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Flexible and Waterproof Micro-Sensors to Uncover Zebrafish Circadian Rhythms: The Next Generation of Cardiac Monitoring for Drug Screening

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Abstract

Flexible electronics are the next generation of sensors for mobile health and implantation. Zebrafish (Danio rerio) is an emergent strategy for pre-clinical drug development and toxicity testing. To address the confounding effects from sedation of fish and removal from the aquatic habitat for micro-electrocardiogram (μECG) measurements, we developed waterproof and wearable sensors to uncover the circadian variation in heart rate (HR) and heart rate variability (HRV)[1]. The parylene-C based ECG sensor consisted of an ultra-soft silicone integrated jacket designed to wrap around the fish during swimming. The Young's modulus of this silicone jacket matched with the fish surface, and an extended parylene cable connected the underwater chest electrodes with the out-of water electronics. In addition, embedded micro-glass spheres in the silicone effectively reduced the effective density of the jacket to ~ 1 $g \cdot cm^{-3}$. These innovations enabled physiological ECG telemetry in the fish's natural habitat without the need for sedation. Furthermore, a set of non-linear signal processing techniques filtered out the breathing and electromagnetic artifacts from the recorded signals. We observed a reduction in mean HR and an increase in HRV over 24 hours at 10 dpa, accompanied by QT prolongation as well as diurnal variations, followed by normalization in mean HR and QT intervals at 26 days post ventricular amputation (dpa). We revealed Amiodarone-mediated QTc prolongation, HR reduction and HRV increase otherwise masked by sedation. The novel features of the flexible silicon jacket for μECG

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telemetry unraveled the biological clock and normalization of QT intervals at 26 dpa, providing the first evidence of new physiological phenomena during cardiac injury and repair as well as cardiac drug-mediated aberrant rhythms. Thus, the light weight and waterproof design holds promise to advance the next generation of mobile health and drug discovery.

Keywords

Zebrafish; Circadian Rhythm; Flexible and waterproof multi-electrode arrays; drug screening; toxicity testing

1. Introduction

The first generation of flexible electronics was established for micromachine-based shear stress sensor arrays mounted on the non-planar surface of air foils for turbulence control[2]. Over the last decade, the advent of flexible microelectronic membranes is evidenced by the biomedical applications to interrogate electrical depolarization in the small vertebrate hearts[3, 4], and the deployment of intravascular flexible shear stress sensors to assess atherosclerotic plaque[4]. These parylene-based high-density electrode arrays have further enabled electrical stimulation in the retina to restore vision and spinal cord to restore locomotion[5]. Stretchable multi-electrode arrays (MEA) further unravel aberrant electrophysiological phenotypes of small animal models of heart regeneration[6]. The MEA membranes adhere to the non-planar body surface, identifying spatial variations in cardiac injury currents from zebrafish hearts[6]. The PDMS-based epidermal electronics revolutionized non-invasive monitoring for mapping cardiac conduction and brain activity[7, 8]. These high density arrays offer precise spatial control of stimulation and recording otherwise challenging with the traditional fine-wire electrodes[5].

Developmental genes involved in zebrafish heart repair are highly conserved in higher vertebrates. The average length of an adult fish is at 2 to 4 cm, accessible for relatively low-cost and high-throughput small molecule screening[9]. Their physiological complexity also provides conserved models of human disease for in vivo validation studies[10]. The biological characteristics of zebrafish are suitable for toxicity testing, including eco-toxicology[11]. However, the Clock gene involved in the central oscillation to coordinate endogenous rhythms is linked to the generation of circadian rhythms[12]. Thus, sedation of zebrafish influences the circadian variations in heart rate (HR) and heart rate variability (HRV) in response to cardiac injury or to drug testing.

The first micro-electrocardiogram (μECG) signals obtained from adult zebrafish required muscle paralysis[13-15]. The gill motion was arrested to reduce electromagnetic (EMG) artifacts while oxygenation was provided to prevent hypoxia and arrhythmias via a needle-to-mouth resuscitation[13-15]. Our group avoided paralytic agents to establish high signal-to-noise ratios for μECG signals via wavelet transform with Tricaine-based sedation[14, 15]. However, translating the zebrafish model to unequivocal drug screening and toxicity testing in the absence of sedation remained a challenge.

To address circadian rhythm-associated heart rates (HR) and heart rate variability (HRV), we have designed stretchable parylene cable and microelectrodes to establish 24 hour telemetry of adult zebrafish. We performed real-time recording and analyses of μECG signals at 10, 18 and 26 days post ventricular amputation without sedation. We compared the nocturnal and daytime HR and HRV prior to and post ventricular amputation. We further identified changes in QTc intervals in response to Amiodarone, a class III anti-arrhythmic agent[16] in the presence versus absence of sedation. Our findings revealed HR and HRV in response to cardiac circadian rhythms, providing a physiological basis to advance drug development and toxicity testing as well as future mobile health and sleep research[17, 18].

