

MICROFABRICATION AND CHARACTERIZATION OF A NOVEL 3D MITOCHONDRIA BIOSENSOR

Randall James¹, Isaac Johnson¹, Ji Chang¹, Jorge Manrique Castro¹, and Swaminathan Rajaraman¹

¹University of Central Florida, Orlando, FL 32816

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 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 sensing mitochondrial electrophysiology using electrochemical impedance spectroscopy (EIS) and electrophysiology recordings. Data obtained through these measurements represent a major functional output of cellular respiration, the electron transport chain (ETC).

KEYWORDS

3D MEAs, Mitochondria, Biosensor, Electrophysiology, Metabolism, Electrochemical Impedance Spectroscopy

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 diabetes, neurodegeneration, myopathy, and cardiovascular disease [1]. A common factor in these diseases is mitochondrial dysfunction [2]. *Mitochondria are an essential organelle that play a key role in maintaining homeostasis including energy production, calcium storage, redox signaling, and regulation of apoptosis* [3]. 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. Cellular respiration is a fundamental process of all living organisms that involves the oxidation of fuel molecules such as glucose to produce adenosine triphosphate (ATP). Mitochondria serves 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 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 cycle [4]. Individual subunits, Complex I and Complex II, oxidize unique substrates such as nicotinamide adenine dinucleotide (NADH) and Succinate, respectively while Complex I concomitantly shuttles protons across the IMM. Methods for assaying such membrane potentials or electrophysiological phenomena traditionally involve voltage or patch clamp techniques [5], fluorescent dyes [6], or electrochemical impedance spectroscopy (EIS) [7] with large needle-type electrodes. 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 challenging because they do not adhere to extracellular membrane

components and are on the scale of 0.5 μ m to 3.0 μ m in diameter [8]. Studies performed using fluorescent probes report IMM potentials ranging from 108mV to 158mV depending on metabolic demands and 139mV at rest however still depend on indirect metrics and kinetic models derived from the Nernst equation [9]. EIS approaches are non-invasive as it only relies on placing an electrode directly in the solution containing the sample of interest. EIS has demonstrated the ability to characterize electrical signatures from mitochondria as well as identifying deficits in function using resuspended mitochondria fractions [10]. 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. 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. 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). In this paper, we report on the development of a novel microfabrication process to produce an integrated 3D mitochondria biosensor specialized for monitoring functional outputs of the ETC using EIS measurements from pelleted mitochondria on the 3D microelectrodes. Further these 3D electrodes demonstrate sufficient sensitivity to obtain electrophysiological recordings.

MATERIALS AND METHODS

3D MEA Design and 3D Printing

Single-well microelectrode arrays 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 where UV light intensity, slice thickness and exposure time were varied to obtain optimal micro-tower dimensions (Diameter: 70 μ m. Height: 250 μ m) and through vias (Diameter: 720 μ m) features simultaneously in a single step. Micro-tower parameter ranges for 3D printing; UV light intensity: 25.00-31.00mW/cm², slice thickness: 50 μ m-100 μ m, and exposure time 2.5-5s were evaluated. Through via parameters for 3D printing; UV light intensity: 5.00mW/cm², slice thickness: 100 μ m, and exposure time: 7-14.5s were sequentially evaluated. The final 3D microelectrode chip schematically depicted in **Fig. 1(A)** (24x24 mm² in overall footprint for electronics interfacing, H: 1.5mm) contains a 3x3 array of 3D microelectrodes. The 3D printed chip was then post-processed using an 70% IPA bath for 5 minutes, followed by insertion of a gold spring pressure test probe pogo pin as shown in **Fig. 1(B)**. (Yosoo, Diameter: 680 μ m) 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 a pre-designed, plotter cut Kapton® stencil masks of either 5x5 mm² or 10x10 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 microtower 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).

