Zebrafish ECG Setup

System Includes

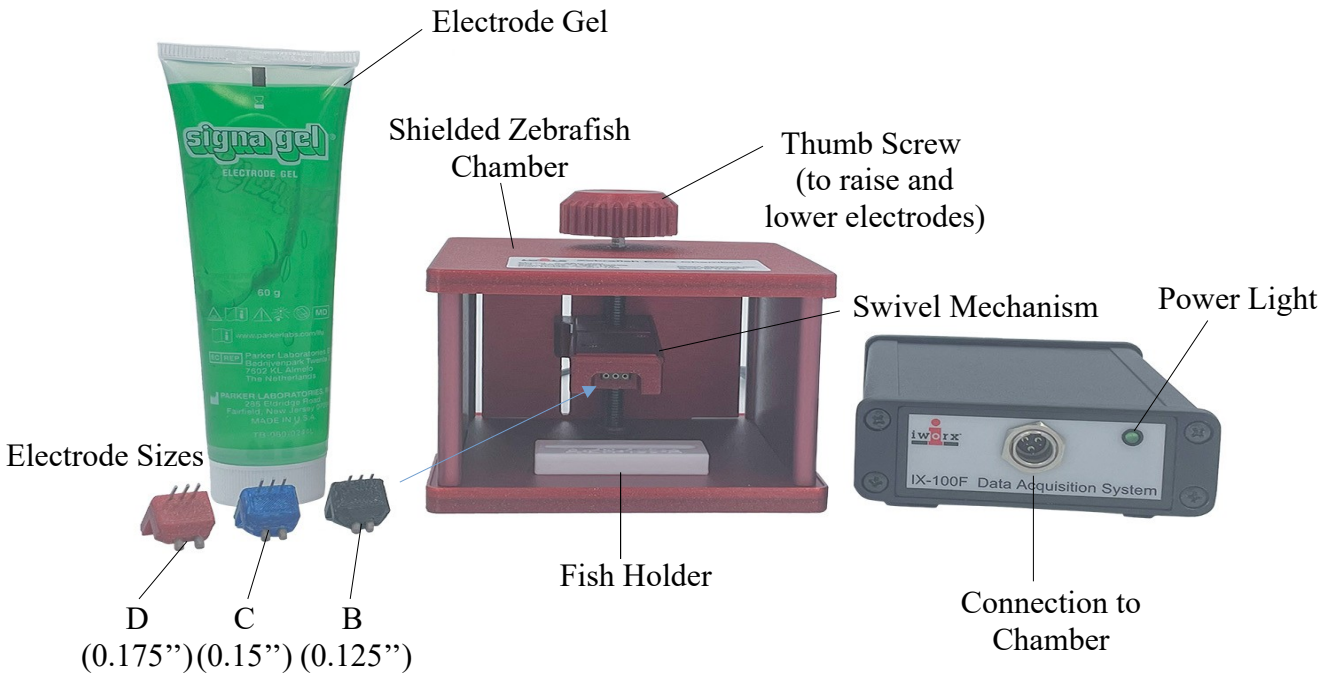
IX-100F Recorder

A-ZEC-200 Recording Chamber

Ag/AgCl Electrodes (0.125″ spacing, 0.15″ spacing, and 0.175″ spacing)

Signa Gel Electrode Gel

LabScribe Software and Advanced ECG Analysis Module



Setup Video

A video of the procedure for recording Zebrafish ECG is available at at: www.iworx.com/Zebrafish
YouTube link: ZECG01 - Zebrafish ECG Set up

Recorder Setup

Plug the cable from the Zebrafish chamber into the front of the IX-100F. Connect the IX-100F to the computer with the USB cable. Use the C-USG-B system-ground banana-jack connection on the rear panel of the IX-100F to ground the recorder to a wall outlet.

Zebrafish ECG Setup

Anesthesia

Determining dosage for the anesthesia for Zebrafish is a bit complex, because there is generally a lack of information, and because the factors that might be used in larger animals—weight, age, health—are not as easily applied to Zebrafish. Following are general guidelines which should provide a starting point to determine what works best for a particular laboratory.

The recommended anesthetic for Zebrafish is a diluted Tricaine-Methanesulfonate solution—sold as MS-222®, or Finquel®. Benzocaine Hydrochloride or 2-phenoxyethanol solutions have been used but are not recommended. Tricaine-Methanesulfonate can is sourced in liquid or powder form. The small volumes required, and the importance of the solution ratios, necessitate using an accurate scale capable of resolving grams when using the powder form.

"**The Laboratory Zebrafish**" by Claudia Harper, and Christian Lawrance, recommends preparing a stock solution of tap-water, sodium-bicarbonate, and Tricaine-Methanesulfonate mixed at a ratio of 1000mL of water, 8000mG sodium-bicarbonate, and 4000mG Tricaine-Methanesulfonate. The sodium-bicarbonate buffers the stock solution at approximately 7.0, which should be measured, so that the ratio can be adjusted as necessary for the local tap-water. Distilled and Reverse-Osmosis water should be avoided. Anesthetic solution is then mixed at a ratio of 4mL of stock solution to 100mL of tap-water[i]. Ideally, water from the fish holding tank should be used when diluting the stock solution. Effectively, this provides a dose approximately equal to 154mG/L(ppm)[ii] of Tricaine-Methanesulfonate.

