

DETECTION OF MITOCHONDRIAL BIOENERGETICS USING A NOVEL BIMODAL 3D MICROELECTRODE ARRAY (MEA)-BASED BIOSENSOR

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1. Supplementary Information

Additional data pertaining to microfabrication timescale, micro-tower height and diameter, along with preliminary EIS characterization from different MEA configurations, initial mitochondrial EIS data utilizing respiration buffer conditions detailed from Prof. John H Miller's group (University of Houston, Houston, TX), pH measurements from various buffer conditions, optical images of cell culture confluency, mitochondrial pellet density and expanded mitochondrial

EIS characterization data in a dose-dependence manner.

1.1 Microfabrication Timescale

Supplementary Table 1 details the individual microfabrication steps with the approximate time it takes to complete each step.

1.2 Micro-tower Dimensions

Supplementary Tables 2-4 display the averaged micro-tower height, top diameter and bottom tower diameter obtained using laser confocal microscopy across N=3 devices from the 3x3 micro-tower array (Total N = 27).

1.3 MEA Surface Area Characterization

Figure S1 presents an optical image displaying the two different MEA configurations utilized for initial device development. Figure S1A displays the 5x5mm² configuration and Figure S2B displays the 10x10mm² configuration. These were developed to find an optimal surface area to elicit optimal EIS and electrophysiological signals.

1.4 Preliminary Mitochondria Biosensor EIS Spectra

Figure S2 here presents the graphical representation of the impedance magnitude/phase spectra obtained from both MEA configurations and a table comparing their quantitative values at the biologically relevant frequency point of 1kHz. The table shows the inverse relationship between conductive surface area and impedance.

1.5 NIH3T3 and iPSC Culture Confluency

Figure S3 shows images of the NIH3T3 and iPSC models at ~100% confluency prior to mitochondrial isolation.

1.6 Mitochondria Pellet Surface Area

Figure S4 shows the isolated mitochondrial pellet obtained from NIH3T3 cell lines plated at ~100% in three T-75 flasks. The pellet is large enough to cover the diameter of a 2mL centrifuge tube which allows indicating a surface area capable of covering the 3x3 micro-tower array.

1.7 Preliminary Mitochondrial EIS Characterization

Figure S5 initial attempts at mitochondria EIS magnitude/phase utilizing respiration buffer conditions detailed from Prof. John H. Miller group (University of Houston, Houston, TX). While the trends did occur as expected, the physiological activity was questionable to the super

physiological concentration of succinate at 400mM.

1.8 Impedance Magnitude/Phase Experimental Design

Supplementary Figures S6-S9 Display the full experimental conditions for the dose-dependent EIS magnitude/phase characterization study. To assess mitochondria activity in physiological conditions the respiration buffer composition was altered to simplify the mixture and use substrate concentrations from 1mM-10mM. The altered buffer composition contained Mannitol: 225mM, Sucrose: 75mM, Tris-HCl: 25mM, KH_2PO_4 : 1mM, MgCl_2 1mM and ADP 1mM as well as additional respiratory substrates in addition to succinate including glutamate and malate. The substrates were initially added by mixing stock solutions into the RB to achieve the desired concentration of 1mM, after the addition of each substrates EIS measurements were taken after 5-minute incubation time. The dry solutes of succinate and malate were then directly added to obtain concentration of 5mM and 10mM during experimental assays, glutamate is only soluble at either basic or acidic pH when not conjugated with an ion such as monosodium glutamate (MSG) which prevented the addition of its dry solute for the experimental condition. Measurements for 5mM and 10mM conditions were performed after a 3-minute incubation time from the addition of the solutes of each substrate. The final measurement was performed after the addition of Sucrose at a final concentration of 10mM to serve as a negative control as mitochondria do not require this as a substrate for energy production thus there should not be a drop in the impedance magnitude signature. Mitochondrial EIS characterization with the adjusted buffer displayed trends expected of mitochondrial samples where the impedance magnitude decreases in the 1mM, 5mM and 10mM from the increase in conductivity attributed to an increase in ETC activity and protons being shuttled across the inner mitochondrial membrane into the IMS. The impedance phase shifts closer to 0° from the 1mM, to the 5mM and 10mM at high frequencies indicating more resistive behavior from the increase in conductivity and less capacitive behavior.

1.9 NIH3T3 pH Characterization of Respiratory Substrates

Supplementary Table 5 displays the change in pH during the NIH3T3 mitochondrial EIS characterization. EIS experiments were initially performed in a dose dependent manner. The 1mM condition was made by mixing stock solutions of malate, glutamate and succinate which had pH values in the physiological range of 7.4. To reach 5mM and 10mM conditions during the experiment, dry solutes of succinate and malate were added directly to the solution. However, the buffering capacity was unable to maintain the pH in physiological range at higher concentrations as each of the substrates are weakly acidic. Glutamate was only soluble at very acidic or basic pH and could not be increased from 1mM. The pH was measured at the beginning for the 1mM condition and again at the end with 10mM of respiratory substrates.

1.10 iPSC pH Characterization of Respiratory Substrates

Supplementary Table 6 displays the change in pH during the iPSC mitochondrial EIS characterization. EIS conditions for the iPSC model were the same as for the NIH3T3 culture and the pH values were monitored similarly at the beginning of the experiment and at the end.

1.11 NIH3T3 and iPSC EIS Percent Change Analysis

Supplementary Table 7 shows the percent change calculation for the MT and MT 1mM conditions to establish a biologically relevant frequency point with the 100kHz range demonstrating peak sensitivity ETC and mitochondrial membrane dielectric modulation.

1.12 iPSC pH Characterization of Respiratory Substrates

Supplementary Table 8 displays the differences in buffer composition between conditions used from Professor John H Miller's (University of Houston, Houston, TX) in comparison to buffer conditions commonly used in bioenergetic studies as detailed from Springer Protocols.