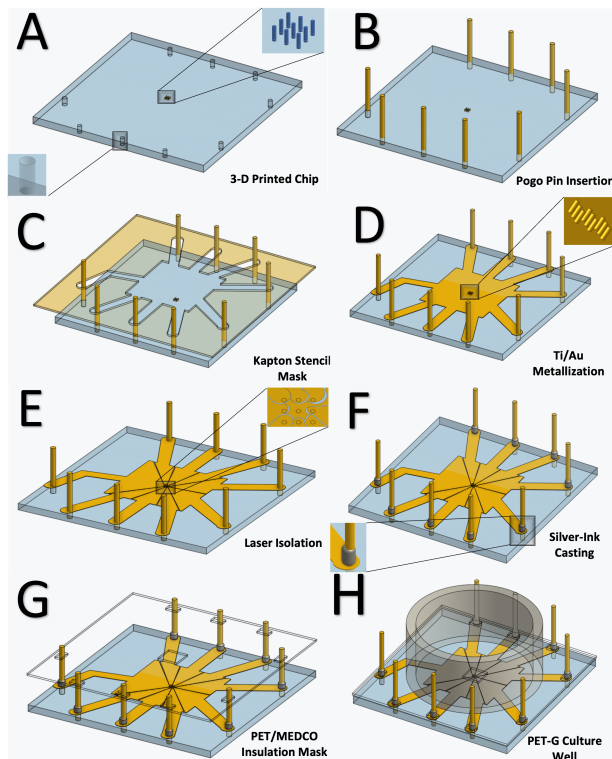


Figure 1: (A) 3D printed chip containing a 3x3 array of micro-towers and through vias. (B) Gold pogo-pins inserted into through vias to interact with Bode Impedance Analyzer and Axion MUSE electrophysiological system. (C) Attachment of adhesive Kapton® stencil mask. (D) Thin film deposition of 100nm Titanium (Ti) and 250nm Gold (Au). (E) Laser ablation of the metalized surface. (F) Silver-ink casting from metalized surface to pogo-pin interconnect. (G) PET Insulation mask adhered to the metalized surface. (H) PET-G Culture well cured onto the surface via PDMS.

Laser Isolation, Insulation and Culture Well Attachment

Laser micromachining using a green light source of 532nm (New Wave Research Quik Lase 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 ~25µm (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 x 24 mm² and an inner area surrounding the electrodes of 2.5 x 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 3x3 MEA area and was attached using PDMS curing at 60°C for 1 hour. (Fig. 1G, H)

Impedance Analysis and Preliminary Electrophysiology Recordings

EIS was performed using a vector network analyzer (Bode 100, Omicron Lab) at single-port configuration in the 1Hz – 10MHz 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 1kHz frequency were recorded. Preliminary electrophysiology recordings (i.e., RMS noise) were obtained on the Axion BioSystems MUSE using PBS as an electrolyte.

Mitochondria Isolation and EIS Characterization

Mitochondria were isolated from NIH3T3 cells at a final cell population of ~9,217,500 cells using a Mitochondria Isolation Kit for cultured cells (ThermoFisher; Fig. 2). The Dounce homogenization method [11] was preservation of mitochondria integrity and pelleted using centrifugation. The mitochondrial pellet was characterized through optical imaging (ANNLOV LCD Digital Microscope). Mitochondria were subsequently transferred to a respiration buffer containing 225mM mannitol, 75mM sucrose, 10mM KCl, 10mM Tris-HCl and 5mM HK₂PO₄ (ThermoFisher) to simulate cytosolic conditions in the 3D MEA for EIS measurements. The mitochondrial pellet was added to the fully fabricated biosensor using a transfer pipette in 1mL of respiration buffer. A solution of 400mM Succinate (ThermoFisher) was added in small doses to the 1mL solution of respiration buffer containing the mitochondrial pellet. Full spectrum EIS recordings were obtained immediately after the addition of Succinate.

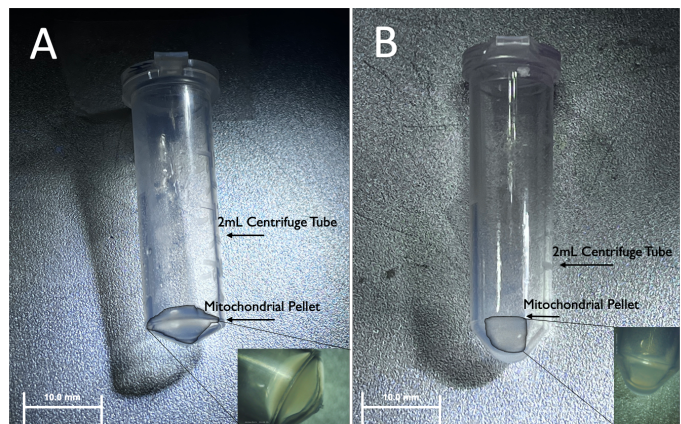


Figure 2: (A, B) Pelleted mitochondria after performing mitochondrial isolation.

RESULTS AND DISCUSSION

Fabrication of a 3D mitochondria biosensor required optimization of several design and microfabrication process parameters. Two 3D MEA configurations were designed to have either a 5x5 mm² (Fig. 3A) or 10x10 mm² (Fig. 3B) central recording area for initial EIS characterization. The total conductive area for each electrode was calculated in AutoCAD software for

each configuration (A: $\sim 13.04\text{mm}^2$, B: $\sim 19.71\text{mm}^2$). These areas were subsequently isolated to defined individual 3D microelectrodes. **Figs. 4 and 5** depict optical, SEM and confocal images of the mitochondria biosensor at various stages of the microfabrication and packaging process.

