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Detection of mitochondrial bioenergetics using a novel bimodal 3D microelectrode array (MEA)-based biosensor

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Abstract

Developing new label-free paradigms for functional assays in biomedical research has the potential to catalyze efforts in drug discovery and improve the understanding of complex disorders. Mitochondria are an essential organelle in nearly every eukaryotic organism that perform vital functions such as adenosine triphosphate (ATP) production, redox signaling, reactive oxygen species (ROS) homeostasis and regulation of programmed cell death. These activities are regulated by electrophysiological processes that occur in the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM) in response to metabolic demands, making them an important physiological marker for bioenergetic studies. Mitochondria dysfunction is an early pathological biomarker of complex diseases, such as diabetes, neurodegeneration, myopathy, cancer, and cardiovascular disease. Built atop a novel microfabrication strategy for 3D Microelectrode Arrays (MEAs), we demonstrate a 3D mitochondria biosensor capable of bimodal sensing of mitochondrial electrophysiology from the OMM and IMM using electrochemical impedance spectroscopy (EIS) and electrophysiology recordings. Data obtained using EIS displays impedance magnitude and phase characterization of mitochondria isolated from NIH3T3 and induced pluripotent stem cells (iPSC) models, these measurements represent the major functional outputs of cellular respiration and electron transport chain (ETC) activity through the detection of conductive and capacitive properties of the IMM. Additionally, time-resolved electrophysiological recordings from an NIH3T3 derived mitochondrial pellet captured sub-millisecond voltage transients, establishing a complementary real-time electrophysiological profile of mitochondrial membrane activity that can be attributed voltage dependent anion channel (VDAC) gating or IMM potential dynamics.

Introduction

Advancements in healthcare and medicine have extended the lifespan of the human population. While these advancements have changed how we live for the better, aging increases the risk of developing several disorders such as neurodegenerative diseases¹, diabetes², cardiovascular disease³, and cancer⁴. A common factor in these diseases is mitochondrial dysfunction^{1,4,5}. Mitochondria

are an essential organelle that play a key role in maintaining homeostasis including energy production, calcium storage, redox signaling, and regulation of apoptosis⁶. Developing methods for assaying mitochondria function can improve the understanding of the bioenergetics of aging and provide a deeper insight into pathological changes throughout disease progression.

Bioenergetics is a fundamental process of all living organisms that involves cellular respiration where the oxidation of fuel molecules such as glucose produces adenosine triphosphate (ATP), the universal cellular energy currency. Mitochondria serve as a cellular battery storing the energy derived from energetically favorable redox reactions in the electron transport chain as a pool of protons in their intermembranous space (IMS). The

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stored charge from protons in the IMS is reflected as a voltage across the inner mitochondrial membrane (IMM). Enzymes embedded in the IMM form super complexes which are responsible for oxidizing electron donors generated from the Krebs cycle⁷. Individual subunits, Complex I and Complex II, oxidize unique substrates such as nicotinamide adenine dinucleotide (NADH) and succinate, respectively. In addition to its oxidative function, Complex I concomitantly shuttle protons across the IMM along with Complex III and Complex IV to create a large proton gradient. The outer mitochondrial membrane (OMM) also contains a high density of voltage dependent anion channels (VDACs)⁸. VDACs regulate apoptosis as well as metabolite flux through the OMM such as ATP, peptides, small molecules and metabolites between the cytosol and IMS. Gating of VDACs have been demonstrated to take a closed conformation at electric potentials around 50 mV and an open conformation at 0 mV⁸. Both the IMM potential and VDAC gating status of the OMM can serve as significant physiological markers for assaying mitochondrial dysfunction in complex disorders^{9,10}. Methods for assaying such membrane potentials or electrophysiological phenomena traditionally involve voltage or patch clamp techniques^{11–13}, fluorescent dyes^{14–17}, ion-sensitive electrodes in a microfluidic on-chip application¹⁸, or electrochemical impedance spectroscopy (EIS)^{19,20} in microfluidic flow cells. Traditional voltage clamp or patch clamp approaches are technically challenging and rely on clamping single mitochondria, stripping the outer membrane or reconstitution of mitochondrial proteins in a lipid membrane. Interfacing directly with mitochondria is also very challenging because they do not adhere to extracellular membrane components and are on the scale of 0.5 μm to 3.0 μm in diameter²¹. Studies performed using fluorescent probes report IMM potentials ranging from 108 to 158 mV depending on metabolic demands, and a potential of 139 mV at rest²². However, these measurements are still dependent on indirect metrics and kinetic models derived from the Nernst equation²³.

While several methodologies have been developed for detecting mitochondrial energy metabolism²⁴, EIS approaches are reliable and advantageous because they are non-invasive as EIS only relies on placing an electrode directly in the solution containing the sample of interest which allows for the real-time detection of electrochemical processes in the inner mitochondrial membrane with high specificity. EIS has been previously demonstrated the ability to characterize electrical signatures from mitochondria as well as identifying deficits in function using resuspended mitochondria fractions^{19,20}. The charge transfer process from the chain of redox reactions and generation of a proton gradient across the

IMM can be monitored by changes in impedance magnitude and phase. Such measurements of mitochondrial electrophysiology and EIS provide unique opportunities for 3D polymer Bio-Microelectromechanical Systems (BioMEMS) devices which can be developed to overcome the hurdle of interfacing directly with singular mitochondria for electrophysiology while providing bimodal sensing capabilities (EIS and electrophysiology) in a single platform. A device demonstrating the capability to achieve both sensing modalities has not yet been demonstrated. Existing mitochondrial isolation protocols can also be modified to obtain a 3D mitochondrial pellet which can be directly placed atop a 3D Microelectrode Array (MEA). The use of 3D microelectrodes to provide an optimal interface with pelleted mitochondria expands on the traditional 2D approach for electrophysiological measurements while exploring new applications for 3D MEA technology²⁵. Unlike adherent cells or monolayer cultures, mitochondrial pellets formed by differential centrifugation have heterogeneous mitochondrial densities throughout their volume, depending on isolation yield, purity, and compaction. A significant fraction of functional mitochondria can reside away from the planar pellet electrode interface. The 3D MEA introduces electrodes that extend into the pellet volume, enabling direct electrical interaction with mitochondria distributed throughout the pellet.

Our group has previously demonstrated the ability to rapidly microfabricate and characterize 3D microelectrode arrays, providing a versatile platform for advanced biosensor applications²⁶. Building on this foundation, we present a hybrid microfabricated/makerspace-driven approach utilizing resin-based 3D printing, selective laser micromachining, and simple insulation strategies to create highly customizable in vitro 3D MEA-based biosensor platform. In this paper, we report the development of an integrated 3D mitochondria biosensing platform designed for the novel application of bimodal electrical characterization of pelleted mitochondria. The device supports electrochemical impedance spectroscopy (EIS) measurements to probe functional outputs of the electron transport chain, while the same 3D microelectrode architecture provides sufficient sensitivity to acquire electrophysiological signals from the mitochondrial pellet. While preliminary data was previously reported at Hilton Head Workshop 2024²⁷, we have expanded on those findings through improvements in the EIS mitochondrial characterization assay comparing NIH3T3 and induced pluripotent stem cell (iPSC) cells models, functional imaging via TMRM fluorescent microscopy displaying mitochondria with an active membrane potential, and electrophysiology recordings from a mitochondrial pellet derived from an NIH3T3 cell model.

