HIGH THROUGHPUT, MULTIMODAL, MICROCHAMBER BIOSENSORS FOR *IN VITRO* SELECTIVE LOCALIZATION OF KILLIFISH CARDIAC MODELS

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ABSTRACT

This paper reports a bioelectronic device for measuring impedance and electrophysiology of fertilized and unfertilized killifish (*Fundulus Grandis*) embryos. We developed a 3D printed, high-throughput (HT) microchamber and integrated microneedle-type microelectrodes within these chambers. We found that graphene coated 3D stainless steel electrodes (GSS) have a 50% reduced impedance compared to uncoated stainless steel (SS) counterparts. We further report for the first time that impedance values at Re(1.712-6.471kHz) and Im(77Hz, 22.382kHz, 12.1 MHz), correlate with fertilization status of embryos and suggest possible muscular movements within the fertilized fish embryos. Electrocardiogram (ECG) measurements were further obtained using the device. Such a bioelectronic system may hold promise as a tool to evaluate fish embryo models as a representative cardiac microphysiological system at various stages of their development, towards *in vitro* toxicology and drug discovery.

KEYWORDS

Multimodal Biosensors, Killifish Electrophysiology, Electrical Impedance Spectroscopy, Graphene Electrodes.

INTRODUCTION

Small alternative model organisms like zebrafish and killifish are trending in heart regeneration, preclinical drug discovery and environmental science testing **[1,2]**. Due to their small size and relatively short lifecycles, these fish species are amenable to high throughput testing that is otherwise not possible with traditional animal models such as mice, dogs, cats, pigs, rabbits, and sheep. These fish models are cost effective and can show correlated effects on human health **[1,2]**. Despite the growing popularity of fish models in biomedicine, there are no commercial devices that can rapidly measure embryo growth and maturity or impacts from various environmental events on these models in an *in vitro* setting **[3]**. Functional endpoints such as electrical potential, and Electrical Impedance Spectroscopy (EIS) are commonly used for rapid assessment of biological membrane permeability in cells and tissues **[4,5]**. Early work in instrumenting primitive life stage fish models spotlighted several design constraints for extracting bioelectric signals from submerged (or wet) fish embryos and larvae. To overcome these constraints electrophysiology, and full frequency EIS need to be integrated in compact 3D HT devices and such a device can enable assessments of biological membrane integrity in cells and cardiography to measure and calculate metrics such as heart rate and blood flow **[2,6]**.

Gulf killifish have a storied history as environmental sentinels of toxicity and environmental health disparity in estuarine land along the Atlantic and Gulf coasts of North America, and as a model for environmentally induced

