Effects of varied stimulation parameters on adipose-derived stem cell response to low-level electrical fields (Supplemental)

Nora Hlavac[1+], Deanna Bousalis[1+], Raffae Ahmad[1], Emily Pallack[1], Angelique Vela[2], Yuan Li[1], Sahba Mobini[1,3], Erin Patrick[2], Christine Schmidt[1]

[1]J. Crayton Pruitt Department of Biomedical Engineering, University of Florida

[2]Department of Electrical and Computer Engineering, University of Florida

[3]Instituto de Micro y Nanotecnología, IMN- CNM, CSIC (CEI UAM+CSIC), Tres Cantos, Madrid, Spain

[+]denotes equal contribution by authors

SUPPLEMENTAL DATA

a. Bioreactor materials and manufacture for cell compatibility

The DC bioreactor design used in this study was created with the goal of building a customizable and affordable system in which cells could be exposed to electrical current via their growth substrate, as the substrate is in contact with the most cell area. Indium tin oxide (ITO) was an ideal choice for the conductive substrate given that cells can grow directly on top of it (without a need for coating) and it has low impedance. For the culture well design, we used 3D printed nylon because it is stable, non-toxic, and easy to adapt for specific applications. To affix wells to the ITO substrate, we used silicone glue rated for aquarium use as this provided a nontoxic material that could withstand being submerged into fluid for multiple days at 37°C. Adipose-derived stem cells (ASCs) were cultured in the assembled bioreactors under similar conditions as were used for electrical stimulation (ES) studies (but were not exposed to any ES). ASCs were seeded in bioreactor wells at $7.5x10^4$ cells/well. After allowing cells to adhere overnight, media was switched to StemPro and cultures were maintained for 48 or 72 hours. Next, samples were processed for Live/Dead staining using calcein AM (Thermo Fisher L3224) and Hoechst (Thermo Fisher 62249). Stain was incubated on cells for 45 minutes at 37°C and then washed off with PBS. Tiled fluorescence images were obtained on a Zeiss Axio Observer microscope and processed for total cells (Hoescht) and live cells (calcein AM) using ImageJ and MATLAB custom scripts. **Figure S1** shows that cells were evenly spreading and >85% alive after 48 or 72 hours in the ITO bioreactor wells.

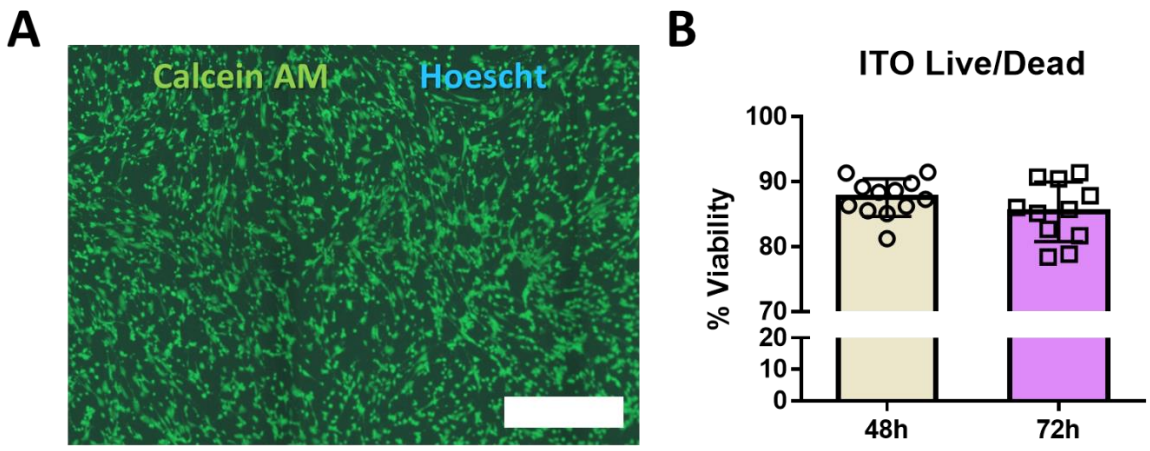


Figure S1*.ASC viability in the ITO wells used for the DC bioreactor. (A) 5X tiled images were obtained to determine viability of ASCs stained with calcein AM (live) and Hoescht (all). Scalebar is 1000 µm. (B) Percent viability after 48 or 72 hours cultured in StemPro secretome media. Data are mean ±std, n=11-12/group across 3 biological replicates*

b. Bioreactor electrochemical impedance and equivalent circuit modeling (AC bioreactor)