Results and discussion

3D Micro-tower printing development

Rapid microfabrication and prototyping of devices for biomedical research is attainable with Computer Aided Design (CAD) software, a scalable design and a well-defined microfabrication process for realizing the necessary structural and functional features of the device. Figure 1a–h depict a schematic of the entire microfabrication process flow and Fig. 2a–f display SEM and optical images

of the 3D printed micro-towers on test chips throughout the optimization process. Micro-tower spacing of 100 μm (Fig. 2a, b) and 200 μm (Fig. 2c–f) as well as heights of 250 μm (Fig. 2b, d, f) and 500 μm (Fig. 2a, c, e) were tested while maintaining a fixed diameter of 70 μm for the micro-towers. At 100 μm spacing, fusion of micro-towers was observed which may be a result of resin being accumulated and confined within a small area with a low exposure time between layers. This low exposure time

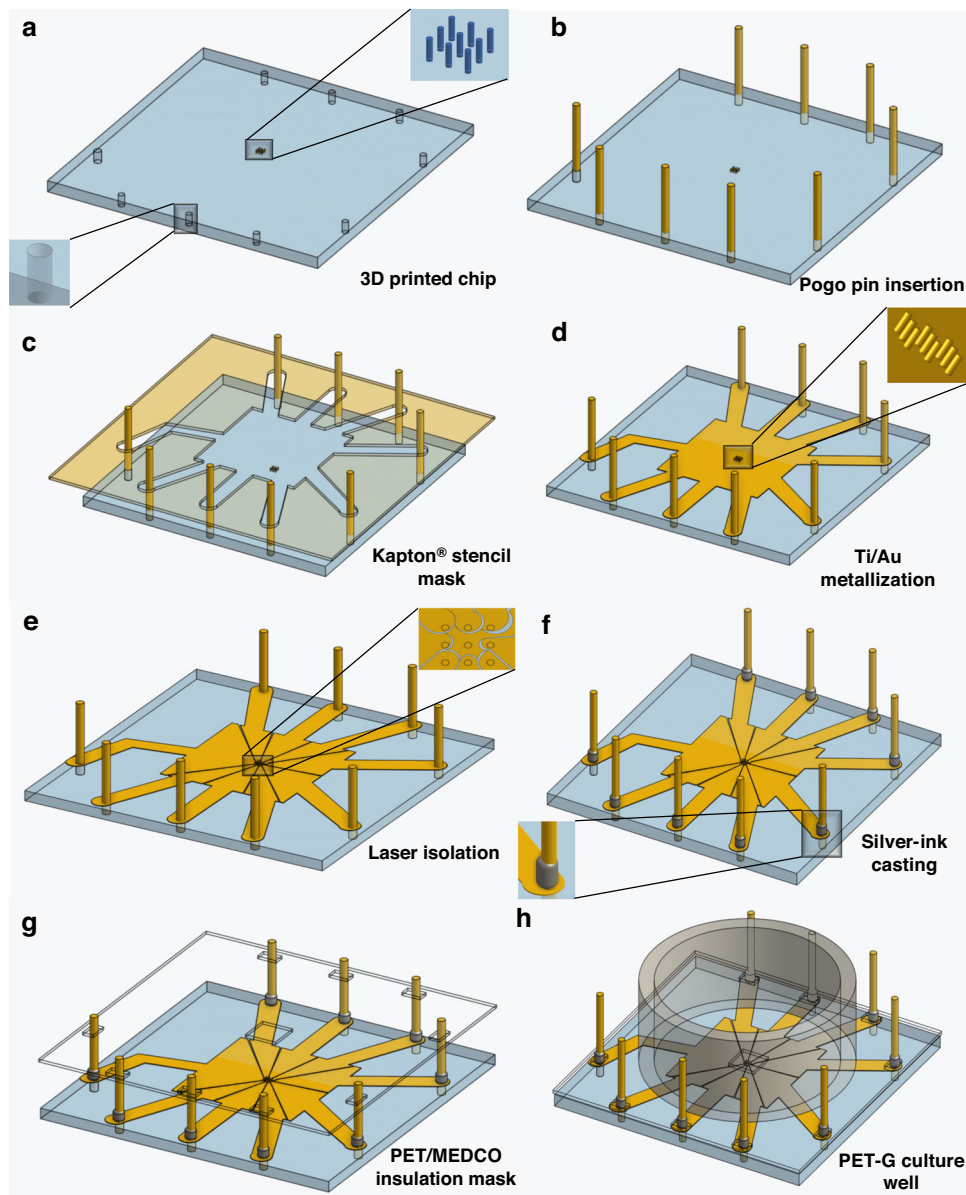


Fig. 1 3D microfabrication process flow **a** 3D printed chip containing a 3×3 array of micro-towers and through vias. **b** Gold pogo-pins inserted into through vias to interact with Bode Impedance Analyzer and Axion MUSE electrophysiological system. **c** Attachment of adhesive Kapton® stencil mask. **d** Thin film deposition of 100 nm Titanium (Ti) and 250 nm Gold (Au). **e** Laser ablation of the metallized surface. **f** Silver-ink casting from metallized surface to pogo-pin interconnect. **g** PET Insulation layer adhered to the metallized surface. **h** PET-G Culture well cured onto the surface via PDMS

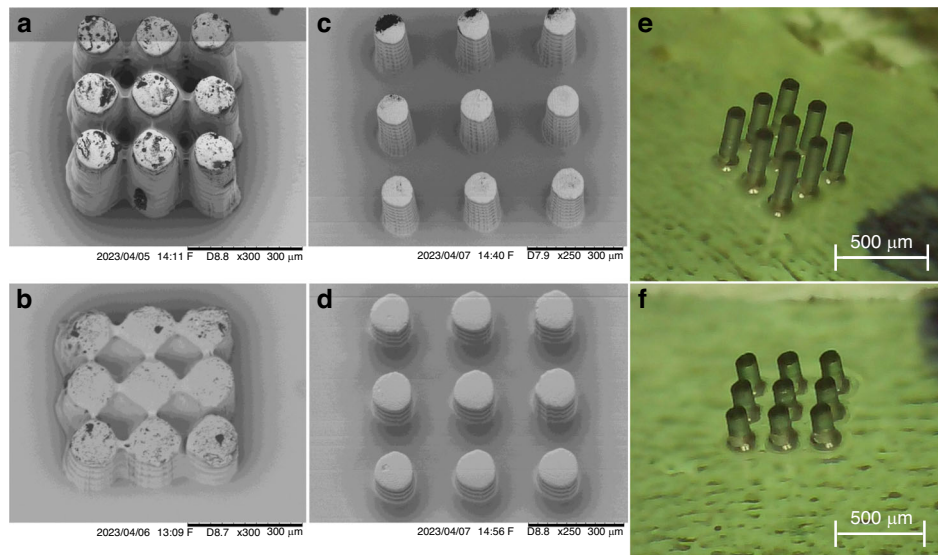


Fig. 2 Micro-tower 3D printing parameter optimization **a** SEM images of 3D printed micro-towers during the optimization process (Height: 500 μm , Spacing: 100 μm , Diameter: 70 μm). **b** Fusion of micro-towers was also observed at a height of 250 μm , while maintaining a diameter of 70 μm and spacing of 100 μm . **c, d** Increasing the spacing distance from 100 μm to 200 μm prevented micro-tower fusion at both 500 μm and 250 μm . **e** Optical image of the 3D printed micro-towers (Height: 500 μm , Spacing: 200 μm , Diameter: 70 μm). **f** Optical image of 3D printed micro-towers (Height: 250 μm , Spacing: 200 μm , Diameter: 70 μm)

and insufficient pitch/spacing was not adequate to allow the resin to completely drain between layers. Increasing the spacing to 200 μm alleviated the fusion of micro-towers and allowed for a well-defined micro-tower array to be produced. Using the ASIGA Composer software, several 3D printing parameters can be tailored to meet the needs for structural integrity of the substrate while obtaining optimal resolution for the desired features. Parameters of interest for obtaining a clean surface and reproducible prints with high fidelity were slice thickness (100–50 μm), exposure time (5–2.5 s), and light intensity (31 mW/cm^2). After printing each chip, post-processing steps were performed to wash off residual resin for a clean surface with 70% Isopropyl alcohol (IPA) bath for 5 min, and a 15-min UV cure at 60 $^{\circ}\text{C}$ to achieve full mechanical integrity of the resin chip. After further analysis, the 250 μm height array with a base diameter of 70 μm and a spacing of 235 μm configuration was chosen for increased spatiotemporal resolution and higher signal-to-noise (SNR). The described 3D microelectrode array, with a 250 μm height, 70 μm base diameter, and 235 μm spacing, offers improved spatiotemporal resolution and signal-to-noise ratio (SNR) compared to standard commercial MEAs. Companies such as Multi Channel Systems²⁸, Microprobes for Life Science²⁹, and PalmSens³⁰ offer MEAs with planar electrode diameters typically ranging from 10 to 30 μm (occasionally as small as 1–6 μm) and interelectrode spacing between 100 and 500 μm . In contrast, the described configuration is optimized for

mitochondrial pellet penetration and incorporates added height for enhanced interaction with 3D cell cultures or organoids, while its compact spacing balances resolution and minimizes signal cross-talk, making it particularly suited for complex in vitro applications³¹.