1.13 Preliminary Electrophysiology Recordings

Supplementary Figure S10 verifies channel-level noise performance. Baseline traces recorded in air; inserting the 3-D MEA into the Axion BioSystems MUSE lowered the noise floor and adding PBS as a conductive medium reduced it further, with several sites (e.g., channels 84 and 74) stabilizing below 10 μ V. Such low intrinsic thermal noise floor confirms that a subset of electrodes is sufficiently sensitive to capture biological field signals, justifying the use of the 3-D MEA for subsequent mitochondrial electrophysiology experiments.

1.14 2D vs 3D MEA Electrochemical Impedance Spectroscopy Characterization

Supplementary Figure S11 displays a comparison of 2D vs 3D MEA EIS signatures. The 3D and 2D MEA devices both contained a 5x5mm² metallization area with a gross central recording area of 2.5x2.5mm² the only difference in surface area came from the 3x3 micro-tower array on the 3D MEA. A volume of 2mL PBS (pH 7.4) was used as the electrolyte solution for each device. The 1kHz frequency point was used to determine sensitivity of each device.

Supplementary Table 1. Timetable of 3D MEA fabrication process flow

	Procedure:	Time:
Day 1	3D Printer	7-8 min ea.
	IPA Bath	5 min ea.
	UV Curing	30 min
	Pogo Pins	1 min ea.
	Air Dry	2 min ea.
	Metallization Mask	2 min ea.
Day 2	E-Beam	1 Day
Day 3	Laser Ablation	12 min ea.
	Silver Ink Casting	2-3 min ea.
	Curing	8 hours
Day 4	Voltmeter	2 min ea.
	PET Insulation Mask	2 min ea.
	Culture Well Attachment	2 min ea.
	Cure	1 hour
	EIS (PBS)	2 min ea.

Supplementary Table 2. Micro-tower heights (n = 27), measurements recorded via laser confocal microscopy showed an average micro-tower height.

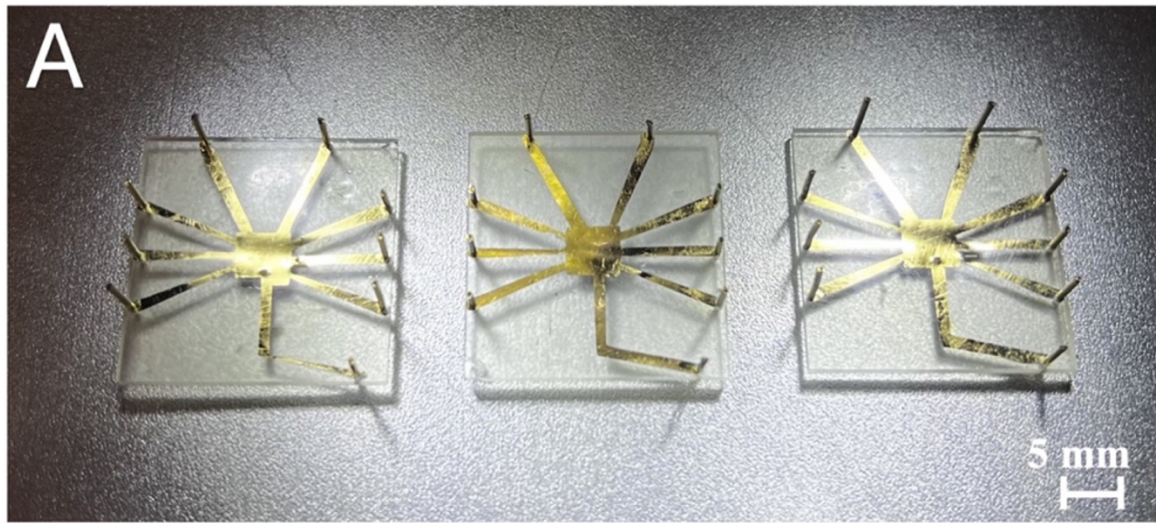
Sample 1 Height (μm)	Sample 2 Height (μm)	Sample 3 Height (μm)	Average (μm)
224.96	236.73	236.74	231.91 (± 6.7)
220.15	229.46	229.46	
221.97	234.66	234.66	
226.7	236.9	236.91	
223.77	248.44	248.45	
227.04	232.7	232.7	
224.99	233.6	233.61	
227.38	232.32	232.32	
227.98	233.49	233.5	

Supplementary Table 3. Micro-tower heights (n = 27), measurements recorded via laser confocal microscopy showed an average micro-tower top diameter of 56.55 μ m.