Multiple AC bioreactor wells were characterized via electrochemical impedance spectroscopy with a potentiostat and frequency response analyzer (Metrohm Autolab). The impedance calculations were performed at open circuit potential and with a two-terminal set-up in which one bioreactor electrode was attached to the working/sense potentiostat input and the other to the counter/ground input. A sinusoidal voltage of 10 mV was applied over a frequency range of 10 Hz-100 kHz. The electrolyte media was 3 mL of that used for ASC electrical stimulation (StemPro MSC SFM XenoFree complete medium, Thermo Fisher Scientific A1067501). The spectra with StemPro media at 37°C for frequencies between 1 and 100,000 Hz are shown in **Figure S2A (n=4)**.

The impedance spectra were then fit to an equivalent circuit matching the model for a blocking (i.e., non-reactive) system, which can be represented by a constant phase element (CPE) in series with an electrolyte resistance (R_e). The CPE is an element whose impedance is given by

$$Z_{CPE} = \frac{1}{(j\omega)^{\alpha}Q} \qquad [Eqn\ 1],$$

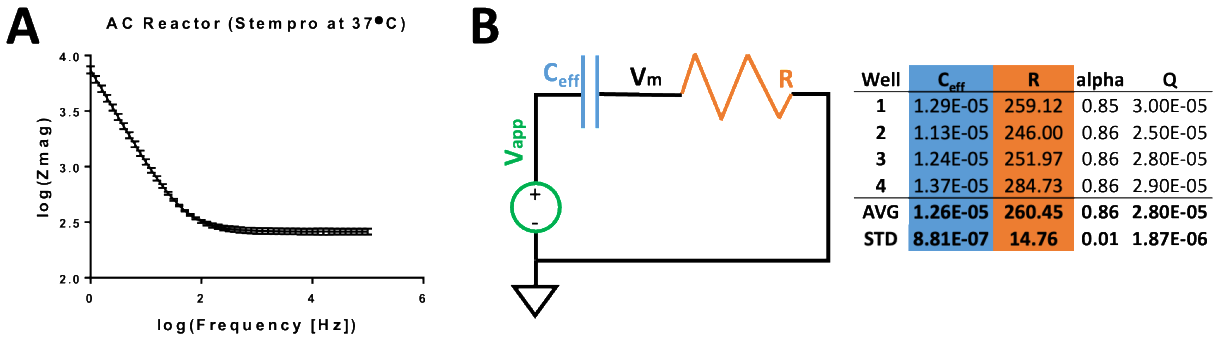
where ω is the angular frequency, and α (a constant between 0 and 1) and Q (with units $F/s^{(1-\alpha)}$) are parameters of the CPE[10]. The CPE represents the capacitive nature of the electrode/electrolyte interface that shows frequency dispersion and cannot be modeled as a pure capacitor.

To analyze the effect of the capacitive nature of the electrode/electrolyte with respect to a voltage-controlled stimulus, we use transient circuit analysis and an effective interfacial capacitance value. We use the CPE model developed by Brug et al. for a surface distribution of time constants (potentially resulting from surface heterogeneities) to calculate an effective double layer capacitance for the blocking electrode system[3]. The equation used for the effective capacitance is

$$C_{eff} = Q^{1/\alpha} {R_e}^{(1-\alpha)/\alpha} \qquad [Eqn\ 2]^{10}.$$

Replacing the CPE element with an ideal capacitor of value equal to C_{eff}, we were able to model the voltage across the resistive media (R_e) using LTSpice (Analog Devices), a standard circuit simulator, for various stimulus waveforms.