2. Methods

2.1 Design and Mounting of the Multi-Electrode Array (MEA) to Zebrafish

The design of MEA highlighted two gold (Au) electrodes (chest electrode and reference electrode) at 3 mm apart (Fig. 1 a-c). Each electrode harbored a micro meshed structure with 25μm × 25μm openings to reduce the stress on the Au thin film in response to stretching. We developed a mounting jacket made with micro-molded partially cured silicone MED4210 (Dow Corning, USA) to closer match the fish skin's modulus of approximately 0.04 GPa for long-term wear[19]. The ultra-soft silicone jacket has a Young's modulus of approximately 0.001 GPa, which significantly reduces strain on the fish surface compared to parylene-C's Young's modulus of 3.2 GPa[20-23]. Moreover, to reduce the long-term stress from extra weight of the jacket, micro glass-spheres are mixed in by weight ratio 15:1 (silicone: micro glass sphere) so that the ultrasoft silicone jacket harbored an effective density close to 1g/cm^3. The ultra-soft silicone jacket utilized a zip-tie structure with multiple teeth to adapt to different sizes of the zebrafish. The extended zip tie length was removed with micro surgical scissors after threading to secure the electrode contact to the ventral part of the animal. A back padding was provided to reduce the stress on the dorsal part of the fish where stress was concentrated in response to forward swimming motion. The chest electrode was threaded through the jacket's center "cross" opening, and placed at the tip of the jacket. The electrode was fixed onto the jacket with an additional application of uncured silicone around the anchor holes on the electrodes. The electrode harbored a PA-C/Ti/Au/PA-C stacked structure which underwent a vacuum oven annealing process at 2000C for 16 hours to enhance long-term parylene-parylene adhesion, and the micro-fabrication process as previously reported[6, 24]. The complete device consisted of PA electrode, cable, silicone jacket, flexible flat connector (Fig. 2 b). The micro electrode cable was fixed to a flat FFC cable as a guide to plug into a zero insertion force FFC connector. The fish was able to comfortably wear the jacket and swam freely in the aquatic environment (Fig. 2 f-g).

2.2. Signal processing

Due to the inherent gill motion artifact in absence of sedation, the recorded raw data required further processing to subtract the large breathing baseline for morphology study. Raw data was first processed with a peak detection algorithm[25, 26] to detect the R-peaks in order to index the data into single ECG wave segments. All the segments were then aligned at their R-peaks and averaged to eliminate the non-correlated breathing baselines. The averaged waveform was subsequently baseline subtracted with a cubic spline fitting to

generate the ECG wave morphology (Fig.3 b-c). The peak detection results were also used to calculate the HR between two consecutive R-peaks as well as HRV. For the raw signals that did not present excessive low frequency artifacts, we further used the adaptive threshold wavelet filter as previously described[6, 14].

2.3. Zebrafish

The studies on the zebrafish were performed in accordance with the Institutional Animal Care and Use Committees (IACUC) at Children Hospital Los Angeles, Los Angeles, CA, USA. The animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Adult zebrafish, 3–5 cm in length, were acquired from Tong's Tropical Fish and Supplies (CA) and maintained under standard laboratory conditions at 24 °C. The individual fish were fed daily with brine shrimp (hatched from eggs in 10 mL in 2 L salt water), kept in constantly circulating water, and isolated from other fish for ECG follow-up post-resection.

2.4. Heart Resection

Eighteen fish were divided into two arms: 6 sham operation and 12 apical ventricular resection. Twelve zebrafish underwent apical ventricular resection according to the previously described method[27]. Zebrafish were sedated in 5% Tricaine methanesulfonate (Tricaine). A midline incision of 0.25 cm in length was created posterior to the ventricle and approximately 20% of the apical ventricle was excised by scissors. The control fish underwent sham operation; that is, ventral midline incision was performed without ventricular resection. The zebrafish were returned to freshwater in the presence of a continuous oxygenator.

2.5. Amiodarone Treatment

Amiodarone (Sigma) was dissolved in 0.3% dimethyl sulfoxide solution and then added to fish system water to either 10 uM or 50 uM final concentration. Adult zebrafish aged 3 – 6 months were exposed to control system water with DMSO, 10 uM Amiodarone, or 50 uM Amiodarone for 24 hours followed by recording of ECG either by our jacket design as described above or traditional needle electrode ECG recording as described previously[14]. Fish were returned to fresh water after the measurement was complete.