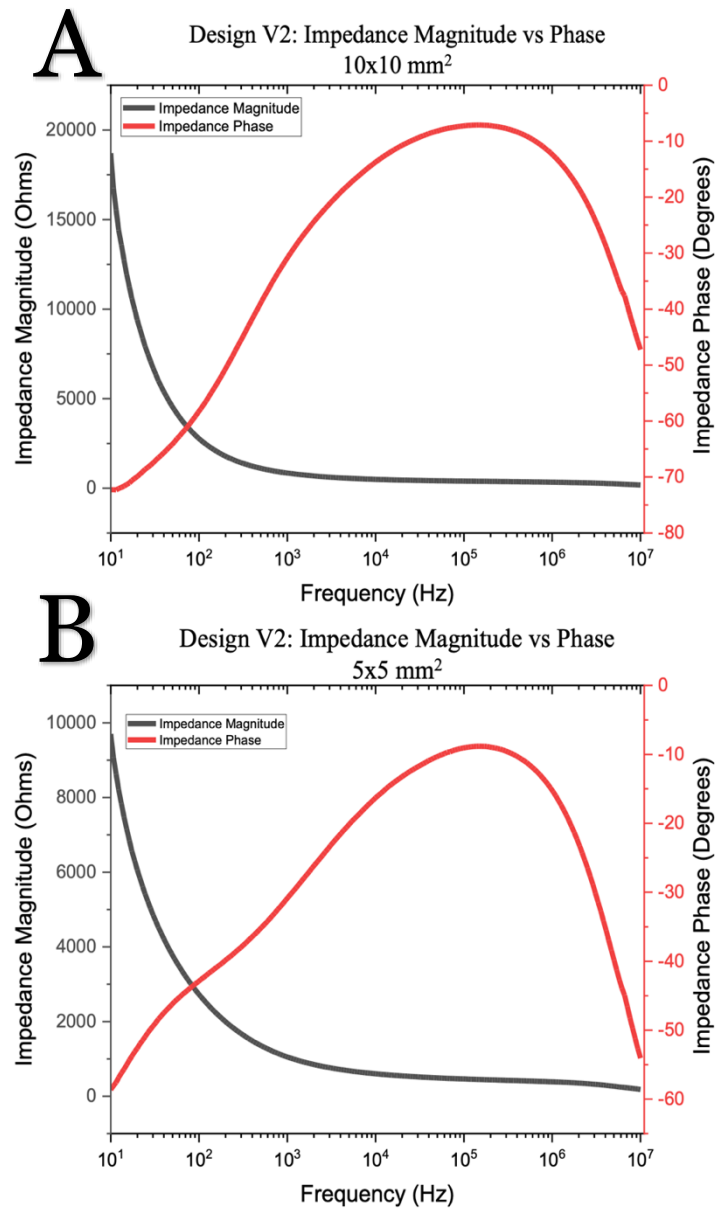
Sample 1 Top Diameter (μm)	Sample 2 Top Diameter (μm)	Sample 3 Top Diameter (μm)	Average (μm)
49.09	64.65	55.93	56.55 ($\pm$7.0)
44.99	60.10	58.82	
40.44	55.66	58.57	
50.17	52.13	62.79	
49.55	57.88	60.46	
41.11	56.12	58.59	
42.08	51.33	59.21	
41.35	51.89	57.72	
46.08	49.41	55.72	

Supplementary Table 4. Micro-tower heights ($n = 27$), measurements recorded via laser confocal microscopy showed an average bottom diameter $68.36\mu\text{m}$.

Sample 1 Bottom Diameter (μm)	Sample 2 Bottom Diameter (μm)	Sample 3 Bottom Diameter (μm)	Average (μm)
62.73	69.86	72.5	68.36 (± 5.9)
60.08	58.02	62.24	
72.58	69.24	63.55	
61.15	65.73	69.75	
57.3	62.69	64.42	
55.67	65.05	66.59	
63.22	66.97	75.01	
59.66	65.10	69.12	
54.26	79.17	64.50	

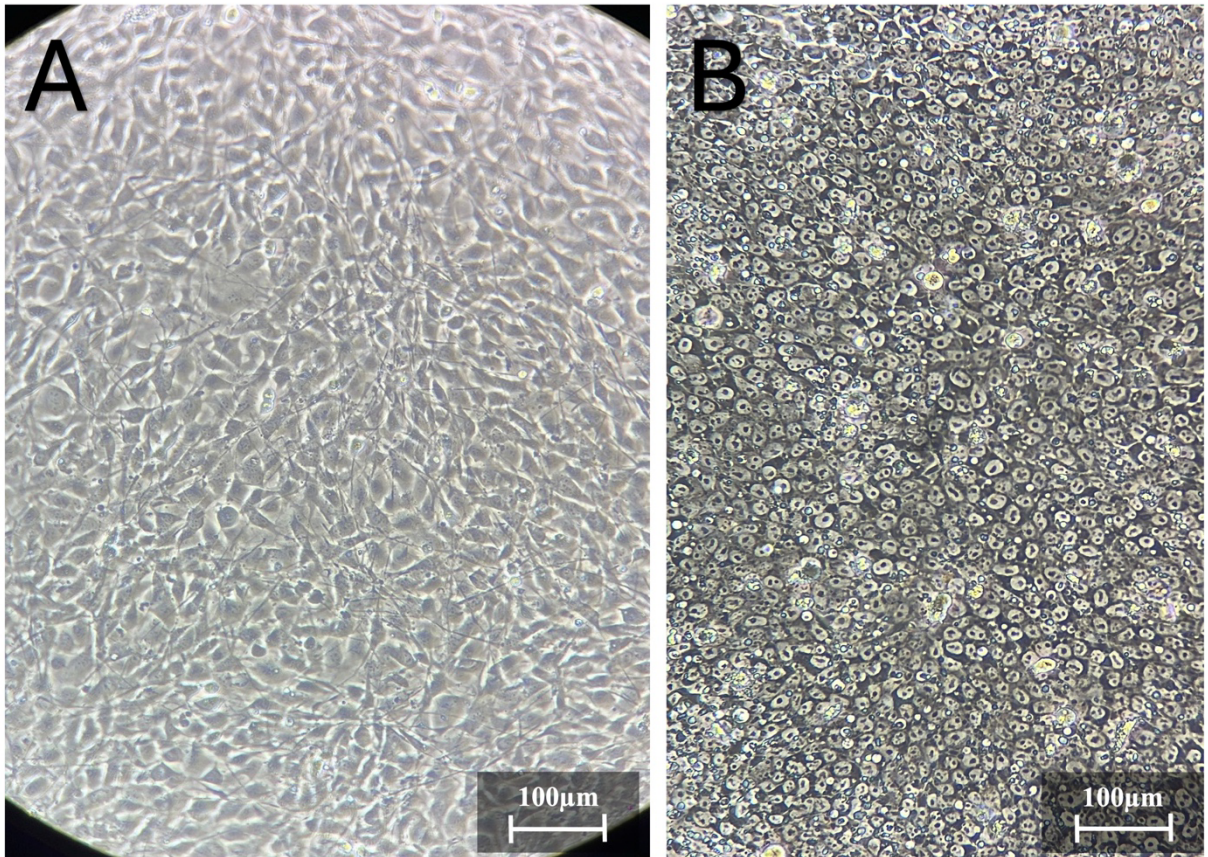


Supplementary Figure 1: A) Optical image of the 5x5mm² MEA configuration with a total conductive area of ~13.04mm². B) Optical image of the 5x5mm² MEA configuration with a total conductive area of ~19.71mm².