Multiparameter 3D printing for micro-towers, micro-vias and 3D MEA microfabrication

In addition to the micro-towers (Fig. 3a), micro, through-via channels were also incorporated into the design to create a monolithic chip that can transduce electrophysiological signals from the mitochondria to the micro-towers, along the conductive surface and finally to the pogo pins inserted into the through-vias which interface with the hardware systems for obtaining electrophysiological recordings. Micro, through-via channels (Fig. 3b) were designed with a diameter of 720 μm to securely fit the pogo pin (Diameter: 680 μm) and account for shrinking of the substrate during the post-processing steps. A multiparameter 3D printing process was developed to successfully attain both positive relief (micro-towers) and negative-relief (micro, through-via) features as designed in SolidWorks. The ASIGA Composer software allows for variation of parameters at specified layer slices during the same 3D printing session. After optimization the substrate portion of the chip containing the through vias had parameters of slice thickness: 100 μm , exposure time: 10 s, and a light intensity of 31 mW/cm^2 . On the other hand, the micro-tower portion had

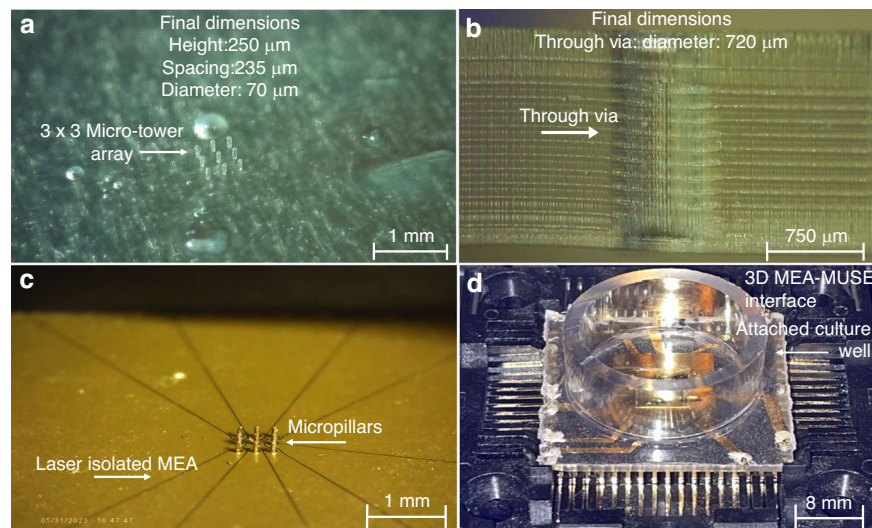


Fig. 3 Optical images of the mitochondria biosensor throughout different stages of the microfabrication process. **a** The final configuration of the micro-towers after the multiparameter 3D printing process. **b** Side profile of through via feature on the same 3D printed chip after multiparameter 3D printing process. **c** Optical image of a laser isolated MEA chip after Ti/Au metallization. **d** Display of the fully fabricated mitochondria biosensor interfacing with the Axion MUSE electrophysiology recording system

parameters of slice thickness: 50 μm , exposure time 2.5 s, and a light intensity of 5 mW/cm^2 . After successful optimization of the 3D printing process, traditional micro-fabrication techniques were employed to deposit conductive metals on the surface of the chip to make functional electrodes and conductive traces to each through via. Full functionalization of the device was achieved after completion of a two-step metallization process involving electron beam deposition of 100 nm titanium (Ti) and 250 nm gold (Au) followed by silver ink casting. Laser ablation of the metallized surface was performed to attain defined conductive areas for each micro-tower (Fig. 3c) followed by the adherence of a polyethylene terephthalate (PET) insulation layer with a $2.5 \times 2.5 \text{ mm}^2$ square area cut out to define a suitable recording area. Lastly, the attachment of a polyethylene terephthalate glycol (PET-G) culture well (Fig. 3d) is required to confine the mitochondria samples during electrical recordings. The finalized microfabrication scheme resulted in a 4-day microfabrication process (Table S1). Upon final completion of the biosensor, characterization of the 3D printed structures was performed via SEM and laser confocal microscopy followed by testing of electrode behavior to determine the optimal surface area for electrochemical impedance spectroscopy (EIS) and electrophysiology recordings (Figs. S1, S2). While a $10 \times 10 \text{ mm}^2$ and $5 \times 5 \text{ mm}^2$ surface areas were both tested, the $10 \times 10 \text{ mm}^2$ configuration displayed lower impedance. The greater surface area may contribute to higher background noise and reduced sensitivity while performing electrophysiological recordings. For the

experimental conditions pursued in this paper, the $5 \times 5 \text{ mm}^2$ configuration was chosen for mitochondrial characterization.

SEM and laser confocal microscopy of micro-tower metallization

Figure 4a depicts an SEM image of the metallized micro-towers to form functional microelectrodes. Figure 4b displays an SEM image of a laser isolated array of microelectrodes with a PET insulation layer adhered. Laser confocal microscopy was utilized to obtain measurements of laser ablation spot size (Fig. 4c), $\sim 25 \mu\text{m}$, as well as micro-tower height and diameter (Fig. 4d) across 27 individual micro-towers ($n = 27$) on 3 different test chips (Tables S2–S4). Micro-tower measurements recorded an average height of 231.91 μm ($\pm 6.7 \mu\text{m}$), average top diameter of micro-tower 56.55 μm ($\pm 7.0 \mu\text{m}$), and an average bottom diameter of 68.36 μm ($\pm 5.9 \mu\text{m}$). These numbers demonstrate remarkable repeatability for the developed microfabrication process. The minor variation from design values could emanate from inconsistent pixel printing in DLP³² as well as shrinking of the resins³³.

Mitochondrial Isolation and Tetramethyl Rhodamine Methyl Ester (TMRM) Imaging

Mitochondria can be derived from most mammalian cell types. Depending on the cell type there are ~ 1000 – 2500 mitochondria per cell to maintain the constant production of ATP to meet their energetic demands. On average a human cell produces $\sim 10^7$ – 10^8 ATP molecules per second³⁴. The NIH3T3 cell line was chosen

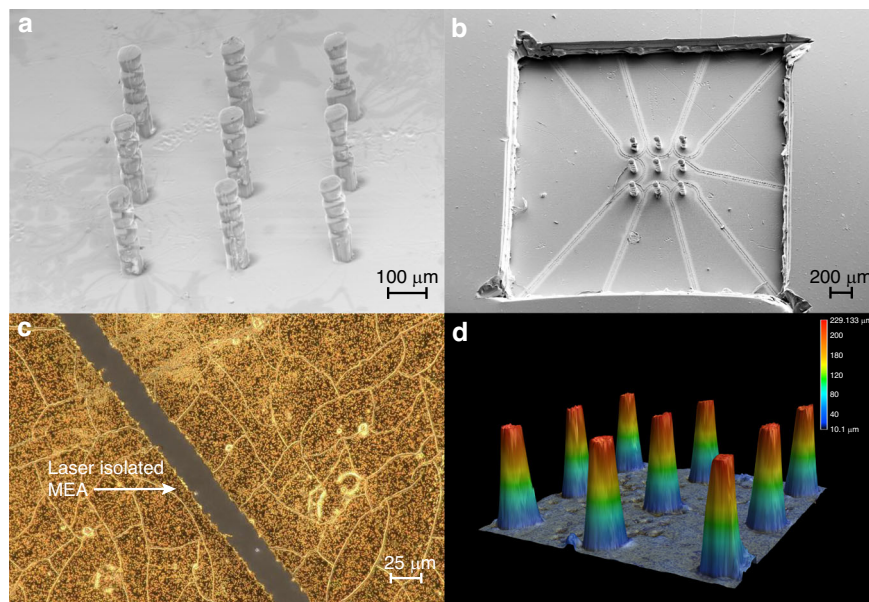


Fig. 4 Physical microelectrode array characterization via SEM and Laser Confocal Microscopy **a** SEM image of a 3×3 micro-tower array post Ti/Au metallization. **b** SEM image of a laser-isolated 3×3 micro-tower array with a polyethylene terephthalate (PET) insulation layer adhered to the surface, central cut out area of $2.5 \times 2.5 \text{ mm}^2$. **c** Laser confocal image of a laser trace on the metallized surface indicating a spot size of $\sim 25 \text{ }\mu\text{m}$. **d** Laser confocal microscopy side profile of a metallized 3×3 micro-tower array with a height of $229.13 \text{ }\mu\text{m}$

for initial characterization due to their robust growth properties as they display rapid proliferation with standard media and cell culture conditions making them efficient and adaptable for developing biological assays³⁵ (Figure S3A). iPSC models were subsequently utilized to provide a more physiologically relevant model compared to the mouse embryonic fibroblast NIH3T3 cell line while boasting similar growth properties (Figure S3B). The mitochondrial isolation process began with harvesting the cells from three T75 flasks and pooling the entire cell population in a 2 mL centrifuge tube. The differential centrifugation steps were followed as described in the mitochondrial isolation protocol in the Materials and Methods section. After performing the centrifugation steps, the supernatant was discarded, and the mitochondrial fraction was stored on ice.