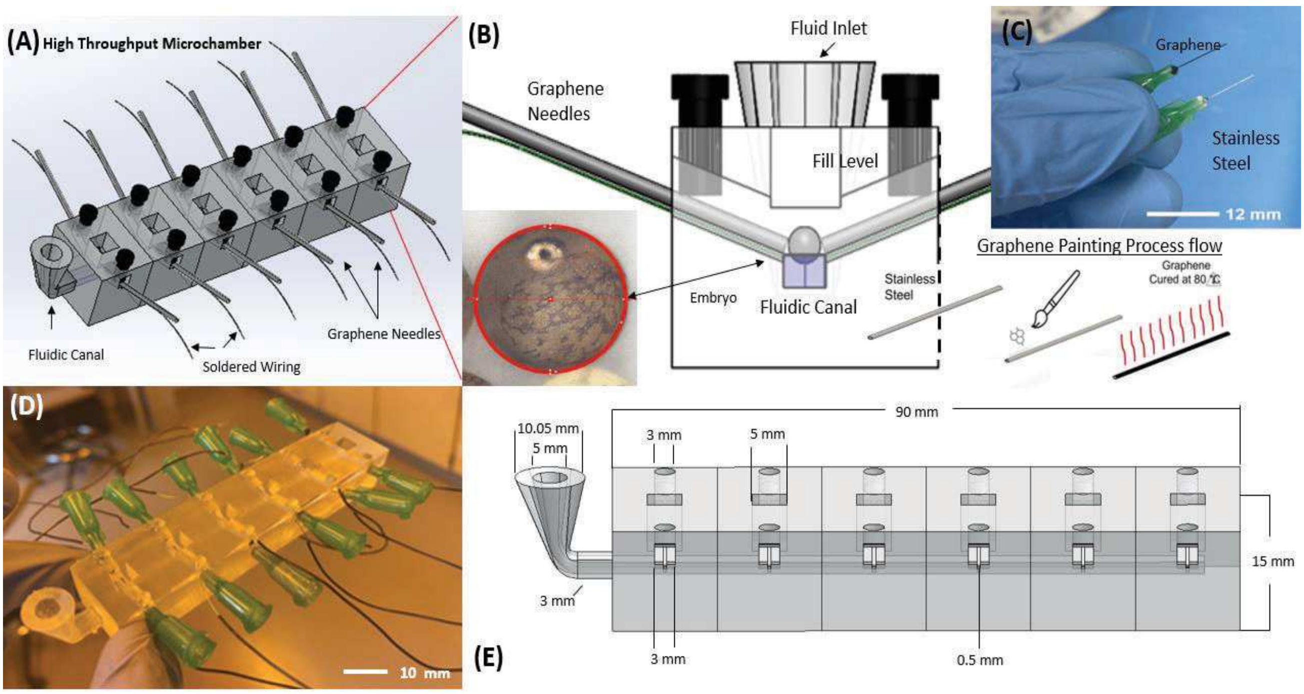

Figure 1. *A) Schematic of High Throughput (HT) Multimodal Biosensors with GSS electrodes. B) Single Microchamber schematic with 3D confocal image of embryo C) Optical Images of GSS and SS electrodes with schematic representation of graphene painting/curing process used. D) Optical image of fully assembled 3D printed HT Multimodal Biosensor device. E) Schematic depiction of dimensions for HT Microchamber.*

cardiotoxicity and ecosystem health. This species is a valuable assessment model for ongoing and future environmental justice and public health crises. Environmental contamination affects all vertebrates through conserved mechanisms such as cardiovascular and other effects observed in developing fish being translatable to human health during early life stages. Thus, understanding how killifish models respond to common environmental toxins informs us on effects on gene expression, cardiovascular, neurotoxicity and several applications in cancer biology **[1,7]**.

This work reports on a new bioelectronic device comprising microchambers integrated with multimodal biosensors to study killifish embryos. To our knowledge, impedance or electrophysiology has not been applied to assess the physiology of fish embryos at higher frequencies. This work develops a new, 3D printed HT microchamber with integrated highly sensitive electrodes for measurement of EIS and electrophysiology of killifish embryos in an artificial seawater solution.

MATERIALS AND METHODS

Design and Microfabrication Process

We designed a HT microchamber that included 6 wells (**Figure 1)** to selectively localize killifish embryos with two electrodes per well, as a platform for measuring impedance and electrophysiology of embryos at various stages of development. The printing of the chamber was performed with a Form Labs stereolithographic resin 3D printer with Form 3 clear resin. The dimensions of the fabricated microchamber are 90 mm in length and 30 mm in width with a height of 15 mm. The microfluidic canal and port are 3mm wide leading to the fill chamber, which is 5 mm wide. The needle inlet is designed as 3x3 mm square with a channel that is 13.5 mm in length with a smaller channel below that is 500 µm in diameter to accommodate the soldered wire inlay.

In **Figure 1A-B**, SS and GSS electrodes are fed through ports defined in the 3D printed microchambers. Embryos are placed between the electrodes fabricated by either native SS or painting graphene on SS/curing as described in the painting process below. The electrodes are soldered, advanced until contacting the embryo's chorion and remain in place with screw holders. The water level, chemistry and embryo position can be adjusted manually during experiments.