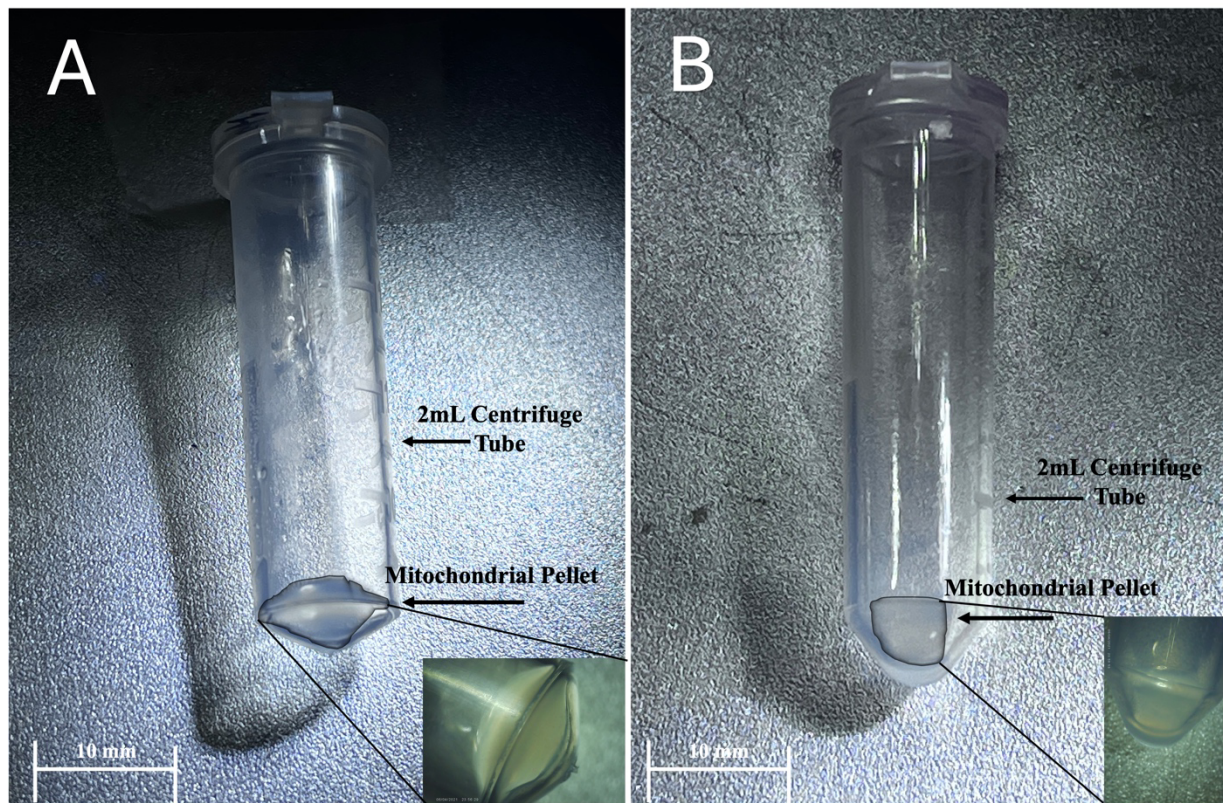


Magnitude/Phase Frequency: 1kHz	Magnitude	Phase
10x10 mm² (PBS)	838.85Ω	-30.61°
5x5 mm² (PBS)	1068.94Ω	-30.71°

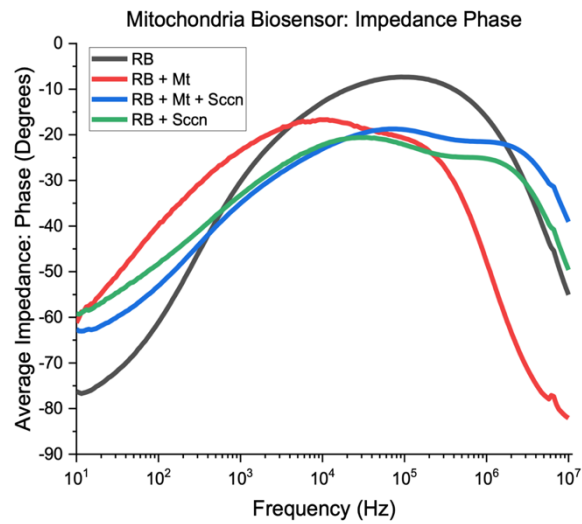
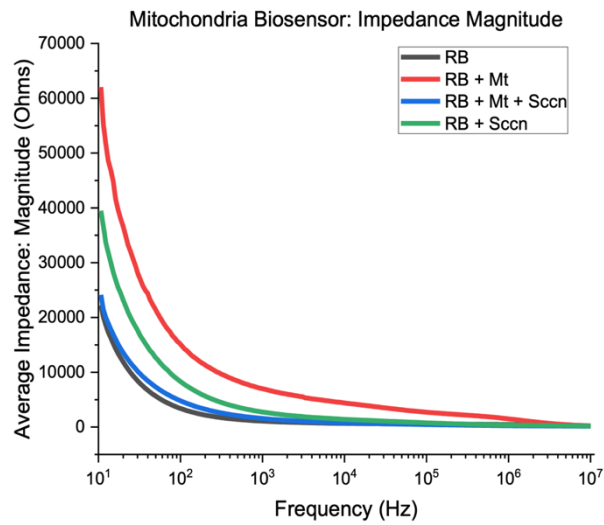
Supplementary Figure 2: A) Impedance Magnitude/Phase micro-electrode (n =9) characterization for two different 3-D MEA configurations of the mitochondria biosensor using PBS (pH 7.4) as an electrolyte for the 10x10mm² B) Shows the micro-electrode characterization for the 5x5mm² configuration.



Supplementary Figure 3: Optical images of A) NIH3T3 cell model at ~100% confluency and B) iPSC model at ~100% confluency.

