First of all we would like to take the opportunity to thank the editor and five reviewers for providing thorough reviews of our paper. It is a pleasure to see that both editor and reviewers have been going into detail in the evaluation of the manuscript and provide excellent comments and thoughts for the improvement of the manuscript. In the following we try to answer the comments/questions raised by the editor and reviewers (response in red) and describe how their suggestions have been incorporated into the revised manuscript (line numbers refer to the revised manuscript with track changes enabled).

**Changes recommended by the JoVE Scientific Review Editor:**

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have thoroughly proofread the manuscript and corrected for spelling and grammatical errors.

• Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Examples NOT in imperative tense: “High quality non-chemically treated tap water can be applied directly.”;” The lip shaped structure can be submerged...”;” General anesthesia will appear within 30 min in all size groups for both benzocaine”; entire section 5.

Protocol text has been rephrased so all text (except that in notes) is in the imperative tense.

• Protocol Detail:Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action.There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) For all software steps: Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

This is tricky. The software steps described in the protocol are too complex to be described as “click that button”, ”open that menu” etc. That would require thousands of steps. Instead we believe the best way to describe the software procedures are to present the math underlying the procedures in the protocol steps and supply the reader with supplementary material (Supplementary material 16 – 20) containing pre-written scripts for software that most researches within biomedical research will be familiar with (Excel and ImageJ)

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

o The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

o The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

o Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

o Notes cannot be filmed and should be excluded from highlighting.

o Please bear in mind that software steps without a graphical user interface/calculations cannot be filmed.

We have evaluated the length of the protocol and it is below the 10 pages limit. We have highlighted <2.5 pages that tell a cohesive story of our protocol steps and should be visualized.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We believe that the discussion covers these subjects.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Milli-Q, Styrofoam, kimwipes, Vevo 2100, etc

1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

Commercial names have been replaced by generic names.

• Table of Materials:Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as axolotl strain, software, imagers, etc.

Table of Materials has been updated.

• Please define all abbreviations at first use.

We believe that all abbreviations are defined at first use.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

We believe that all units are in SI units with correct abbreviations.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We reuse material from one of our own previous publications (Thygesen, M.M., Rasmussen, M.M., Madsen, J.G., Pedersen, M. and Lauridsen, H. Propofol (2,6-diisopropylphenol) is an applicable immersion anesthetic in the axolotl with potential uses in hemodynamic and neurophysiological experiments. Regeneration 0, 1–9, https://doi.org/10.1002/reg2.80 (2017)). Since this material is available under the Creative Commons Attribution License we believe that permission is not required for academic or commercial reuse, provided that full attribution is included in the new work. This is also specified on the publisher’s homepage: <http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)2052-4412/homepage/Permissions.html>

**Comments from Peer-Reviewers:**

**Reviewer #1:**

Manuscript Summary:

The protocol describes several different methods to acquire echocardiographic measurements on the axolotl heart in anesthetized and unanesthetized animals in two and three spatial dimensions. The authors assessed three different anesthetics: benzocaine, trichaine, and propofol. The methods are applicable in heart regenerative experiments where cardiac function can be monitored over the course of a regeneration process. Moreover, the method could be useful for cardophysiological studies in different experimental conditions.

Major Concerns:

None

Minor Concerns:

None

**Reviewer #2:**

Manuscript Summary:

The manuscript by Dittrich et al provides a thorough protocol for the visualization of the axolotl heart by echocardiography. The axolotl is an interesting choice of animal due to its regenerative capacity, and the authors correctly argue that similar studies are currently lacking from the literature. Successful visualizations by echocardiography at times require subtle approaches which are not easily described in words, but may be very well visualized on video. JoVE seems to be a wholly appropriate outlet for the submitted protocol. I have no major concerns with the manuscript. The text is concise and exhaustive, the figures are good.

Minor Concerns:

Besides a number of specific comments, please see below, I only have one general comment. Line 705-706: what to make of the discrepancy between the values derived from Doppler and those derived from the equation assuming a spherical ventricle? It would be nice to see a comparison between the values, for instance a simple scatter plot with stroke volume assuming a sphere versus stroke volume measured by Doppler. Did you attempt to quantify by Doppler cardiac output as flow through the atrioventricular canal? If so, the relation between the cardiac output as assessed from the atrioventricular canal and the outflow tract could be used to the reliability of stroke volume as derived from the equation assuming a spherical ventricle.