Using the impedance spectra from **Figure S2A**, the equivalent circuit model was developed for the AC bioreactor to estimate theoretical electrical field strengths for 1 Hz, 20 Hz, and 1000 Hz square pulse waves as a function of the capacitance model described above (**Figure S2B**). These results were then employed to determine the difference in applied versus actual electrical field strengths in the bioreactor (Figure 2).



Well	C_{eff}	R	alpha	Q
1	1.29E-05	259.12	0.85	3.00E-05
2	1.13E-05	246.00	0.86	2.50E-05
3	1.24E-05	251.97	0.86	2.80E-05
4	1.37E-05	284.73	0.86	2.90E-05
AVG	**1.26E-05**	**260.45**	**0.86**	**2.80E-05**
STD	**8.81E-07**	**14.76**	**0.01**	**1.87E-06**

***Figure S2**. AC Bioreactor impedance characterization and modeling. (A) Impedance spectra for 4 individual wells in the AC bioreactor. (B) Equivalent circuit model for the AC bioreactor and fitted parameters for model to generate simulated electrical fields.*

c. Secretome media selection

In the literature, secretome media varies based on the cell type, as well as the final application of the cells and their secretome. For example, researchers commonly use DMEM with 10% FBS in their electrical stimulation experiments[8,9,14,15] when their goal is to simply observe the effect of ES on the cells. Depending on the application of the secretome, researchers sometimes use more specific media to prime the cells towards a certain phenotype which would increase its secretion of particular growth factors. For example, Claycomb medium was used for myocardial phenotype[12] or StemPro Osteocyte and StemPro Adipocyte was used for osteogenic and adipogenic differentiation [2] in the literature, respectively. Another study used MesenPro RS medium when looking at the effect of ES on ASC differentiation, to ensure that ASCs remained in an undifferentiated state until introduction of electrical stimuli[5]. Researchers working with the secreted products of cells generally use serum-free medium during stimulation[1] to prevent serum from interfering with subsequent protein composition characterization[13]. To determine which media would be more suitable for supporting ASC viability and health during electrical stimulation, we conducted a metabolic analysis of ASCs cultured in either DMEM-F12 or StemPro MSC SFM XenoFree medium. StemPro, although serum free, contains a growth factor supplement that was hypothesized to potentially better support cells during ES. Further, it has been utilized before by other researchers seeking to optimize xeno- and serum-free culture conditions[11,13,16]. After 3 days in culture without electrical stimulation, StemPro promoted significantly higher metabolic activity than DMEM-F12 (**Figure S3A**), as measured by a standard AlamarBlue incubation (Thermo Fisher DAL1100). **Figure S3B** shows that ASCs under electrical stimulation (1 hour x 3 days, 20 mV/mm) had significant reduction in metabolic activity when

cultured in DMEM-F12 but not in StemPro. Thus, StemPro was used in all subsequent assessments.