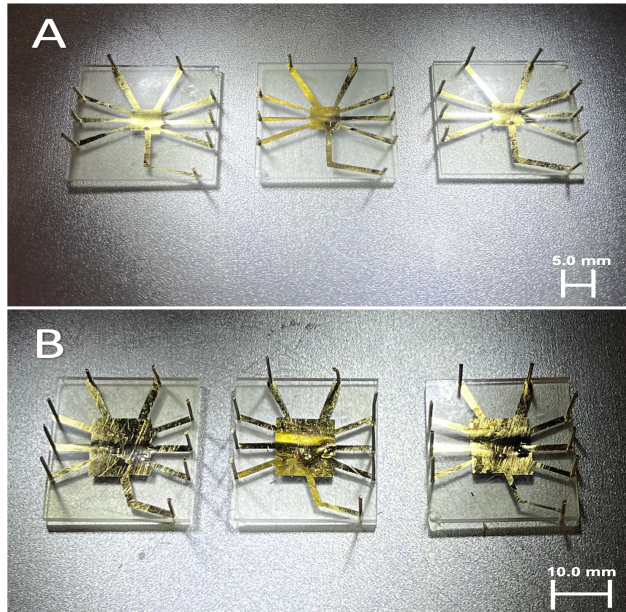


Figure 3: Metallized (100nm Ti; 250nm Au) 3D MEAs with $5 \times 5\text{mm}^2$ (A) and $10 \times 10\text{mm}^2$ (B) central area. Total designed conductive area for each electrode: A: $\sim 13.04\text{mm}^2$, B: $\sim 19.71\text{mm}^2$

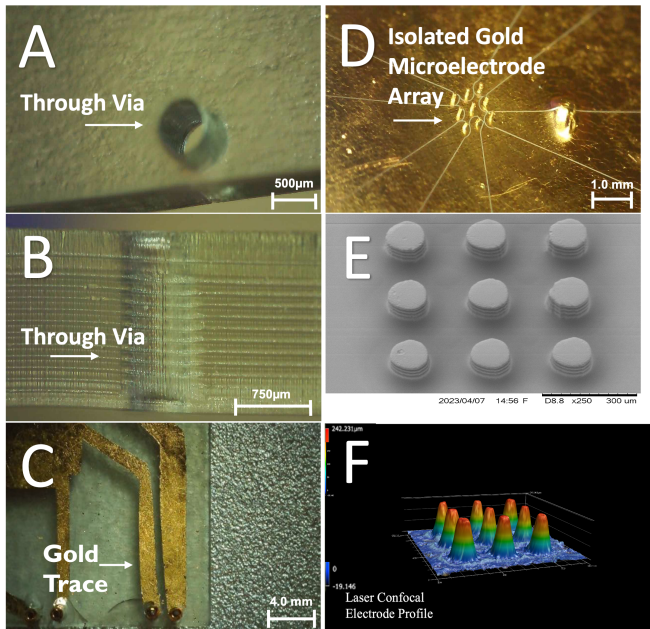


Figure 4: (A, B, C) Optical images of 3D printed through vias and metalized trace to the pogo-pin interconnect. (D) Optical images of metallized 3×3 three-dimensional microelectrode array (MEA). (E) SEM images of the 3×3 MEA. (F) Laser confocal characterization of 3×3 MEA, Height: $242.231\mu\text{m}$

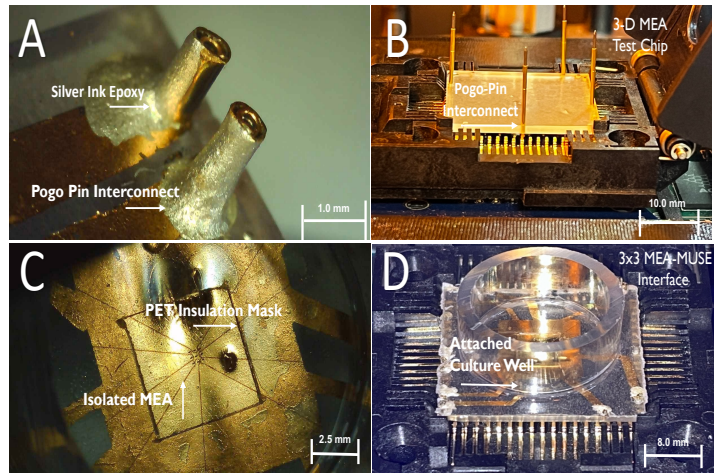


Figure 5: (A, B) Optical images of silver-ink casted pogo-pin interconnects and pogo-pin interconnects aligned with the external electronics (Axion MUSE) for mitochondria electrophysiological recordings. (C) Isolated biosensor surface with PET insulation layer attached to the surface. (D) Fully fabricated mitochondria biosensor integrated with Axion MUSE.