Rosalind Franklin University recommends a slightly lower dosage range, between 75 and 125mG/L(ppm)[iii].

Kenneth Wingerter has a very helpful article that details the use of Tricaine-Methanesulfonate. He recommends a dosage of approximately 140mG/L(ppm) to achieve full anesthesia. He also recommends aggressively aerating both the immersion tank and the recovery tank. He also recommends withholding food for 12-24 hours before anesthetizing the fish[iv].

Dosage is impacted by the amount of anesthetic used, and the duration the fish is immersed in the solution. It also depends on fish size and other factors, including the age of the anesthetic used. Using Tricaine-Methanesulfonate, immersion for 2.5-3 minutes is typically adequate to immobilize the fish without harming it. Observe that the fish gill motion is minimally noticeable. It is recommended to begin with a shorter dosage time and increase the time as required such that EMG does not significantly degrade the ECG recording quality.

Tricaine-Methanesulfonate provides the greatest margin between the required dose for anesthesia and the dose that will result in euthanasia. For perspective, note that the recommended dosage for euthanasia requires the fish be immersed for 5-10 minutes in a solution with the volume of Tricaine-Methanesulfonate between 200-500mG/L(ppm). This is an increase of approximately twice the immersion duration in a solution that has between 30%, and 660%, more Tricaine-Methanesulfonate per volume of water.

If using 2-phenoxyethanol, the recommended ratio is 1 part 2-phenoxyethanol to 1000 parts tap-water[v]. The same dosage concerns exist. Dosage times are recommended at approximately 1.25 to 1.5 minutes —again starting at lower dosage and increasing as necessary.