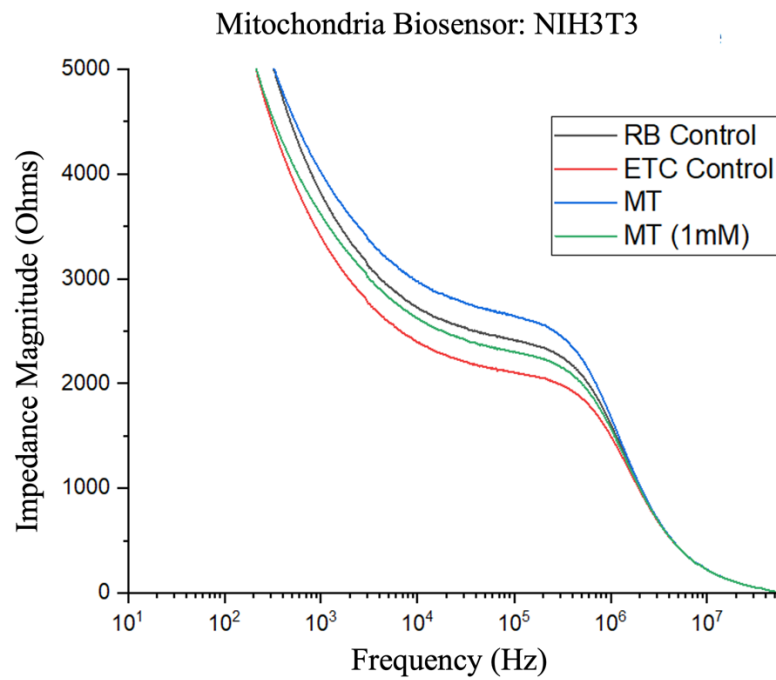
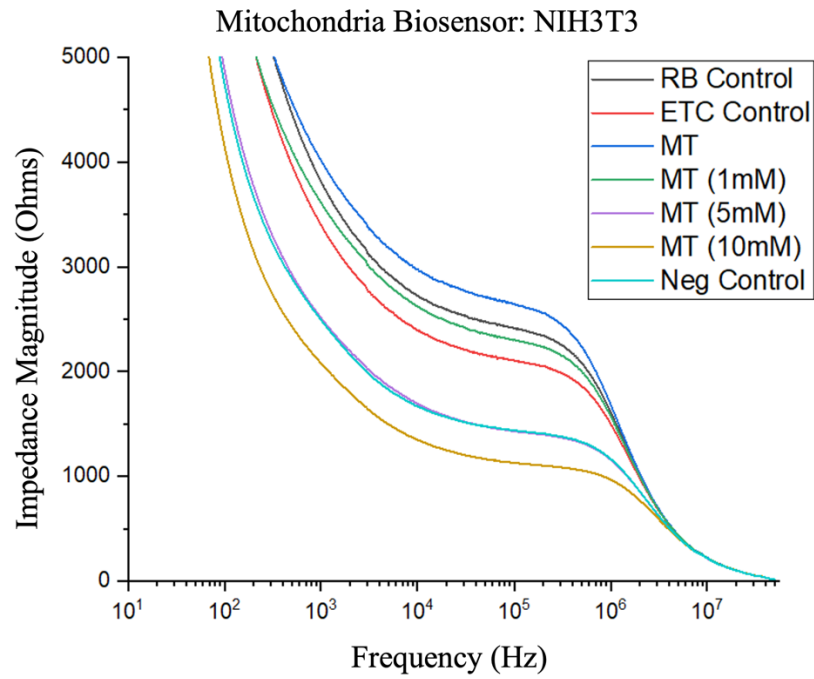


Supplementary Figure 4: A&B Optical images of isolated mitochondria pellets obtained from NIH3T3 cells. Final cell population was ~9,217,500

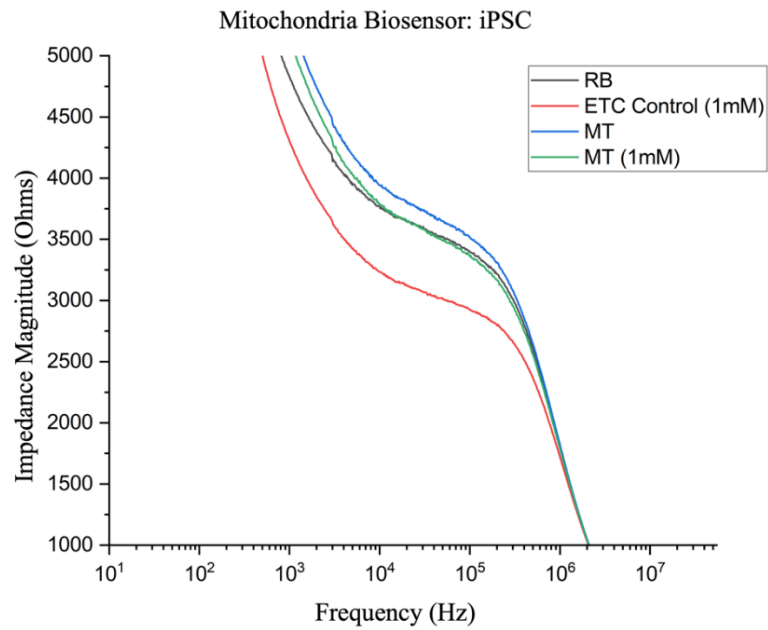
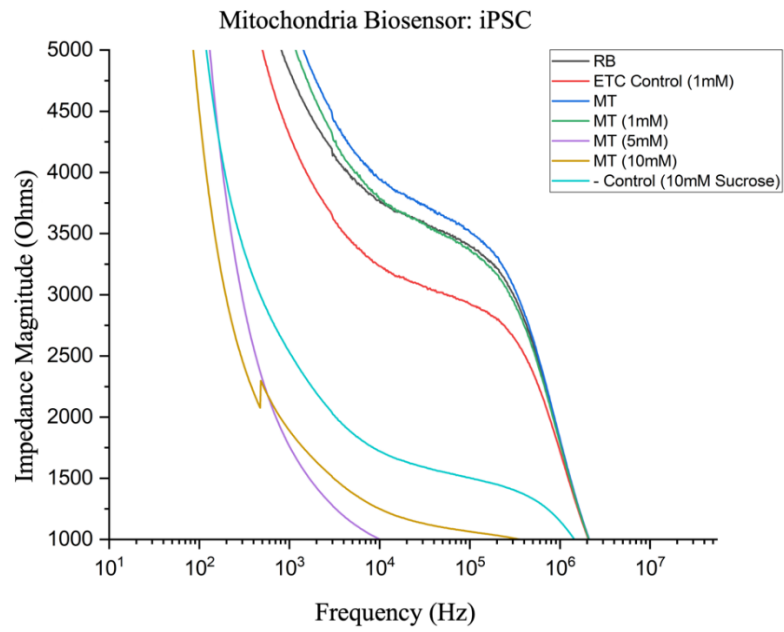