Painting Process and Testing

The SS acupuncture needle (Spring Ten) and 34-gauge (34G) SS needle (BSTEAN) were left either in their native state or sanded using 180 sandpaper (SP) and then painted with water based conductive graphene carbon paint from Semicro. The painted needles were either plasma treated and/or subsequently placed in an oven to cure the painted graphene layer for 24, 48 hours at 80°C. After the curing process, a Scotch® tape peel test was performed (N=3) to qualitatively inspect graphene removed from the surface of the electrodes for the different test conditions (**Table I**). An agitated solution test consisted of vigorously swirling the GSS needles for 30 seconds in saltwater solution, to determine if there was any flaking of the graphene layer left in the saltwater solution.

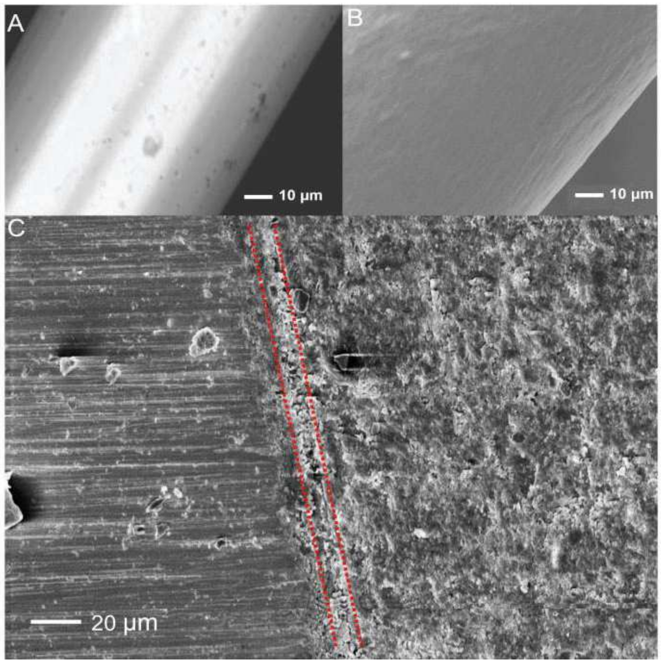

Figure 2. *A and B) SEM image of SS and GSS electrodes, C) SEM Cross section image of the boundary painting of SS with Graphene shown with dotted line. Outside remaining boundary, Left) stainless steel, Right) resin from mold.*

EIS and ECG Measurement

Impedance was measured using a BODE impedance spectroscopy system and electrophysiology was measured using CardioPhys^{TM} ECG differential amplifier set up for monopolar recordings according to manufacturer's specifications. The SS and GSS electrodes were advanced to make contact with the sides of the embryo for EIS measurements from 1 Hz to 40 MHz using a series-through measurement setup.

SEM and Confocal and Optical microscopy SEM and Measurements

SS and GSS needles were cut using wire cutters and placed into a Form Labs clear resin and then cured in the resin for 1hr. This layer was sanded with fine grained 180 SP grit, until the cross section of the graphene and stainless steel were exposed for viewing on the Zeiss ULTRA-55 FEG SEM as shown in **Figure 2**. *F. Grandis Killifish* embryos were viewed with the Keyence Confocal Microscope with a 10x lens shown in **Figure 1B**. *Killifish* embryos preserved in DMSO and were sectioned on a Leica cryostat at a thickness of 60 µm tissue slices without the use of sucrose and placed under a bright field Nikon 81 microscope and captured with an iPhone 11 (**Figure 3**).