Zebrafish ECG Setup

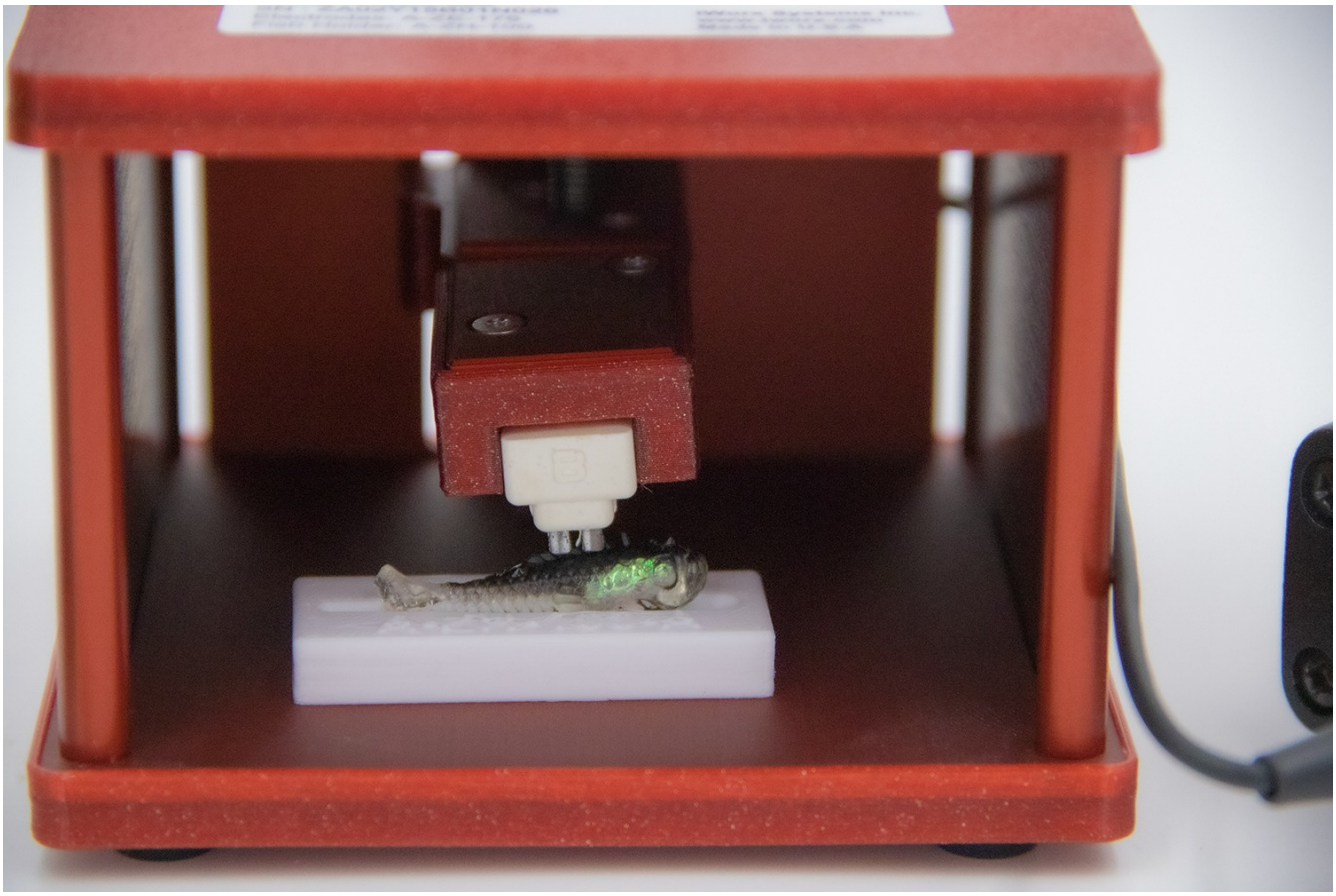
Electrode Preparation

Labscribe software can be used to time the immersion duration. This is an opportune time to apply an electrically conductive gel to the tips of the two electrodes. Clean the Electrodes with isopropyl alcohol and distilled water. Apply a small amount of electrode gel to the electrodes. Make sure that you do not short out the electrodes with gel between them. Care should be exercised when applying gel—the close proximity of the two electrodes means that an excessive amount of gel can provide an electrical path between the electrodes, attenuating the already small ECG signal.

Fish Preparation

The fish is removed from the immersion tank, and positioned on its back on a fish-bed. Use a paper towel to gently remove excess water. Gently use the paper towel, or a cotton swab, to ensure that the fins are not crossing the belly of the fish as this would interfere with the electrode connections.

Place the fish-bed with the fish, head to the right, in the chamber and position it under Ag/AgCl surface electrodes. The electrodes are easily maneuvered up and down with a thumb screw. The swivel mechanism allows the electrodes to make uniform contact with the fish body. The swivel mechanism allows ±10 degree rotation, and will tend to align with the body of the fish, as the electrodes are lowered onto the fish. It can also be manually aligned for best contact.



Zebrafish ECG Setup

Electrode Placement

The two electrodes are placed axially along the center-line of the fish's belly. It is best that each sits on the skin of the belly—demarcated by the color change—and not on the fins, or other non-belly skin. The forward electrode should be placed close to the gills, along the center-line of the fish. The heart of a Zebrafish is very close to the gills as can be seen in this image from Nature publication. The closer to the gills you place it, the larger the amplitude of the recorded ECG from a paralyzed fish, but as you approach the gills, for a less paralyzed fish, the motion artifact due to gill motion increases.

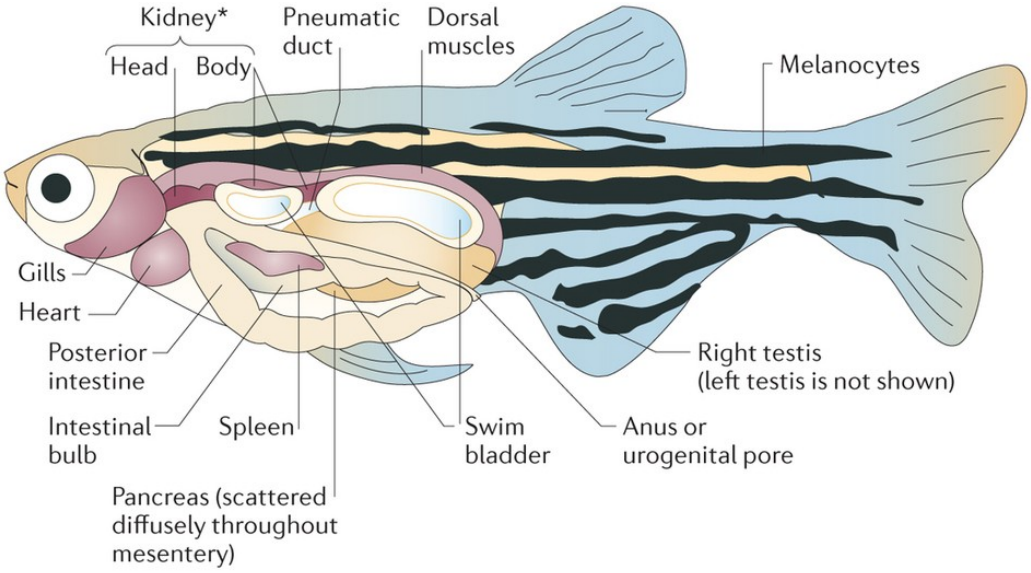


Illustration 1: Zebrafish anatomy image from: From Zebrafish cancer: the state of the art and the path forward Richard White, Kristin Rose & Leonard Zon Nature Reviews Cancer 13, 624–636 (2013) doi:10.1038/nrc3589

Several electrode sizes are available, increasing the distance between the centers of the two electrodes in 0.025” intervals. The electrodes are marked with a letter denoting the electrode pitch. The Black “B” electrode pitch is 0.125”, the Blue “C” is 0.150”, and the Red “D” is 0.175”. The various electrode sizes allow the system to be configured to best fit various sizes of Zebrafish.

When recording ECG, the pellet electrodes require no significant pressure. Ideally, they will only gently touch the skin of the belly of the fish. Increasing the pressure does not result in better recording, and will cause discomfort and ultimately damage to the fish. Note that the electrodes need not even touch the fish at all if adequate connection is provided by the electrically conductive gel. When the gel is stretched significantly, the signal will become attenuated, and this can be observed by lifting the electrodes after a good connection has been established. This attenuation results from increased impedance between the fish and the electrodes as the geometry of the gel narrows.

