The Influence of Therapeutic Ultrasound Stimulation on Schwann Cell Plasticity for Peripheral Nerve Regeneration

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Abstract

Each year, over 67,000 of patients in the United States suffer from peripheral nervous system (PNS) damage, which can result from inherited disorders, diseases, and traumatic injuries. Damaged peripheral nerves possess a limited amount of regenerative potential due to the presence of Schwann cells within the PNS, which can transdifferentiate into a regenerative cell type to guide axons across nerve lesions and support nerve recovery. Because Schwan cells have been shown to play a critical role in the regeneration of peripheral nerves, finding efficient methods to modulate the phenotype and behavior of Schwann cells is an important strategy for developing clinical solutions for nerve repair. Application of low intensity ultrasound stimulation (US) to cells has gained focus as a non-invasive method for controlling cell behavior. However, the exact impact of US on Schwann cells is not well defined. Additionally, the effects of applying US in tandem with other microenvironmental cues such as electric stimulation has not been investigated. In this study, we analyzed the effect of US on Schwann cells, as well as Schwann cells on a piezoelectric PVDF-TrFE scaffold, in order to deter the effect of US on Schwann cell regenerative behavior and how this effect is mediated by the introduction of a piezoelectric substrate capable of electric stimulation to the cells. Our preliminary results indicate that US did not have a significant effect on proliferation and elongation when cells were cultured on glass coverslips, but US did significantly increase cell elongation when cells were cultured on PVDF-TrFE. Our results indicate that US may have a positive effect on Schwann cell regenerative behavior, and that this effect may be enhanced when used in combination with a piezoelectric scaffold. The development of therapeutic US treatments can ultimately lead to the production of safer and more effective peripheral nerve injury therapies.

1. Introduction

Peripheral nerve damage is a significant healthcare concern, with over 67,000 Americans suffering from peripheral nerve system damage each year (Taylor, Rice, & Dillingham, 2008). Unlike in the central nervous system, damaged peripheral nerves possess a limited amount of regenerative potential due to the presence of Schwann cells within the peripheral nerve system, which can transdifferentiate into a regenerative cell type to support nerve recovery (Orkwis, et al., 2020). However, when injuries are

too severe, medical interventions such as nerve grafts and nerve guidance conduits may be necessary to achieve functional recovery.

Because Schwann cell reprogramming plays a critical role in the peripheral nerve regeneration process, when developing tissue engineering technologies to enhance nerve repair it is critical that researchers understand how Schwann cells react to their environment. Previous literature suggests that ultrasound waves ranging from 0 to 750 mW/cm^2 can be used to induce regenerative behaviors in biological tissues, and support axon regeneration for nerve repair (Acheta, Stephens, Belin, & Poitelon, 2022). However, the exact impact of US on Schwan cells is not well defined. Additionally, the effects of applying US in tandem with other microenvironmental cues such as electrical stimulation has not been thoroughly examined. The main objectives of our research were to investigate the effect of US stimulation on Schwann cell regenerative behavior, specifically examining cell proliferation and elongation, as well as investigate the interplay between US stimulation and the piezoelectric response of a PVDF-TrFE scaffold for promoting a regenerative Schwann cell phenotype.

Ultrasound stimulation was hypothesized to provide two key functions. The first function was to induce a regenerative phenotype in cells that are important to the regeneration of nerves, specifically Schwann cells and fibroblasts. The second function was to activate the piezoelectric PVDF through the sound waves emitted which will cause the fibers to produce the electrical response. This method is a feasible tool that can be used in clinical settings that does not require any invasive techniques. If these PVFD-TrFE scaffolds were implanted in a living organism as a conduit for a nerve repair remedy, this technique may provide a non-invasive solution for activating the piezoelectric properties of the material. Our hypothesis is that the ultrasound stimulation on PVDF-TrFE material seeded in cell culture will promote a favorable regenerative environment for cells and subsequently impact cell viability and cell spreading.

Polyvinylidene fluoride-trifluoro ethylene (PVDF-TrFE) electrospun fibrous scaffolds were used as the piezoelectric substrate in this experiment due to previous work illustrating it’s biocompatibility and suitability for nerve repair applications (Orkwis, et al., 2020). This material can be deposited in an aligned formation which can function as a piezoelectric material. Previous work suggests that when a mechanical deformation is applied to the piezoelectric material, the electric current generated promotes a favorable regenerative environment for nerves (Orkwis, et al., 2020).

The first aim of the project is to examine the effect of various ultrasound waves on Schwann cells. In our research, cells were examined after different time points which include 36 hours to 40 hours. The phenotype markers that were analyzed included proliferation, alignment and cell elongation. Proliferation was analyzed using MTT assays for analyzing the cell’s metabolic activity. This technique is based on the NAD(P)H-dependent cellular oxidoreductase enzymes which indicate proliferation, cytotoxicity, and cell viability. Cell elongation was analyzed using immunofluorescent staining with monoclonal antibodies, anti-rabbit and rhodamine phalloidin. Cell

alignment was quantified using the full width half maximum of the radial sums obtained from FTT images of samples.

