

Supplementary Materials for

NCX-IP3R Crosstalk Maintains Calcium Oscillations to Regulate Angiogenic Signaling in Endothelial Cells

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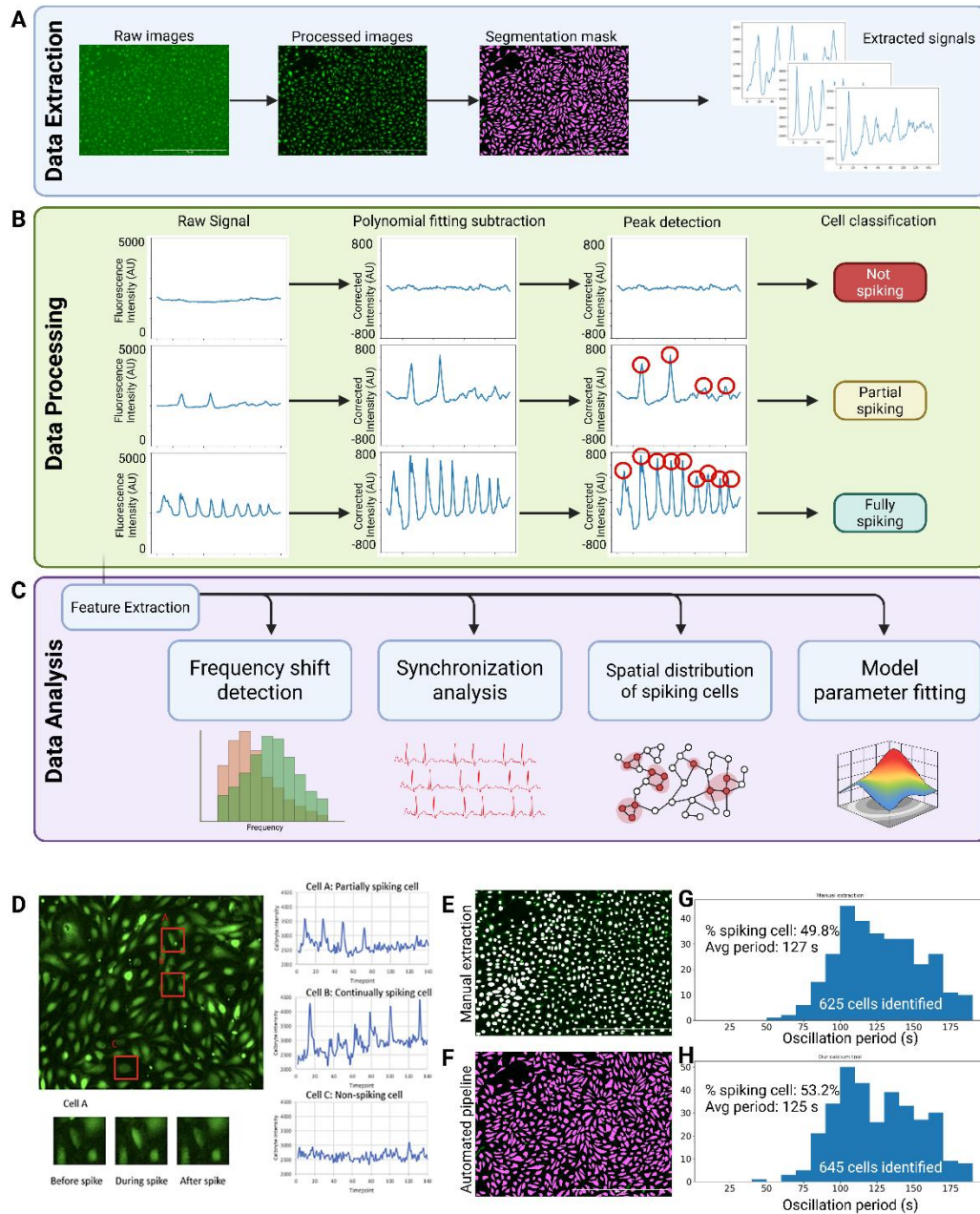
This PDF file includes:

Supplementary Figures S1 to S5

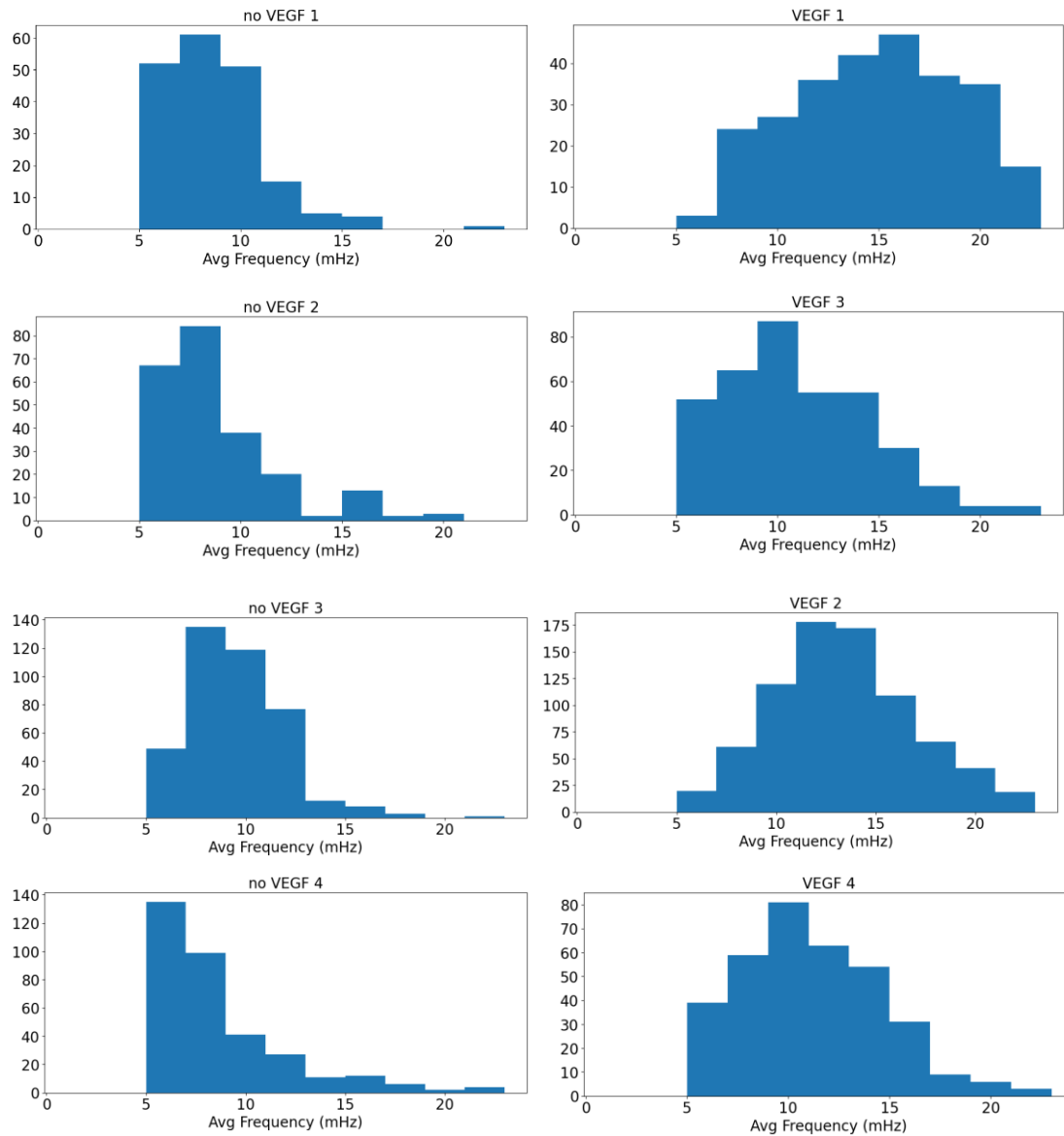
Supplementary Table S1

Other Supplementary Materials for this manuscript include the following:

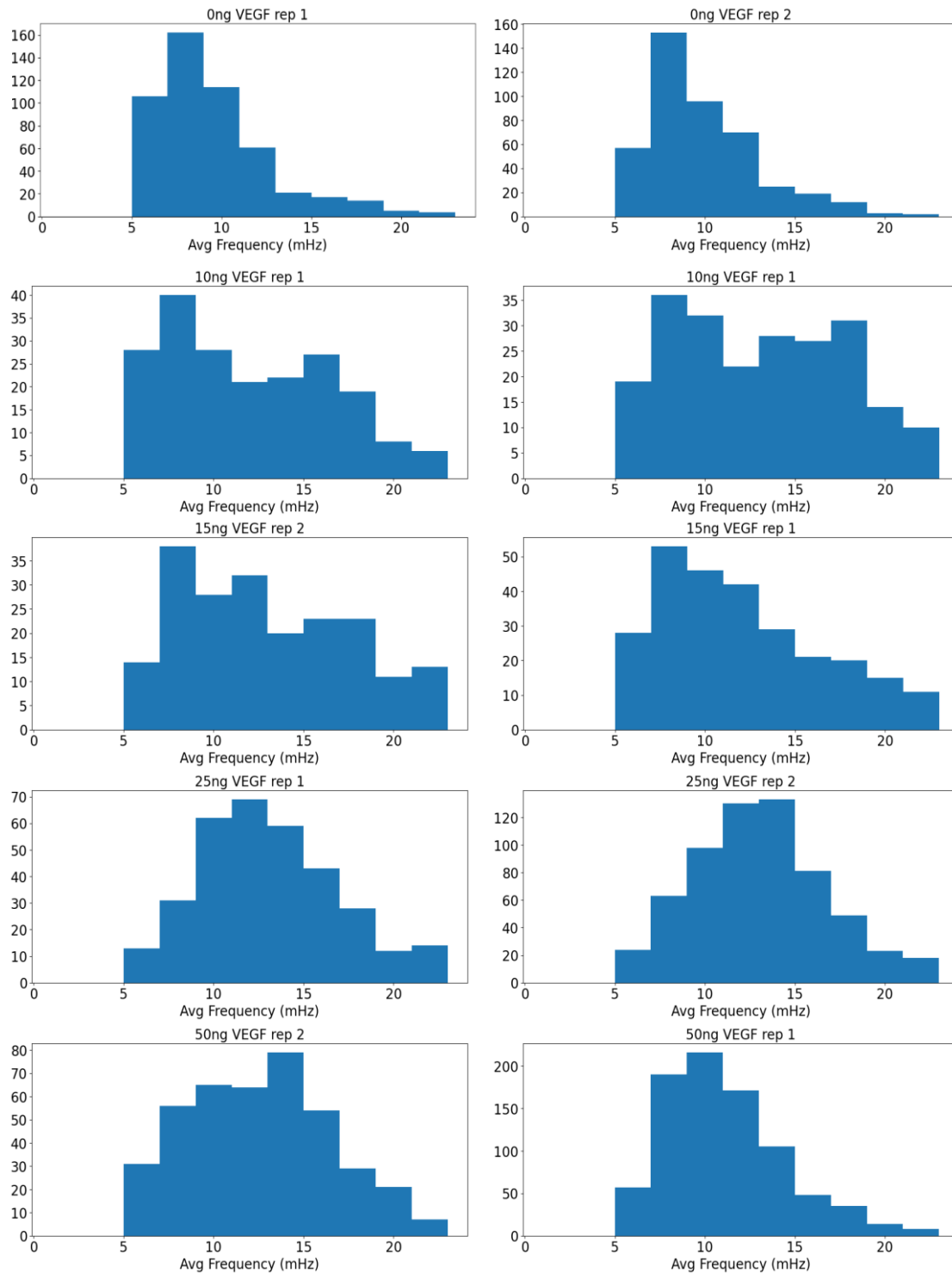
Movies S1 to S13



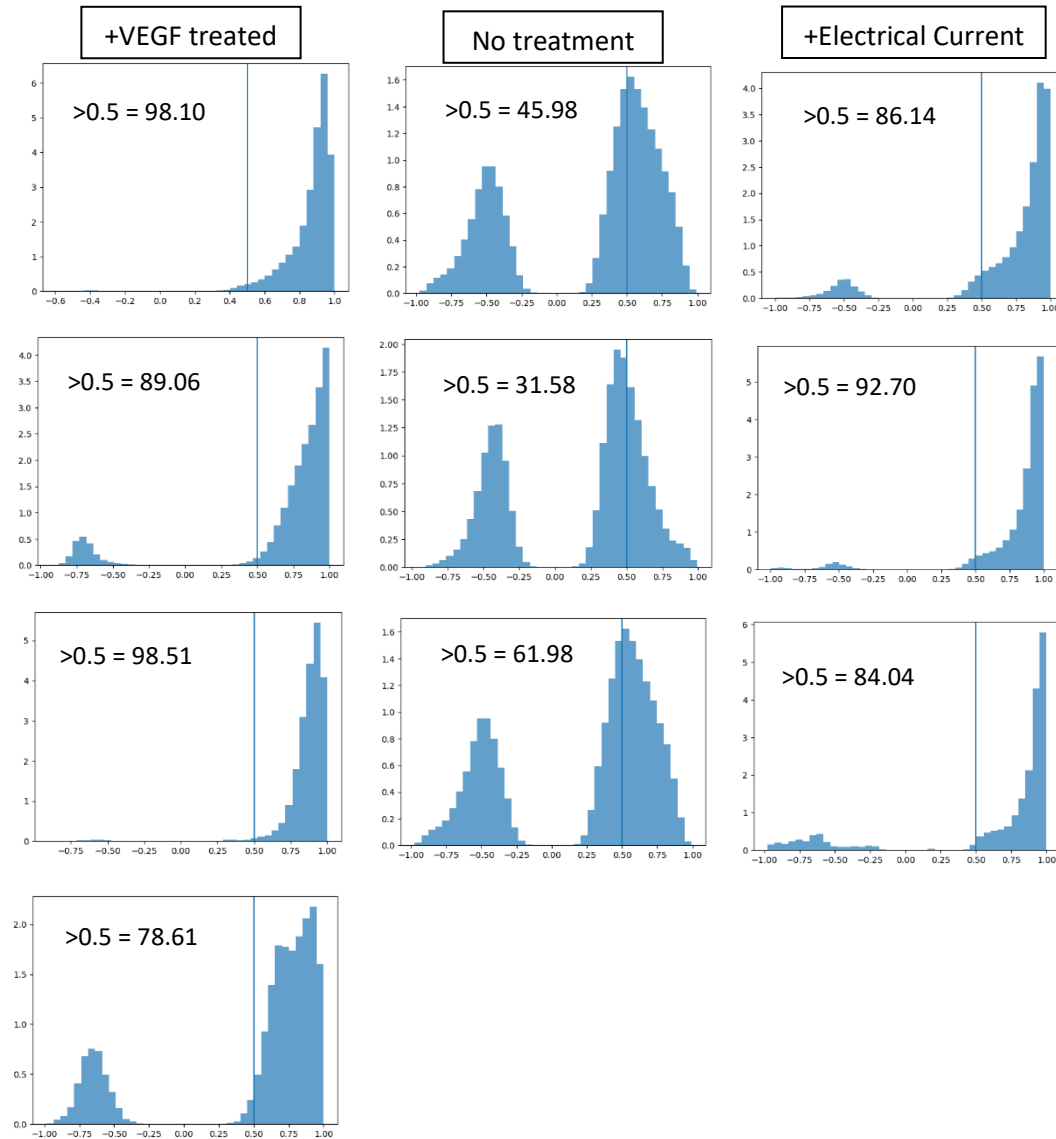
Supplemental Figure S1. Outline of the automated calcium data analysis tool and its performance comparison with manual segmentation and extraction. **A.** Steps for initial image data processing and extraction. **B.** Extracted temporal calcium data is further processed and filtered. **C.** The processed oscillatory data is used for various analysis downstream, such as frequency shift or synchronization. Scale bars represent 1 mm. **D.** An example of a calcium staining image with 3 sample cells identified as partially, continuously, and non-spiking cells. **E-F.** Cell segmentation results from manual ROI identification and Cellpose-based automated algorithm. **G-H.** Distribution of average oscillation period in the cells identified by each method.