Zebrafish ECG Setup

Recording with LabScribe

1. Click on the **LabScribe** shortcut on the computer's desktop to open the program. The LabScribe Main window will appear once the program opens and a dialog window will pop up recognizing the device.
2. From the Main window, select the ***Settings*** tab and then click on "***Load Group...***".
3. Locate the **Research Settings** folder (default location is: **C:Program Files:iWorx:Labscribe:Research Settings**...), which contains the folder "Zebrafish". Open the folder and select the settings group, "Zebrafish". Click open.
4. Again, select the **Settings** tab. Select the **Zebrafish-ECG** settings file from the list.
5. LabScribe will be configured by the **Zebrafish-ECG** settings file, and a pdf setup document will open.

   Note*: if you do not see the Research Settings folder in the LabScribe folder, Reinstall LabScribe, making sure that the Research settings are installed.*
6. When ready to record Zebrafish ECG—after anesthetizing and placing the fish, and locating the electrodes—initiate recording by pressing the red icon labeled "**REC**". The icon will now change to black and be labeled "**STOP**".

Recognizing Issues And Optimizing ECG Recording

Good connections to an adequately anesthetized fish will provide ECG signal recordings that typically exhibit peak amplitudes—generally the R-wave—on the order of 12 to 25uV. Signals as low as 4uV and as large as 100uV have been observed, but are less prevalent. For most applications, signal amplitude is not itself important, beyond providing adequate resolution so that periodic measurements may be made between the constituent ECG components. The system resolution is approximately $750nV_{peak\text{-}to\text{-}peak}$ with inputs shorted, and approximately $1.5uV_{peak\text{-}to\text{-}peak}$ in use with a Zebrafish, because of the source impedance of the Zebrafish, which is typically in the low tens of kilo-Ohms.

For ECG recordings where the R-wave is limited to less than 10uV, the P-wave begins to be lost in the system noise, and the T-wave may not be discernible at all. The baseline of the recording should be stable, and near zero due to the data acquisition systems 0.5Hz cutoff High-Pass Filter.

Electrode Care

Avoid touching the sensing area of the electrodes at the bottom. The Ag/AgCl pellet electrodes are best cleaned with alcohol and distilled water.

The electrode can be resurfaced if needed with a fine grit sand paper. With proper care the electrodes should last for a long time. Replacement electrodes (A-ZE-175) can be ordered from iworx.com. Note the size of the electrode—marked on each electrode—when ordering replacements.

Zebrafish ECG Setup

Troubleshooting

The main source of noise in the recording is low frequency noise resulting from the acquisition system input amplifier, in conjunction with the relatively high source impedance of the fish as a voltage source. Mains pickup is minimized by the provided aluminum ECG chamber. Where Mains frequency noise does couple, using a well grounded desktop instead of a Laptop may help. A banana jack Ground connector on the back of the IX-100F allows optional grounding as needed. It is best to minimize other equipment near the IX-100F. The IX-100F has an input shorted system noise of about 750nV at 2k Samples/sec. Mains coupled noise should not be obvious in the time-domain signal, though it may be seen using the FFT function.

Zebrafish like humans have different levels of skin conductance. The Electrode Gel helps with the connection, but if the signal picked up from the fish is 10-20 uV then it will be hard for the system to resolve the P and T waves. ECG signals greater than 25uV can be typically recorded from Zebrafish. If you are seeing a smaller signal, try re-positioning the electrodes. Applying too much gel to the fish can cause a short across the electrodes, reducing the ECG signal being recorded.

Make sure that the fish is properly anesthetized, since any motion will be picked up by the electrodes.

Notes, Links, and Speculation Regarding Zebrafish Physiology

The shape and amplitude of the ECG recording is of some interest, because the result typically leaves the Q, S, and T-Waves difficult to resolve. Per Karlsson's undergraduate thesis titled "**Myocardial Regeneration In Fish Models**[vi]", touches upon this because ECG is a method used to monitor the progress of cardiac regeneration. Karlsson differentiates between the human and Trout cardiac anatomy, and states that anatomy is consistent amongst the Teleost fish family. The Zebrafish is a member of the Teleost family. Of particular interest, Karlsson provides a comparison of anatomy and circulation of the mammalian and Teleost fish hearts. He also provides a comparative view of the depolarization of human and Teleost fish hearts, the Ventricular depolarization of the Teleost fish heart, and the approximate geometry of the Teleost fish heart, relative to the body of the fish.

These figures are included here to aid in determining the approximate Zebrafish heart vector, which speaks to the shape of the recorded ECG signal—in the same way that the in human ECG recording, "Lead I", Lead II", "aVL", "aVR", and "aVF" recording configurations each show a different perspective, and certain configurations may better resolve certain ECG components. It is worth noting, that the relationship of the fish heart to the electrodes must be considered, and that requires understanding the relationship of the fish heart to the body of the fish. Figure 1 of the publication *"**Cardiac Morphology and Blood Pressure in the Adult Zebrafish**[vii]"* provides some insight by showing details of the Zebrafish cardiac anatomy, including the Afferent Branchial Arteries which provide context since they interface with the gills of the fish, and thus allow an understanding of the physical relationship of fish heart to fish body. That figure, along with those provided by Karlsson, suggest an approximate Cardiac Vector for Zebrafish, as depicted below.