RESULTS

Figure 1B-D depict optical and confocal images of the fabricated microchambers, and SS and graphene coated SS needles. **Figure 2** depicts SEM images of the SS and GSS needles with the cross sectioning depicting regions with clear coating of graphene and the enhanced surface roughness imbibed from the painting process. The graphene painting process was tested using five different methods in order to evaluate its performance. SS needles which were sanded with SP 180 grit, followed by graphene being directly painted on the 34G SS needle and cured for 48 hours, and also placed in the plasma treatment for 1 and 5 mins as suggested in Williams et al. **[8]**. Surprisingly, both plasma treated graphene needles failed the scotch tape peel test (N=3) and solution test which consisted of multiple agitations after solution incubation and running impedance tests thereafter. The plasma treated and painted graphene layer was brittle and disintegrated upon solution stirring. The GSS electrodes that passed the two conducted tests were ranked in order as shown in **Table I**.

Table I. Graphene Enhancement Adhesion Test

Graphene Adhesion	**Electrode Test**	
	Tape	**Solution**
GSS w/ SP 180 48 hr. cure	Pass	Pass
GSS w/ Plasma & SP 180 48 hr. cure	Fail	Pass
GSS w/ Plasma 5 min 48 hr. cure	Fail	Pass
GSS w/ Plasma 1 min 48 hr. cure	Fail	Pass
GSS 24 hr. cure No Plasma or SP	Fail	Fail

Figure 3 showcases a 60 µm slice of the embryo depicting spine and sliced eye along with other organelles which are under further study and interpretation.

Figure 4 showcases the enhanced sensitivity due to the graphene coating on the SS electrodes with a reduced impedance signature for the 3D electrodes by ~50%. Furthermore, compared to traditional 2D electrodes **[4]** impedance was lowered by ~33%. The fertilized embryo has a lower impedance than the unfertilized egg by ~64% because impedance enabled fast sampling frequencies capturing alterations in the complex resistance value across the microchamber, which indicate change in physiological and behavioral events such as heart activity, fin, and muscle movement, hatching events, etc.

The electrical properties depicted in **Figure 4** further captures the comparison of EIS between unfertilized eggs with GSS, and fertilized eggs with GSS in saltwater showcasing the difference in Impedance at 1 kHz is Re(Ω)=500 and Re(Ω)=256. There was a strong change in the imaginary impedance for all plots comparing fertilized to unfertilized eggs. For GSS electrodes the real impedance data followed a similar trend to SS electrodes yet values were 46% lower when comparing the unfertilized and fertilized eggs at 300 Hz a retracement in data occurs when

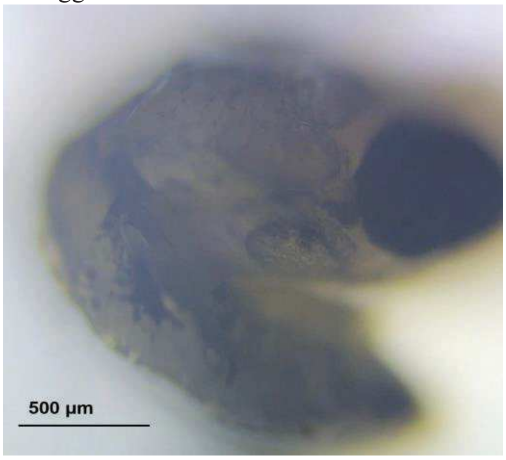

Figure 3*. Showcases a stage of a fertilized embryo development sliced 60 µm thick no sucrose stored in DMSO for 4 months.*

testing with GSS; fertilized egg shows an increase in capacitance, showing enhanced sensitivity for GSS.

