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In Vivo Surface Electrocardiography for Adult Zebrafish

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Corresponding Author:	Thao P. Nguyen, M.D., Ph.D. University of California Los Angeles Los Angeles, CA UNITED STATES
Corresponding Author's Institution:	University of California Los Angeles
Corresponding Author E-Mail:	tpnguyen@mednet.ucla.edu
Order of Authors:	Yali Zhao, M.D., Ph.D.
	Michelle N Tran
	Morgan Yun
	Sean A Nguyen
	Thao P. Nguyen, M.D., Ph.D.
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Cover Letter

DAVID GEFFEN SCHOOL OF MEDICINE AT UCLA

Thao P. Nguyen, M.D., Ph.D.
Assistant Professor-in-Residence of Medicine
Office: (310) 825-9465
Laboratory: (310) 825-6713
Fax: (310) 206-5777
Email: tpnguyen@mednet.ucla.edu

Department of Medicine, Division of Cardiology
Room 3770 MRL
675 Charles E Young Drive South
Box 951760
Mail Code 176022
Los Angeles, CA 90095-1760

May 3, 2019

Re: Revision MS#60011
Vineeta Bajaj, Ph.D.
JoVE Editorial Board

Dear Dr. Bajaj,

We would like to submit a second revision for our manuscript #60011, titled, "*In Vivo* Surface Electrocardiography for Adult Zebrafish" for publication in *JoVE*. To date, this manuscript, or part of it, has never been published. We are not currently submitting this manuscript to any other journals. All authors have read the manuscript and approved its submission to *JoVE*.

We have included in this resubmission a point-by-point response to the Editor' comments.

Please contact me if any questions or concerns.
Thank you very much for your consideration, Dr. Bajaj

Best regards,

Thao P. Nguyen, M.D., Ph.D.

TITLE

In Vivo Surface Electrocardiography for Adult Zebrafish

AUTHORS AND AFFILIATIONS

Yali Zhao[1], Michelle Tran[1], Morgan Yun[1], Sean A. Nguyen[1,2], Thao P. Nguyen[1]

[1]Department of Medicine, Division of Cardiology, the Cardiovascular Research Laboratory, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
[2]California State Polytechnic University Pomona, Pomona, CA, USA

Email Addresses of Co-Authors:
Yali Zhao (YaliZhao@mednet.ucla.edu)
Michelle Tran (michellenhutran@gmail.com)
Morgan Yun (morganyun@g.ucla.edu)
Sean A. Nguyen (seann6556@gmail.com)

Corresponding Author:
Thao P. Nguyen (tpnguyen@mednet.ucla.edu)

KEYWORDS

Electrocardiography; electrocardiogram; ECG; EKG; zebrafish; *Danio rerio*; myocardial ischemia; myocardial infarction

SUMMARY

Here, we present a reliable, minimally invasive, and cost-effective method to record and interpret electrocardiograms in live anesthetized adult zebrafish.

ABSTRACT

The electrocardiogram waveforms of adult zebrafish and those of humans are remarkably similar. These electrocardiogram similarities enhance the value of zebrafish not only as a research model for human cardiac electrophysiology and myopathies but also as a surrogate model in high throughput pharmaceutical screening for potential cardiotoxicities to humans, such as QT prolongation. As such, in vivo electrocardiography for adult zebrafish is an electrical phenotyping tool that is necessary, if not indispensable, for cross-sectional or longitudinal in vivo electrophysiological characterizations. However, too often, the lack of a reliable, practical, and cost-effective recording method remains a major challenge preventing this in vivo diagnostic tool from becoming more readily accessible. Here, we describe a practical, straightforward approach to in vivo electrocardiography for adult zebrafish using a low-maintenance, cost-effective and comprehensive system that yields consistent, reliable recordings. We illustrate our protocol using healthy adult male zebrafish of 12-18 months of age. We also introduce a rapid real-time interpretation strategy for quality validation to ensure data accuracy and robustness early in the electrocardiogram

recording process.

INTRODUCTION

The zebrafish (*Danio rerio*) heart is located anteroventrally to the thoracic cavity between the operculum and the pectoral girdles. The heart is enclosed rather loosely within a silver-colored pericardial sac. Anatomically, the zebrafish heart is different from the four-chambered human and other mammalian hearts because of its diminutive scale (100-fold smaller than the human heart) and its two-chambered structure consisting of only one atrium and one ventricle. Nonetheless, the electrocardiogram (ECG) waveforms and the duration of the QT interval of both species are remarkably similar (**Figure 1**). Accordingly, zebrafish has emerged as a popular model for studying human inherited arrhythmias[1-3] and for high-throughput drug screening of potential human cardiotoxicities[4,5], such as QT prolongation.

In the routine evaluation of human cardiac diseases, the body-surface ECG has become the most extensively used first-line non-invasive diagnostic tool since its invention by Einthoven in 1903. In contrast, since the first adaptation of the body-surface ECG recording method for adult zebrafish in 2006[6] and several modifications thereafter[7], this technique has remained largely inaccessible to many researchers in the field despite the popularity of this animal model. For other researchers who performed in vivo ECG interrogation for adult zebrafish, wide variations among operators led to inconsistency in ECG findings from different studies. Common reasons include cumbersome and expensive specialized devices and software, low signal-to-noise ratio, and confusion regarding the electrode placement, all further aggravated by an incomplete understanding of the adult zebrafish ECG features and underlying tissue mechanisms. Given that in vivo ECG is the only diagnostic tool to electrically phenotype live zebrafish, there is a clear need for a standardized method to improve sensitivity and specificity, reproducibility and accessibility.

Here, we present a practical, reliable, and validated approach to record and interpret zebrafish in vivo electrocardiograms (**Figure 2**). Using a single bipolar lead in the frontal plane, we investigated the changes in ECG waveforms and interval durations of live anesthetized healthy wild-type AB adult zebrafish.

PROTOCOL

All experiments in this study were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols in this study were approved by the UCLA Institutional Animal Care and Use Committee.

1. Prepare the experimental set up

1.1 Maintain zebrafish in flow-through aquarium systems on a 14 h light, 10 h dark photoperiod at 28 °C ± 0.5 °C. Feed with flake food daily and live brine shrimp (*Artemia*

nauplii) twice daily. Zebrafish in this study were maintained and fed by the UCLA Zebrafish Core.

1.2 Set up the in vivo ECG recording system by connecting the essential pieces of equipment and inserting the three color-coded stainless-steel electrodes into the three color-matched access portals of the amplifier (**Figure 3**). Start the system at the start of an ECG recording and/or analysis session.

1.3 Procure zebrafish and other necessary tools, such as a wet sponge with a slit to hold the fish, forceps, scissors, Pasteur pipettes, and culture dishes (100 mm x 20 mm).

2. Induction of anesthesia

2.1 Prepare immersion anesthesia for pain control and fish immobilization to avoid motion artifacts during ECG data acquisition. For example, immersion tricaine (ethyl 3-aminobenzoate methanesulfonate, MS-222).

2.1.1 To make the tricaine 0.4% stock solution, combine the following items in a screw-capped dark glass bottle: 400 mg of tricaine powder, 98 mL of double distilled water, and 2 mL of 1 M Tris (pH 9). Adjust to pH 7.0 using 1N NaOH or 1N HCl as needed[8].

2.1.2 To make the tricaine final immersion solution, determine the minimum concentration that is appropriate for the zebrafish age[9], size, metabolic state, strain, disease model, scientific objectives, and procedural duration.

2.1.3 Perform a tricaine concentration-response study, titrating up or down from the recommended concentration of 168 mg/L (or 0.0168%)[9] if necessary, to attain the level 4 of anesthesia within 3 min with the fewest possible cardiorespiratory toxicities. For example, in this study, the immersion of wild-type AB zebrafish of 12-18 months of age into a 0.02-0.04% tricaine solution will induce level 4 of anesthesia within 3 min.