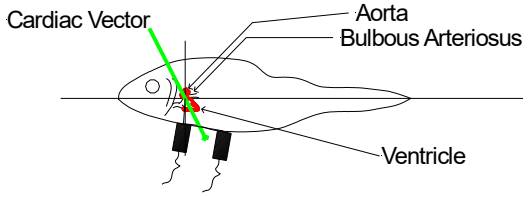


Illustration 2: Proposed Zebrafish Cardiac Vector

Zebrafish ECG Setup

Karlsson proposes a "biophysical model of the heart" based upon the electrical dipole moment. The approximation treats the heart—and body—as perfect spheres. The formula developed for surface recordings proposes that the maximum amplitude of the QRS complex:

$$\textbf{QRS-Complex}_{MAX} = \tfrac{3}{4} \times \text{Membrane\_Potential} \times (\mathbf{R}_{HEART}/\mathbf{R}_{BODY})^2.$$

For the Zebrafish, the membrane-potential of ventricular myocytes equals -70.4mV[viii] (versus -83.9mV for Rainbow Trout used by Karlsson). Karlsson mentions that the mathematical model proposed typically results in a value much larger than recorded stating "***The upper limit value for the non invasive ECG method calculated by the model, is of much greater magnitude than the experimental result in this study.***" His results are tabulated for surface and invasive recordings in his Table 6.1, page 33, but of key interest, the maximum QRS complex measured 0.01mV, whereas 0.20mV was predicted by the formula. Note that Karlsson expresses greater satisfaction with modeling invasive recording amplitude using a modified formula, but actual recordings provided only 27% of the predicted amplitude—correlating better than the formula for surface recording, but still significantly smaller than predicted. Karlsson attributes the difference between predicted and recorded amplitude to "***the rather small QRS amplitude found in this study might be a result of improper placement of electrodes or isolation of electrical components. The model predicted a QRS amplitude of much greater magnitude than that measured by the non invasive ECG monitoring method, this is likely to be a result of improper delimitations in the model.***"

According to Karlsson, the relative geometry of the radius of the heart to the body should be very similar between members of the Teleost family, so the Zebrafish and Rainbow Trout predicted maximal amplitudes should differ, according to the equation proposed, only as a ratio of the membrane-potential of ventricular myocytes values. That is, the Zebrafish predicted maximal value should be 70.4mV/83.9mV = 0.84 that of Rainbow Trout.

Zebrafish ECG surface recordings, however, are typically 15-25uV in maximal QRS complex amplitude, and vary according to electrode placement, amongst other factors, exceeding the typical values recorded for Rainbow Trout, recorded by Karlsson, despite having only 84% of the membrane-potential of the Rainbow Trout. Clearly, this speaks to limitations in the proposed formula.

The typical radius of an adult Zebrafish heart is approximately 0.5mm. The radius of a sphere that could sit within the typical body is between 1.5 and 2.0mm.

$$\textbf{Zebrafish QRS-Complex}_{MAX} = \tfrac{3}{4} \times -70.4\text{mV} \times (\mathbf{0.5mm/2.0mm})^2 = 3.30\text{mV}$$

The predicted value for Zebrafish is significantly larger than the model predicts—approximately 100 times larger. Various electrode placements on the belly, the back, and the sides of Zebrafish, reveal no significant shift in amplitude—although variation in the characteristic shape of the ECG is noticeable.

One potential explanation is that modeling the heart as a dipole, borrowed from Karlsson—is predicated on the idea that the surrounding body is a sphere of electrically conductive material. For human ECG recording, Lead-II configuration, measures the heart's "net dipole" best because the electrodes are placed most approximately along that vector. The electrodes are small, relative to the human heart. The electrodes are connected to the heart via relatively electrically consistent body mass —that is, the electrical path provided by the left-arm is not significantly different from that provided by the right-arm, or by either leg. Re-positioning the Lead-II electrodes closer to the heart—on the chest,

and belly—will typically provide larger amplitude recordings because the electrical connection to the heart by limiting the amount of body mass—and thus impedance—required used to connect the electrode to the heart, while still providing connection from the differential amplifier inputs most directly to points on the heart that have a differential ECG potential. If, however, the electrodes were moved closer to the heart, but instead of being placed in the classic Lead-II configuration, the two differential input electrodes were essentially co-located, they would each best connect electrically to the same part of the heart, and thus some large portion of the signal would shift from differential to common-mode—that is, the two inputs would have very little differential potential to amplify.

The model Karlsson proposed is too because the electrodes are constrained from being ideally placed along the net dipole vector of the Zebrafish. If the placement was not constrained, the underlying body mass between each electrode and the heart would likely not be of uniform, due to bone, fat, or other organ material between the heart and the electrode providing electrically different connections.