3. Results

3.1. Parylene C-based Flexible Electrode Design and Fabrication

Two gold (Au) electrodes were embedded into parylene-C 3 mm apart as the recording and reference electrodes (Fig 1 A-B, E). The flexible electrode has a parylene-gold-parylene sandwich structure as illustrated in the fabrication process (Fig 1 C). The front recording electrode was placed near the heart, and the second placed on the abdomen as reference. Differential ECG voltages were collected by the electrode pair and were processed through the filtering and amplification circuitry. In addition, the electrodes were integrated into a new ultra-soft silicone jacket designed to comfortably wrap around the swimming fish to secure the chest electrodes. The design of the jacket consisted of a zip tie structure to adapt to various fish sizes, back padding to reduce the stress on the fish, and anchoring for the

electrode to integrate with the jacket via “wings” on the electrode, which were removed once integrated (Fig 2 A). The electrode was connected to an adapter board via a stretchable MEMS cable (Fig 2 B).

3.2. ECG Recording Signal Processing

The wearable jacket design allowed for the electrodes to be securely adhered to the ventral surface of the zebrafish for ECG recording. Zip ties secured the jacket to the size of the particular fish, and there was padding to reduce stress on the fish, allowing for ECG telemetry for 24 hours. (Fig 2 C-E.). Over the 24 hour recording period, no sensitivity loss of the sensor was observed. The fish was able to swim while tethered to the recording system via a stretchable MEMS cable (Fig 2 F, Supp Fig 1, Supp Video 1). During signal recording, the fish was confined in a transparent cylinder to restrict movement and to reduce mechanical noise (Fig 2 G). Raw ECG data were notable for noise emanated from gill motion and low frequency baseline wandering (Fig 3 A). Approximately 500 ECG segments were superimposed at their R peaks, and then averaged, to produce the gill-motion-subtracted ECG (the gill motion was assumed to be non-correlated to the heart beats, and thus were “averaged out”). Individual waveforms were in black, and the averaged ECG waveform in red (Fig 3 B). The gill motion subtracted ECG was further subtracted with a manually fitted spline curve (blue) at its iso-electrical line to eliminate the residual gill motion and low frequency baseline. The resulting ECG demonstrated the P-wave, QRS-complex, and T-wave components (Fig 3 C).

3.3. QT Prolongation and Heart Rate Decrease in Response to Ventricular Resection

Having established ECG telemetry, we investigated the electrical phenotypes in response to ventricular resection. We unraveled circadian rhythm variations in terms of HR and HRV from the non-sedated fish (Fig 4 A). Non-sedated HR varied over the course of heart regeneration, with significantly reduced HR at 10 days post-amputation (dpa) (Fig 4 C) accompanied by increased HRV (Fig 4 D) in the amputated fish compared to control, and normalized to baseline at 26 dpa. QT interval prolongation was observed in the amputated fish at 10 dpa, and normalized to baseline by 26 dpa (Fig 4 B). Thus, our 24 hour telemetry identified physiological significance in the heart rate and heart rate variability of the regenerating fish.

3.4. QT Prolongation in Response to Amiodarone Treatment

We assessed the effects of sedation on Amiodarone-mediated QT prolongation. We compared non-sedated analysis using our jacket design with sedated regimen using needlepoint electrodes. In the absence of sedation, we observed a decrease in mean HR ($n=3$, $p< 0.01$) and increase in QT interval (Fig 5 A-C, Supp Fig 3), whereas in the presence of sedation, the change in HR was masked ($p < 0.7$, $n=4$), HRV amongst measurements increased, and the change in QTc was not detected (Fig 5 D-F, Supp Fig 4). Thus, the data highlighted the effect of sedation to mask physiological rhythms and electrical repolarization for cardiac toxicity testing.

4. Discussion

The novelty of this study is to establish waterproof wearable sensors for long-term continuous monitoring of zebrafish ECG signals in the absence of confounding sedative effects. Our group has previously developed sensors to detect zebrafish ECG signals to characterize cardiac regeneration under sedation [28] as well as wearable microelectrode membranes for the neonatal mouse model[29]. However, the design of the current wearable sensors is the first to enable long-term non-sedated monitoring of the zebrafish ECG signal for applications such as circadian rhythm detection.. For the first time, the new ultra-soft silicone jacket was designed to comfortably wrap around the swimming fish to secure the chest electrodes. We uncovered circadian rhythm variations in terms of HR and HRV from the non-sedated animals. Specifically, we observed a reduction in mean HR and an increase in HRV at 10 dpa, accompanied by QT prolongation as well as diurnal variations, followed by normalization in mean HR and QT intervals at 26 dpa. We further revealed Amiodarone-mediated QTc prolongation, HR reduction and HRV otherwise masked by sedation. Identifying these physiologically relevant effects otherwise masked by traditional needle electrodes advances future toxicity testing and drug screening.