NOTE: At level 4 of anesthesia, equilibrium and muscle tone are completely lost and opercular movement rate is reduced[8].

2.1.4 If necessary, consult the veterinarian in the Institutional Animal Care and Use Committee (IACUC) for additional guidance on the appropriateness of the selection of anesthetic(s) and route of administration.

2.2 Immerse an adult zebrafish into a dish containing tricaine solution of the lowest predetermined and IACUC-approved concentration (e.g., 0.02-0.04% in this study) to induce level 4 of anesthesia within 3 min (**Figure 2**).

2.2.1 For survival ECG protocol, keep the ECG recording session as brief as possible (under 10 min). For brief ECG recording sessions lasting less than 15 min, anesthesia maintenance is not necessary.

2.2.2 For long ECG recording sessions lasting hours, use a long-acting intramuscular paralytic and an oral perfusion system to provide ample hydration and oxygenation[6].

3. Place the ECG lead electrodes

3.1 Once the zebrafish maintains level 4 of anesthesia for 3 s, use a pair of blunt forceps to transfer the fish immediately onto the damp sponge slit with its ventral surface uppermost for placement of ECG lead electrodes (**Figure 4**).

3.2 Gently insert the three ECG lead electrodes into the fish musculature to approximately 1 mm in depth to establish a bipolar lead in the frontal plane that parallels the left caudal-right cranial orientation of the cardiac main axis.

3.3 Position the positive (red) electrode in the ventral midline at the level of the bulbus arteriosus, i.e., at 1-2 mm above an imaginary line connecting the two lower edges of the operculums (**Figure 4A**).

3.4 Using the positive electrode as the reference, position the negative (black) electrode caudally and 0.5-1.0 mm left laterally, at a distance greater than the maximal apicobasal length of the adult zebrafish ventricle (**Figure 4A**).

3.5 Position the reference (green) electrode caudally, near the anal region.

NOTE: Since the cardiac main axis varies somewhat from fish to fish, to maximize the R and T wave amplitudes, adjust the lead positions by making only small, systematic changes through trial and error. For example, change one electrode (positive or negative), instead of both electrodes, at a time and make gradual changes in one specified direction before changing to another direction instead of making erratic changes in random directions.

4. Record ECG

4.1 Open the ECG data acquisition program. Select a desired setting from the drop-down menus for range, low pass, and high pass. For example, the following setting in the in vivo ECG recording system used in this experiment yields consistent, satisfactory signal-to-noise ratio for a normal adult zebrafish: range “2 mV”, low pass “120 Hz”, and high pass “0.03 s”.

4.2 Press **Start** to start continuous gap-free ECG recording at a sampling rate of 1 kHz.

4.3 To optimize lead positioning, press **Stop** to stop ECG recording and review the ECG trace for the satisfaction of all following four validating criteria in a normal ECG (**Figures 1**):

4.3.1. Criterion 1: Ensure all ECG waveforms (P, QRS, and T) are distinct and readily visible.

4.3.2. Criterion 2: Ensure the P wave is positive.

4.3.3. Criterion 3: Ensure the R wave is positive.

4.3.4. Criterion 4: Ensure the T wave is positive.

4.4 Reposition the electrodes (try the negative electrode first) if necessary, until all four criteria are satisfied if a normal ECG is expected.

4.5 At the end of the ECG recording session, save the ECG sweeps for subsequent analysis.

5. Recovery from anesthesia

5.1 Carefully remove the electrodes ensuring not to injure the fish. Return the fish from the damp sponge to fresh, oxygenated fish water free of tricaine.

5.2 To facilitate recovery from anesthesia, squirt water over the gills vigorously with a Pasteur pipette until the fish resumes regular gill movement or swimming.

5.3 Monitor for full recovery from anesthesia (typically 1-2 min), as indicated by the ability to swim upright for at least 5 s.

6. Analyze the ECG recordings

6.1 Define the analysis settings.

6.1.1 Know the software interface (**Table of Materials**) by reading the operating manual of the ECG data analysis software.

NOTE: Although the directions below are specific to the commercial software used in our laboratory, the basic tasks to accomplish are essentially the same in any software package for ECG analysis.

6.1.2 Open the ECG data analysis program. From the **File** menu, select **Open** to open the ECG file of interest and display the full ECG trace. Use the mouse to drag out a section of interest in the ECG trace to analyze.

6.1.3 From the **ECG Analysis** menu, select **ECG Settings** to open a dialogue box to pre-define various parameter settings for software automatic analysis (**Figure 5A**).

6.2 Analyze the heart rhythm and rate.

NOTE: Heart rate depends on several factors, including zebrafish age and strain, anesthesia agents (e.g., tricaine, isoflurane, etc.) and concentration, anesthesia usage (single agent[5,7] vs. combined agents[5]) and exposure time[5]. For example, the heart rate of 12-18 month-old wild-type AB zebrafish in this study after 3-5 min of immersion in 0.02-0.04% tricaine solution was 116 ± 17 beats per minute (*n* = 9), consistent with literature reports of heart rate for this age group and anesthetic[5,7].

6.2.1 Determine whether the heart rhythm is sinus or not, regular or irregular.

NOTE: The presence (or absence) of sinus rhythm is based on the presence (or absence) of an upright P wave preceding each QRS by a normal PR interval (e.g., 60-65 ms for Liu et al.'s 10-12 month-old[7] and 12-18 month-old wild-type AB zebrafish in this study). The atrial and ventricular rhythm regularity (or irregularity) is based on the regularity (or irregularity) of successive PP or RR intervals, respectively.

6.2.2 Determine the appropriateness of the software automatically generated atrial (or ventricular) rate by verifying that the software correctly identify all the P waves (or R waves). The atrial rate is the average PP interval whereas the ventricular rate is the average RR interval.

NOTE: The software automatically identifies the P and R waves. Based on these automatic identifications (or manual corrections) of the P and R waves, the software automatically measures all the PP and RR intervals in the ECG selection, calculates the interval averages to generate the atrial and ventricular rate. Therefore, to determine the heart rate, correct identification of the P and R waves is critical.

6.2.3 Correct any auto-identification mistakes by moving the misplaced cursors to the appropriate waves (**Figure 5B**).

NOTE: If the heart is in sinus rhythm, the atrial rate and ventricular rate are the same because of the one-to-one correspondence between the sinus P waves and the QRS complexes. However, in the case of atrioventricular dissociation (e.g., in ventricular tachycardia or third-degree atrioventricular block), this one-to-one correspondence between the P waves and QRS complexes is lost; therefore, there are two heart rates because the atrial rate is different from the ventricular rate.

6.2.3 Determine the heart rate based on at least five consecutive complete cardiac cycles

if the heart rhythm is regular, or a strip of at least six seconds if the heart rhythm is irregular.

6.3. Calculate the intervals and wave durations.

6.3.1. Go to **ECG Analysis** > **Averaging View** to concatenate *n* (e.g., 5) consecutive cardiac cycles into a single average signal (**Figure 5C**).

NOTE: If the ECG waveforms of an individual cardiac cycle diverge substantially from the average signal, study that cardiac cycle separately without concatenation.

6.3.2. Ensure that the software automatically identifies the start and end of the P wave, QRS complex, and T wave. Based on these automatic identifications (or manual corrections) of these waves and intervals, the software automatically measures the durations as defined conventionally.