Further, the electrodes used have a 1mm radius, some portion of which makes contact with the fish. The typical Zebrafish heart has a 0.5mm radius, half that of the electrodes. The body mass connection between heart and each electrode—were the electrodes able to be placed directly in line with the net dipole cardiac vector, across the heart—would at such close range imply that each electrode had varying degrees of electrical connection to some large portion approaching one half of the fish heart, rather than to some small point on that half of the heart, and the signal amplitude is attenuated because the conductive material provides not just a path between a single point on the heart and the electrode, but also between the various points that can be drawn between the heart and the electrode.

Effectively, it is easier to detect the ECG where the electrodes are so relatively large, but the ECG signal amplitude will be diminished at the same time. The illustration below provides a depiction of the fish, cardiac vector[ix], and electrodes, with emphasis shown on the paths between the Zebrafish heart and each electrode, depicting that the signal connected to each electrode—represented with Yellow and Cyan areas—has large overlap, shown in dark Green. Where the dark Green overlapping area touches the heart, indicates the points on the heart with some degree of connection to each electrode, and a cancellation, to the degree that the overlapping connection is equivalent.

The relative size of electrodes to fish heart does not shift significantly by decreasing the electrodes radius, but the manufacturability—and ability to view the electrode when connecting to the fish—becomes tremendously more difficult in each case. Thus, the larger electrodes are utilized because their benefits outweigh their limitations.

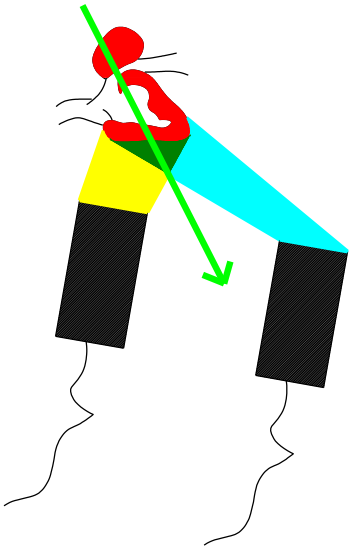


Illustration 3: Overlapping Electrode To Heart Connections

Zebrafish ECG Setup

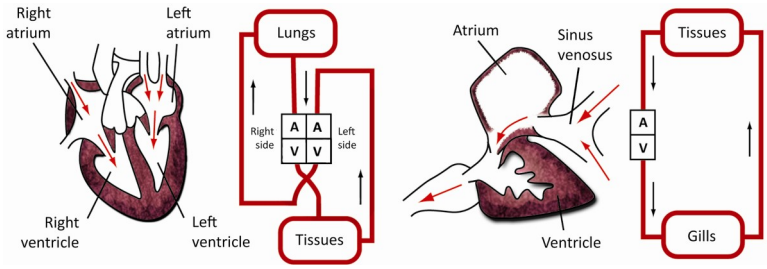


Illustration 4: "Myocardial Regeneration In Fish Models", Figure 1.1, "Comparative anatomy and circulation of the (a) mammalian heart and (b) the teleost fish heart (modified from Farrell, 2000)"

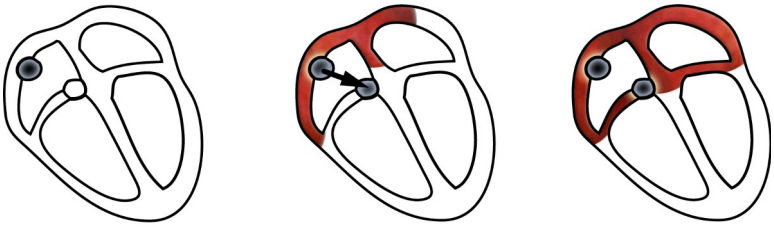


Illustration 5: "Myocardial Regeneration In Fish Models", Figure 1.2, "Depolarization sequence in atrium of the human heart. (a) SA node initiation, followed by (b) atrial depolarization. The signal is then slowed at the AV node (c), before continuing to ventricular depolarization (lower region), along the ventricular septum. (Modified from Sörnmo and Laguna, 2005)"

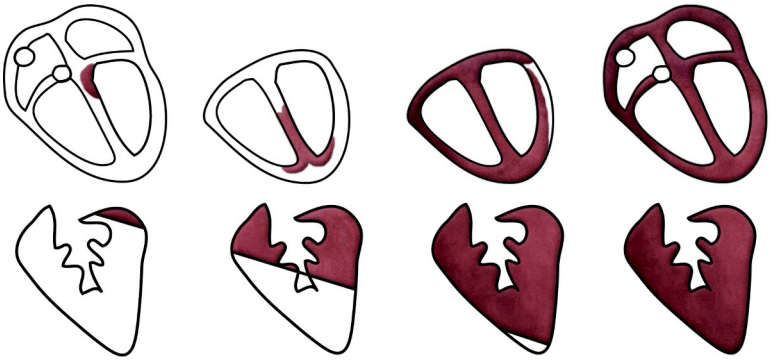


Illustration 6: "Myocardial Regeneration In Fish Models", Figure 1.3, "Comparative view of ventricle depolarization the human and fish heart.Depolarization starts at the His bundle (a), then continues through left and right bundles to Purkinje fibers (b-d). Note that this figure is not drawn to scale. (Modified from Hobbie, 1973; Rantin et al, 1995)"