Magnitude/Phase Frequency: 1kHz	Magnitude	Phase
RB	1062.18Ω	-30°
RB + Mt	6857.2Ω	-23.37°
RB + Mt + Sccn (400mM)	1507.69Ω	-34.85°
RB + Sccn (400mM)	2658.19Ω	-33.07°

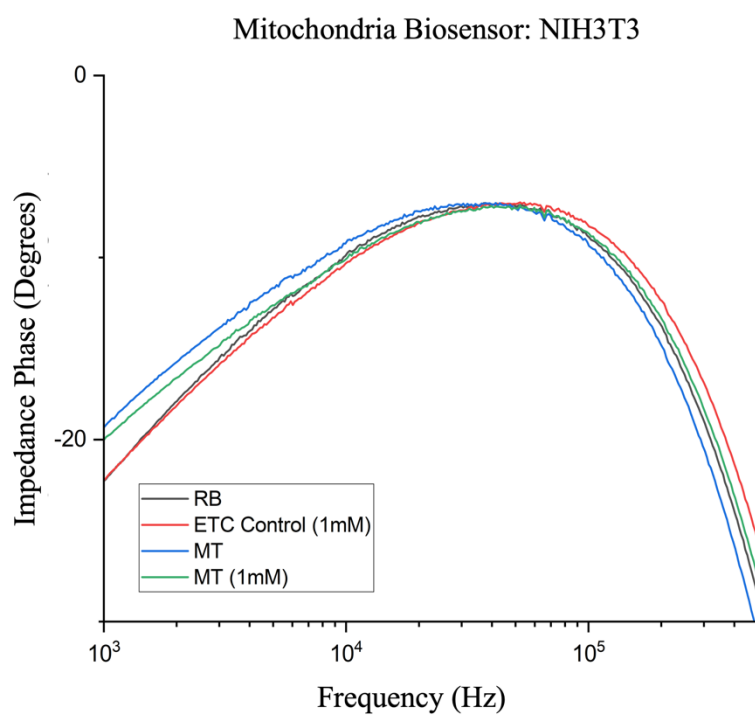
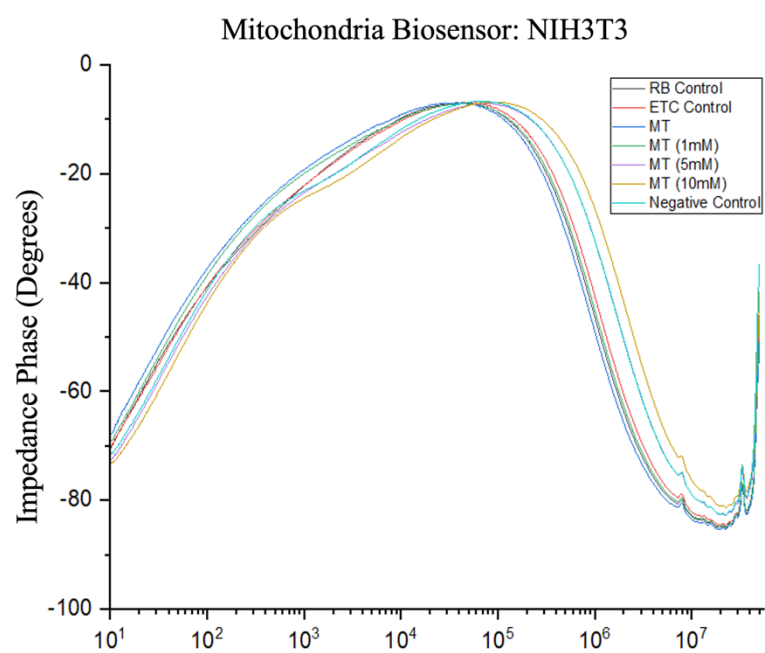
Supplementary Figure 5: Initial mitochondrial biosensor EIS magnitude/phase characterization. (n = 9) Frequency ranges from 10Hz-10MHz.