NOTE: The PR interval extends from the start of the P wave to the start of the QRS complex (or the RS complex if the Q wave is not visible). The QRS duration extends from the start of the Q wave (or the R wave if the Q wave is not visible) to the end of the S wave (i.e., the J point; **Figure 1**). The QT interval extends from the start of the Q wave (or the R wave if the Q wave is not visible) to the end of the T wave. Therefore, to calculate intervals and durations, correct identification of the start and end of the P wave, QRS complex, and R wave is critical.

6.3.3. Determine the appropriateness of the software automatically generated intervals and wave durations by verifying that the software correctly identifies the start and end of the P wave, QRS complex, and T wave displayed in the Averaging View window (**Figure 5C**). Correct any auto-identification mistakes by moving the misplaced cursors to the appropriate positions.

6.3.4. Select the negative peak of the S wave as the end of the QRS complex[7] because the zebrafish J point that signals the end of the S wave can be particularly difficult to identify accurately. This will cause a slight underestimate of the true QRS duration.

NOTE: The ECG analysis software automatically corrects the QT interval to the ventricular rate (or RR interval) to generate the corrected QT interval QTc using the method pre-selected by the user in step 6.1.3, for example, Bazett (**Figure 5A**). The Bazett's formula (1920) $QTc = QT \div \sqrt{RR}$ is the most popular and the first of several methods proposed to correct the human QT interval for heart rate. Because the accuracy of the Bazett's formula has been questioned, refer to other methods proposed for humans[10,11] and zebrafish[6] (**Figure 5D**).

6.4. Interpret ECG abnormalities by recognizing exceptions for the four validating criteria in step 4.3.

6.4.1 Recognize exceptions for criterion 1. In the absence of any P waves (which indicates the absence of sinus rhythm), rely on the RR intervals and QRS duration to diagnose the heart rhythm. For example, if the RR intervals are irregularly irregular, diagnose atrial fibrillation; if the RR intervals are regular and the QRS is normally narrow, diagnose junctional escape rhythm; on the other hand, if the RR intervals are regular and the QRS is abnormally prolonged, diagnose ventricular escape rhythm.

6.4.2 Recognize exceptions for criterion 2. When the P wave is negative (or inverted), diagnose retrograde atrial activation from an ectopic pacemaker (such as an atrial site downstream of the sinus node, the atrioventricular node, or the ventricle).

6.4.3 Recognize exceptions for criterion 3. When tall and narrow Q waves present with negative P and negative T waves, diagnose lead reversal due to an erroneous switch of the positive and negative electrode positions because those tall and narrow Q waves were true R waves mistakenly inverted (**Figure 6D**). In contrast, when broad Q waves present with positive P waves following significant cardiac injury, diagnose myocardial infarction because those broad Q waves are true pathologic Q waves.

6.4.4 Recognize exceptions for criterion 4. When the T wave is inverted, inspect ventricular activation to identify whether the ventricular repolarization abnormality is primary or secondary. Rely on the clinical scenario to narrow down the correct diagnosis from a differential list of primary ventricular repolarization abnormality (from drug effects or myocardial ischemia; **Figures 6C**) vs. secondary ventricular repolarization abnormality (due to aberrant ventricular activation from pre-excitation, ventricular ectopy, or ventricular pacing).

6.5 Export ECG findings.

6.5.1 Select **Table View** to review all ECG measurements. Select the measurements of interest to copy and paste into the desired document (e.g., Excel spreadsheet).

6.5.2 To export an ECG trace, highlight a section of interest in the ECG sweep using the magnifier icon. Copy and paste into the desired document (e.g., Word or PowerPoint).

REPRESENTATIVE RESULTS

Figure 1 illustrates the clinical relevance of the method presented here. In vivo surface electrocardiography for adult zebrafish is an essential electrical phenotyping tool because of the remarkable similarities between the zebrafish and human ECG despite their vast anatomical differences. The zebrafish heart has only one atrium and one ventricle in

contrast to the human heart with two atria and two ventricles (top row; right and left, respectively). However, despite its apparent anatomical simplicity, the zebrafish heart shares several ECG features with the human heart (bottom row; right and left, respectively) Therefore, the zebrafish heart has emerged as a surrogate model for human cardiac electrophysiology[5,12,13]. **Figure 1** illustrates a small but distinct Q wave from a live, healthy 14-month-old zebrafish. However, in zebrafish ECG, lead positioning is not commonly optimized to demonstrate the Q wave. Therefore, the Q wave is commonly invisible, and an RS complex is more commonly seen than the full QRS complex in zebrafish ECG.

Figure 2 summarizes the four essential action steps to conduct minimally invasive in vivo electrocardiography for adult zebrafish. Following anesthesia induction (step 1) and electrode placement (step 2), we recorded baseline ECG signals (step 3) from healthy wild-type AB zebrafish of 12 to 18 months of age (*n* = 9). Our electrode insertion technique was only minimally invasive because we did not need to peel fish scales or perform pericardiotomy. Following data acquisition, we manually reviewed and verified each ECG recording (step 4) to avoid potential misinterpretation by software automatic analysis.

Figure 3 shows the three indispensable components of a typical ECG data acquisition and processing system: a high-performance data acquisition hardware, a high-gain differential amplifier, and a computer uploaded with software for ECG data acquisition and analysis. In our laboratory, we adapted an existing commercial in vivo ECG recording system originally designed for small mammalian models (such as mice, rats, and rabbits) to accommodate the adult zebrafish model.

Figure 4 demonstrates that proper lead placement requires aligning the lead with the presumed cardiac main axis. In zebrafish *in vivo* ECG recording, because only one single lead is used, proper lead positioning to maximize concurrently both R and T wave amplitudes is critical. To maximize R and T wave amplitudes, we aligned the positive and negative lead electrodes with the cardiac main axis, presumably in the left caudal to right cranial orientation. Following thoracotomy and pericardiotomy to open the pericardial sac and expose the heart, the cardiac main axis becomes apparent (**Figure 4B** white dashed line). In fact, pericardiotomy to expose the heart is a commonly used strategy to increase the signal-to-noise ratio[7] at the cost of converting the ECG recording from a minimally invasive into a highly invasive procedure.

Figure 5 illustrates critical steps in ECG analysis. First, we pre-defined the various parameter settings for software automatic analysis using the ECG Settings dialogue box (**Figure 5A**). Because we repurpose an existing ECG recording equipment designed for mammalian models to accommodate adult zebrafish, the Detection and Analysis setting for zebrafish is not available. We selected the Human Preset instead, given the remarkable similarity of zebrafish ECG to human ECG (**Figure 5A**). Second, we manually verified (red) the software automatic ECG identification (black) of the R wave peaks and correct any R wave auto-

identification mistakes prior to commanding the software to recalculate the average ventricular rate. For example, in **Figure 5B**, a large P wave in relation to the R wave fooled the software into misidentifying the R waves, leading to the subsequent automatic miscalculation of the RR interval or ventricular rate. Therefore, human verification and appropriate corrections as needed are critical in ECG analysis. Third, we quickly assessed rhythm regularity and calculated the average duration of waves and intervals using the **Averaging View** (**Figure 5C**) to concatenate several consecutive cardiac cycles (green) into one single average signal (black). Here in **Figure 5C**, the negligible deviation between each of the nine cardiac cycles and the average signal argues for the excellent rhythm regularity of this zebrafish heart. Lastly, we enabled the software to automatically correct the QT interval for heart rate using Bazett, one of the seven different methods available (**Figure 5D**).