Validation of isolated mitochondria was performed with a pelleted fraction obtained from an NIH3T3 cell population of $\sim 9,217,500$. The pelleted fraction was large enough to cover the diameter of a 2 mL centrifuge tubes which is $\sim 10 \text{ mm}$ (Figure S4) and fully envelope the open 3D microelectrode array (MEA) recording areas, $2.5 \times 2.5 \text{ mm}^2$ (Fig. 5a, b). To confirm that the electrochemical impedance spectroscopy (EIS) signatures are emanating from isolated mitochondria, functional imaging was performed using tetramethyl rhodamine methyl ester (TMRM) (Fig. 5c–f). TMRM is a lipophilic cation which allows it to diffuse through membranes and accumulate where there is a net negative charge. The inner

mitochondrial membrane (IMM) potential is generated by an electrochemical gradient of protons that are stored in the intermembranous space (IMS) which results in a negative charge on the side of the inner mitochondrial matrix²³. TMRM protocols are typically performed on whole cell cultures in a culture well where incubation periods of 15–30 min are necessary to allow the dye to diffuse throughout the entire cell. To adapt the imaging process to isolated mitochondria, 1.3 mm glass concavity slides were used to mix TMRM at 1 nM, isolated mitochondria and respiration buffer components in a volume of $100 \text{ }\mu\text{L}$ as described by Pandey et al.³⁶ and imaged via fluorescence microscopy.

The reaction mixture was composed of a respiration buffer to support enzymatic respiratory activity of mitochondrial proteins using concentrations of respiratory substrates at 1 mM, 5 mM and 10 mM and the mean fluorescence intensity (MFI) was measured from each condition. Figure 5c displays the fluorescence intensity measurements with mitochondria from 1 mM substrate concentration having an MFI of 56.42 (S.D. = 20.64), 5 mM substrate concentration have an MFI of 70.61 (S.D. = 8.81), and 10 mM substrate concentrations had an MFI of 89.15 (S.D. = 10.59), showing signs of an active membrane potential in response to respiratory substrates. Figure 5d shows the 5 mM respiratory condition with an active mitochondrial population under fluorescent signal. Figure 5e shows an active mitochondrial population at the 1 mM respiratory condition at 10x magnification while

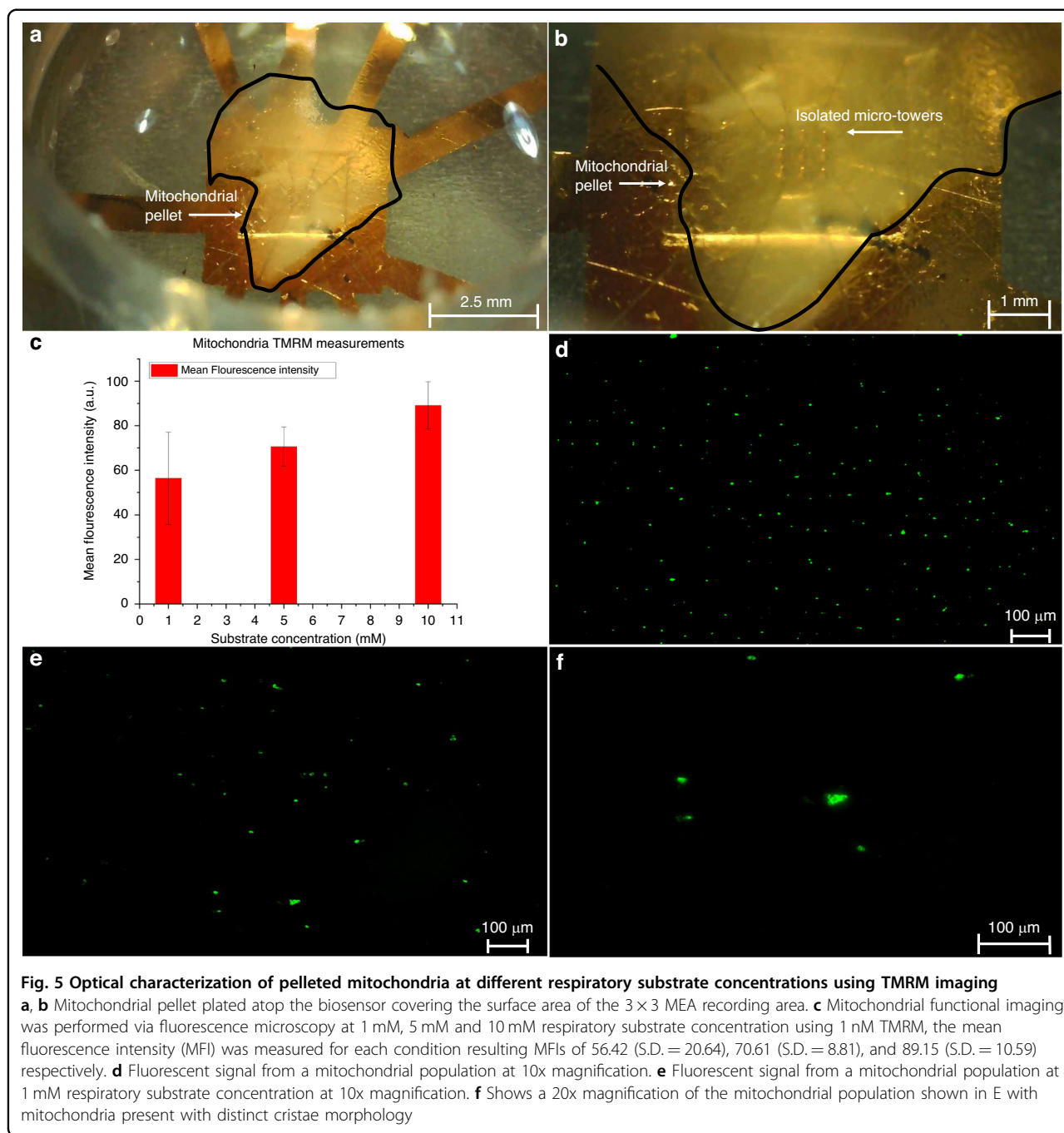


Fig. 5f displays a 20x magnification of the 1 mM mitochondrial population showing an isolated mitochondria with distinct cristae.

Electrochemical Impedance Spectroscopy Characterization of Mitochondria Derived from NIH3T3 and iPSC Models

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is used to assay electrochemical and biological systems^{37–43} by using a broad-spectrum AC current or voltage frequency

sweep (1 Hz–50 MHz) as the input signal to measure changes in the output current or voltage from the sample to determine the overall impedance of the system. Impedance is the AC equivalent of resistance, and it is a combination of all the circuit components that prevent the flow of current such as resistors, capacitors, and inductors (depending on the type of circuit used). Mathematically impedance can be expressed as a complex number (Z) with a real and imaginary part, where the real part (Z') corresponds to resistance while the imaginary

component (Z'') reflects the reactance (coming from other parts of the circuit such as capacitance and inductance) of the electrochemical system as shown in Eq. 1⁴⁴:

$$Z(\omega) = |Z|(\cos \varphi + j \sin \varphi) = Z' + jZ'' \quad (1)$$

Where:

$Z(\omega)$ = The impedance at a specific frequency

$|Z|$ = The magnitude of impedance

$|Z| \cos \varphi$ = Real Impedance or Z'

$|Z| \sin \varphi$ = Imaginary Impedance or Z''

φ = phase difference between the input and output signal.

Using electromagnetic waves to interrogate electrochemical systems can serve as an invaluable noninvasive diagnostic tool for biological systems and samples such as mitochondria. Biological systems including organs, cells, and organelles have dielectric, capacitive, and conductive properties⁴⁵. Dielectrics are insulative materials that do not display conductivity. However, once exposed to an electric field the material polarizes due to the formation of electric dipoles, therefore a material that is easily polarizable can store more electrical energy from the electric field⁴⁶. The dielectric property of biological samples emerges from lipid membranes which separate the electrically conductive extracellular and intracellular fluids. The mobility of bound charges, proteins and free ions in the physiological environment such as cations (H^+ , Na^+ , K^+ , Ca^{2+} , Mg^{2+}) and anions (HCO_3^- , Cl^- , HPO_4^{2-} , SO_4^{2-}) contribute to the polarizability and capacitive properties of electrochemical membranes^{45,46}. Lastly the semi-permeability of lipid membranes and ionic transport mechanisms across the membrane are responsible for the conductive features of biological samples. Each of these properties will display frequency-dependent behavior that can significantly impact the impedance measurements. Conductive properties affect resistance, capacitive properties introduce frequency-dependent reactance and phase shifts, and dielectric properties influence the material's interaction with the electric field. Together, these properties determine the overall impedance, which varies with frequency and is crucial for interpreting bioimpedance data and various medical diagnostic techniques.