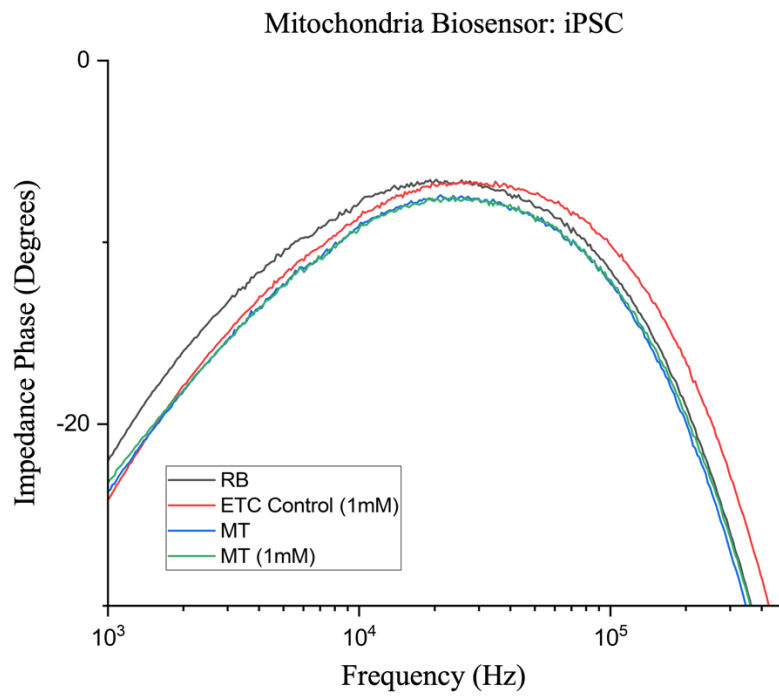
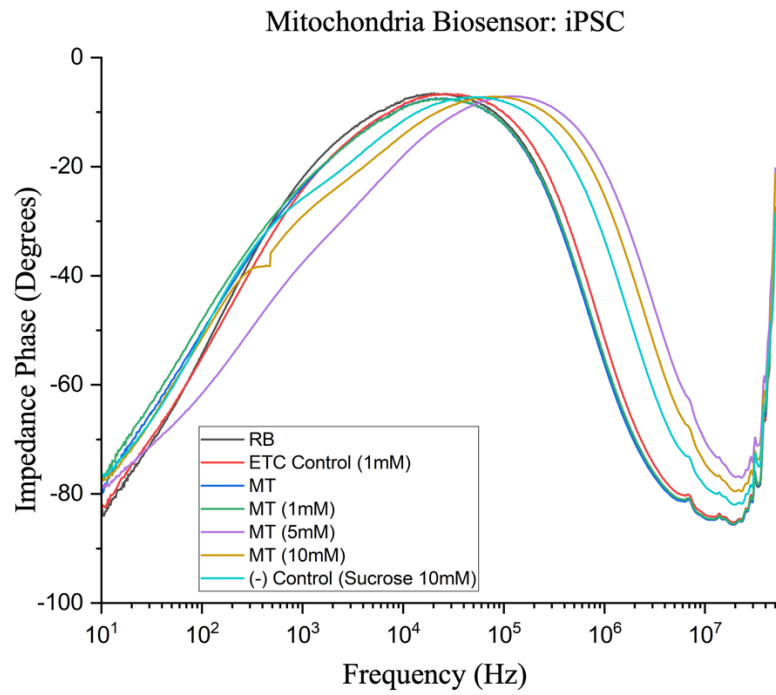
Supplementary Figure 6: EIS Impedance magnitude characterization of mitochondrial biosensor electrodes ($n = 9$) for NIH3T3 cell line. The buffer conditions were adjusted closer to physiological conditions. Full frequency ranges from 10Hz-50MHz.



Supplementary Figure 7: EIS impedance magnitude characterization of mitochondrial biosensor electrodes ($n = 9$) for the iPSC model. The buffer conditions were adjusted closer to physiological conditions. Full frequency ranges from 10Hz-50MHz.



Supplementary Figure 8: EIS impedance phase characterization of mitochondrial biosensor electrodes ($n = 9$) for NIH3T3 cell line, the buffer conditions were adjusted closer to physiological conditions. Full frequency ranges from 10Hz-50MHz.



Supplementary Figure 9: EIS impedance phase comparison of mitochondrial biosensor electrodes ($n = 9$) for NIH3T3 cell lines and the iPSC model. The buffer conditions were adjusted closer to physiological conditions. Full frequency ranges from 10Hz-50MHz.

Supplementary Table 5. pH measurements of the buffer condition as the beginning and end of the EIS experiment for the NIH3T3 cell line.

Respiration Buffer	Initial pH (ETC Control 1mM)	Final pH (MT 10mM)
7.46	7.28	5.58

Supplementary Table 6. pH measurements of the buffer condition as the beginning and end of the EIS experiment for the iPSC model.

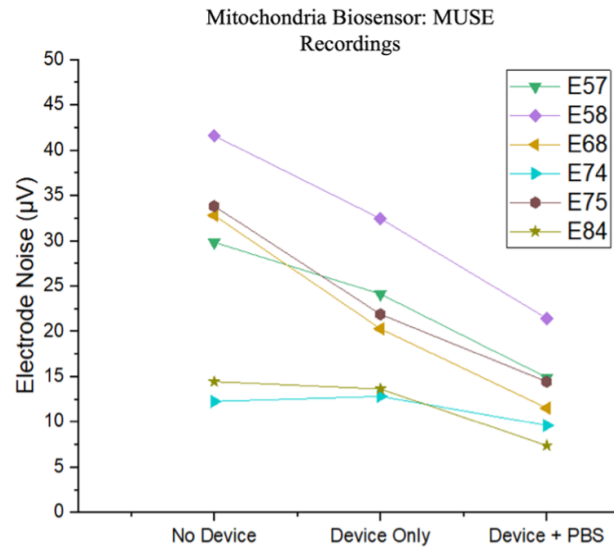
Respiration Buffer	Initial pH (ETC Control 1mM)	Final pH (MT 10mM)
7.46	7.33	5.48

Supplementary Table 7: Percent change calculations for MT and MT 1mM conditions at selected frequency points over throughout the impedance spectra.