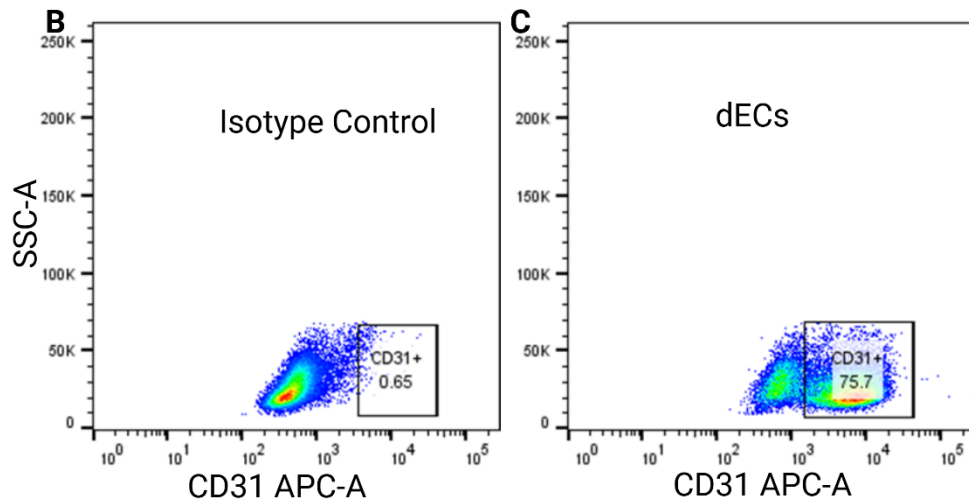
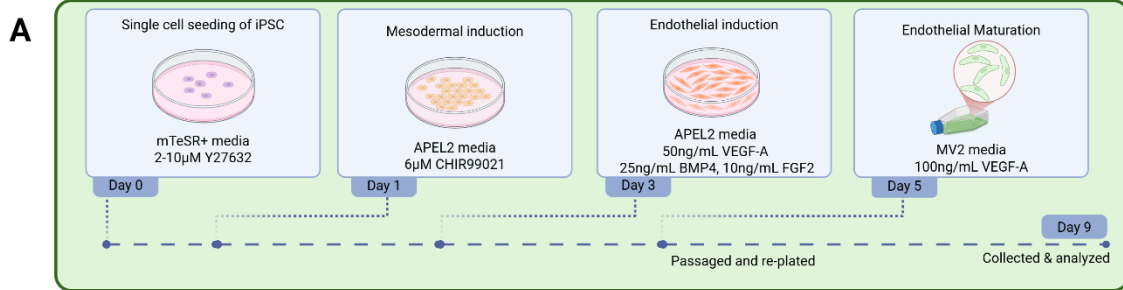
Supplemental Figure S2. Calcium oscillation frequency shift when treated with VEGF. Each histogram consists of cells collected from a distinct experimental setup with cells cultured on separate days. Each VEGF-no VEGF pair was run on the same day.



Supplemental Figure S3. Calcium oscillation frequency shift when treated with various concentrations of VEGF. Each concentration was tested in duplicates (n=2). All concentrations tested above 10ng/mL resulted in the oscillation frequency shift.