NIH3T3 mitochondrial EIS

To assess mitochondria activity in physiological conditions EIS was performed on isolated mitochondria resuspended in respiration buffer with respiratory substrate concentrations of 1 mM. The respiratory substrates included were succinate, glutamate, and malate. Succinate will provide electrons to Complex II of the ETC by directly generating the electron donor flavin adenine dinucleotide ($FADH_2$) while glutamate and malate can

provide electrons to Complex I of the ETC through generation of the electron donor nicotinamide adenosine dinucleotide (NADH)⁷. The substrates were added by mixing stock solutions into the respiration buffer to achieve the desired concentration of 1 mM. Mitochondrial EIS characterization with the respiration buffer displayed trends expected of mitochondrial samples (Fig. 6). Using the respiration buffer as a baseline, the addition of a mitochondrial pellet derived from an NIH3T3 cell population resulted in an increase in the overall impedance magnitude across the spectrum (Fig. 6a, b), which can be attributed to the presence of the double membrane system in mitochondria serving as an insulator and increasing the resistive properties of the solution. The impedance phase also shifted the closest towards -90° (Fig. 6c, d) in the mid to high frequency range, which can be observed at ~ 100 kHz indicating the most capacitive behavior of -9.36° (Table 1) at that frequency point.

This behavior in impedance phase is also expected because as the frequency increases the membranes become more permeable to the AC voltage frequency which is better able to penetrate through⁴⁷. While the mitochondria are still inactive without the metabolic substrates the inner and outer membrane have capacitive properties, phase shifts closer to -90° , due to the presence of free charges around the inner mitochondrial membrane and outer mitochondrial membrane⁴⁸. The OMM is permeable to small ions and molecules allowing for ions to accumulate on either membrane⁴⁹. Once the mitochondria are in the presence of glutamate, malate and succinate (MT 1 mM), they can generate electron donors which can begin ETC activity allowing for the transfer of protons across the IMM allowing for a larger movement of free ions, increasing the resistive properties of the solution, resulting in impedance phase shifts closer to 0° . The movement of free ions increases the conductivity which can be reflected in the decrease of the impedance magnitude from 2642.38Ω to 2302.1Ω (Table 1) while also reducing the capacitive reactance leading towards more resistive properties of the membrane shown in the phase shift from -9.36° to -8.69° (Table 1) when comparing MT and MT (1 mM) samples. The ETC control served as a conductive model with 1 mM of each substrate in the absence of mitochondria which showed the lowest magnitude impedance for 100 kHz at 2101.88Ω and the least capacitive reactance with a phase of -8.3° . The pH of the 1 mM ETC control and the final solution containing the mitochondria after the experiment were recorded. The 1 mM control had a pH of 7.28, which was in physiological range (Table S5).

iPSC mitochondrial EIS

The mitochondrial EIS characterization was repeated with an iPSC cellular model. Observing similar trends but

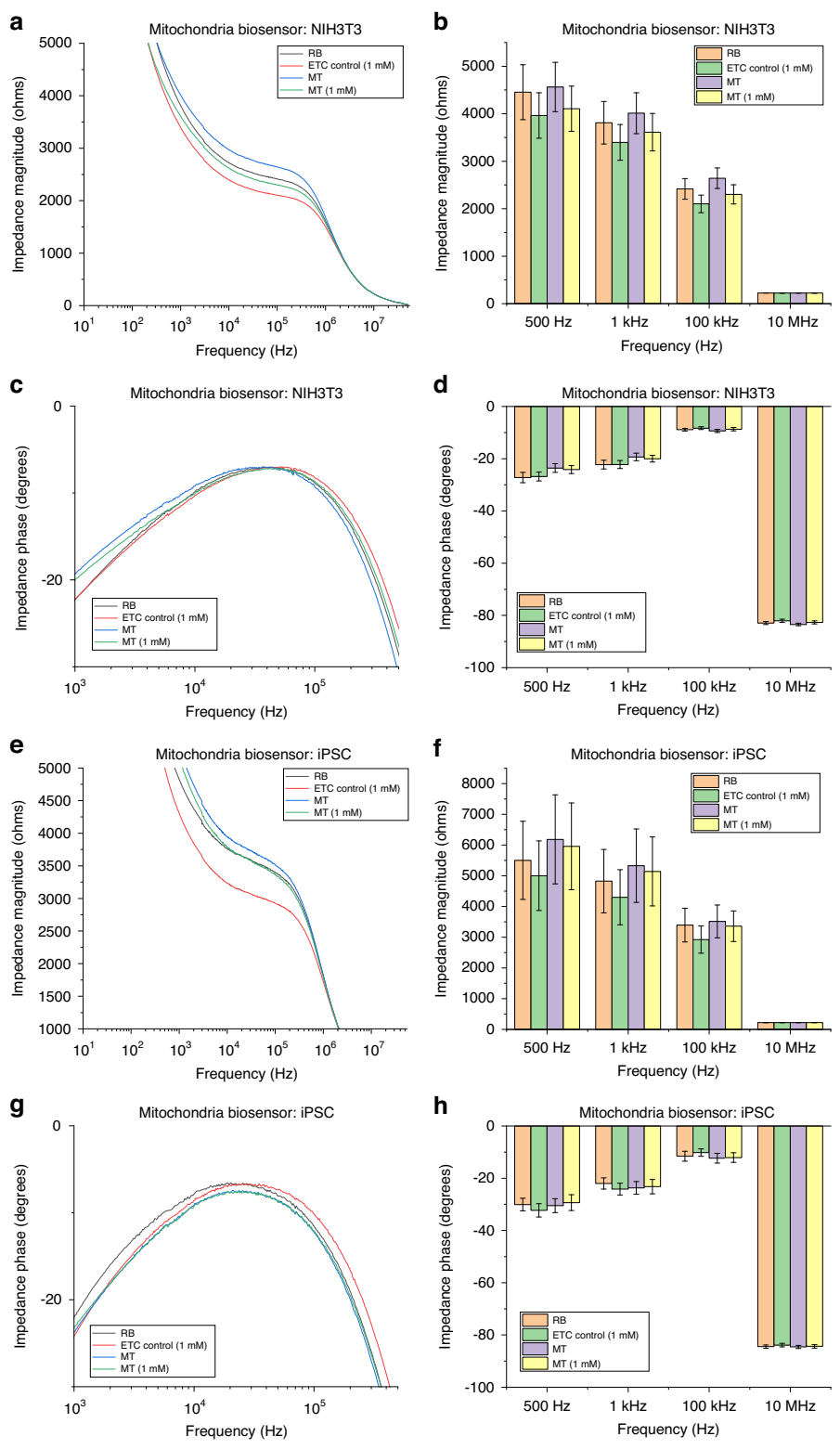


Fig. 6 (See legend on next page.)

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Fig. 6 Electrochemical impedance spectroscopy (EIS) magnitude and phase comparison of the mitochondrial biosensor across NIH3T3 and iPSC-derived mitochondrial models under respiratory metabolic conditions. **a** Continuous impedance magnitude spectra (10 Hz–50 MHz) for NIH3T3 mitochondria under RB, ETC control, MT, and MT (1 mM) conditions ($n = 9$ electrodes). **b** Discrete frequency impedance magnitude comparison for NIH3T3 at 500 Hz, 1 kHz, 100 kHz, and 10 MHz with standard deviation error bars. **c** Continuous impedance phase spectra for NIH3T3 across the full frequency range, demonstrating frequency-dependent capacitive behavior of mitochondrial membranes. **d** Discrete frequency phase comparison for NIH3T3 at selected frequencies (500 Hz–10 MHz). **e** Continuous impedance magnitude spectra for iPSC-derived mitochondrial pellets (10 Hz–50 MHz) under identical experimental conditions ($n = 9$ electrodes). **f** Discrete frequency impedance magnitude comparison for iPSC mitochondria at 500 Hz, 1 kHz, 100 kHz, and 10 MHz with standard deviation. **g** Continuous impedance phase spectra for iPSC mitochondria showing phase shifts associated with frequency-dependent capacitive behavior. **h** Discrete frequency phase comparison for iPSC mitochondria across selected frequencies. Collectively, the spectra demonstrate condition-dependent impedance shifts consistent with mitochondrial metabolic state and membrane integrity

Table 1 Shows a comparison of the quantitative values of the mitochondrial EIS characterization for NIH3T3 and iPSC models