Our novel design incorporating parylene-C based ECG sensors with an ultra-soft silicone wearable jacket enables ECG telemetry. The extended parylene cable connecting the underwater chest electrodes and out-of water electronics allows the fish to swim freely through its aquatic habitat, and eliminates the needs to disturb the cardiac physiology prior to ECG recording. The zip-tie design of the jacket accommodates varying sizes of fish to maintain adherence of the chest electrodes while the fish is moving. The jacket is made of micro-molded partially cured silicone to match the Young’s modulus of the fish surface and to minimize potential tissue strain for long-term wear. Matching elastic modulus properties of the sensing platform with the contacted tissue is important to address patients’ acceptance and adherence to flexible and wearable biosensors[30]. Another innovation is to embed micro glass-spheres in the thin film of silicone to reduce the effective density of the jacket to close to 1 g·cm-3; thereby, eliminating the additional weight of the jacket. Non-linear signal processing techniques further filters out the breathing and electromagnetic artifacts arising from gill and muscle movements. Together, these advances provide the opportunity for long-term monitoring and observation of circadian phenomena in response to heart resection, repair, and drug toxicity.

Circadian variation in the frequency of onset of acute myocardial infarction has been widely recognized in humans, with a peak incidence between 6 a.m. and 12 noon[31]. Long-term monitoring also allows for a higher chance of observing cardiovascular events - nocturnal cardiovascular events are more frequent at the beginning and end of the night[32]. As a corollary, Zebrafish constitutes the ideal vertebrate system to study the complexity of the circadian clock in relation to behavioral, sleep cycle, cellular, and molecular responses. The National Institutes of Health (NIH) has ranked the role of zebrafish as the third most important experimental organism[33]. It has become a widely used model organism because of its fecundity, its and physiological similarity to mammals, the existence of many genomic tools, and the ease with which large, phenotype-based screens can be performed[17]. They have an immune system, bones, and digestive, nervous and cardiovascular systems. In

addition to developmental process and disease modeling, transgenic mutants have provided the opportunity for optogenetic control of cardiac function[34], hemodynamic stress-mediated developmental genes for vascular repair[10], and a platform for in vivo drug discovery[17].

The application of zebrafish to preclinical drug development and toxicity testing, including recent advances in mutant generation, strengthens the capabilities this model organism in drug discovery. It is widely known that anesthesia drugs affect both the heart rate and the morphology of ECG measurements. The quantified effects of anesthesia are unknown and heavily based upon individual response, and thus cannot be systematically subtracted. By enabling non-sedated measurement, our wearable and waterproof ECG electrodes uncovered prolonged QT intervals and reduced heart rates in response to Amiodarone at concentrations where traditional sedated needle ECG measurements failed to detect any difference. This enhanced acuity is essential as a preclinical model to recognize potentially dangerous electrocardiac phenotypes.

5. Conclusions

In this paper, we introduce the next generation of waterproof and wearable micro-ECG telemetry for mobile health. The ultra-soft jacket for long-term monitoring, the zip-tie design of the jacket to maintain adherence of the electrodes, and the embedded micro-glass spheres for light weight are the technical advances to uncover physiological phenomena for small animal models of tissue regeneration, and drug discovery. The technical innovation further paves the way for mobile health, wearable sensors, and sleep research[17, 18].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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highlights

- Waterproof wearable sensors for long-term non-sedated ECG measurement
- Ultra-soft silicone jacket secures parylene-C based micro-ECG multi-electrode array
- Uncovered circadian rhythm variation in response to ventricle resection and repair
- Revealed drug-mediated cardiac toxicity and heart rate reduction
- Implications for future toxicity testing, drug screening, and mobile health