Figure 6A-C demonstrates how the depth of electrode placement affects the amplitudes of the ECG signals. When we incorrectly inserted the electrodes too superficially in the dermis (**Figure 6A**), the lead was "indirect"-like (more than two cardiac diameters from the heart, similar to the indirect standard human ECG limb leads I, II, and III) and the voltage signals were small. When we appropriately inserted the electrodes 1 mm deeper into the pectoralis musculature (**Figure 6B**), the lead became "semidirect" (in close proximity but not in direct contact with the heart) and the voltage signals increased. The ECG waveforms became readily visible. However, when we incorrectly inserted the electrodes even deeper into the ventricle (**Figure 6C**), the lead became "direct" (in direct contact with the heart) and the voltage signals increased further. The R wave amplitude in **Figure 6C** increased by eight-fold compared to **Figure 6A** and by four-fold compared to **Figure 6B**. However, the ECG trace in **Figure 6C** revealed new signs of injury to the ventricular myocardium, such as new ST depression and new T wave inversion.

Figure 6D demonstrates how the unusual inversions of all ECG waveforms (P, Q, R, S, and T) should signal a lead reversal mistake, in which the positive and negative electrodes switched place. Note that by definition Q and S are always negative whereas R is always positive.

Figure 6E-F shows how inappropriate anesthesia depth can impair the quality of *in vivo* ECG recording. In **Figure 6E**, inadequate anesthesia (0.017% tricaine) led to failure to immobilize the zebrafish completely. The resultant motion artifacts lowered the signal-to-noise ratio by both contaminating the signal (asterisk) and increasing the noise (arrows). In contrast, in **Figure 6F**, overdosed anesthesia (0.08% tricaine) induced severe sinus bradyarrhythmia as well as changes of the ST segment and T wave.

FIGURE LEGENDS

Figure 1: Contrasting anatomy and ECG of human and zebrafish hearts. In contrast to the human heart with two atria and two ventricles, the zebrafish heart has only one atrium and

one ventricle (top row). Abbreviations: RA, right atrium; LA, left atrium; RV, right ventricle; LV: left ventricle. The zebrafish heart shares several common ECG features with the human heart (bottom row).

Figure 2: Minimally invasive in vivo ECG recording protocol. A schematic flow chart illustrates four critical action steps in conducting an in vivo ECG interrogation: induce anesthesia, place ECG lead electrodes, record ECG, and analyze the ECG recordings.

Figure 3: ECG data acquisition and processing system. The three key components of an integrated in vivo ECG recording system include a hardware to acquire data, an amplifier, and computer software for data acquisition and analysis. The amplifier comes with three ready-to-use 29-gauge stainless steel microelectrodes.

Figure 4: ECG lead placement. Three 29-gauge color-coded stainless steel electrodes are inserted securely into the fish musculature to approximately 1 mm in depth. Placement of the negative (black) electrode and the positive (red) electrode establishes a bipolar lead in the frontal plane, along a left caudal to right cranial orientation. Abbreviation: ref, reference electrode

Figure 5: Critical steps in ECG analysis. (**A**) Pre-define the various parameter settings for software automatic analysis. (**B**) Manually correct (red) two automatic misidentifications by the software (black) of the P and R waves to rectify software miscalculation of the atrial and ventricular rate. (**C**) Concatenate nine consecutive cardiac cycles (green) into a single average signal (black) to quickly assess rhythm regularities/irregularities and calculate average durations of waves and intervals. (**D**) Correct the QT interval for heart rate using one of the various methods, such as Bazett.

Figure 6: Effects of lead placement and anesthesia depth on ECG signals. Two most critical steps that determine the success of in vivo ECG recording are lead placement (**A-D**) and anesthesia depth (**E-F**).

DISCUSSION

When recording in vivo ECG for adult zebrafish by means of a single lead as we demonstrated in this study, there are a number of caveats concerning the quality and validity of the ECG recording results. First, in choosing the appropriate anesthetics and determining the minimal needed anesthesia concentration, depth, and duration, balance the anesthetic cardiotoxicities against the critical need to suppress motion artifacts and the a priori determination for a survival vs. terminal experimental design. Capitalizing on the synergistic potency of a combination of multiple anesthetics from different drug classes[5,14] and paralytics[1,6] to lower the dose of individual agents[5] or administering a low maintenance dose following a higher induction dose are typical strategies. However, despite its well-known potential cardiorespiratory toxicities, including death[8], tricaine is still the most

widely used, the best available, and the only anesthetic approved by the US Food and Drug Administration (FDA) for zebrafish anesthesia. Tricaine has been popularly used in ECG recording of adult zebrafish either as a single agent or in combination with other anesthetics or paralytics.

Second, lead placement accuracy can be ensured at least for healthy normal zebrafish using our four validating criteria for a normal adult zebrafish ECG. Of the four validating criteria that we propose here, the last two criteria together confirm the fundamental concordance between the polarity of the R wave and that of the T wave in a normal ECG[5,7,15]. This R and T wave concordance is a fortuitous, yet critical, similarity between zebrafish and human[16,17] normal ECG that contributes to the clinical relevance of the zebrafish heart model as a surrogate for human cardiac electrophysiology. However, several benign or malignant conditions may invalidate any of the four validating criteria. For example, the R and T wave concordance is lost in myocardial ischemia[7,15]. This loss of R and T wave concordance in myocardial ischemia is another striking resemblance between zebrafish and human ECG that contributes to the clinical relevance of the zebrafish myocardial infarction model.

Lastly, we recommend a standard practice in ECG analysis. With the advent of technology, ECG analysis software can generate automatic ECG interpretation. However, we strongly recommend that trained humans should always re-interpret and verify all ECGs based on the respective clinical scenario leading to ECG recording. Routine over-reliance solely on automatic interpretation by an ECG analysis software is inadvisable, particularly in the presence of common normal ECG variants, cardiac pathologies, or suboptimal lead placement.

This study focuses on the minimally invasive method for brief ECG recording sessions. However, should the need arise for terminal prolonged ECG recording sessions lasting hours, modifications are necessary to provide adequate oxygenation, hydration, and anesthesia by continuous perfusion[6].

Additionally, enhance the signal-to-noise ratio by one of at least three ways. Choosing a more powerful amplifier is often a costly, if not impractical, option. Opening the pericardial sac to reduce the volume conductor is a reasonable, although invasive, approach that has been adopted[7]. Strategic lead placement to align the lead axis in a direction parallel to the main cardiac axis (**Figure 4B**) will maximize the ECG voltage signals but may require trial and error, especially in the absence of pericardiotomy.

The in vivo ECG interrogation method for adult zebrafish that we presented here offers four main advantages. First, our minimally invasive approach requires only electrode insertion, but no fish scale removal or thoracotomy-pericardiotomy. Therefore, by minimizing pain for the fish, our approach enables repeated ECG interrogations in longitudinal survival studies. Second, when anesthetics adequately suppress fish motion, the in vivo ECG recording

system in our study consistently yields a satisfactory signal-to-noise ratio with noise-free raw signals. Third, the four-criterion quality validation that we propose here ensures data accuracy and robustness early in the ECG data acquisition and minimizes operator-dependent variations. Lastly, in particular, our last validating criterion (the normal T wave is upright) encapsulates the concordance of the R wave and T wave, an important human-like feature of zebrafish normal ECG (**Figure 1**).

However, there still exist four major limitations to current in vivo ECG methodology for adult zebrafish by our group and others.

First, the lack of subject cooperation necessitates the need for anesthesia with its limiting cardiorespiratory toxicity consequences. Whereas human patients never need sedation for the in vivo ECG interrogation, zebrafish require anesthetics or paralytics, all of which cause variable cardiorespiratory toxicities.

Second, the need to secure the attached ECG leads slightly elevates the invasiveness of an otherwise non-invasive procedure. Whereas lead placement in body-surface ECG recording of humans is entirely non-invasive because electrodes adhere to the human epidermis, lead placement for in vivo ECG recording of zebrafish is more invasive because, at the minimum, steel electrodes must puncture the fish skin for secure insertion into the fish musculature.