Magnitude/Phase Frequency: NIH3T3	500 Hz	1 kHz	100 kHz	10 MHz	Magnitude/Phase Frequency: iPSC	500 Hz	1 kHz	100 kHz	10 MHz
RB	4453.97Ω/ -27.20°	3809.31Ω/ -22.26°	2416.66Ω/ -8.88°	224.04Ω/ -82.94°	RB	5501.22Ω/ -30.08°	4826.65Ω/ -22.0°	3395.38Ω/ -11.56°	222.02Ω/ -84.47°
ETC Control	3963.33Ω/ -26.81°	3396.57Ω/ -22.23°	2101.88Ω/ -8.3°	223.13Ω/ -82.09°	ETC Control	5001.13Ω/ -32.30°	4299.43Ω/ -24.17°	2924.76Ω/ -10.18°	221.31Ω/ -83.87°
MT	4564.25Ω/ -23.58°	4011.71Ω/ -19.32°	2642.38Ω/ -9.36°	224.0Ω/ -83.46°	MT	6184.55Ω/ -30.47°	5327.53Ω/ -23.70°	3514.63Ω/ -12.31°	221.40Ω/ -84.58°
MT (1mM)	4105.75Ω/ -24.14°	3610.75Ω/ -20.0°	2302.1Ω/ -8.69°	222.76Ω/ -82.68°	MT (1mM)	5957.77Ω/ -29.34°	5141.55Ω/ -23.21°	3358.25Ω/ -12.08°	220.86Ω/ -84.36°
Standard Deviation: RB	±579.14/ ±1.98	±447.95/ ±1.66	±218.04/ ±0.50	±1.51/ ±0.57	Standard Deviation: RB	±1271.57/ ±2.40	±1031.37/ ±2.17	±548.25/ ±1.88	±1.35/ ±0.65
Standard Deviation: ETC Control	±478.01/ ±1.68	±376.84/ ±1.54	±184.56/ ±0.55	±1.24/ ±0.63	Standard Deviation: ETC Control	±1131.10/ ±2.56	±895.90/ ±2.27	±443.82/ ±1.41	±1.32/ ±0.67
Standard Deviation: MT	±520.28/ ±1.67	±430.73/ ±1.45	±216.25/ ±0.61	±1.14/ ±0.49	Standard Deviation: MT	±1448.44/ ±2.67	±1195.16/ ±2.42	±535.04/ ±1.87	±1.15/ ±0.60
Standard Deviation: MT (1mM)	±475.83/ ±1.54	±395.51/ ±1.29	±203.22/ ±0.65	±1.06/ ±0.60	Standard Deviation: MT (1mM)	±1411.55/ ±3.03	±1121.85/ ±2.74	±499.11/ ±1.86	±1.43/ ±0.63

Two different devices were used for each experiment, The respiration buffer (RB) serves as a baseline for electrochemical behavior (yellow) across both cell models. Similar trends can be identified between both models; mitochondrial membranes increase impedance due to their insulative properties (Red). While respiratory substrates are weakly acidic and may trigger conductive processes within the mitochondria, decreasing impedance (Green). Inert mitochondria will have more capacitive behavior, pushing the phase closer to -90° (Red). While active mitochondria generate protons leading to more resistive behavior closer to 0° (Green). The 100 kHz and 10 MHz frequency points are highlighted as the electromagnetic signal is more permeable to the outer membrane at higher frequencies allowing for detection of respiratory activity in the inner membrane. Standard deviations for both magnitude and phase are reported to reflect electrode-to-electrode variability and device reproducibility across the two biosensor configurations

dissimilar values from a mitochondrial pellet derived from iPSC cells allows for electrophysiological assessment of more physiologically relevant models (Fig. 6e–h). As observed with NIH3T3 cells, the addition of the mitochondrial pellet increased the impedance magnitude across the spectrum and induced a capacitive phase

response from the respiration buffer baseline, 3395.38 Ω/ -11.56° to 3514.63 Ω/ -12.31° (Table 1). The addition of 1 mM of metabolic substrates also resulted in a decrease in the impedance magnitude and a slight phase shift towards more resistive behavior as the frequency increases from 1 kHz to 100 kHz (3358.25 Ω/ -12.08°), which is

consistent with the AC voltage frequency penetrating through the membrane. Differences in mitochondrial pellet density, integrity, and composition may have contributed to the higher impedance magnitude above the respiration buffer at lower frequencies as well as the more subtle phase shift in the high frequency range compared to the NIH3T3 cell line. The 1 mM ETC control without mitochondria stayed consistent as a conductive model compared to the MT (1 mM). The 1 mM ETC control pH was 7.33 (Table S6), indicating a physiological environment. The MT (1 mM) condition was viable for physiological conditions in both experimental conditions and allowed for a direct comparison of the mitochondrial EIS characterization between NIH3T3 and iPSC models (Table 1).

While mitochondrial responses to higher concentrations of ETC substrates were assayed (Figs. S5–S9), addition of solutes to reach the 5 mM and 10 mM exceeded the buffering capacity leading to pH values outside of physiological ranges (Table S5 and S6). Further statistical analysis of micro-electrode performance revealed low electrode-to-electrode variability across the 3D MEA (Table 1), with standard deviations that decreased at higher frequencies and remained below reported benchmarks for conventional mitochondrial assays^{50,51}. This consistency reflects strong device-sample coupling and supports the platform's capacity for reproducible signal acquisition. Ongoing refinements, including electrode miniaturization and surface equalization, are expected to further reduce variance and enhance specificity. Mid-frequency impedance of ~10–300 kHz reflects composite mitochondrial membrane dielectric properties modulated by ETC activity with 100 kHz demonstrating peak sensitivity towards ETC and mitochondrial membrane dielectric modulation (Table S7), whereas high-frequency >1 MHz measurements, while still following expected trends, display less sensitivity and may be increasingly dominated by bulk electrolyte and device parasitics rather than organelle-specific membrane contributions.

Preliminary electrophysiological recordings

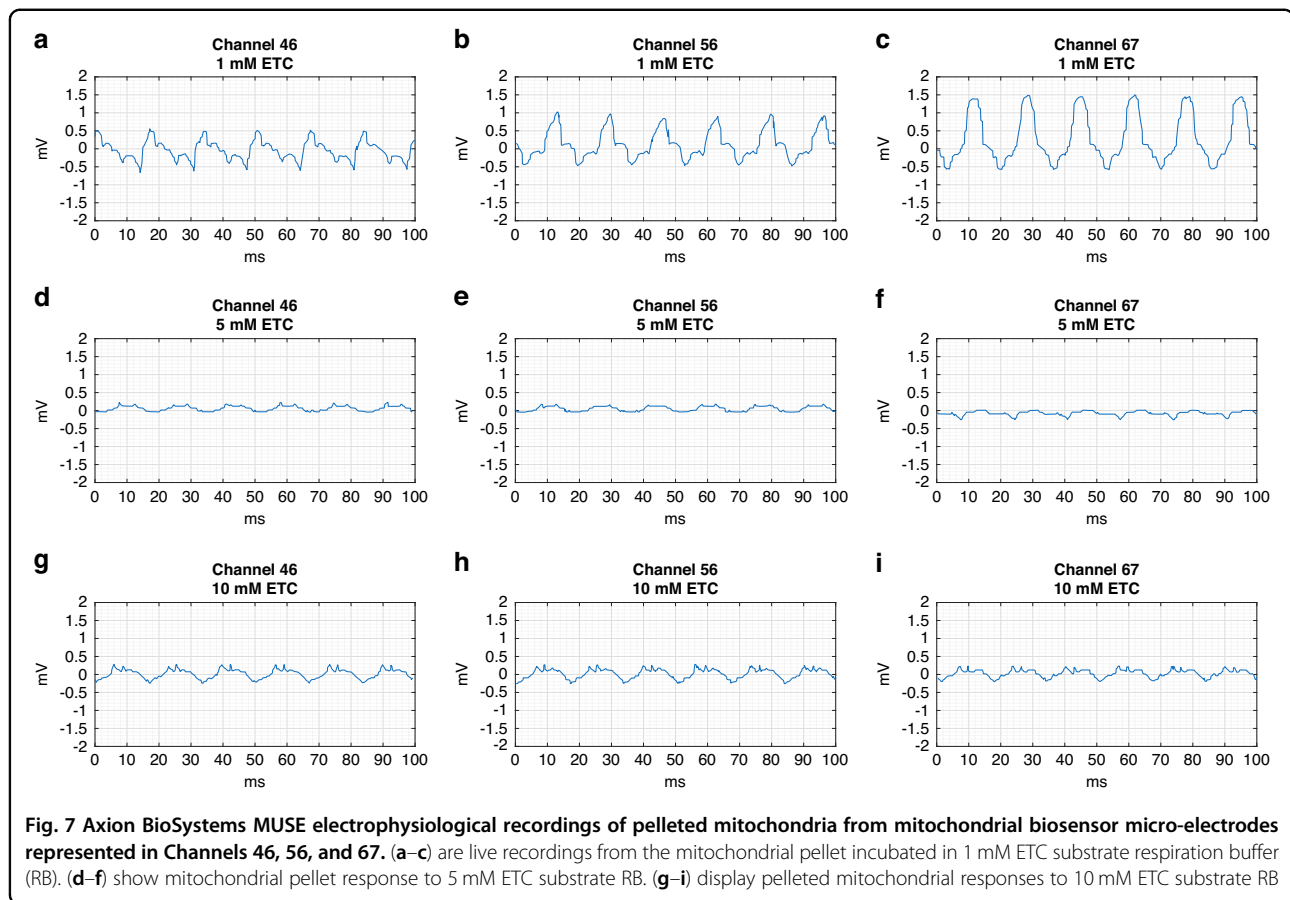
Preliminary electrophysiology recordings were performed to observe the changes in μV noise values of the 3D microelectrodes. Baseline noise recordings (air) were obtained in the absence of the biosensor. The biosensor was then placed in the housing chamber of the Axion BioSystems MUSE recording system allowing for a conductive circuit to be created and subsequently causing a drop in the μV noise signatures. Phosphate buffered saline (PBS) was added as an electrolyte solution allowing for the conductive medium to complete the circuit and hence reduce the noise recording even further (Fig. S10). In the device used for this measurement, Electrodes 84