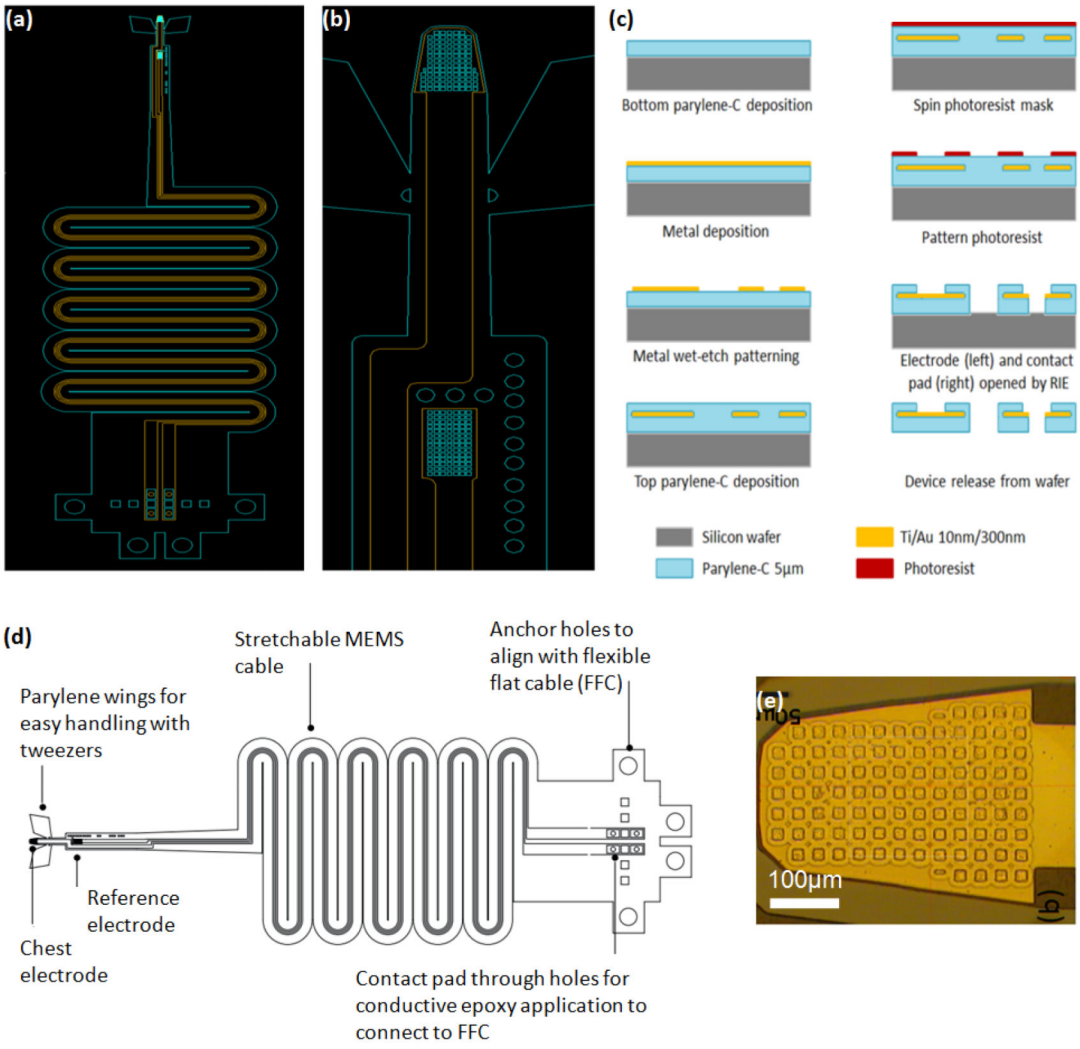

Figure 1. Parylene C-based electrode and cable design, micro-fabrication, and device integration

(a) The blue pattern illustrates the Parylene outline, and yellow pattern is the gold (Au) trace. Two Au electrodes (chest electrode and reference electrode) are patterned at 3 mm apart. Each electrode harbors a micro meshed structure with 5 μm × 25 μm openings designed to reduce stress on the Au thin film in response to stretching. The “wings” next to the chest electrode are designed to thread the zip tie ribbons into the silicone jacket, and are removed after the integration with jacket is complete. The PA holes around the reference electrode allow for anchoring the MEMS electrode/cable onto the jacket. Uncured silicone will be applied at these holes to adhere the electrode onto the jacket. (b) The zoomed-in view reveals the chest and reference electrodes. (c) Micro-fabrication process of the PA-Au/Ti-PA electrodes highlight the use of parylene C to embed electodes (d) Schematic diagram of the flexible cable and electrodes. (e) The zoomed-in view reveals the micro mesh openings in the chest electrode. Each opening is at 25 μm ×25 μm.