The last two limitations stem from the anatomical constraints of the zebrafish chest and heart. Third, the minuscule size of the adult zebrafish heart necessitates a drastic reduction in the number of ECG leads. While humans readily accommodate twelve leads in a standard ECG recording, adult zebrafish can typically accommodate only a single unipolar or bipolar lead. The ramification of a single ECG lead is the challenge to optimize concurrently the amplitudes of all three P, R, and T waves. Hence, the importance of optimal and accurate lead placement in zebrafish ECG interrogation cannot be overstated. In zebrafish, the T wave presents a unique detection challenge because it is often the smallest of these three waves. Therefore, the zebrafish T wave amplitude should receive optimization priority over the typically larger P and R waves.

Fourth, determining the zebrafish main cardiac axis to maximize the R wave amplitude can be challenging. The reason is that the zebrafish heart has more freedom of motion within its loose pericardial sac compared to the human heart within its form-fitting glove-like pericardium.

Overall, these limitations will stimulate future method innovation. With the advent of 3D printing and deformable electronics[18], there is hope for direct lead implantation one day in awake, alert, swimming zebrafish using a ‘cardiac sock’ of wireless electrode sensors.

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DISCLOSURES

The authors have nothing to disclose.

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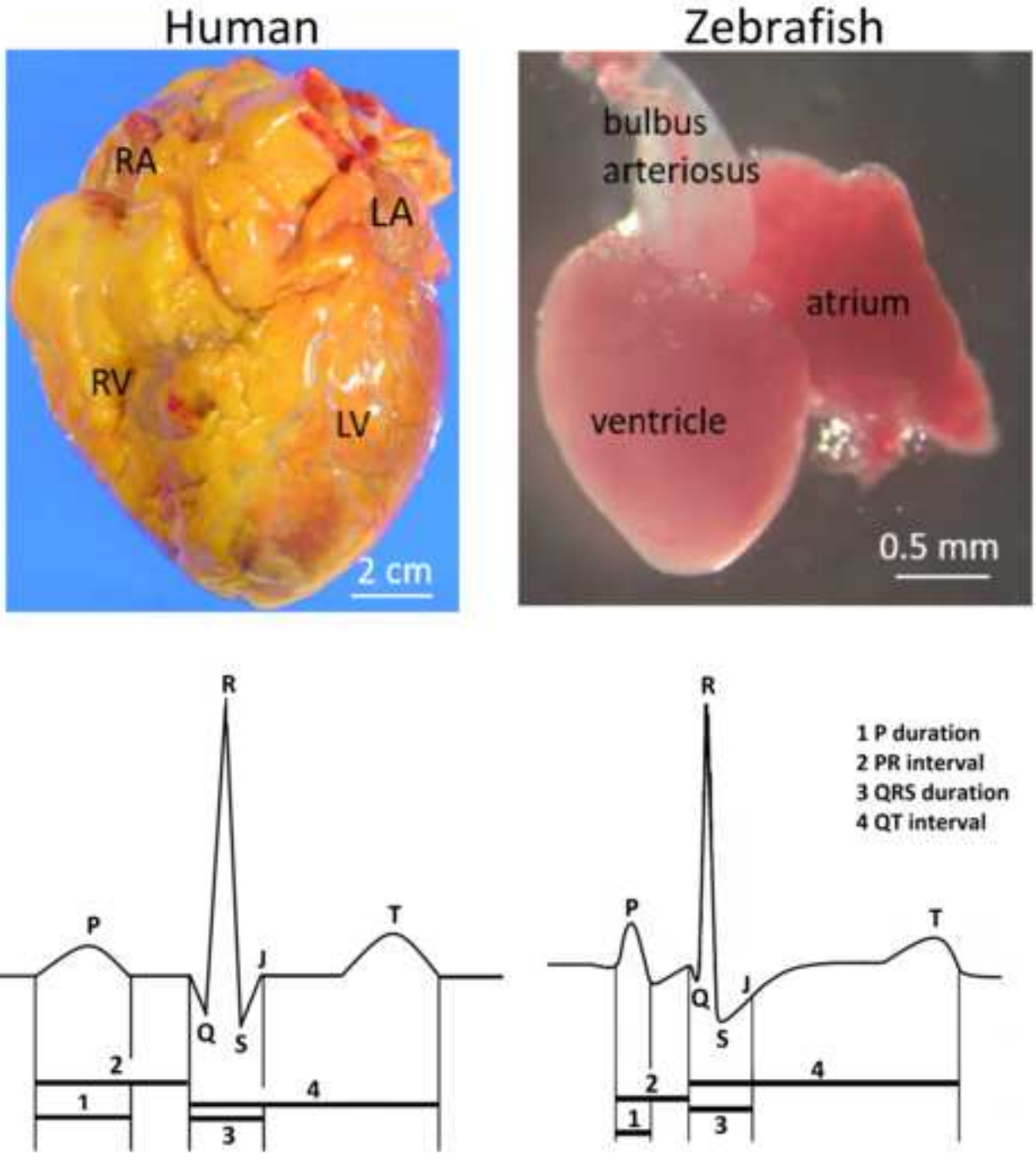
Figure 1


Human
RA
LA
RV
LV
2 cm
Zebrafish
bulbus
arteriosus
atrium
ventricle
0.5 mm
R
P
T
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Q
S
2
1
4
3
1 P duration
2 PR interval
3 QRS duration
4 QT interval