In **Figure 5** the *in vitro* changes in embryogenesis

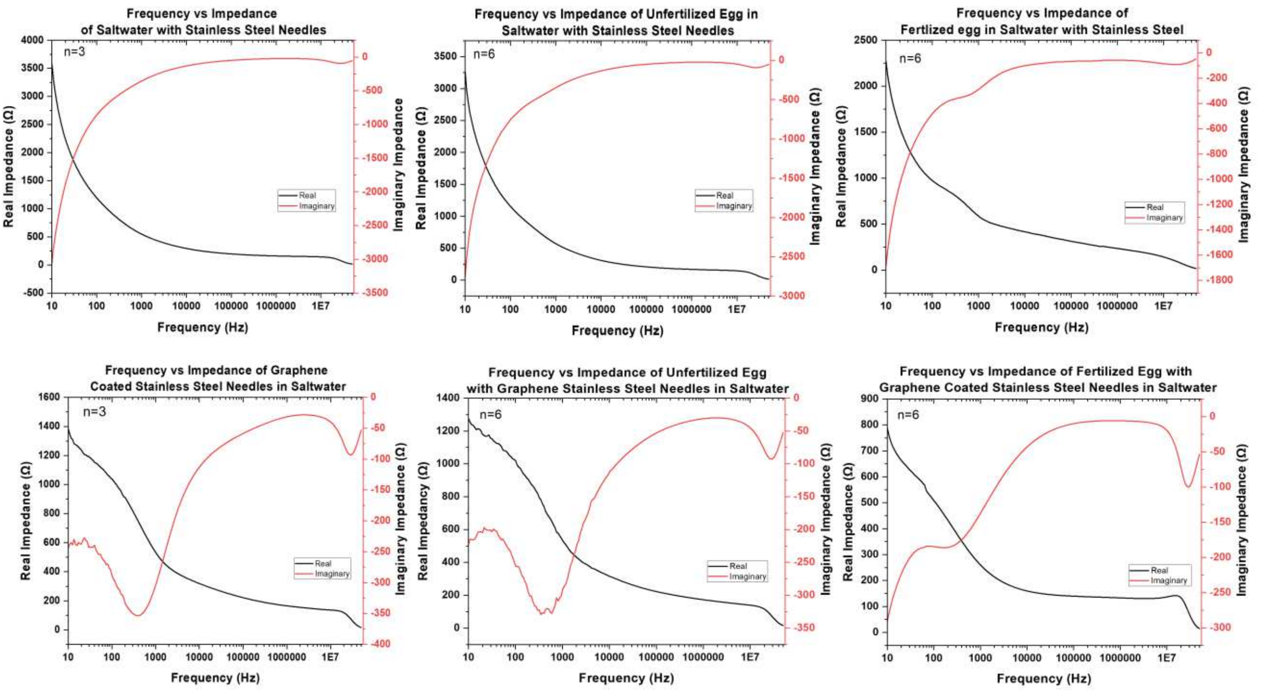

Figure 4*. Full spectrum Impedance Graphs of Stainless Steel Needles and Graphene Coated Stainless Steel Top Left) SS Needles with saltwater, Top Center) SS Needles with Unfertilized Eggs in Saltwater, Top Right) SS Needles with Fertilized Egg in saltwater, Bottom Left) GSS Needles in saltwater, Bottom Center) GSS Needles of Unfertilized Eggs in saltwater, Bottom Right) GSS Needles of Fertilized Egg in saltwater.*

monitoring in saltwater showcasing features at Re(1.712-6,471kHz) and Im(77, 22.382Khz, 12.1 MHz). Growth seems to be depicted within the shift in phase between the days likely associated with the change in the size of the embryo increasing with time and morphological changes with positioning. An outcome anticipated considering the drastic transformation of space within the egg as the embryo develops rapidly from a single cell going through the blastomere, morula, and blastula stages. **[9]**

Figure 6 depicts the ECG for the killifish larvae showcasing the foundational PQRST waveforms**.** The QRS complex (duration:200ms) with an R amplitude of 3.9 mV which correlated directly to Ventricle Depolarization. These results indicate that this system may be a viable platform to detect sensitives of fish embryos to drugs and environmental stressors using multimodal impedance and electrophysiology-based detection system that measures fine scale changes within the embryo and larvae. With both segments of PR and ST underlined in blue in the positive