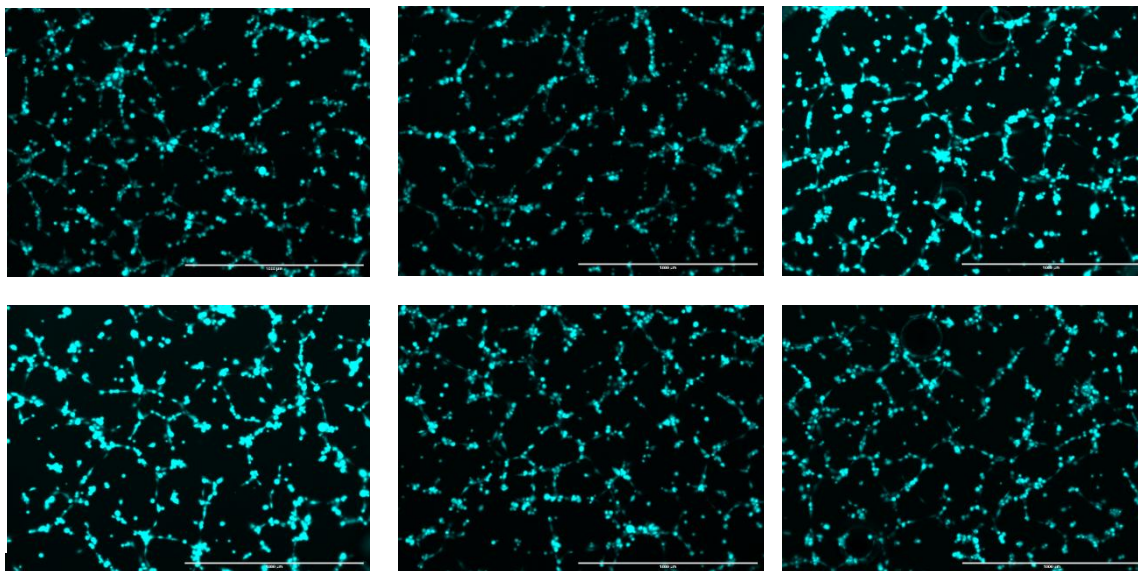


Supplemental Figure S4. Histograms of cross-correlation values for each cell-cell pair within the imaging window. Left column shows cell correlation immediately following treatment with 50ng/mL of VEGF. Right column shows cell correlation during electric current treatment of 10mA and 5V. The center column shows correlation for untreated cells undergoing natural oscillation. N=4 for VEGF and n=3 for control and electric current.

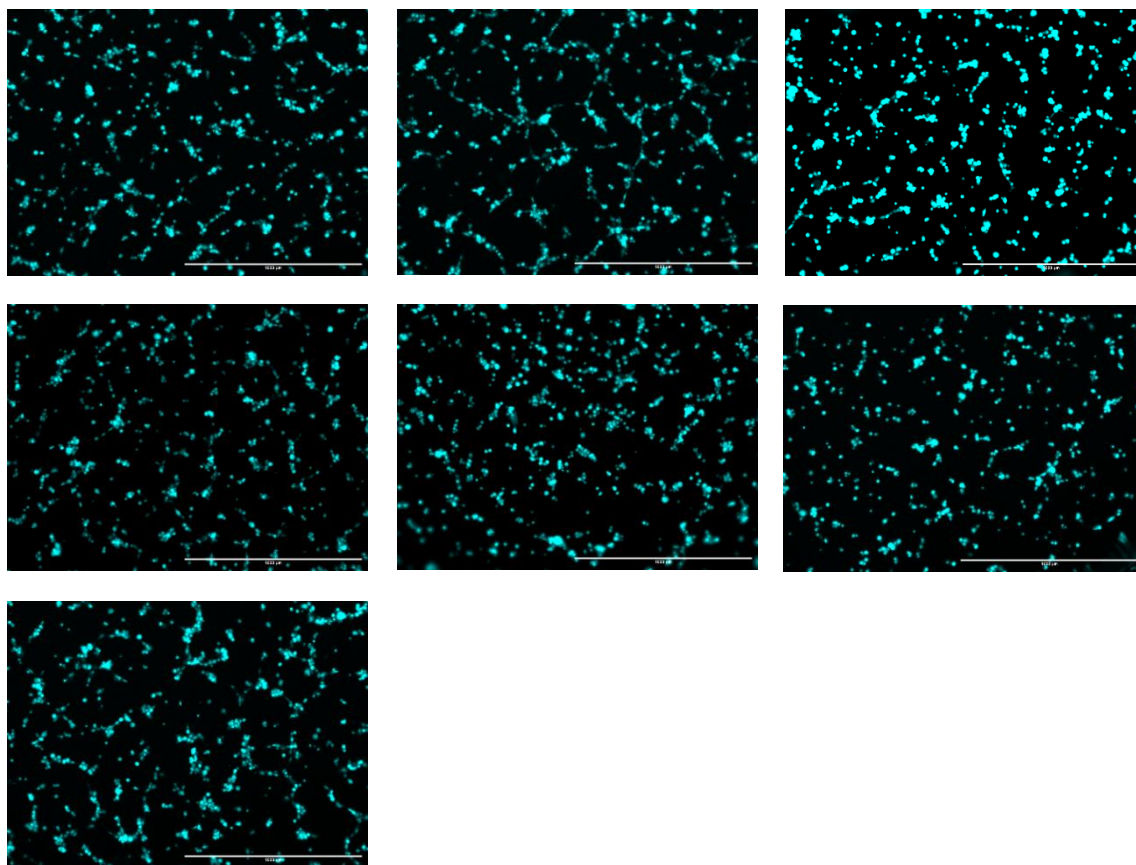


Supplemental Figure S5. A. Schematic of the differentiation process used, based on the Harding et al. protocol. **B-C.** FACS plot of differentiated ECs showing CD31 expression compared with isotype control-stained samples as baseline.