Figure 2

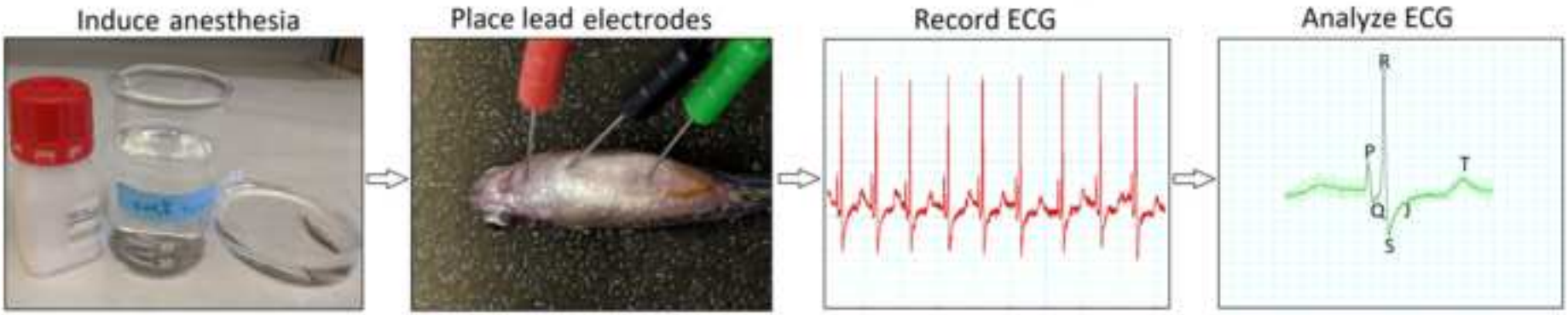


Figure 3

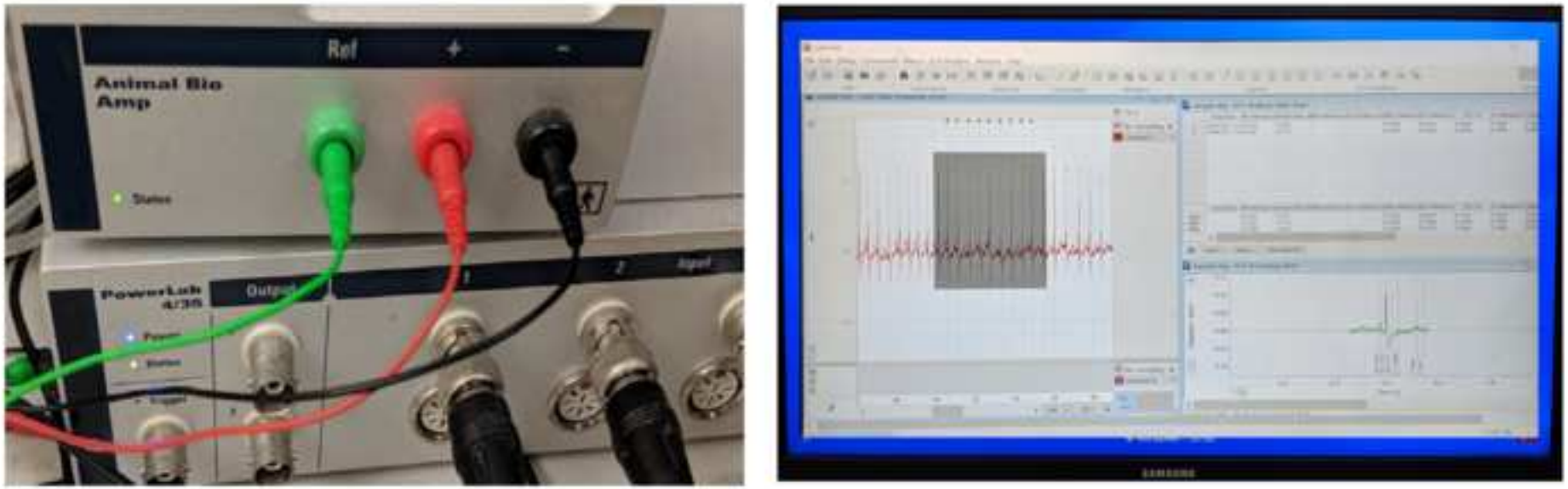


Figure 4

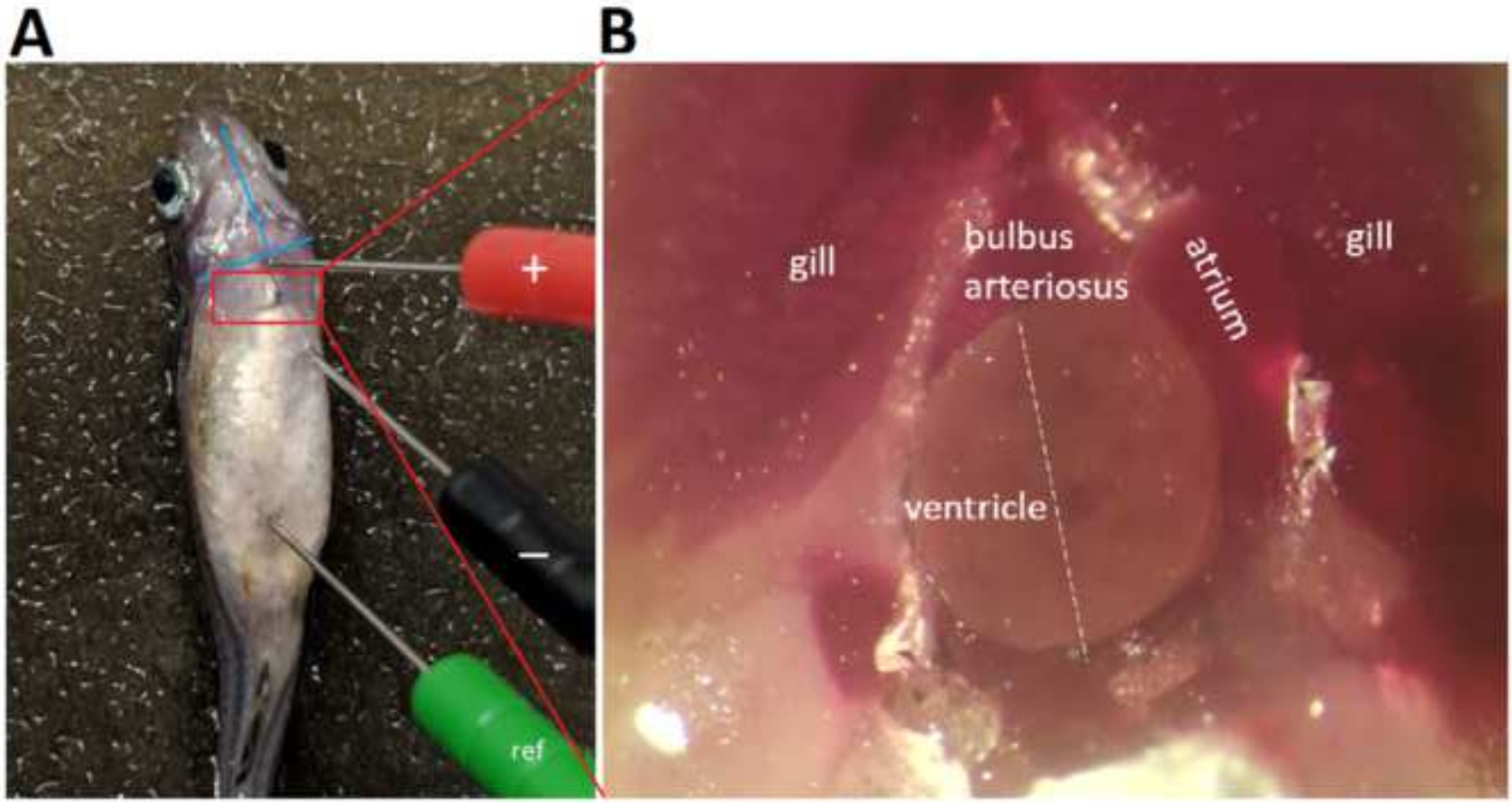


Figure 5

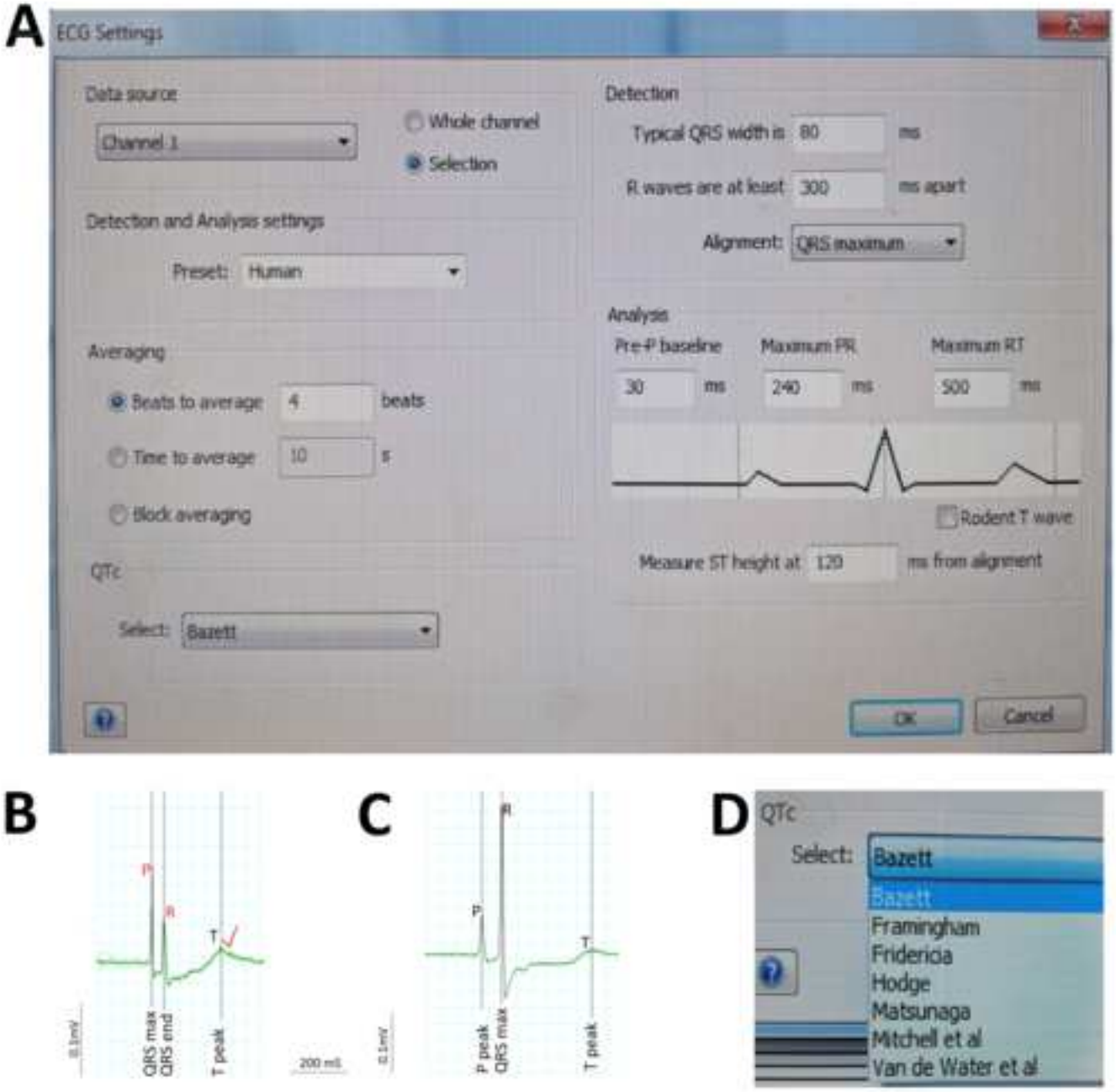
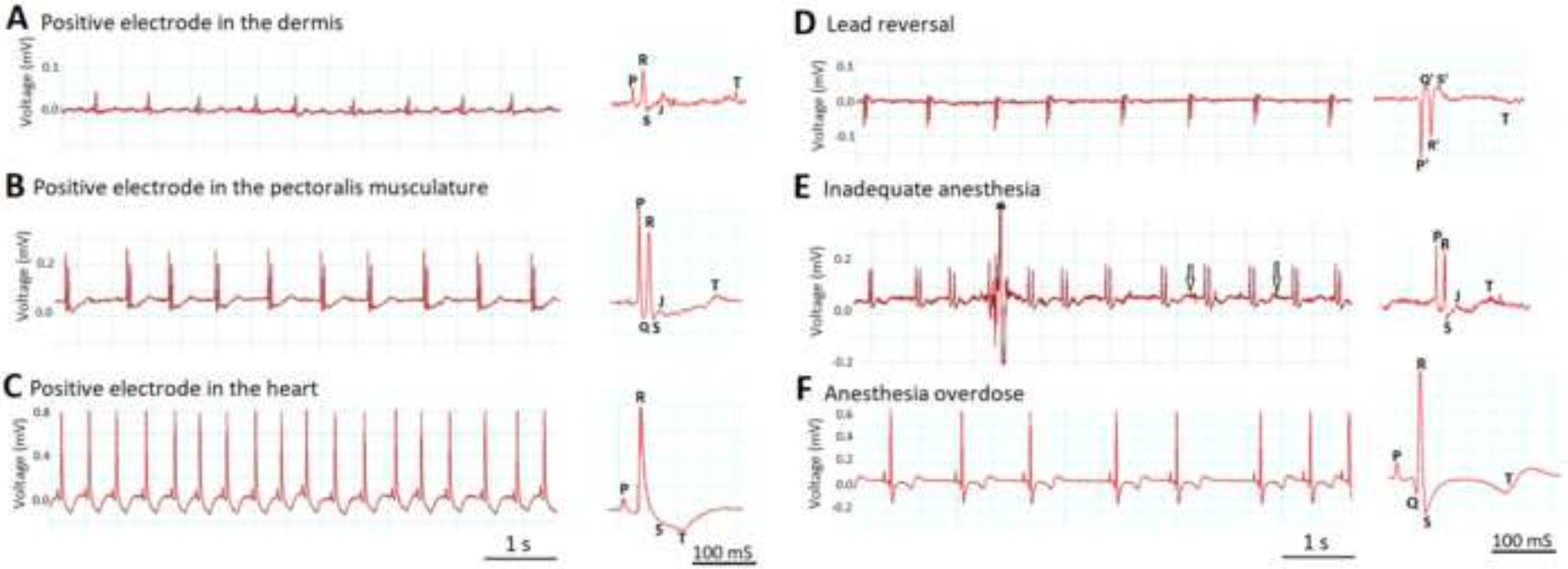

A
ECG Settings
Data source
Channel 1
Whole channel
Selection
Detection
Typical QRS width is 80 ms
R waves are at least 300 ms apart
Alignment: QRS maximum
Detection and Analysis settings
Preset: Human
Analysis
Pre-P baseline
Maximum PR
Maximum RT
30 ms
240 ms
500 ms
Averaging
Beats to average 4 beats
Time to average 10 s
Block averaging
Rodent T wave
Measure ST height at 120 ms from alignment
QTc
Select: Bazett
OK
Cancel
B
QRS max
QRS end
T peak
C
P peak
QRS max
T peak
D
QTc
Select: Bazett
Bazett
Framingham
Fridericia
Hodge
Matsunaga
Mitchell et al
Van de Water et al

Figure 6



Name of Material/ Equipment	Company
Culture dishes	Fisher Scientific
Dumont Forceps	Fine Sciense Tools
FE136 Animal Bio Amp	AD Instruments
Iris Forceps	Fine Sciense Tools
LabChart 8 Pro	AD Instruments
Needle electrodes for Animal Bio Amp	AD Instruments
Plastic Disposable Transfer Pipets	Fisher Scientific
PowerLab 4/35	AD Instruments