and 74 attained values lower than $10\ \mu\text{V}$ which is sufficient to capture reliably electrophysiological recordings from even neuronal samples⁵². The low baseline noise of the 3D microelectrodes verified that the system may be sensitive-enough to detect live recordings of mitochondrial outer membrane electrophysiology. Further live electrophysiological measurements from the mitochondrial pellet were attempted for the first time to our knowledge.

For live mitochondrial pellet electrophysiology recordings, mitochondria were isolated by differential centrifugation from an NIH3T3 cell model, concentrated into a cohesive pellet, incubated for 5 min at 37°C in respiration buffer of 1 mM ETC substrates (glutamate, malate and succinate), and transferred intact onto the 3D MEA ($2.5 \times 2.5\ \text{mm}^2$ active area) mounted in the very same **Axion BioSystems MUSE** system. To ensure effective coupling with the pelleted mitochondria and maximize contact surface area for signal fidelity, we employed 3.57:1 aspect ratio 3D microelectrodes ($250\ \mu\text{m}$ tall, $70\ \mu\text{m}$ wide), a 2D MEA configuration was fabricated using the same processes and tested against the 3D MEA using the same central recording area of $2.5 \times 2.5\ \text{mm}^2$, without the 3×3 micro-tower array, to assess baseline sensitivity for performing EIS using PBS. The 3D configuration had a lower impedance of $575.12\ \Omega$ compared to the $826.30\ \Omega$ impedance of the 2D configuration supporting that 3D MEA configurations can attain a higher SNR^{53–55} (Figure S11) and during live electrophysiological recordings can attain signatures by providing tighter spatial coupling²⁵. Sequential substrate titrations (1, 5, and 10 mM) were applied dropwise onto the active pellet which equilibrated to room temperature, and signals were recorded at different ETC substrate concentrations.

These results obtained in Channels 46, 56, and 67 of one particular device used in the experiments represent first pass evidence that three-dimensional microelectrode arrays can capture bona-fide electrophysiological signals from a compacted, cell-free mitochondrial preparation. When the pellet was dosed with a low (1 mM) glutamate/malate/succinate mixture the traces displayed prominent, low-frequency oscillations (Fig. 7a–c), whereas elevating the ETC substrate mixture concentration to 5 mM sharply attenuated the waveform (Fig. 7d–f). A further increase to 10 mM restored a fraction of the activity (Fig. 7g–i), indicating that the recorded field potential is dose-responsive to electron-transport-chain substrates rather than an artefact of mechanical contact or buffer exchange. The experiment therefore confirms the feasibility of monitoring electrochemical behavior from pelleted mitochondria with sub-millivolt resolution.

We hypothesize that the signal reflects outer-membrane VDAC activity that is gated directly or indirectly by oxidative phosphorylation and the resulting changes in



membrane potential. While canonically VDAC gating is regulated by binding partners such as tubulin and hexokinase⁵⁶, in this view the robust oscillations at 1 mM correspond to a “flicker” regime of VDAC openings⁵⁷, the quiescence at 5 mM marks channel closure under hyperpolarizing conditions⁵⁷, and the partial rebound at 10 mM arises from leak-induced depolarization that re-licenses a subset of channels⁵⁸. Discriminating between these possibilities will require cross-correlational assays such as Seahorse respirometry, inner mitochondrial membrane potential imaging with potentiometric dyes, and pharmacological modulation of VDAC and ATP-synthase, ideally performed simultaneously with MEA recording. These recordings that will be performed in the future, but we believe are out of scope for the current investigation.

This simple protocol yields clear, ETC substrate dependent electrical readouts which underscores the promise of **pellet based mitochondrial electrophysiology** as a rapid screening platform. Follow-up studies that combine the electrical channel with orthogonal bioenergetic metrics should establish whether VDAC gating, proton-pump dynamics, or an interplay of both underlies the field potentials and, whether the approach can be utilized to dissect mitochondrial

pathophysiology or screen small-molecule modulators that impact mitochondrial function.

Materials and Methods

3D MEA design and 3D printing

Microelectrode arrays in a single well were designed using CAD software (SolidWorks 2022, Dassault Systems) and microfabricated using Digital Light Processing (DLP) 3D printing (ASIGA Max, ASIGA) with High Temp resin (FormLabs). A multiparameter 3D printing process was developed where UV light intensity, slice thickness and exposure time were varied to obtain optimal micro-tower dimensions (Diameter: 70 μm , Height: 250 μm) and through vias (Diameter: 720 μm) features simultaneously in a single step. Micro-tower parameter ranges for 3D printing; UV light intensity: 25.00–31.00 mW/cm^2 , slice thickness: 50 μm –100 μm , and exposure time 2.5–5 s were evaluated. Through via parameters for 3D printing; UV light intensity: 5.00 mW/cm^2 , slice thickness: 100 μm , and exposure time: 7–14.5 s were sequentially evaluated. The final 3D microelectrode chip schematically depicted in Fig. 1a (24 $\times$ 24 mm^2 in overall footprint for electronics interfacing, Height: 1.25 mm) contains a 3 $\times$ 3 array of 3D microelectrodes. The 3D printed chip was then

postprocessed using an 70% Isopropyl alcohol (IPA) bath for 5 min, followed by insertion of a gold spring pressure test probe (pogo pin) as shown in Fig. 1b. (Diameter: 680 μm) The final post-processing step was a 15-minute UV cure at 60 °C for attainment of full mechanical strength of the resin material.

Two-step metallization

Microelectrode array and package metallization required a two-step process starting with electron beam metallization (Ti and Au evaporation, Kurt J. Lesker Company) using pre-designed, plotter cut Kapton[®] stencil masks of either 5 $\times$ 5 mm² or 10 $\times$ 10 mm² (Fig. 1c). Precision definition of Kapton[®] was carried out with an automatic plotter cutter (Cameo 4, Silhouette) using settings – Force: 10, Speed: 1, Passes: 2, Acceleration: 1, and Blade Depth: 3. Ti/Au deposition was performed using an electron beam deposition system (Thin Film Deposition System, Thermionics E-Beam & RDM Thermal Evaporator) under vacuum conditions of 10⁻⁷ torr (Fig. 1d). Ag ink (Prima-Solder EG8050, AiTechnology) was subsequently manually casted onto the metallized surface to connect with the pogo pin interconnect (Fig. 1f). This two-step metallization process allowed 100 nm Ti, and 250 nm of Au to form a conductive thin film over the 3D micro-tower array and package surface, while Ag ink casting allowed for full conductivity from package surface to the pogo pin interfacing with the external data acquisition system for electrophysiology (chip bottom side). The external system used for electrophysiological measurements was a modified Axion BioSystems MUSE system (Axion BioSystems Inc.).

Laser isolation, insulation and culture well attachment

Laser micromachining using a green light source of 532 nm (New Wave Research QuikLaze 50ST2 System) for the ablation of the Ti/Au layer was performed to define distinct conductive areas for each electrode with a laser spot size of ~25 μm (Fig. 1e). A plotter cut polyethylene terephthalate (PET, MEDCO Coated Products) insulation layer was attached to the isolated conductive areas surface with an outer area of 24 $\times$ 24 mm² and an inner area surrounding the electrodes of 2.5 $\times$ 2.5 mm². Insulation layer designs were generated in AutoCAD (Autodesk CAD software) and subsequently fabricated using an automatic plotter cutter (Fig. 1g). A polyethylene terephthalate glycol (PET-G) culture well to contain the 3 $\times$ 3 MEA area and was attached using PDMS cured at 60 °C for 1 h (Fig. 1h).