We have made a scatterplot (Fig. 7) showing stroke volume values acquired by the geometrical equation and the Doppler method for all three anesthetics described in the study (benzocaine, MS-222, and propofol). We haven’t attempted to quantify Doppler cardiac output as flow through the atrioventricular canal.

Specific comments

With the described setup, can the echocardiography be synchronized with electrocardiograms?

We have attempted to synchronize echocardiography with electrocardiograms previously, but haven’t been successful, hence we have not included any comments regarding this in the manuscript.

The literature on Axolotl regeneration is well cited, but you may also be interested in the recent study by Nakamura et al in Development, Growth & Differentiation 58, no. 4 (2016): 367-382.

This is a relevant paper and we have referenced it in the revised manuscript (l. 64 and l. 836-837).

Line 21: try to avoid that the verbs are placed at the end of the sentence

We are not sure what the reviewer is getting at here. We cannot find any verbs in original line 21 (“Long abstract”) and cannot find examples of verbs placed at the end of sentences in the adjacent sentences.

Line 29: "to meaningfully and reproducibly" - reproducible is good enough. I suggest to leave it to your readers whether something is meaningful.

We agree and have left out “meaningfully” (l. 29 and l. 70).

Line 32: "Due to the lack in dimensionality, " I don't think this sentence makes sense, and it can be deleted without the loss of any meaning.

Sentence has been deleted (l. 32-33).

Line 52-55: this sentence is redundant

We disagree and are inclined to leave the sentence. We believe it is important to introduce the zebrafish as an important model in heart regeneration but also put emphasis on both pros and cons in this model in order to justify the use of a larger model like the axolotl.

Line 60: delete "to be"

“to be” is deleted (l. 61.)

Line 163-164: "Ensure positioning of cranial to the right" - why?

This is the traditional practice in echocardiography and ensures standardized acquisitions that are easy to interpret for other. We have extended the sentence to clarify this (l. 178).

Line 185: how much does the ventricle approximate the spherical shape? Consider on the basis of this recent paper whether there should adjustments to your assumption of complete sphericity: Perrichon, Prescilla, Martin Grosell, and Warren W. Burggren. "Heart Performance Determination by Visualization in Larval Fishes: Influence of Alternative Models for Heart Shape and Volume." Frontiers in Physiology 8 (2017).

This is a very interesting paper. The best evidence that the spherical assumption is not perfect comes from our own 3D data (Supplementary material 13-15). Especially in the end-diastolic phase the spherical assumption is not exactly accurate. But we do believe that the spherical model is reasonable to use for its simplicity, though a better model encompassing different life stages and sizes of the axolotl would be interesting to develop as derivative work.

Line 294: haunted is an odd choice of word

Sentence has been rewritten and “haunted” left out (l. 307-310).

Line 703-704: "Likewise, echocardiographic measurements should be viewed more as index values rather than absolute values." Indexed to what? In cardiology, functional measures are typically indexed to body mass or the calculated body surface, but this has nothing to do with the reproducibility of the primary data and instead concerns comparisons between individuals.

Our use of index is in the meaning of something that serves to guide, point out, or otherwise facilitate reference between measurements made under different circumstances, i.e. values that can be used to study relative changes. We have tried to highlight that point in the new version of the sentence (l. 734-735).

**Reviewer #3:**

Manuscript Summary:

The focus of the current manuscript is to exhibit different methods of measuring cardiac function in the Mexican Axolotl. This is a powerful model to study regenerative processes, such as heart regeneration, and the authors speculate that these protocols will be useful in making measurements on regenerating axolotl hearts in the future. The authors discuss how different methods of anesthesia are used on the axolotl in addition to 2D echocardiography (in anesthetized and unanesthetized animals), how to evaluate 2D echocardiography, and 3D echocardiography. The representative data seems appropriate and the protocols appear to have all of the appropriate details to perform similar experiments -however since this technology is outside of my realm of expertise, I can't be sure that sufficient detail is provided. I think that the protocols provided here will be useful to those that stay cardiac function and regeneration in axolotl.

Minor Concerns:

1) Figure are referenced in text out of order. For example, figure 6 is referenced before figure 2. It is common practice to have figures numbered in the order that they are referenced in the text.

We agree with the reviewer. The reason is the multifaceted nature of the figures that makes is very difficult both to combine different representative results in appealing figures and still strictly adhere to the order of the figures when presenting them in the text. We have now initially introduced all the figures in the introduction in such way the later referencing to specific figures can follow the logics of the text (l. 69 – 72).