Scissors	Fine Sciense Tools
Tricaine (Ethyl 3-aminobenzoate methanesulfonate)	Sigma

Catalog Number	Comments/Description
FB087571	100 mm x 20 mm
11253-20	0.1 x 0.06 mm
FE231	
11064-07	0.6 x 0.5 mm
	Software with ECG Module
MLA1213	29 gauge
13-669-12	6 in., 1.2 mL
4//35	
15000-08	2.5 mm, 0.075 mm
E10521-10G	MS-222

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CORRESPONDING AUTHOR

Name:	Thao P. Nguyen, M.D., Ph.D.
Department:	Medicine/Cardiology
Institution:	David Geffen School of Medicine at UCLA
Title:	Assistant Professor-in-Residence of Medicine
Signature:	Thao P. Nguyen, M.D., Ph.D. Digitally signed by Thao P. Nguyen, M.D., Ph.D. DN: cn=Thao P. Nguyen, M.D., Ph.D., o=UCLA David Geffen School of Medicine, ou, email=tpnguyen@mednet.ucla.edu, c=US Date: 2019.04.30 14:34:36 -07'00'
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Title: In Vivo Surface Electrocardiography for Adult Zebrafish
Authors: Yali Zhao, Michelle Tran, Morgan Yun, Sean A. Nguyen, Thao P. Nguyen

Correspondence to: Thao P. Nguyen at tpnguyen@mednet.ucla.edu

RESPONSE TO EDITOR

We thank the Editor for prompt and constructive comments and valuable editing assistance. Below are our responses.