Zebrafish ECG Setup

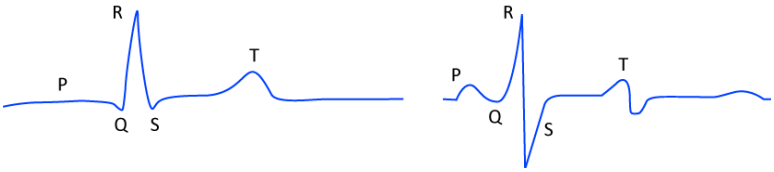


Illustration 7: "Myocardial Regeneration In Fish Models", Figure 1.4, "Electrocardiograms from (a) human and (b) fish.Note that specific intervals relevant for ECG morphology are indicated in the figure. (Adapted from Farrell, 2001)"

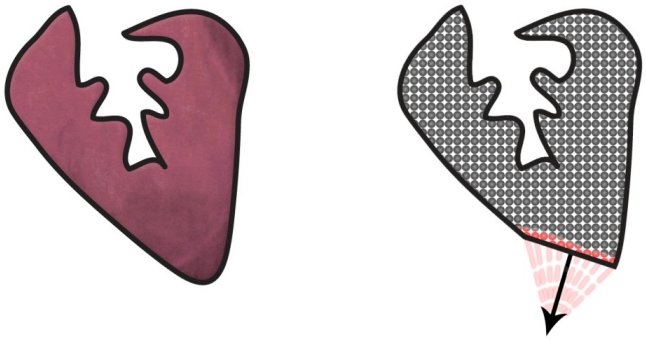


Illustration 8: "Myocardial Regeneration In Fish Models", Figure 5.3, "(a) Illustration of the heart ventricle in fish. (b) Summation of individual dipole moments from depolarizing myocytes (coloured in red) forms the heart vector at a given time of the cardiac cycle. Note cancelling segments are left out and already depolarized cells are coloured in gray. (Hobbie, 2007)"

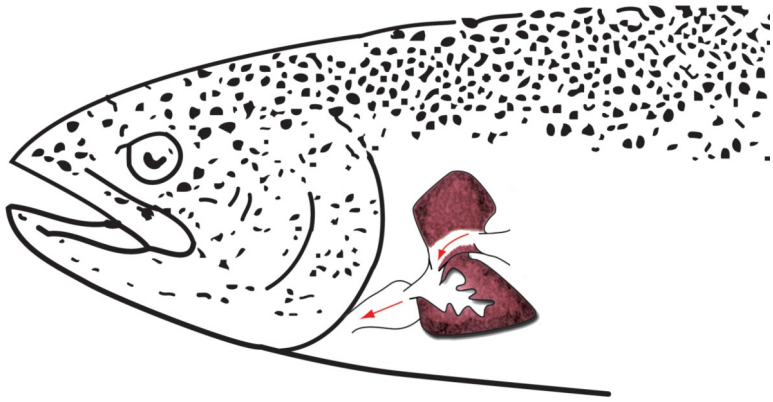


Illustration 9: "Myocardial Regeneration In Fish Models", Figure 5.4, "Illustration of the heart in rainbow trout in its ventral anterior location."

Zebrafish ECG Setup

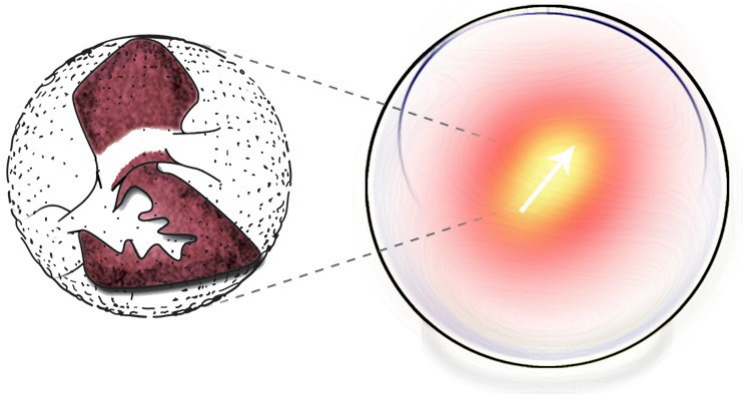


Illustration 10: Myocardial Regeneration In Fish Models", Figure 5.5, "The heart approximated as a net dipole, located in the center of a spherical cavity (the thoracic cavity)."

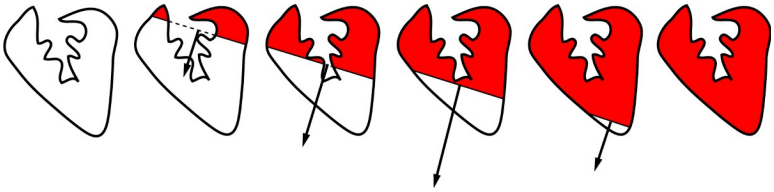


Illustration 11: "Myocardial Regeneration In Fish Models", Figure 5.6, "Ventricular depolarization of the teleost fish. (modified from Rantin et al, 1995)"