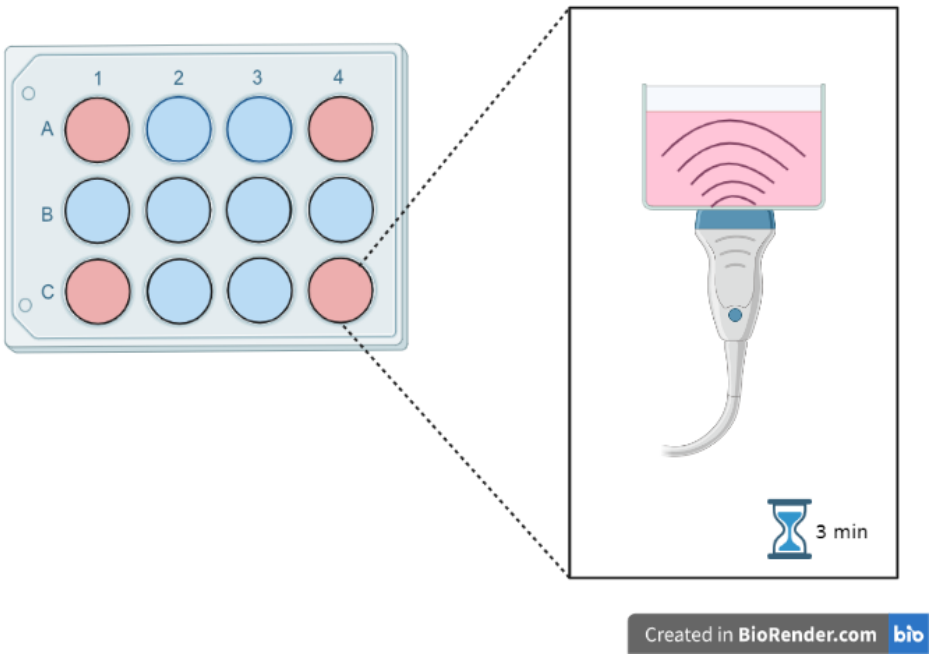

Figure 1. *Ultrasound application setup.*

2. Experimental Methods

2.1. PVDF-TrFE Fabrication and Preparation

PVDF-TrFE scaffolds were prepared via electrospinning by one of the graduate students in the lab prior to beginning the experiment. Detailed descriptions of scaffold fabrication are summarized elsewhere (Orkwis 2020). Briefly, an aligned electrospinning configuration (FLuidnatek LE-50) was used to fabricate the PVDF-TrFE scaffolds. The 20% (w/v) PVDF-TrFE (70/30) (PolyKTechnologies, State College, PA) was dissolved in a mixture of dimethylformamide and acetone (6:4). The material was then loaded into a syringe using a 20-gauge needle and ejected at a flow rate of 1 mL h−1. The collector was wrapped with a conductive polymer liner (McMaster-Carr) and set 10 cm from the needle tip.The aligned fibers were created using a rotation speed of 2000 rpm.[80][1]

After obtaining the electro spun scaffold, the polymer was cut into 2 cm by 2 cm segments. The segmented PVDF-TrFE scaffolds were rinsed with 70% ethanol then twice with phosphate buffered saline (PBS). The scaffolds were carefully placed on UV-sterilized 18 mm coverslips (Fischer) and placed in a 12-well plate. The entire plate

containing the scaffolds was then placed under UV light for 7 minutes for further sterilization.

2.2. Cell Culture

High glucose Dulbecco's modified eagle medium (DMEM) (SH30022) (GE Healthcare) supplemented with 10% fetal bovine serum (Thermo Fisher) was used to culture RT4-D6P2T Schwann cells (ATCC). Cells were grown roughly 72 hours before passing into 12-well plates for experimental conditions using phosphate buffered saline (PBS) (Thermo Fisher) for washing the cells before using Trypsin in versine solution to remove cells from plate. Experimental conditions include transferring cells onto a sterilized coverslip or onto a sterilized coverslip with PVDF-TrFE scaffold and O-ring. A separate 12-well plate was used for each condition, with four wells per condition. For the ultrasound culture conditions, the four far corners of the 12-well plate were used to reduce interference of the ultrasound waves between wells.

2.3. Ultrasound Application

Ultrasound was administered to cells using US Pro 2000TM 2nd Edition Portable Ultrasound unit. This device transmits pulsed high frequency (1MHz) sound waves through the sound head probe. The three intensity settings on the device are summarized in Table 1. The US application was done in a sterile setting under the biological safety cabinet. A small pea-sized portion of the transmission gel was applied to the bottom of the culture well plate under each sample well. Then the probe was firmly placed under each well for three minutes to ensure optimal contact between the plate and the probe head. The load detection system within the device will cause the device to stop treatment and the time indicator light to flash when there is insufficient contact between the probe head and the treatment area. After treatment was applied to each cell culture well, the bottom of the plate was wiped clean from the US gel and the plates were placed back in the incubator. Table 2. outlines each experimental run including the cell culture time before US application, US application intensity and time, and incubation time after US.

Two sets of experiments were run using these US stimulation conditions: for the first set of experiments, cells were stimulated once with US 16 hours after seeding, and then were left in the incubator for an additional 24 hours. For the second set of experiments, cells were stimulated once with US 24 hours after seeding, and then were left in the incubator for an additional 12 hours. For both sets of experiments, the control group consisted of cells that were cultured either on coverslips or PVDF-TrFE scaffolds without any US stimulation. The experimental groups included a 0.08 W/cm^2 US group, a 0.8 W/cm^2 US group, and a 1.6 W/cm^2 US group each on both coverslips and PvDF-TrFE scaffolds. Not all experiments included a 1.6 W/cm^2 US group.