Magnitude Frequency: NIH3T3	MT (Ω)	MT 1 mM (Ω)	Δ Mean (Ω)	% Change	Phase Frequency: NIH3T3	MT ($^{\circ}$)	MT 1 mM ($^{\circ}$)	Δ Mean ($^{\circ}$)	% Change
500 Hz	4654.25	4105.75	-458.5	-10.05%	500 Hz	-23.58 $^{\circ}$	-24.14 $^{\circ}$	-0.56	-2.37%
1 kHz	4011.71	3610.75	-400.96	-9.99%	1 kHz	-19.32 $^{\circ}$	-20 $^{\circ}$	-0.68	-3.52%
100 kHz	2642.38	2302.19	-340.19	-12.87%	100 kHz	-9.36 $^{\circ}$	-8.69 $^{\circ}$	0.67	7.16%
1 MHz	224.02	222.76	-1.26	-0.56%	1 MHz	-83.46 $^{\circ}$	-82.68 $^{\circ}$	0.78	0.93%
Magnitude Frequency: iPSC	MT (Ω)	MT 1 mM (Ω)	Δ Mean (Ω)	% Change	Phase Frequency: iPSC	MT ($^{\circ}$)	MT 1 mM ($^{\circ}$)	Δ Mean ($^{\circ}$)	% Change
500 Hz	6184.55	5957.77	-226.78	-3.67%	500 Hz	-30.47 $^{\circ}$	-29.34 $^{\circ}$	1.13	3.71%
1 kHz	5327.53	5141.55	-185.98	-3.49%	1 kHz	-23.7 $^{\circ}$	-23.21 $^{\circ}$	0.49	2.07%
100 kHz	3514.63	3358.28	-156.35	-4.45%	100 kHz	-12.31 $^{\circ}$	-12.08 $^{\circ}$	0.23	1.87%
1 MHz	221.4	220.86	-0.54	-0.24% $^{\circ}$	1 MHz	-84.58 $^{\circ}$	-84.36 $^{\circ}$	0.22	0.26%

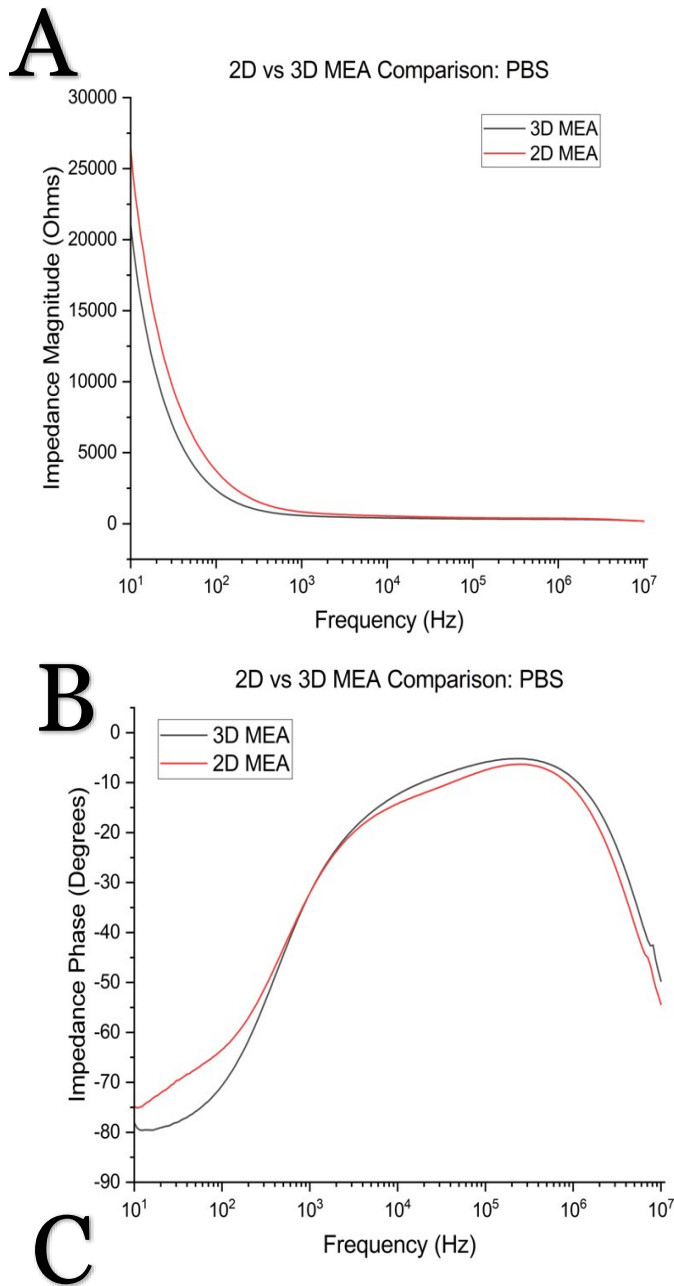
Supplementary Table 8. Comparison of respiration buffer conditions for performing EIS from Professor John H. Miller's group at the University of Houston, Houston, TX vs. buffer conditions from Springer Protocols

Respiration Buffer	Adjusted Respiration Buffer
225mM Mannitol	225mM Mannitol
75mM Sucrose	75mM Sucrose
10mM HCl	25mM Tris HCl
10mM Tris-HCl	1mM MgCl₂
5mM KH₂PO₄	1mM KH₂PO₄
1mM MgCl₂	1mM ADP



Electrode	No Device	Device Only	Device + PBS
E57	29.82µV	24.11µV	14.89µV
E58	41.58µV	32.44µV	21.42µV
E68	32.79µV	20.27µV	11.52µV
E74	12.25µV	12.82µV	9.61µV
E75	33.81µV	21.88µV	14.43µV
E84	14.45µV	13.62µV	7.39µV

Supplementary Figure 10: Axion BioSystems MUSE electrophysiological response of mitochondrial biosensor electrodes with PBS. E74 and E84 attain noise values below 10µV, which is suitable for neural recordings and all electrodes should be suitable for mitochondrial electrophysiology.



Magnitude/Phase Frequency: 1kHz	Magnitude	Phase
3D MEA (PBS)	575.12Ω	-31.78°
2D MEA (PBS)	826.30Ω	-31.8°

Supplementary Figure 11: A) Displays the EIS impedance Magnitude comparison of mitochondrial biosensor electrodes ($n = 9$) of the 2D and 3D MEA configurations. B) Shows the EIS impedance Phase comparison of mitochondrial biosensor electrodes ($n = 9$) of the 2D and 3D MEA configurations. C) Shows the impedance magnitude/phase data at 1kHz.