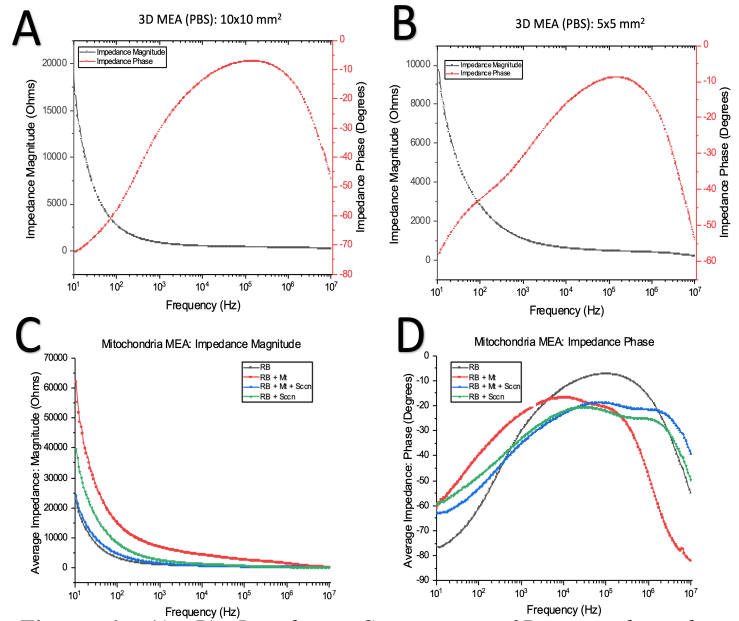
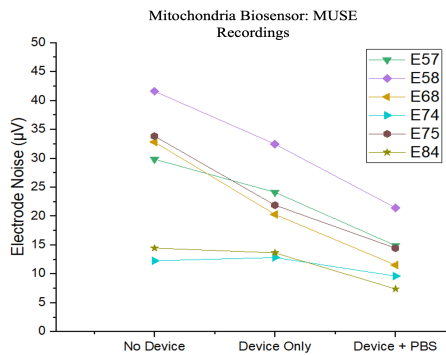


Figure 6: (A, B) Impedance Spectroscopy 3D microelectrode characterization (Impedance Magnitude vs Impedance Phase) of mitochondria biosensor in PBS (pH: 7.4) for a $10 \times 10\text{mm}^2$ and $5 \times 5\text{mm}^2$ configuration. (C, D) Impedance magnitude/phase response of the respiration buffer (RB), and subsequent addition of Mitochondria (Mt) and Succinate (Scn) on an $10 \times 10\text{mm}^2$ 3D MEA.

Laser confocal surface characterization indicated the 3D printed micro-towers **printed to a height of $242.231\mu\text{m}$ consistent with the designed height of $250\mu\text{m}$** . Baseline impedance (PBS) magnitude/phase sweep at **1kHz measured $838.9W/-30.6^\circ$ from the $10 \times 10\text{mm}^2$ configuration and $1068.94W/-30.7^\circ$ from the $5 \times 5\text{mm}^2$ configuration (Fig. 6)**. The $10 \times 10\text{mm}^2$ configuration demonstrated a lower impedance at the 1kHz frequency which may be attributed to the greater surface area of the Ti/Au thin film. EIS characterization of isolated mitochondria was performed on the $10 \times 10\text{mm}^2$ configuration. Detection of mitochondrial respiratory activity was obtained by performing EIS under different biochemical conditions as presented in **Table 1**. Respiration buffer

Table 1: Impedance Magnitude/Phase Data recorded from the Mitochondria Biosensor.