***Table 1**. Ultrasound settings.*

Program	Modulation Duty Factor	Wave Character	Output Power	Effective Intensity
L	5%	Low	0.32± 20%	0.08 W/cm^2 ± 20%
M	50%	Medium	3.20± 20%	0.80 W/ cm^2 ± 20%
H	100%	High	6.4± 20%	1.60 W/ cm^2 ± 20%

2.4. Proliferation Assay

The cellular response to the US stimulation was analyzed 24 hours after stimulation or 12 hours after stimulation using Invitrogen™ CyQUANT™ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay kit (CyQUANT MTT Cell Viability Assay, 2022). This assay measures metabolic activity of cells by quantifying the absorbance at 540 nanometers using a multi-well spectrophotometer. The theory of this assay is based on the chemistry of the MTT solution which changes from a yellow to purple color when NAD(P)H is reduced by the oxidoreductase enzymes of viable cells. This is a result of the formation of formazan from the reduced tetrazolium MTT salt (CyQUANT MTT Cell Viability Assay, 2022).

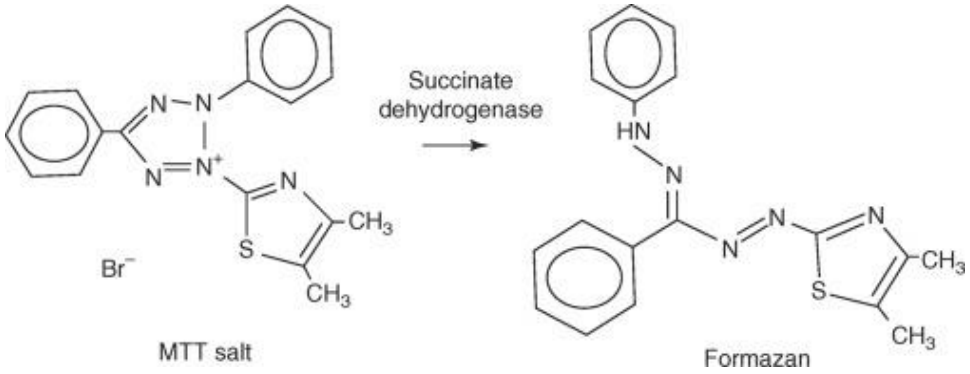

***Figure 2**. Reduction of MTT salt to purpole formazan (CyQUANT MTT Cell Viability Assay, 2022).*

Dimethyl sulfoxide (DMSO) was added to solubilize and release the formazan for the analysis. The result of the fluorescent intensity of the pigment indicates the viability and the proliferation of the cells in the sample. The solution incubated for ten minutes at 37 degrees Celsius then 75 microliter aliquots were transferred into a 96-well plate for analysis by the microplate reader.

2.5. Elongation Assay

An elongation quantification assay was also performed either 24 hours post-US stimulation or 12 hours post-US stimulation. An Immunofluorescent staining protocol was performed for each sample coverslip including negative controls, controls, and

PVDF-TrFE scaffold samples. The cells were fixed using diluted formaldehyde then permeabilized with 0.1% Triton-X. A 1° Ab FNR457 solution was added to the coverslips then incubated at 37 degrees Celsius for thirty minutes. Then the samples were rinsed thrice with phosphate buffered saline (PBS) before the addition of 2° Ab monoclonal antibodies, anti-rabbit 488 and rhodamine phalloidin in order to obtain F-actin labeled images. Cells incubated a second time at 37 degrees Celsius for thirty minutes then rinsed thrice with phosphate buffered saline (PBS). The coverslips were mounted on glass slides with DAPI then sealed with clear nail polish (Sally Hansen Clear Coat).

The cells were imaged using NIS Elements software and fluorescence filters on Nikon Eclipse Ti2 Inverted Microscope at 40x magnification with a Nikon DS-Qi2 camera. Images were obtained for each slide using a randomized feature which captured 10 images within an 8-millimeter radius for each slide. The automated detection feature was utilized for each image to select cells for quantifications via the built-in software's elongation calculation (Equation 1) (NIS Elements). Roughly 15 cells total were quantified per slide.

$$Elongation = \frac{MaxFeret}{MinFeret} \qquad \textit{Eq 1.}$$

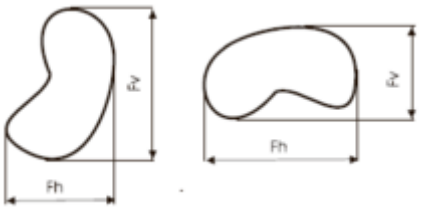

Figure 3. *Example of Feret Dimensions (Feret Diameter, 2022).*

2.6. Alignment Quantification

To quantify cell cytoskeleton alignment, we performed fast Fourier transformations (FFT) on F-actin labeled representative images of our samples, then used oval profiles generated in ImageJ from the FFT images to obtain the radial sums from 0 to 180 degrees. We then obtained the full width at half maximum (FWHM) values and used these to quantify cell alignment.

2.7. Statistical Analysis

Data were reported as mean values ± 95% confidence interval using a t-distribution for proliferation data, and mean values ± 95% confidence interval using a normal distribution were reported for elongation data. The statistical significance of data was determined based on confidence intervals of each condition. Outliers in the data were identified and removed using an interquartile range (IQR).