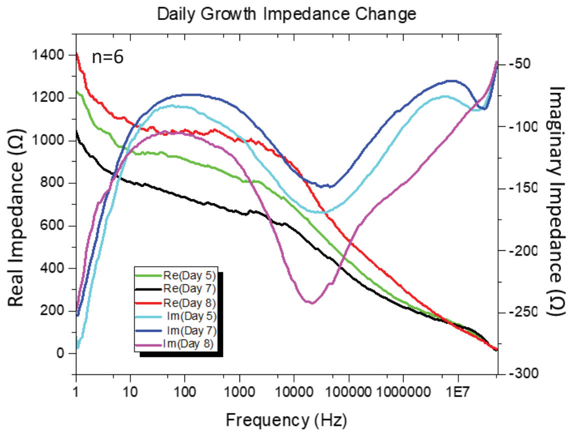

__Figure 5.__ A comparison of Impedance Spectroscopy in Unfertilized vs Fertilized Killifish Growth during days 5,7 and 8 after Embryo Egg Release.

axis and the interval PR in green and QT in green under the negative axis. The QRS complex is underlined and shown in a separated parallelogram box in red and measured ~200 ms with an R amplitude of 3.9 mV. The QT interval was ~300 ms and corresponded to cardiac depolarization and repolarization. The PR interval lasted ~230 ms showing the Atrial Depolarization. A continuous ECG signal spanning 5 seconds is shown in the inset graph in the lower left-hand corner of **Figure 6**.

CONCLUSION

We developed a 3D printed, multimodal sensor device for the high throughput measurement of EIS, ECG, and visual development of multiple killifish embryos. We were able to show that adherence of graphene was increased with proper surface preparation of the stainless steel electrodes, and greatly reduced the impedance and thus increased the sensitivity for these stainless steel electrodes.

The embryogenesis change shows that there are regions of interest in EIS data for days 5,7,8 *in vitro* between 1-40kHz. Beyond this range, 100kHz becomes a region of interest for real impedance. For the imaginary impedance we see a daily change between 1-40kHz. The lines intersect with one another at 30kHz. The physiological implications for these changes are currently being further explored.

The intervals and segments from the classic ECG signature composed of the PQRST waveform was measured. Throughout the entirety of this paper, we have demonstrated powerful technologies that enable us to use small killifish models to look at the health of the ecosystems and test new pharmaceutical compounds. Complex impedance values measured across the microchamber change, due to the development of new tissues within the embryo, fin and muscle activity and physiological signatures. Detailed knowledge and modeling combined with mechanical analysis of heart function within the embryo will allow for planning and

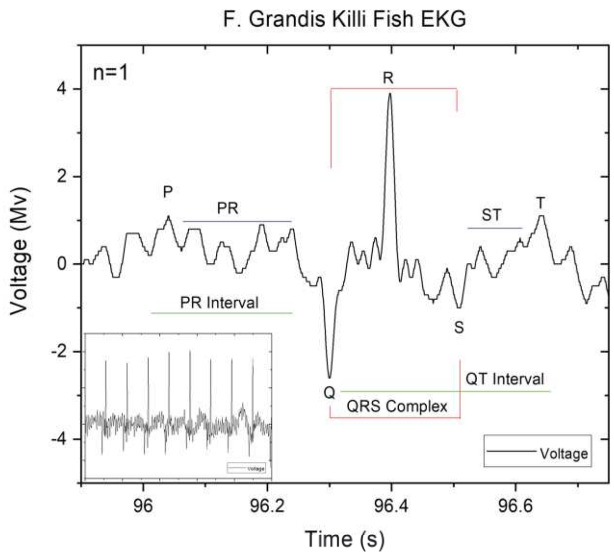

__Figure 6.__ ECG of a freshly hatched Fundulus Grandis fish larvae with the GSS electrodes. The intervals and segments from the classic ECG characteristic signature composed of the PQRST waveform is depicted.

analyses of clinical evaluation of this model system for the general understanding of normal and abnormal cardiac functions during early vertebrate development.

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