Magnitude/Phase Frequency: 1kHz	Magnitude	Phase
10x10 mm ² (PBS)	838.85Ω	-30.61°
5x5 mm ² (PBS)	1068.94Ω	-30.71°
RB	1062.18Ω	-30°
RB + Mt	6857.2Ω	-23.37°
RB + Mt + Sccn	1507.69Ω	-34.85°
RB + Sccn	2658.19Ω	-33.07°



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

Figure 7: Axion BioSystems MUSE electrophysiological response of 10x10 mm² 3D MEAs with PBS. E74 and E84 attain noise values below 10μV, which is suitable for biological

(RB) resulted in **magnitude/phase readings of 1062.2Ω/-30.0°**. The components of the buffer were then varied with Mitochondria (Mt) and Succinate (Sccn). RB and Mt resulted in an increased magnitude/phase response: **6857.2Ω/-23.37°**. Further addition of Sccn decreased the magnitude/phase response to **1507.7Ω/-34.9°**. The effect of Sccn only on the RB was recorded with a magnitude/phase response of **2658.2Ω/-33.1°**. **Alterations in magnitude/phase responses likely reflect the changes in the mitochondrial membrane potential.** Mitochondria begin respiration in the presence of succinate, directly activating Complex II of the ETC, succinate dehydrogenase, and indirectly causing an increase in IMM potential. This triggers redox reactions to begin in the ETC and increases the capacitance across the IMM which reduces the impedance and decreases the phase towards -90° which is characteristic of an ideal capacitor. Background noise recordings

resulted in a decrease in noise (μV) upon addition of PBS. **3D microelectrodes E74 and E84 attained noise values below 10μV, which is suitable for biological recordings and may be sensitive to detect live recordings of mitochondrial outer membrane electrophysiology (Fig. 7).** These recordings are currently underway.

CONCLUSIONS

The invention of a mitochondrial biosensors for biomedical researchers will enhance the scientific discovery of disease mechanisms and lead compound development. Standardization of new bioenergetic parameters, improvements in experimental design and biochemical optimization are necessary for the device to be fully realized however, continued advancement of the mitochondria biosensor will allow for dynamic non-invasive physical measurements of mitochondrial electrophysiology. The potential bimodal sensing capabilities of 3D mitochondria biosensor will provide invaluable data for the functional outputs of cellular respiration which may serve as a new standard for biomarkers in the fields of cell biology, biotechnology, and bioenergetics.

ACKNOWLEDGMENTS

The authors would like to thank the NBSS Group, NSTC, GSA at UCF and Hilton Head MEMS2024 workshop for travel funding. Additional financial support for the work was provided by NIH grant R01HL164936.

REFERENCES

- [1] Srivastava S, "The Mitochondrial Basis of Aging and Age-Related Disorders." *Genes*, 8, 12, (2017).
- [2] Brand, M. D., et al. "The Role of Mitochondrial Function and Cellular Bioenergetics in Ageing and Disease: Mitochondrial Function in Ageing and Disease." *British Journal of Dermatology*, 169, (2013).
- [3] Pucadyil, Thomas J., et al. "The Multifaceted Roles of Mitochondria." *Molecular Cell*, 83, 6, (2023).
- [4] Martínez-Reyes, et al. "Mitochondrial TCA Cycle Metabolites Control Physiology and Disease." *Nature Communications*, 11, 1, (2020).
- [5] Kumari, Anshu, et al. "Patch-Clamp Technique to Study Mitochondrial Membrane Biophysics." *Journal of General Physiology*, 155, 8, (2023).
- [6] Sorvina, Alexandra, et al. "Mitochondrial Imaging in Live or Fixed Tissues Using a Luminescent Iridium Complex." *Scientific Reports*, 8, 1, (2018).
- [7] Rc, Mitra, et al. "Evidence from Impedance Spectroscopy That Elevated Dopamine Reduces Mitochondrial Membrane Potential." *Journal of Biosensors & Bioelectronics*, 08, 02, (2017).
- [8] Hayden, Melvin R. "The Mighty Mitochondria Are Unifying Organelles and Metabolic Hubs in Multiple Organs of Obesity, Insulin Resistance, Metabolic Syndrome, and Type 2 Diabetes: An Observational Ultrastructure Study." *International Journal of Molecular Sciences*, 23, 9, (2022).
- [9] Gerencser, Akos A., et al. "Quantitative Measurement of Mitochondrial Membrane Potential in Cultured Cells: Calcium-induced De- and Hyperpolarization of Neuronal Mitochondria." *The Journal of Physiology*, 590, 12, (2012).
- [10] Padmaraj, Divya, et al. "Mitochondrial Membrane Studies Using Impedance Spectroscopy with Parallel pH Monitoring." *PLOS ONE*, 9, 7, (2014).
- [11] Bahnmann, Janina, et al. "In Search of an Effective Cell Disruption Method to Isolate Intact Mitochondria from Chinese Hamster Ovary Cells." *Engineering in Life Sciences*, 14, 2, (2014).