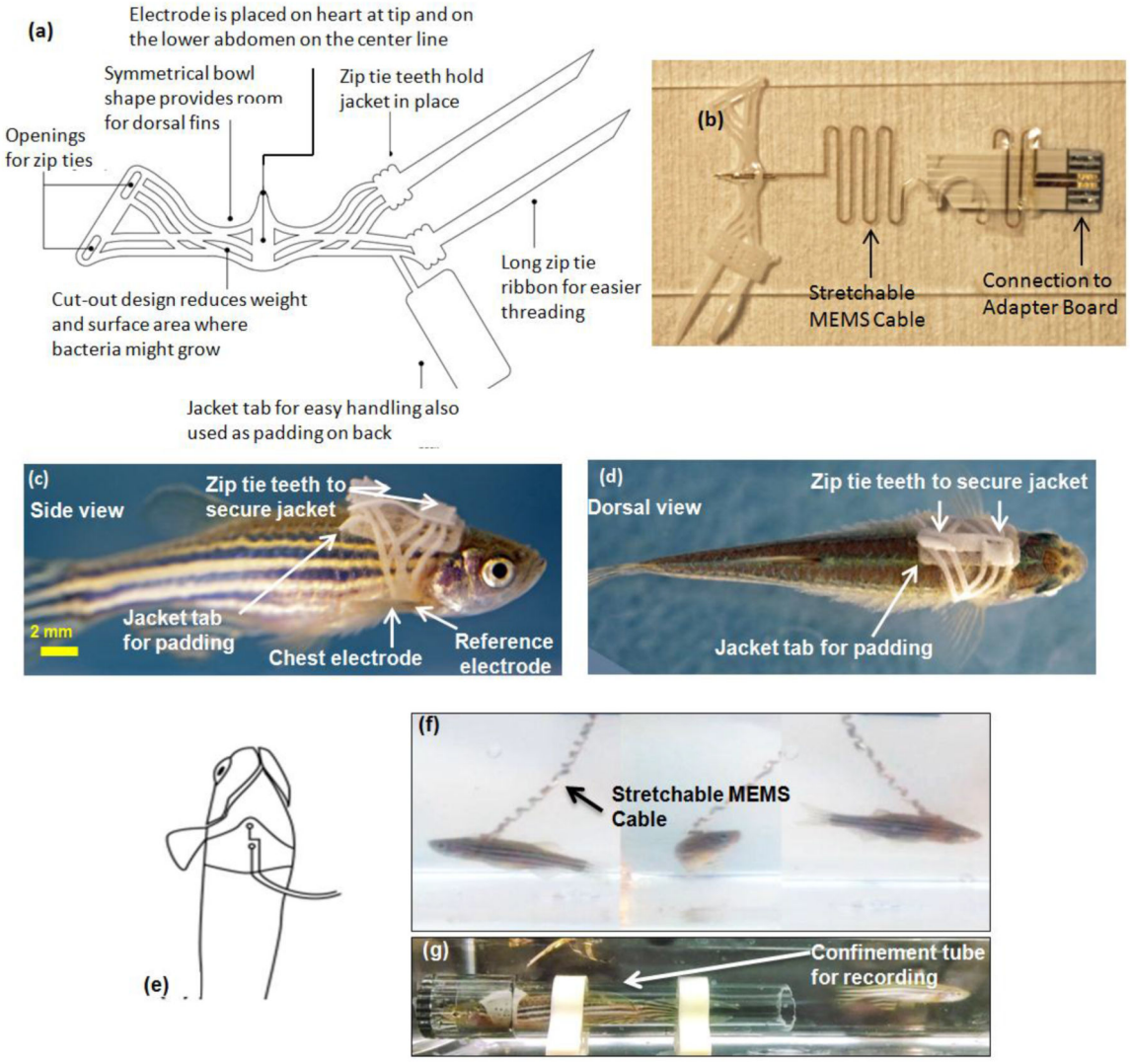

Figure 2. The PDMS-based jacket allows the multi-electrode array (MEA) to securely adhere to the ventral surface of zebrafish for ECG recording

(a) Schematic diagram of the ultra-soft silicone jacket reveals a zip tie structure with multiple teeth to adapt to various fish sizes; the extended zip tie length will be cut after threading. A back padding reduces the stress on the fish skin in response to the Young's modulus mismatch between the skin and the silicone jacket. The chest electrode will be threaded through the center "cross" opening at the tip of the jacket. The electrode is anchored at the PA opening holes. (b) Stretchable cable and micro electrodes integrated with PDMS jacket and FFC connection. (c) and (d) Wearable silicone jacket is made of a dry film micro-casted with a medical grade PDMS elastomer (MDX4210, Dow Corning, USA) at a mixing ratio of 20:1 to reduce Young's modulus, and is mixed with hollow micro glass beads (3M-S38 Microspheres, 3M, USA) to minimize the weight at 1g/ml. . (e) A schematic diagram illustrates the placement of electrodes on the ventral part of zebrafish. (f) The fish is wearing the silicon jacket tethered to the recording system. (g) Fish is confined in a tube for real-time ECG recording.

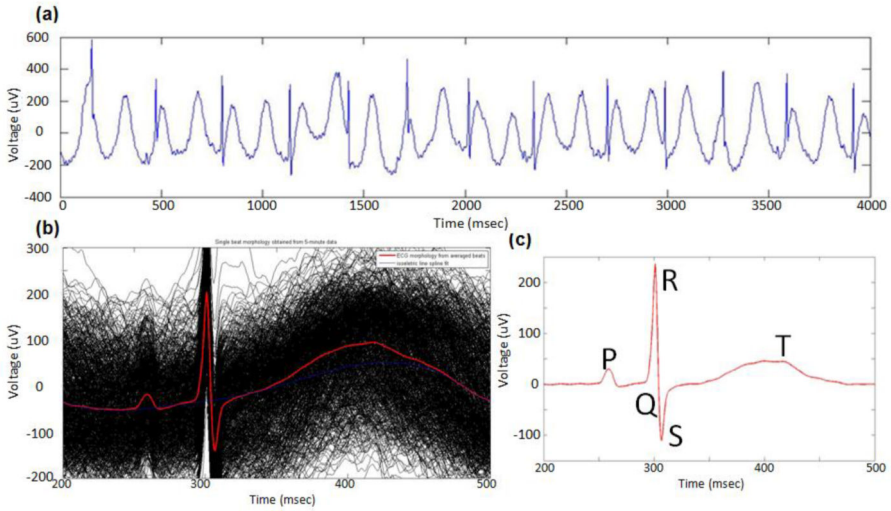

Figure 3.