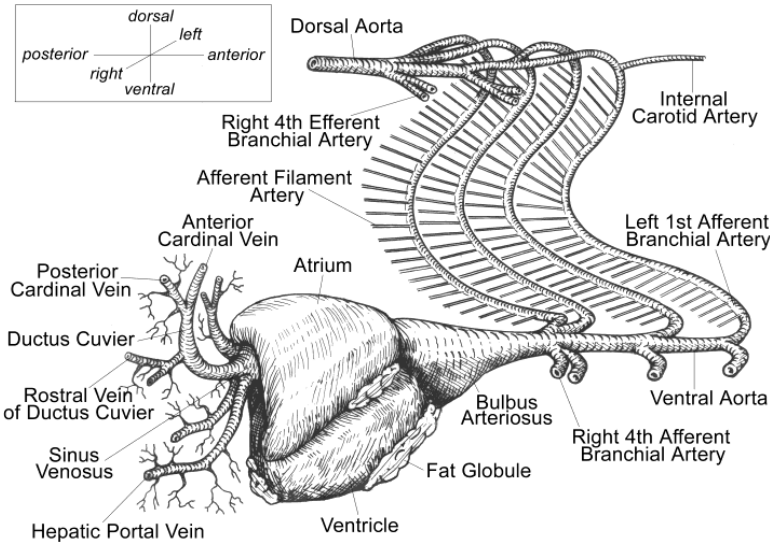


Illustration 12: "Cardiac Morphology and Blood Pressure in the Adult Zebrafish"--Figure 1, "Posteroanteriro View Of Adult Zebrafish Heart"

i Anesthesia is detailed in "**The Laboratory Zebrafish**", by Claudia Harper, and Christian Lawrance,and states:

"Anesthesia Tricaine (MS-222) is the anesthetic agent most commonly used for Zebrafish. Although there are many other anesthetics available for fish, they will not be discussed in this chapter (Iwama and Ackerman 1994; Noga 1996; AVMA 2007). The recommended stock solution of MS-222 for Zebrafish is 4 g/L; the anesthetic dose is 4 mL of stock solution in 100 mL of water, or 40 µg/mL (Westerfield 2007). MS-222 is light sensitive and may develop a brown tinge if exposed to light. If this is the case, it should be discarded (Noga 1996; Iwama and Ackerman 1994).

The buffering capacity of the water has a direct effect on the impact of MS-222 on water pH. Water with no buffering capacity has zero alkalinity, and the anesthetic or euthanasia solution can lead to acidosis in fish since the water pH could decrease to 5 (Houston 1990; Iwama and Ackerman 1994; Noga 1996). Distilled water and reverse-osmosis water are suboptimal when reconstituting MS-222 since they have very little to no buffering capacity. Sodium bicarbonate or sodium hydroxide are both commonly used as buffering agents (Noga 1996). Sodium bicarbonate should be added to the solution at a ratio of 2:1 (sodium bicarbonate (wt) to MS-222 (wt)) and adjusted to a pH of 7 (Noga 1996). Fish are immersed in buffered water with dissolved MS-222. Continuous delivery of anesthetic in water to the gills of fish is necessary for long procedures. Various delivery systems have been devised and are applicable for Zebrafish (Harms and Lewbart 2000; Harms 2005; Weber and Innis 2007). However, anesthetic regimens in fish should be conducted under the advisement of a veterinarian and reviewedand approved by the IACUC."

ii 4mL x (4000mG Tricaine-Methanesulfonate/1000mL Tap-Water) = 16mG Tricaine-Methanesulfonate in a solution of 104mL (4mL Stock Solution + 100mL Tap-Water). (16mG/104mL) x (1000mL/1L) = 153.85mG/L(ppm) Tricaine-Methanesulfonate.

iii "**Institutional Animal Care and Use Committee: Guidelines and Policies**", Rosalind Franklin University Of Medicine And Science, available in pdf format, at: http://www.rosalindfranklin.edu/Portals/0/Documents/research/Zebrafish%20Use.pdf.

iv **Aquarium Fish: Use of MS-222 (Tricaine Methanesulfonate) to Induce Sedation and Anesthesia in Ornamental Fishes**, By Kenneth Wingerter. Published at "Advanced Aquarist", Volume IX, November 2010. This is available online at: http://www.advancedaquarist.com/2010/11/fish

v Rosalind Franklin University Of Medicine And Science's document, titled "**Institutional Animal Care and Use Committee: Guidelines and Policies**".

vi "**Myocardial Regeneration In Fish Models**", Per Karlsson, Department of Applied Physics, CHALMERS UNIVERSITY OF TECHNOLOGY, Göteborg, Sweden, 2007, available in pdf format at: http://www.pkarlsson.com/wp-content/uploads/2013/02/perkarlsson_bsceng_thesis.pdf

vii "**Cardiac Morphology and Blood Pressure in the Adult Zebrafish**", Hu, Yost, Clark, http://www.ncbi.nlm.nih.gov/pubmed/11505366

viii "**Characterization of isolated ventricular myocytes from adult Zebrafish (Danio rerio)**", Brette, Luxan, Cros, Dixey, Wilson, and Shiels, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2581121/

ix "**Basic ECG Theory, Recordings, and Interpretation**", Dupre, Vincent, Iaizzo, http://eknygos.lsmuni.lt/springer/675/191-201.pdf