Microelectrode impedance analysis and preliminary electrophysiology recordings

EIS was performed using a vector network analyzer (Bode 100, Omicron Lab) at single-port configuration in

the 1 Hz–50 MHz frequency range. Phosphate buffered saline (Gibbs PBS (10X), pH 7.4, ThermoFisher) served as electrolyte on top of the 3D microelectrodes. A platinum electrode served as the counter electrode which was inserted into culture well filled with PBS. Alligator clips were attached to the pogo pin interconnect serving as the working electrodes. Baseline values of impedance magnitude and phase at the electrophysiologically relevant 1 kHz frequency were recorded. Preliminary electrophysiology recordings (RMS noise; Figure S10) were obtained using a modified Axion BioSystems MUSE recording system using PBS as an electrolyte.

NIH3T3 and iPSC cell culture

Mouse embryonic fibroblast NIH3T3 cells were passaged up to P16 and P17 using NIH recommended protocols and cryopreserved. P16 and P17 cells were thawed in a 37 °C water bath. A 5 mL pipette was attached to a pipette gun and brought over to fill a 15 mL conical vial with 10 mL of DMEM-F12 (ThermoFisher) media and to pre-wet the pipette to prevent cells from sticking onto the sides of the pipette. The pipette was then brought over to the cryovial and used to extract 1 mL of cells to place into the 15 mL of media-filled conical tube. 1 mL of the media was pipetted out, used to rinse the cryovial, and pipetted back into the 15 mL tube to ensure all cells were collected. The cells were then centrifuged at 1000 revolutions per minute (rpm) for 5 min at 37 °C. Next, the excess media was carefully removed to prevent disruption of the cell pellet. The cell pellet was then re-suspended in 1 mL of DMEM-F12 media and plated to 90–100% confluency in three T75 flasks for use in experiments.

Human iPSCs (line ND41865) were obtained from the Coriell Institute for Medical Research and denoted as passage 0 (P0). The cells were passaged up to P16 using NIH-recommended protocols and cryopreserved. P16 iPSCs were thawed and expanded in mTeSR E8 medium (ThermoFisher, A1517001) to 90–100% confluency in a T75 flask for use in experiments.

TMRM functional imaging

Attempts to confirm that the electrochemical impedance spectroscopy (EIS) signatures were emanating from isolated mitochondria, functional imaging using tetramethyl rhodamine methyl ester (TMRM) (ThermoFisher) was carried out. 1.3 mm glass concavity slides (FisherScientific) were used to mix TMRM at 1 nM, isolated mitochondria and respiration buffer components in a volume of 100 μL to be imaged via BZ-X series All-in-One fluorescence microscope (Keyence). The 1 mM, 5 mM, and 10 mM concentrations were reached by mixing 100 mM stock solution with respiration buffer to achieve each concentration. The initial pH of each stock solution was measured to be within physiological range prior to

imaging. TMRM imaging was performed over 4 biological replicates and the mean fluorescent intensity measurements were recorded over 3 technical replicates averaged from each respiratory substrate condition, $n = 3$ for each condition. Fluorescent image analysis was performed using ImageJ software (ImageJJS version 1, JavaScript ImJoy Browser). Split channel was used to isolate the green channel while the area, max/min grey and mean gray value measurements were selected to obtain the mean fluorescence intensity.

Mitochondria Isolation and EIS Characterization

Mitochondria were isolated from NIH3T3 and iPSC models after reaching ~100% confluency in three T75 flasks using a Mitochondria Isolation Kit for cultured cells (ThermoFisher). The Dounce homogenization method⁵⁹ was utilized for preservation of mitochondria integrity and pelleted using differential centrifugation. The mitochondrial pellet was characterized through optical imaging (ANNLOV LCD Digital Microscope). Mitochondria were subsequently transferred to a buffer composition contained Mannitol: 225 mM, Sucrose: 75 mM, Tris-HCl: 25 mM, KH_2PO_4 : 1 mM, MgCl_2 : 1 mM and ADP: 1mM⁶⁰ (ThermoFisher) to simulate cytosolic conditions in the 3D MEA for EIS measurements. For initial mitochondrial EIS characterization the mitochondrial pellet was re-suspended and added to the fully fabricated biosensor using a transfer pipette in 1 mL of respiration buffer. A solution of 400 mM Succinate (ThermoFisher) was added in small doses to the 1 mL solution of respiration buffer containing the mitochondrial pellet (Figure S5). The buffer conditions and concentrations of respiratory substrates were adjusted for physiological conditions at 1, 5, and 10 mM of glutamate, succinate and malate. (Table S8). For experimental conditions, mitochondria were re-suspended in respiration buffer without ETC substrates to obtain a baseline. Next, ETC substrate stock solutions of malate, glutamate, and succinate were added to reach a 1 mM concentration in 2 mL. Two separate devices were used for mitochondrial characterization from each cell line.

Mitochondrial pellet electrophysiology recordings

Electrode noise was characterized on an Axion Bio-Systems MUSE platform by recording first in air, then with the 3D array seated in its chamber, and finally after adding phosphate-buffered saline; the latter reduced background to $<10 \mu\text{V}$ on the best channels, confirming adequate sensitivity (Figure S10). NIH3T3 mitochondria were isolated by differential centrifugation, pelleted, pre-incubated 5 min at 37°C in respiration buffer containing 1 mM glutamate, malate, succinate, and gently placed on the $2.5 \times 2.5 \text{ mm}^2$ micro-tower array (250 μm tall, 70 μm wide pillars) with a histology brush (Fisher Scientific).

Sequential ETC substrate solutions of 5 mM and 10 mM were then applied dropwise, and voltage traces were captured in real time. Raw MUSE files were then exported and processed in MATLAB, where traces were plotted and quantitative metrics were extracted for downstream analysis.

Conclusions and Perspectives

The development of a bimodal 3D MEA-based mitochondria biosensor requires special consideration of the microfabrication techniques to craft the microelectrodes, substrate, and signal transduction pathway necessary to detect the electrophysiological signatures from mitochondria and carry them to signal amplifiers interfacing with the biosensor for data acquisition. In this paper, advancements in microfabrication processes including computer aided design (CAD) digital light processing (3D Printing), thin film deposition, non-conventional metalization, laser micromachining, and packaging allow for rapid development and prototyping of novel designs and structures for a 3D mitochondria biosensor. Integrating electrophysiological sensing modalities of MEA and EIS-based technologies into a single device will provide a comprehensive measurement of mitochondrial electrophysiology by characterizing the electrical and energy-transducing properties of the OMM and IMM. While performing EIS to obtain IMM potential signatures is straightforward in practice, obtaining voltage signatures from the OMM in vitro via a 3D MEA is a novel approach for performing electrophysiology. We have at present demonstrated that impedance magnitude and phase can be utilized to observe trends in solution conductivity and indicate changes in membrane capacitance at high AC frequencies which allow for detection of electrophysiological activity of isolated mitochondria. Further development of design improvements and biochemical assay optimization are currently underway; however, the successful completion of a mitochondria biosensor marks a step towards label free paradigms in biomedical research and a way to assay metabolism in real time. Integration of these sensing modalities into existing BioMEMS constructs such as organ on a chip or lab on a chip can provide a more wholistic method of determining cell function and viability that can empower biomedical researchers to explore new methods of studying the pathogenesis and pathophysiology of complex disorders involving mitochondrial dysfunction.

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Author contributions

R.J. is the main contributor of this work, including conception of device design, experimental plan, experimental work, and writing of the manuscript. T.H. performed microfabrication steps, preliminary testing, cell harvesting and data processing with the assistance of R.J.; J.C. performed cell culture maintenance and passaging of the NIH3T3 cell line. A.P. performed cell culture maintenance and passaging of the iPSC model. F.B.K. processed the Axion MUSE files in MATLAB for data processing. I.J. assisted with creation of microfabrication designs in SolidWorks and AutoCAD. J.M.C. performed experimental work in the micro-tower 3D printing, device design, early-stage microfabrication, characterization, and parameter optimization. J.H. contributed to iPSC model culture and harvesting. S.R. contributed to the experimental plan, funding (co-I of the NIH grant), and interpretation of results. All authors contributed to the writing and final editing of the manuscript where appropriate.

Conflict of interest

J.H. is a co-founder of Hesperos Inc. but declares no funding conflicts of interest.

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