3. Results and Discussion

3.1. Proliferation

The results for both US conditions (stimulation after 16 h culture and stimulation after 24 h culture) are summarized in Figures 4 and 5 respectively. In summary, there was no statistically significant difference between US conditions regardless of the substrate used. It is noted that when US was applied 16 h after seeding (with a total culture time of 40 hours), cell proliferation was significantly higher on PVDF compared to coverslips. However, this difference was not observed when US was applied 24 h after seeding (with a total culture time of only 36 hours). This may be because the cells were given less time in total to grow, meaning that the differences between coverslip and PVDF samples was not yet distinguishable at the time the assay was performed.

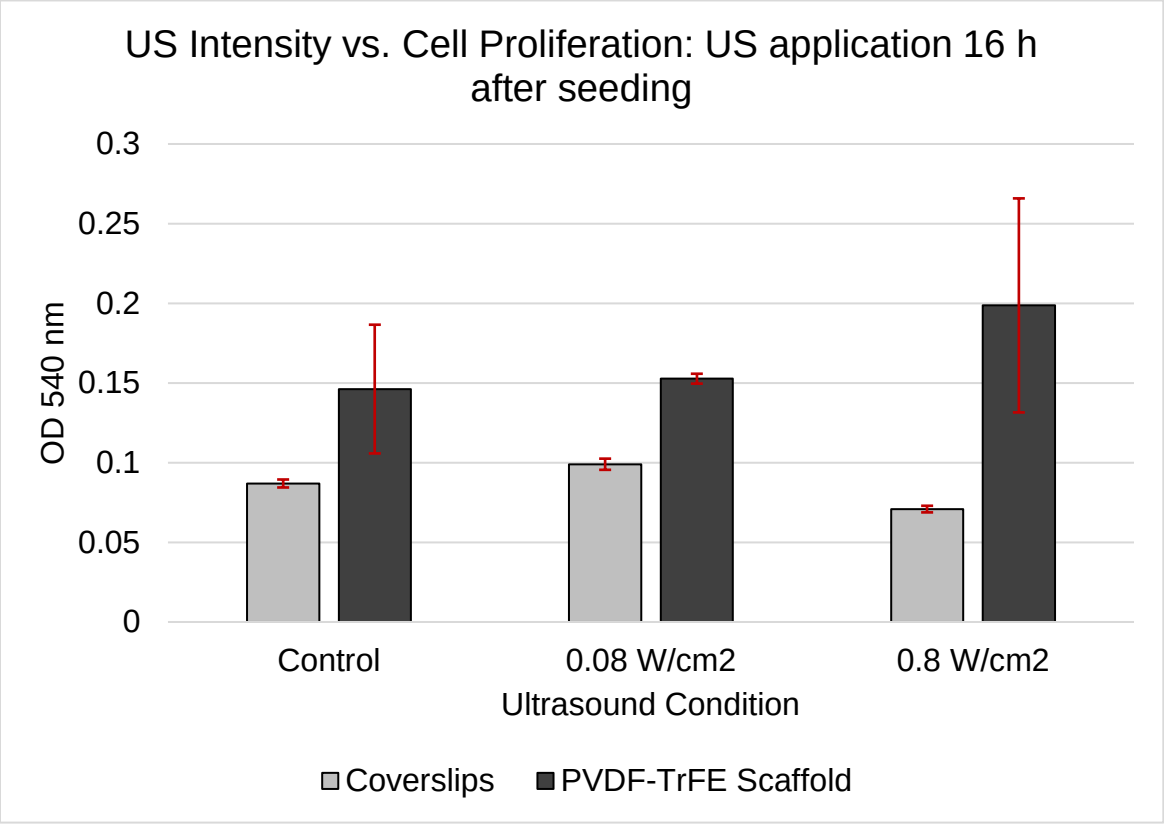

***Figure 44**. Proliferation on coverslips and PVDF-TrFE scaffold (US applied 16 h after seeding, total culture time 40 h).*