Editorial comments:

1. Please do not change the page layout and size.
   Answer: We thank the Editor for the change of page layout and size.

2. Please include this here as well in the format shown for Corresponding Author.
   Answer: Done.

3. Protocol: The highlighted section is the one which will be used for filming purpose as directed by you. We need to highlight complete sentences.

   However, presently the highlight is more than our upper limit of 2.75 pages including headings and spacing. Please adjust the highlights to fit the required page length. Some of the smaller steps can be combined to make one so that there are 2-3 actions per step.

   Also, anesthesia steps cannot be filmed so not highlighted.
   Answer: We have revised the protocol and the highlight is now within 2.75 pages including headings and spacing.

4. This figure (Fig. 3) doesn't show this step (1.2.1). So maybe the reference to the figure can be removed.
   Answer: We have revised the action step, but would like to keep the reference to Fig. 3.

5. "2.4.4 Validate the accuracy of lead positioning based on the satisfaction of all following four criteria in a zebrafish normal ECG (**Figures 1B & 6B**):
   - 2.4.4.1 Confirm that all ECG waveforms (P, QRS, and T) are distinct and readily visible.
   - 2.4.4.2 Confirm that the P wave is positive.
   - 2.4.4.3 Confirm that the R wave is positive.
   - 2.4.4.4 Confirm that the T wave is positive."

   How is this done, do you look for something in the waveform. Please detail the same. Please refer figure 5 before figure 6.
   Answer: Yes, by looking at the ECG waveforms in the ECG recordings. We have revised this paragraph. We have removed the reference to Fig. 6.

6. "3.1 Mastering software (Table of Materials) and interface"
   Please ensure that you include the name of the software in the table of materials.
   Answer: We thank the Editor for the kind reminder. We did so with the last resubmission.

7. "Step 3.2 Analyzing heart rate and rhythm"
   How ? what should the rate and rhythm be?
   Answer: How to analyze the rate and rhythm is detailed in the substeps that follow the new section 6.2. We have expanded this section to add more "how" explanations. The rate and rhythm are what they are in the fish; we have no control over how they should be (although all anesthetics slow the heart rate).

8. "Step 3.2.1 Identify whether the rhythm is sinus or not, regular or irregular."
   How do you do so in the software? Do you see some irregularities in the waveform? Please provide all the details.
   Answer: As answer above. How to sinus rhythm and regularity is detailed in the new substep 6.2.1. We have expanded this subsection to add more "how" explanations.

9. "Step 3.2.3 Verify the software automatic ECG identification of the R wave peaks."
   Needs rewording.

Answer: Done

10. "Step 3.2.3 Correct any R wave auto-identification mistakes by moving the cursors to the appropriate peaks prior to automatic re-generation of the average ventricular rate (**Figure 5B**)"
    What are the appropriate peaks? what is done after moving the cursors to the appropriate peak?
    Answer: Peaks of the R waves. To avoid confusion, we have revised 'peak's to 'waves'. We have clarified this question in the new section 6.2.2. Moving the cursor is manual. After the cursor is moved by the human operator, the software will re-calculate the heart rate based on the human corrected R wave identification.

11. "Step 3.2.3. In the case of atrioventricular dissociation during which the relationship between the P waves and QRS complexes is lost, calculate in addition the average atrial rate based on P wave peaks."
    How do you do this?
    Answer: The average ventricular rate is the average RR interval. Calculating the atrial rate is done the same way as for the ventricular rate, but using the average PP interval instead of RR. Please see new section 6.2.2.

12. "Step 3.3.2 Verify the software automatic ECG identification of the start and end of the P, QRS, and T wave displayed in the Averaging View window (**Figure 5C**)"
    Verify the software automatically identifies start and end of P waves in the ECG ?
    Answer: We have revised this sentence and expand the explanation in section 6.3.2

13. "3.3.3 Measure the PR interval from the start of the P wave to the start of the R wave)."
How? do you perform any button clicks?
Answer: We explain "how" in the new section 6.3.2 and NOTE. Manually moving the cursor to the correct start and end of the wave and the software takes care of the automatic recalculation.

14. "3.3.4 Measure the QRS duration from the start of the Q wave (or of the R wave if the Q wave is not visible) to the end of the S wave (i.e., the J point; **Figure 1B**)".
How is this done?
Answer: Same as above.

15. "3.3.5 Measure the QT interval from the start of the R wave to the end of the T wave.
Answer: Same as above.

16. Correct the QT interval to heart rate (or RR interval) to generate the corrected QT interval QTc."
How is this done?
Answer: We explained how in the 2nd NOTE of section 6.3.2 as well as in Fig 5A and Fig 5D. The human user pre-select the method of correction in step 6.1.3 (Fig 5A, 5D) and the software will automatically applies the mathematical formula of that method to generate the QTc.

17. **"3.4.1 Recognize exceptions for criterion 1:**
Instead mark the step number to be more clear. ..step 2.4.4.2?
Answer: We have now indicated the criterion numbers in the new section 4.3. In the new section 6.4, we have marked the step number 4.3 when referring to these criteria.

In the **absence of any P waves** (which indicates the absence of sinus rhythm), rely on the RR intervals and QRS duration to diagnose the heart rhythm."
How do you diagnose the same?
Answer: We do not understand the editor's question. We have already explained "How to diagnose" all the different scenarios of non-sinus heart rhythms in the subsequent sentence and have nothing more to add. "For example, diagnose atrial fibrillation if the RR intervals are irregularly irregular, junctional escape rhythm if the RR intervals are regular and the QRS is normally narrow, or ventricular escape rhythm if the RR intervals are regular and the QRS is abnormally prolonged." We have now reversed the sentence order of the scenarios and diagnoses to clarify.

18. Representative Results
Please describe the result with respect to your experiment, you performed an experiment, how did it helped you to conclude what you wanted to and how is it in line with the title.
I have reworded in some case. Please check.
Answer: Done for each figure.

19. **"Figure 1** contrasts the anatomy and ECG of human and zebrafish hearts.
    This is redundant and can be removed.
    Answer: Done.

20. The zebrafish heart has only one atrium and one ventricle in contrast to the heart of humans and other mammals with two atria and two ventricles (**Figure 1A**). However, despite the apparent simplicity of its two-chambered structure, the zebrafish heart shares several common ECG features with the human heart and has therefore emerged as a surrogate model for human cardiac electrophysiology."
    Any citation for this?
    Answer: We have provided 3 new citations.

21. "**Figure 1B** illustrates a small but distinct Q wave from a live, healthy 14-month-old zebrafish."
    Isn't the first panel of figure 1b is from human heart?
    Answer: Yes. We have removed the labels A and B in Figure 1 so that it becomes more apparent that the label "Human" applies for column 1 and the label "Zebrafish" applies for column 2.

22. "Pericardiotomy to expose the heart is a commonly used strategy to increase the signal-to-noise ratio at the cost of converting the ECG recording from a minimally invasive into a highly invasive procedure."
    Citation?
    Answer: Done

23. Figure 3. We cannot have commercial terms in the figure. Please either zoom the figure to not show ADI instruments or cover it.
    Answer: We have covered the vendor name.

24. Of the four validating criteria that we propose here, the last two criteria together confirm the fundamental "concordance of the R wave and T wave" in a normal ECG.
    Does this needs to be in quotes? Citation?
    Answer: We have removed the quotation marks. We have provided 3 new citations.

25. "First, is the subject cooperation." "Second, lead placement." " Third, number of leads."
    Please describe in complete sentences and in paragraph style
    Answer: Done

26. "a "cardiac sock" of wireless electrode sensors."
    Please do not use quotes without citations.
    Answer: We have changed to single quotation marks to indicate that the word 'cardiac sock' here has a particular meaning that is unique to this specialized cardiac device, unlike the meaning of the common terminology 'sock' as a clothing item.