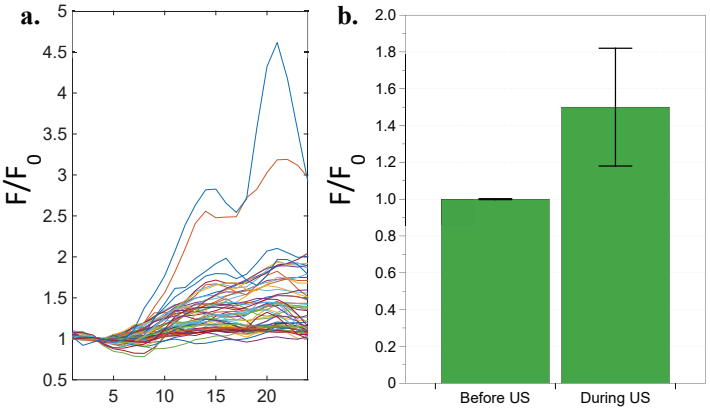

Fig. 3. a. Time course of regional average of activated differentiated SH-SY5Y with normalized and background subtracted fluorescence and 2 second smoothing window. Stimulus starts at 5s and lasts until end. b. Time point averages at 5s (Left), 20s (Right).

III. Results

A. 1.05 GHz Ultrasonic Stimulus

The data demonstrates the effects of stimulation using 1.05 GHz continuous wave ultrasound, with 5 sec of baseline recording and 20 sec of during stimulus recording. To further confirm that the baseline activity without ultrasonic stimulation is low, negligible activity of the cells during a separate observation with no ultrasonic stimulus over 120 sec was observed. Additionally, there is no significant activity of neurons with or without stimulation with the administration of Gentamicin, a known Ca^{2+} ion channel blocker [7]. Separate data suggests that the activity after ultrasonic stimulation is removed (post-stimulus) is preserved upwards of 60 sec within the in vitro network of neurons.

Figure 3a shows regional averages through time series data, with 57 identified activated regions, determined by before and during stimulation thresholding as described in the methods section. The during action potential normalized fluorescence intensity for Fluo-8 has been shown in the literature to be at an average of upwards of ~4 for cells loaded per protocol, however slightly lower values were observed with the methods presented in the previous section for a few plausible reasons [4,6]. As presented in much of the literature with *in vitro* calcium imaging agent loading, there is heterogeneity in the initial loaded fluorescence intensity. The camera has limitations in vertical resolution, intensity sensitivity, frame rate, and exposure time. The unconventional setup with a reflective silicon oxide surface contributes to noise and high background

values, leading to a potentially diminished peak signal magnitude. The administration of ultrasonics, especially at the GHz frequency range, on the activity of Fluo-8 and other similar calcium chelators is uncharacterized. Stimulus could contribute to the lower peak signal values. Regardless, the data is in range of literature values and demonstrates statistical significant increases in neural activity during ultrasonic stimulation. While here data is shown from one experiment with 57 ROIs, repeated trials, to be later published, validate the results further. Figure 3b shows the time series averages for two data points, one at the 5 second time point before ultrasound stimulus, and one at the 20 second time point during ultrasound stimulus. The during stimulus time point average of activated regions of normalized fluorescence is 1.5 ± 0.328. The pre-stimulus time point average is 1.0 ± 0.003. A significant difference with $p<0.01$ is observed. The plausible mechanisms of the observed stimulation include acoustic radiation force, acoustic streaming, heating, and electrical stimulation given the two-sided transducer design schema.

B. Dosimetry

The first echo-return signal is a function of the energy transmitted into the liquid, and is a measure of the delivered dose to tissue. The first echo signal was measured for various biologically relevant materials at the interface shown in Fig. 1. Using the return signal normalized to that from the air interface, a relative comparison of delivered dosage was obtained (Fig. 1). This is useful in applications where a precisely delivered ultrasound intensity is important for monitoring of *in situ* conditions of tissue for clinical and tissue engineering applications is necessitated.

Figure 4a and 4b shows that the voltage amplitude response is linear with pulse voltage, with almost linear input to output voltage relations, and significant differences among the varying return signal to transmitted signal ratio with $p<0.01$. The data is normalized to the 3.1 V amplitude input of the air interface. Among the different interface materials, the average first echo amplitude normalized to the respective air interface value for each input amplitude is graphed in Figure 4c. Statistically different return amplitudes for all the samples (tested at $\alpha = 0.01$) with the exception of the noted samples on the graph (* and **) are seen. The acoustic impedance is given by $Z = \rho c\left(1 - j\frac{\alpha\lambda}{2\pi}\right)$, where ρ is density, c is the wave propagation speed, α is attenuation, and λ is acoustic wavelength, all qualities in the medium. ρc is a good predictor of the effects for the samples shown. However, density can be challenging to predict for heterogeneous and dynamic materials. The interface material and the transducer surface may also have insubstantial contact or roughness. Propagation velocity could have frequency and wave number dependence through the dispersion relation and mode conversion.

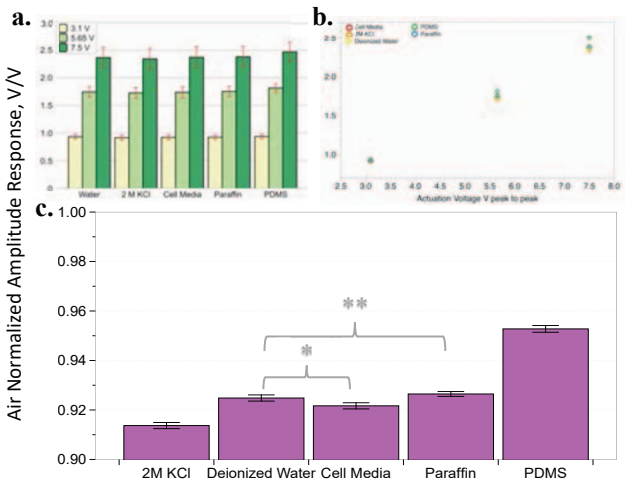

Fig 4. a. Echo return amplitudes for various interface medium with different input amplitudes. $p<0.01$ significant difference amongst different input amplitudes within one sample. b. Data from 4a plotted to show linear trend. c. Comparison of first echo amplitude for different samples, all samples show $p<0.01$ significant difference, except * and ** which show no significant difference at $\alpha = 0.01$. Standard error on error bars.

IV. Conclusions and Future Directions

This paper demonstrates one of the first studies of GHz ultrasonic stimulation of an in vitro neural preparation, with the potential for localization of stimulus at the cellular level. Analysis of pulse-echo signals provides a pathway towards dosage regulation. Dynamic impedance monitoring with high resolution is in progress. Future research will provide details into controls and computational models that elucidate the mechanism of neural stimulation.

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