ECG Recording and Signal Processing

(a) Raw data of a representative ECG waveform in the presence of gill motion and low frequency baseline wandering. R waves were detected by using an in-house R wave detection algorithm. Specifically, all of the individual ECG signals were aligned with the peak R waves and were summed together to filter the unsynchronized breathing artifacts and wandering baselines. (b) A summed ECG waveform over 5 minutes was highlighted as the red ECG pattern while the black waveforms were the individual ECG signals. (c) The residual baseline was then fitted with a cubic spline curve (blue) and subtracted from the summed ECG waveform.

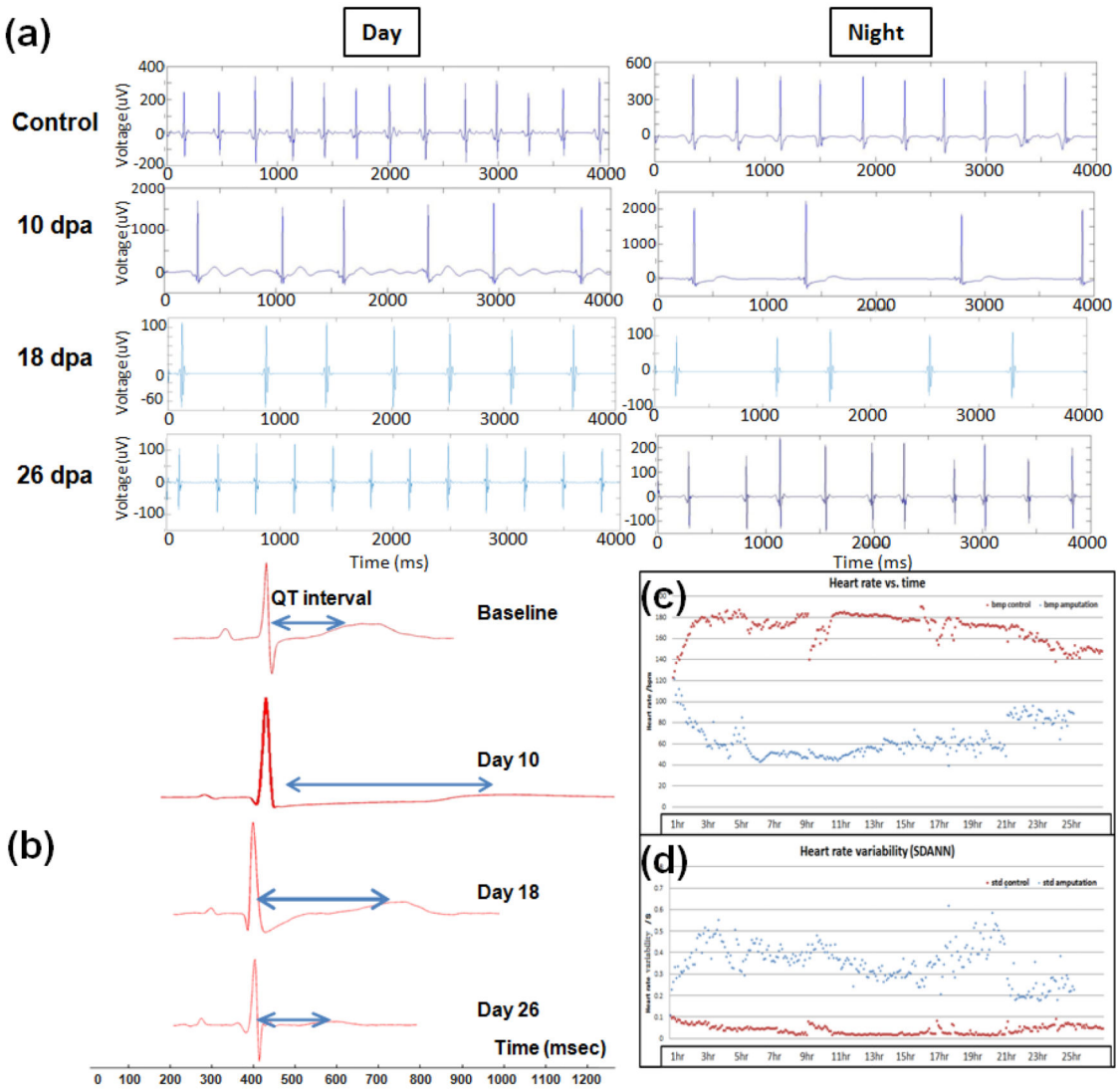

Figure 4.