2) For individuals that are not familiar with making measurements on cardiac function -explaining a little more on axolotl cardiac anatomy, and what exactly is being measured with an echocardiograph, would be very helpful. I think that adding this to the introduction makes the most sense.

We have added a more in-depth description of cardiac anatomy in the axolotl (amphibians in general) to the introduction (l. 72-76). We prefer to leave out a very general introduction to echocardiography/ultrasound imaging from the manuscript since this is widely available information in textbooks and imaging courses.

3) In Figure 3, the insets of the images of the exalt are very hard to see. I would either make a larger version of these, or draw a simple cartoon with the appropriate positions indicated.

We agree with the reviewer that these photos were hard to see. We have instead inserted simple cartoon drawings as suggested (Fig. 3 – Fig. 5 and l. 538-539, l. 554-5556, l. 570-571).

4) Line 474-475; in addition to putting standard weights of axolotl at the different developmental ranges, also indicate the average length of the animals (snout to tail tip), which is the standard way that many refer to axolotl size.

We have added indications of length (snout to tail tip) and sexual maturity of animals. We believe that referring to length when describing the sizes of axolotls is slightly imprecise since this measure is highly dependable on the feeding status of the animal, but it is correct that length is often referred to in the litterature when describing size, so we have included it in the manuscript nonetheless (l. 143-144 and l. 463-467).

5) Since this work is performed on vertebrate animals, some statement of ethical conduct of research (ex. IACUC approval, or approval from another regulatory agency) should be indicated.

We have added an Ethics statement section after the Acknowledgements and Disclosures with the relevant ethical approval (l. 777-780).

**Reviewer #4:**

Manuscript Summary:

This manuscript describes a protocol for study of cardiac function in an organism capable of organ regeneration. This is a very useful protocol since it gives the field a way to measure the regeneration of function whereas so far we often only observe regeneration of structure and pattern. This manuscript is highly recommended.

Major Concerns:

I have no major concerns.

Minor Concerns:

I have only 3 minor concerns which involve simple clarifications in the text.

1. It is standard in the axolotl field to describe the size of axolotls based on length in centimeters (cm) and maturity stage (larva, juvenile, sexually mature adult). This protocol uses grams (>20g, <20g). It would be useful for the field if the authors state somewhere in the manuscript the general stage and size of a 20g axolotl (sexually mature adult? 12-14 cm?).

We have added indications of length (snout to tail tip) and sexual maturity of animals. We believe that referring to length when describing the sizes of axolotls is slightly imprecise since this measure is highly dependable on the feeding status of the animal, but it is correct that length is often referred to in the litterature when describing size, so we have included it in the manuscript nonetheless (l. 143-144 and l. 463-467).

2. Although most axolotl research is done on white axolotls (leucistic), the manuscript would be more useful if the authors added a sentence describing the strains of axolotls available (wildtype, melanoid, white, albino, transgenic white with GFP) and which strains would be compatible with this echocardiography protocol. Can the more darkly colored wildtype or melanoid strains be used with this procedure or does it have to be altered?

The protocol is compatible with all different strains of axolotls. We have added a sentence stating this in the introduction (l. 83-86).

3. The authors should spell out the full name and common name for MS222 somewhere in the protocol (Ethyl 3-aminobenzoate methanesulfonate, also known as 'tricane').

Full chemical names of the three anesthetics have been spelled out in the protocol (l. 107, l. 111-112, l. 115)

**Reviewer #5:**

Manuscript Summary:

This manuscript demonstrates methods for assaying cardiac function in axolotls. It will be of interest to researchers using axolotls to study physiology of the heart and, more likely, those studying heart regeneration. Developing tools for assaying regenerative function while the animal is alive is importnat to advance the field and should result in more researchers assaying cardiac function over the course of regeneration, which could potentially improve our understanding of how the process occurs.

Major Concerns:

none

Minor Concerns:

Are the anesthetic solutions buffered? Do they need to be checked for pH and adjusted before use? There are a few grammatical errors and syntax errors that should be caught by the in-house editor.

We are aware that some anesthesia protocols pay detailed attention to adjustments of small variations in pH, especially after reuse of the same anesthesia solution on multiple animals, however in our experience this does not have any practical implications on axolotl wellbeing and physiology if each solution is only used once, so we have left out a complicating discussion on this from the manuscript.