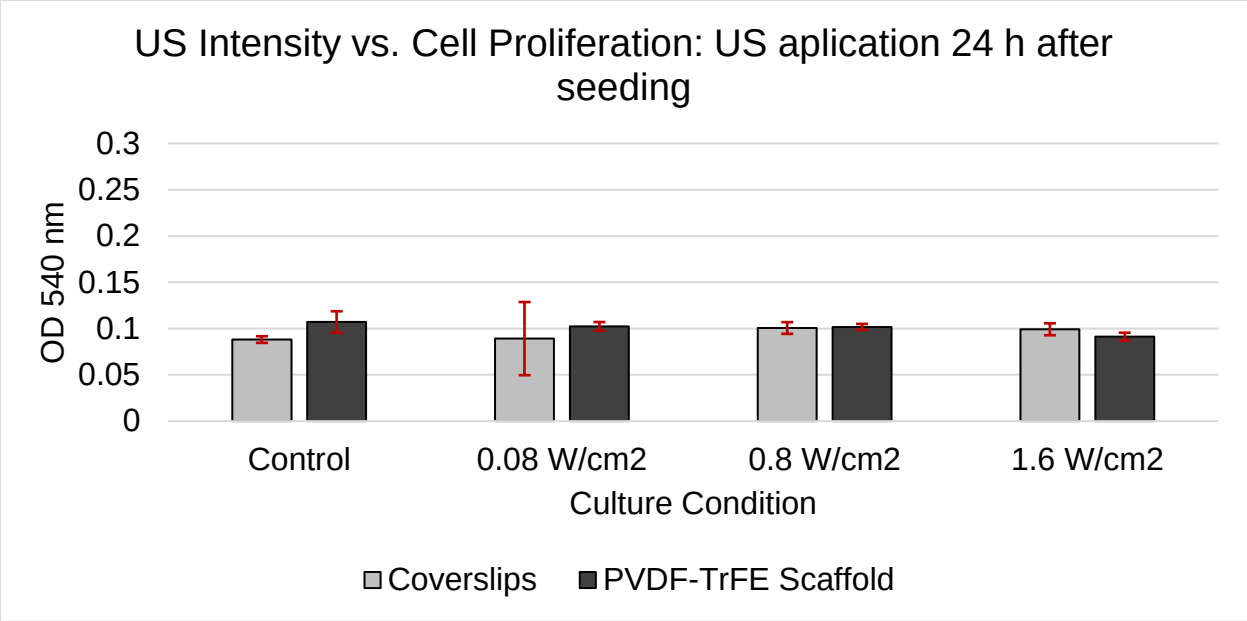

***Figure 55**. Proliferation on coverslips and PVDF-TrFE scaffold (US applied 24 h after seeding, total culture time 36 h).*

3.2. Alignment

Based on our results, there was no statistically significant difference in alignment between all conditions, even when comparing between coverslip and PVDF-TrFE scaffold conditions. This is not consistent with the established literature showing a clear relationship between scaffold alignment and cell alignment (Orkwis, et al., 2020).

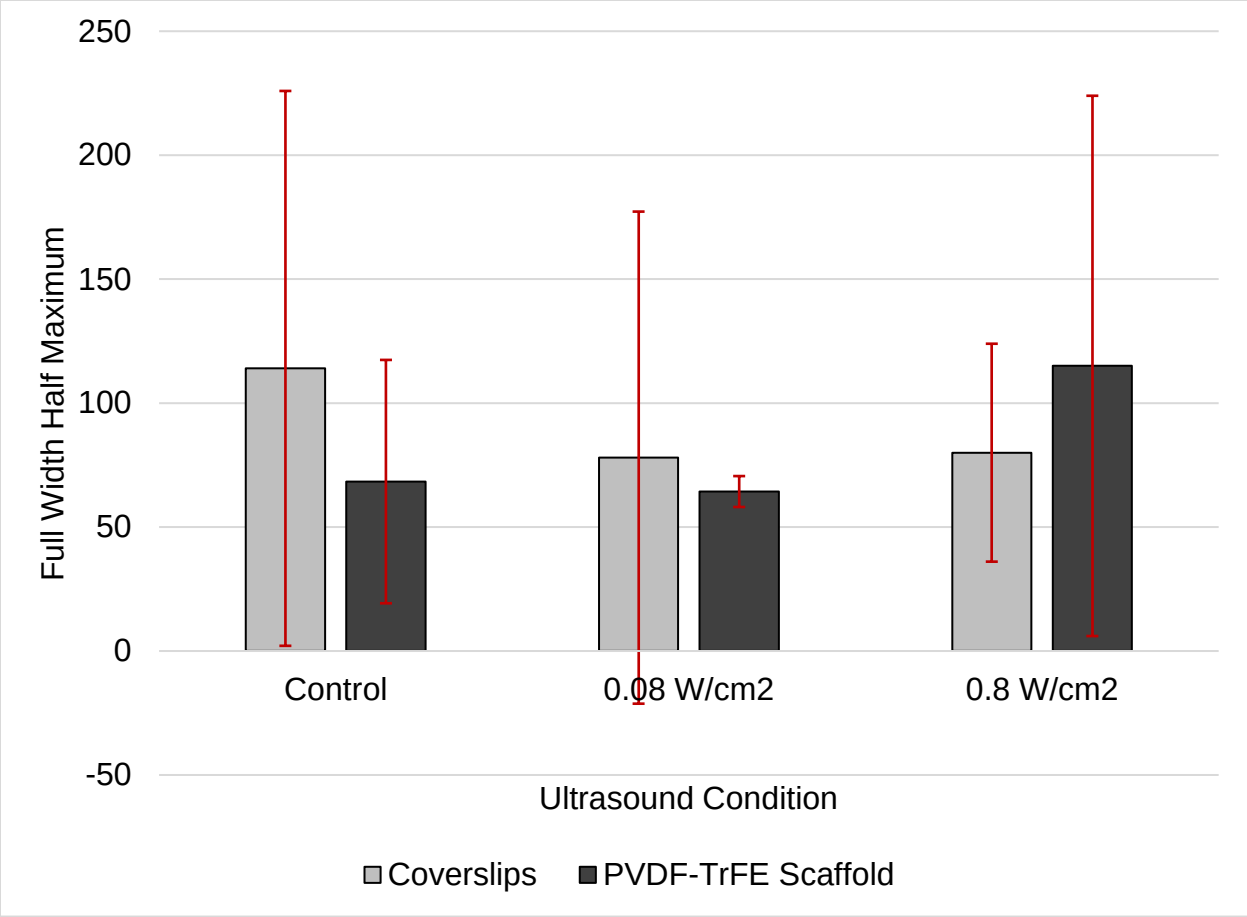

***Figure 66**. Full width half maximum values for cells cultured on coverslips and PVDF-TrFE scaffold (US applied 24 h after seeding, total culture time 36 h).*

3.3. Elongation

Ultrasound stimulation applied 16 h after cell seeding did not significantly increase cell elongation when cells were cultured on glass coverslips (Figures). In fact, for our second set of data where we applied US 24 h after cell seeding on coverslips, there was a slight decrease in cell elongation for cells stimulated with 0.08 W/cm^2 US. However, when cells were cultured on PVDF-TrFE scaffolds, elongation was significantly increased in both the 0.08 W/cm^2 US and 0.8 W/cm^2 US samples compared to the control samples (Figure 8-10). There was no statistically significant difference in cell elongation between the 0.08 W/cm^2 US and 0.08 W/cm^2 US samples on PVDF-TrFE scaffolds.