Circadian variation in heart rates.

(a) At day 0 (baseline), the mean heart rate at day time was 192, and at night was 154. At day 10, the mean heart rate at day time was 88, and at night was 51. At day 18, the mean heart rate at day time was 103, and at night was 77. At day 26, the mean heart rate at day time was 168, and at night was 154. At night when fish is resting, the heart rate is slightly slower. However the heart rate difference in the 10 day post surgery case is much more pronounced. At 18 day post surgery, the heart rate has increased and at 26 day post surgery the heart rate has approximately recovered back to normal. (b) QT intervals were prolonged in response to ventricular injury at day 10 and 18, followed by normalization at day 26. The ECG monitoring captured the electrical phenotypes to recapitulate the regenerative capacity of adult zebrafish. We applied the R wave detection algorithm to extract all of the heart rate intervals. We calculated the mean heart rates (HR) and heart rate variability (HRV) at every 5 minutes. Next, we plotted the HR and HRV as a function of time for 24 hours. (c) Comparison of HR between the sham-treated and the ventricular injury fish over 24 hours at

10 day post resection. Interestingly, HR tends to be higher in the sham group (n=5) (d) Comparison of HRV between sham- and amputated-treated animals for 24 hours at 10 day post amputation (dpa). HRV is higher in the amputated than the sham-treaed group (n=5).

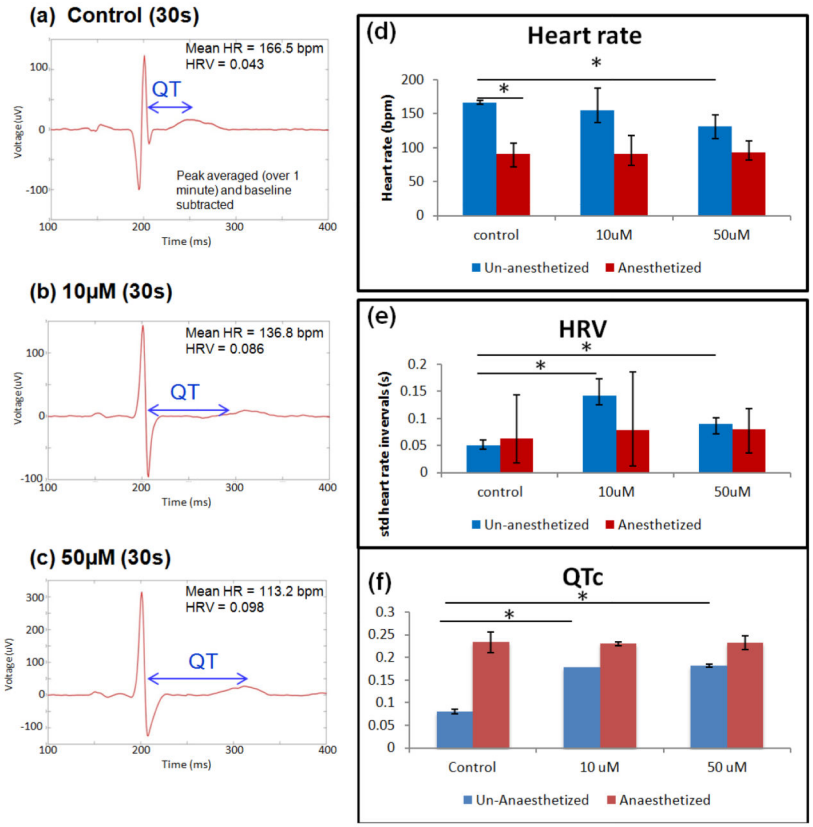

Figure 5.

ECG Analysis in response to amiodarone treatment.

The left panels represent the signals obtained from control (a), treatment with 10μM (b), and 50μM (c) of Amiodarone in the non-sedated fish (n=5), respectively, to highlight changes in heart rate (HR) and prolonged QT intervals in response to 10μM and 50μM of Amiodarone. (d) In the non-sedated animals, the mean HR significantly decreased in response to 50 μM of Amiodarone (* $P < 0.01$, n=3). In the sedated animals, the mean HR is significantly lower than those of non-sedated animals (* $P < 0.01$, n=3), but it is not responsive to an increase in Amiodarone concentration ($P > 0.7$, n=4). (e) In the non-sedated animals, HRV is responsive to 10 μM and 50 μM of Amiodarone (* $P < 0.01$, n=3). In the sedated animals, HRV is statistically insignificant ($P > 0.6$, n=4). (f) QTc reveals significant change in corrected QT interval between control and both 10μM and 50μM Amiodarone treatment (* $P < 0.05$, n=3).