Current-treated ECs



Non-treated ECs



Supplementary Figure S6. Matrigel tube formation assay results. Top 6 images correspond to ECs treated with electrical current, and the bottom 7 images correspond to untreated ECs. Scale bar represents 1 mm.

Gene (Entrez Gene ID)	Reporter type	Taqman Probe ID (Thermo Fisher)
Human <i>AKT1</i> (207)	FAM-MGB	Hs00178289_m1
Human <i>MAPK1</i> (5594)	FAM-MGB	Hs01046830_m1
Human <i>KDR</i> (3791)	FAM-MGB	Hs00911700_m1
Human <i>Wnt5a</i> (7474)	FAM-MGB	Hs00998537_m1
Human <i>CTGF</i> (1490)	FAM-MGB	Hs00170014_m1
Human <i>GAPDH</i> (2597)	FAM-MGB	Hs99999905_m1

Supplementary Table 1. List of RT-qPCR primers used in the study.

Supplemental Movie S1. Acute calcium response to VEGF. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. Imaging was conducted for 10 minutes before 100ng/mL VEGF solution was added in-between timepoints. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S2. Baseline calcium oscillation of BECs. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. The BECs are in regular MV2 media with no additional VEGF supplementation. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S3. Chronic calcium response to VEGF. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. The BECs were treated with 50ng/mL VEGF 24 hours prior to imaging, and was placed in VEGF-containing media for the duration of the imaging. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S4. Calcium oscillations in immature dECs. Differentiated ECs at day 6 were stained with Calbryte 520AM and imaged every 5 seconds. The dECs were left in the 100ng/mL VEGF-A differentiation media for the duration of the imaging. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S5. Calcium oscillations in mature dECs. Differentiated ECs at day 8 were stained with Calbryte 520AM and imaged every 5 seconds. The dECs were left in the 100ng/mL VEGF-A differentiation media for the duration of the imaging. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S6. Calcium synchronization with ion depletion. P6 BECs were seeded into the depletion chip and stained with Calbryte 520AM, then imaged every 5 seconds. Imaging was conducted for 5 minutes before first depletion was conducted for 50 seconds, followed by second depletion at 15 minutes. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S7. Calcium synchronization with direct electrical stimulation. P6 BECs were seeded into the Ibidi single channel chip and stained with Calbryte 520AM, then imaged every 5 seconds. Imaging was conducted for 2 minutes before electrical current was applied for 50 seconds. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S8. Calcium and membrane potential changes in BECs. P6 BECs were stained with Calbryte 520AM and RH421 dye and imaged every 5 seconds. The BECs are supplemented with 50ng/mL VEGF 24 hours prior to staining and during the imaging. Green channel represents the Calbryte staining and magenta channel represents membrane potential. Scale bar represents 0.5mm and video is compressed to 25 frames per second.

Supplemental Movie S9. Effect of external sodium spiking on calcium oscillation. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. After 8 minutes, 1M NaCl solution was added to the cell culture media such that the resulting Na⁺ concentration reached 300mM. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S10. Reversal of the effect of external sodium. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. The cells were placed into cell culture media at 300mM Na⁺ concentration, then after 8 minutes, the media was diluted using deionized water

to reach 190mM Na⁺ concentration. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S11. Effect of external sodium spiking on calcium oscillation. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. After 8 minutes, 1M KCl solution was added to the cell culture media such that the resulting K⁺ concentration reached 50mM. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S12. Effect of NCX inhibitor on calcium oscillation. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. The cells were treated with 50μM SEA0400 for 20 minutes prior to imaging and during the imaging. Scale bar represents 1mm and video is compressed to 25 frames per second.

Supplemental Movie S13. Effect of external sodium and VEGF on calcium oscillation. P6 BECs were stained with Calbryte 520AM and imaged every 5 seconds. The cells were placed into cell culture media at 300mM Na⁺ concentration 5 minutes prior to imaging, then imaged for 10 minutes. After 10 minutes, media containing 300mM Na⁺ and 100ng/mL VEGF was added to adjust the VEGF concentration in the cell media to 50ng/mL. Scale bar represents 1mm and video is compressed to 25 frames per second.