Coverslips PVDF-TrFE Scaffold

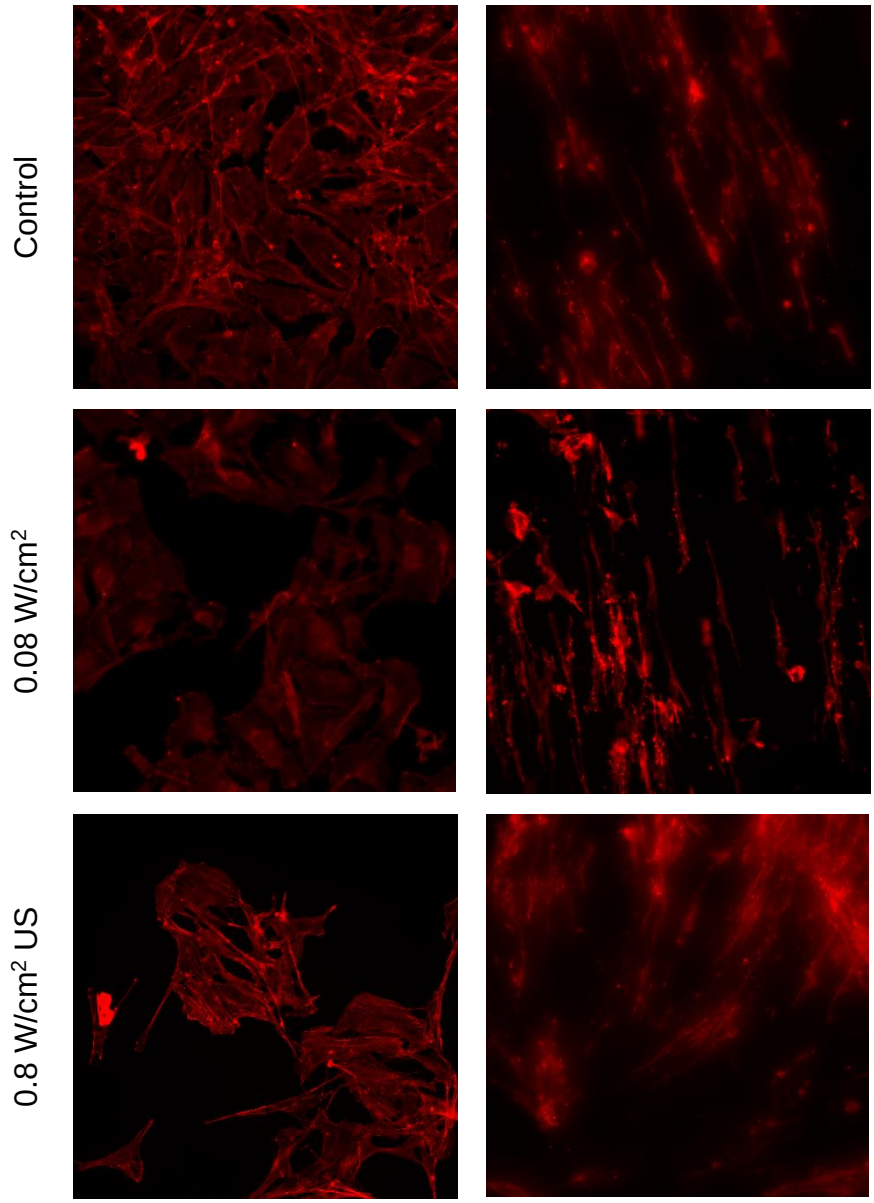

***Figure 77**. Representative confocal microscopy cells seeded on coverslips and PVDF-TrFE scaffold (US applied 16 h after seeding, total culture time 40 h).*