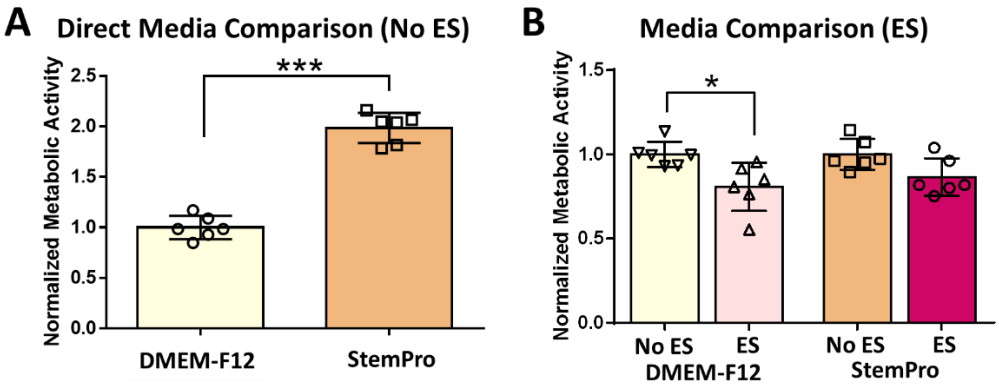


Figure S3*. Secretome media comparison for optimal ASC metabolism. (A) ASCs cultured under static conditions in StemPro media for three days as compared to DMEM-F12. (B) ASC metabolic activity measured 24 hours after applied ES in either DMEM-F12 medium or Stempro medium. ES parameters included 20 mV/mm, 500 µs pulses applied at 1000 Hz using the AC bioreactor for 1 hour x 3 days. Data are mean ± std, n=6/group and were normalized to respective NoES controls. *p-value<0.05, ***p-value<0.0001 compared to NoES DMEM-F12*

d. Schwann cell response to low-level ES

Human Schwann cells were used as a neural cell comparison for ASCs, given their better characterized response to ES[4,6,7]. Schwann cells were purchased from Sciencell (1700) and cultured in the recommended medium from Sciencell (1701). After seeding, cells were cultured overnight in their growth media before being switched to StemPro secretome media. ES applied in the AC bioreactor at 1 Hz (500 µs, square pulse, 20 mV/mm) for 24 hours (followed by 24 hours of non-stimulated incubation) resulted in similar patterns of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) upregulation at the gene level as compared to ASCs. As with the ASCs, gene expression changes were less than 1.5-fold different from the NoES control but were statistically significant (**Figure S4**). Only a single biological replication (n=4) was conducted for this study, so future studies will be necessary to confirm this comparison to ASCs. Overall, these results indicate that ASCs are comparably responsive to low-level, low-frequency stimulation as the electrically responsive Schwann cells.

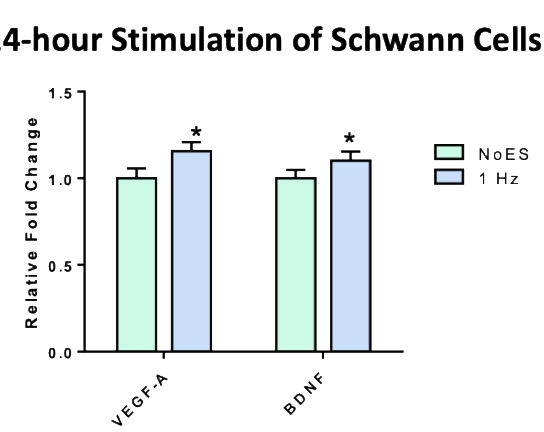


Figure S4*. AC Bioreactor: Continuous ES was applied to Schwann cells with square pulses (500 µs width) at a peak voltage of 674 mV (20 mV/mm) and a frequency of 1 Hz. Schwann cell gene expression results for VEGF and BDNF 24 hours post-stimulation. Data are mean ± std, n=4/group for PCR with 1 biological replicate. *p-value<0.05 compared to NoES*

References for Supplemental Data Section

1. Beugels, J., D. G. M. Molin, D. R. M. G. Ophelders, T. Rutten, L. Kessels, N. Kloosterboer, A. A. P. de Grzymala, B. W. W. Kramer, R. R. W. J. van der Hulst, and T. G. A. M. Wolfs. Electrical stimulation promotes the angiogenic potential of adipose-derived stem cells. *Scientific Reports* 9:12076, 2019. doi:10.1038/s41598-019-48369-w

2. Bicer, M., J. Sheard, D. Iandolo, S. Y. Boateng, G. S. Cottrell, and D. Widera. Electrical stimulation of adipose-derived stem cells in 3D nanofibrillar cellulose increases their osteogenic potential. *Biomolecules* 10(12):1696, 2020.doi:10.3390/biom10121696

3. Brug, G. J., A. L. G. van den Eeden, M. Sluyters-Rehbach, and J. H. Sluyters. The analysis of electrode impedances complicated by the presence of a constant phase element. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry* 176:275–295, 1984.