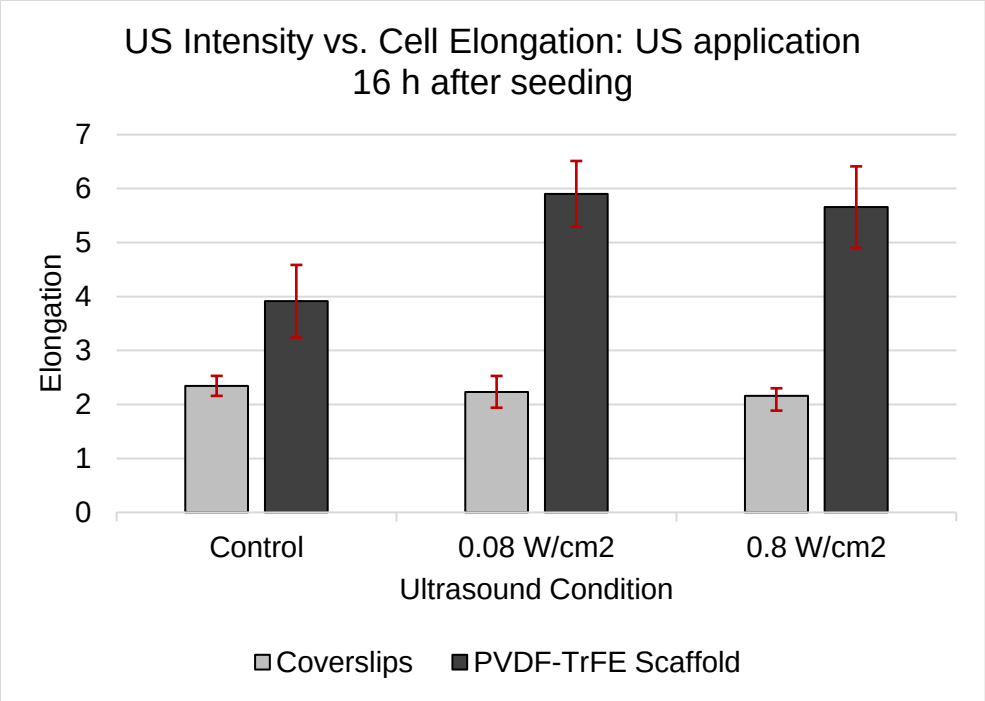

Figure 88. *Elongation on coverslips and PVDF-TrFE scaffold (US applied 16 h after seeding, total culture time 40 h).*

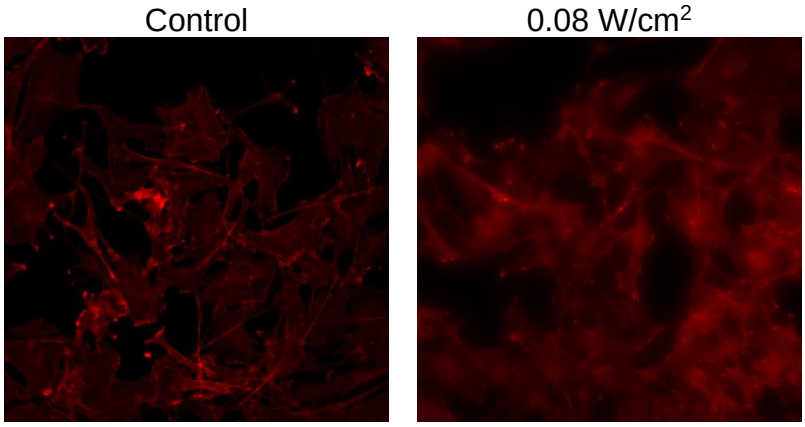

Figure 99. *Representative confocal images showing cells seeded on coverslips (US applied 24 h after seeding, total culture time 36 h).*

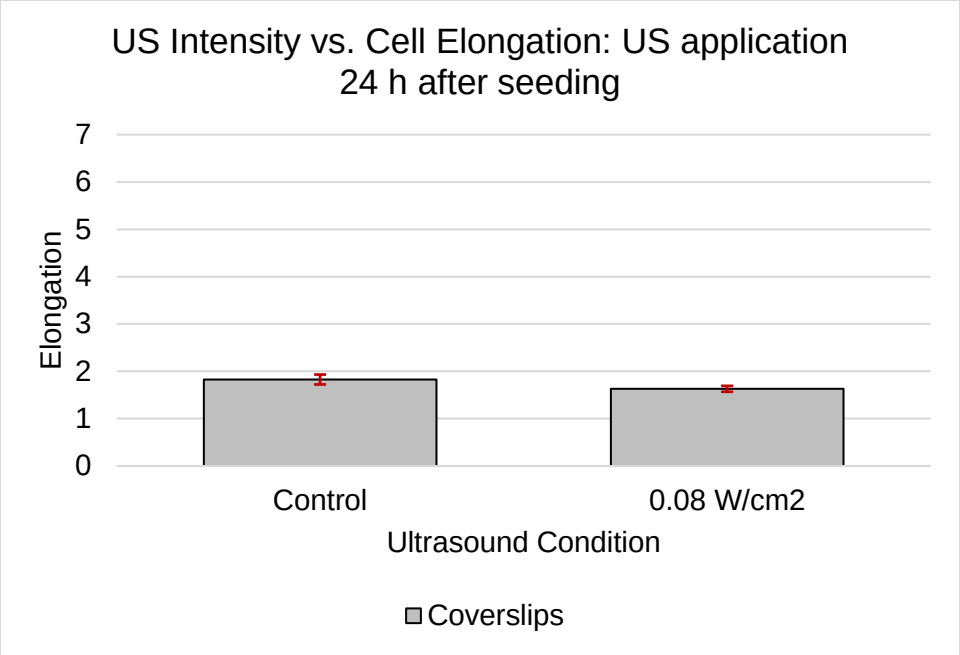

Figure 1010. *Elongation on coverslips (US applied 24 h after seeding, total culture time 36 h).*

4. Discussion

The Results from our proliferation, elongation, and alignment assays are summarized in Table 2.