4. Forciniti, L., J. Ybarra, M. H. Zaman, and C. E. Schmidt. Schwann cell response on polypyrrole substrates upon electrical stimulation. *Acta Biomaterialia* 10(6):2423-33, 2014.doi:10.1016/j.actbio.2014.01.030

5. Heo, D. N., N. Acquah, J. Kim, S. J. Lee, N. J. Castro, and L. G. Zhang. Directly Induced Neural Differentiation of Human Adipose-Derived Stem Cells Using Three-Dimensional Culture System of Conductive Microwell with Electrical Stimulation. *Tissue Engineering - Part A* 24(7-8):537-545, 2018. doi:10.1089/ten.tea.2017.0150

6. Huang, J., X. Hu, L. Lu, Z. Ye, Q. Zhang, and Z. Luo. Electrical regulation of Schwann cells using conductive polypyrrole/chitosan polymers. *Journal of Biomedical Materials Research - Part A* 93(1): 164-74, 2010. doi:10.1002/jbm.a.32511

7. Koppes, A. N., A. L. Nordberg, G. M. Paolillo, N. M. Goodsell, H. A. Darwish, L. Zhang, and D. M. Thompson. Electrical stimulation of schwann cells promotes sustained increases in neurite outgrowth. *Tissue Engineering - Part A* 20(3-4):494-506, 2014. doi:10.1089/ten.tea.2013.0012

8. Li, J., X. Liu, J. M. Crook, and G. G. Wallace. Electrical stimulation-induced osteogenesis of human adipose derived stem cells using a conductive graphene-cellulose scaffold. *Materials Science and Engineering: C* 107:110312, 2020. doi:10.1016/j.msec.2019.110312

9. Mobini, S., L. Leppik, V. Thottakkattumana Parameswaran, and J. H. Barker. *In vitro* effect of direct current electrical stimulation on rat mesenchymal stem cells. *PeerJ* 5:e2821, 2017.

10. Orazem, M. E., and B. Tribollet. Electrochemical Impedance Spectroscopy. Pennington, NJ: Wiley, 2008.

11. Patrikoski, M., M. Juntunen, S. Boucher, A. Campbell, M. C. Vemuri, B. Mannerström, and S. Miettinen. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. *Stem Cell Research and Therapy* 4(2):27, 2013.doi:10.1186/scrt175

12. Pavesi, A., M. Soncini, A. Zamperone, S. Pietronave, E. Medico, A. Redaelli, M. Prat, and G. B. Fiore. Electrical conditioning of adipose-derived stem cells in a multi-chamber culture platform. *Biotechnology and Bioengineering* 111(7):1452-63, 2014. doi:10.1002/bit.25201

13. Riis, S., A. Stensballe, J. Emmersen, C. P. Pennisi, S. Birkelund, V. Zachar, and T. Fink. Mass spectrometry analysis of adipose-derived stem cells reveals a significant effect of hypoxia on pathways regulating extracellular matrix. *Stem Cell Research and Therapy* 7:52, 2016. doi:10.1186/s13287-016-0310-7

14. Tandon, N., B. Goh, A. Marsano, P. H. G. Chao, C. Montouri-Sorrentino, J. Gimble, and G. Vunjak-Novakovic. Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. Conference Proceedings of IEEE Engineering Medical Biology Society 1:6517-6521, 2009. doi:10.1109/IEMBS.2009.5333142

15. Yang, G., H. Long, X. Ren, K. Ma, Z. Xiao, Y. Wang, and Y. Guo. Regulation of adipose-tissue-derived stromal cell orientation and motility in 2D- and 3D-cultures by direct-current electrical field. *Development, Growth and Differentiation* 59(2):70-82, 2017. doi:10.1111/dgd.12340

16. Yang, S., L. Pilgaard, L. G. Chase, S. Boucher, M. C. Vemuri, T. Fink, and V. Zachar. Defined xenogeneic-free and hypoxic environment provides superior conditions for long-term expansion of human adipose-derived stem cells. *Tissue Engineering - Part C Methods* 18(8): 593-602, 2012. doi:10.1089/ten.tec.2011.0592