Table 2*. Results Summary.*

Therapeutic Regiment								**Outcomes**	
Transducer	**Parameters**	**Culture time pre US**	**Application**	**Duration**	**Culture time post US**	**Number**	**Substrate**	**Proliferation**	**Elongation**
Bottom of 12 well plate	0.08 W/cm2, 1 MHz, 5% pulsed	16 h	Once	3 min	24 h	41,149 cells/well	coverslip	n.d.	no statistically significant difference
Bottom of 12 well plate	0.08 W/cm2, 1 MHz, 5% pulsed	16 h	Once	3 min	24 h	41,140 cells/well	PVDF	n.d.	Increased elongation

Bottom of 12 well plate	0.8 W/cm2, 1MHz, 50% pulsed	16 h	Once	3 min	24 h	41,140 cells/well	coverslip	no statistically significant difference	no statistically significant difference
Bottom of 12 well plate	0.8 W/cm2, 1MHz, 50% pulsed	16 h	Once	3 min	24 h	41,140 cells/well	PVDF	no statistically significant difference	Increased Elongation
Bottom of 12 well plate	0.08 W/cm2, 1 MHz, 5% pulsed	24 h	Once	3 min	12 h	40,000 cells/well	coverslip	no statistically significant difference	Decreased elongation
Bottom of 12 well plate	0.08 W/cm2, 1 MHz, 5% pulsed	24 h	Once	3 min	12 h	40,000 cells/well	PVDF	No statistically significant different	n.d.
Bottom of 12 well plate	0.8 W/cm2, 1 MHz, 5% pulsed	24 h	Once	3 min	12 h	40,000 cells/well	coverslip	No statistically significant different	n.d.
Bottom of 12 well plate	0.8 W/cm2, 1 MHz, 5% pulsed	24 h	Once	3 min	12 h	40,000 cells/well	PVDF	No statistically significant different	n.d.
Bottom of 12 well plate	1.6 W/cm2, 1 MHz, continuous	24 h	Once	3 min	12 h	40,000 cells/well	coverslip	No statistically significant difference	n.d.
Bottom of 12 well plate	1.6 W/cm2, 1 MHz, continuous	24 h	Once	3 min	12 h	40,000 cells/well	PVDF	No statistically significant difference	n.d.

While our alignment results were not statistically significant, Schwann cells cultured with 0.08 W/cm^2 US on PVDF-TrFE did exhibit the highest average cell alignment of all conditions (Figure 4), while cells cultured on coverslips without US stimulation exhibited the lowest average alignment of all conditions (Figure 4). These preliminary results seem to point to a possible relationship between low (0.08 W/Cm2) ultrasound stimulation and cell alignment, but there were significant limitations to the preliminary data collected, namely that average FWHM values were calculated with only 3 images per condition, and there was a high degree of variability between images for each condition. Increasing image sample size would be beneficial to yield more conclusive results.

Our proliferation results indicate that US did not significantly affect cell proliferation. Previous literature has shown low intensity US (≤1 W/cm2) may be used to induce regenerative effects in biological tissues (Acheta, Stephens, Belin, & Poitelon, 2022), while our results indicate that US did not influence cell proliferation or elongation unless cells were also cultured on PVDF-TrFE scaffolds. One possible explanation for this difference may be that we only applied US once to our cells, while other studies have stimulated cells multiple times over a longer period.

Our elongation results indicate that US may be successfully used to enhance Schwann cell elongation when used in combination with a piezoelectric substrate. It has previously been established that PVDF-TrFE releases an electric charge after undergoing mechanical deformations, and that electrical stimulation has been used to modulate Schwann cell behavior (Orkwis, et al., 2020). Based on this previous information, our results seem to indicate that the vibrations caused by the US waves may have caused mechanical deformations in the scaffold. These mechanical deformations may have elicited a piezoelectric response, thus stimulating the cells and promoting elongation. Further quantification of the piezoelectric response generated by different US intensities and quantification of the relationship between piezoelectric response and Schwann cell elongation would be useful for future investigations.

While the elongation results for cell cultured on PVDF-TrFE scaffolds are promising, there were several limitations to the methods used in this study that call for further investigation. A major limitation for all experiments was that the US was applied using a handheld device which was held up to the bottom of the culture plate. While this method has been used in other studies (Acheta, Stephens, Belin, & Poitelon, 2022), a significant drawback to this method is that the US must pass through the glass of the culture plate before reaching the cells. This can result in the US waves bouncing off the glass wells, making it difficult to accurately control the level of US stimulation each sample received. Additionally, the US handheld device was not designed for application on plastic culture plates. Because of this, it would frequently shut off for several seconds during US applications due to overheating, so some samples likely did not receive the full three minutes of US stimulation that was expected.

Another limitation to this experiment was that we did not stain for specific protein expressions that might help us prove whether changes in cell proliferation and elongation because of US stimulation were in fact indicative of a regenerative cell phenotype. Further experiments may examine expression of regenerative markers such as c-Jun to further investigate how US stimulation affects Schwann cell reprogramming.

5. Conclusion

The results of this preliminary research suggest that ultrasound stimulation was successfully used to promote Schwann cells elongation when used in combination with a piezoelectric substrate. Future work should include further quantification of the

piezoelectric response generative by US stimulation, including trials with multiple ultrasound exposures and ultrasound via water bath. Further investigation of the relationship between piezoelectric response and Schwann cell phenotype should also be further studied in accordance with other assessments such as alignment, cell migration, and gene expression to support this research.

6. References

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7. Supplementary Material

Please see attached file “US Project Supplementary Material.”