We would like to thank the editors and the reviewers for their helpful comments to improve our protocol.

NOTE: We have removed any line numbers that the reviewers themselves referenced in their comments from this document since those numbers refer back to an earlier version. Any line numbers referenced in this document refer to the edited version (not the clean version) of the document that we are resubmitting along with these comments.

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have done our best to proofread the manuscript.  
2. Please remove the embedded figure(s) and Table of Materials from the manuscript.

We have removed the embedded figure(s) and Table of Materials from the manuscript.  
3. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

Done.  
4. Figure 1: Please note that Figure 1 embedded in the manuscript (page 17) and Figure 1 uploaded separately (page 30) are not the same. Please upload a final version of Figure 1 that matches the Figure 1 legend and has its scale bar defined in the figure or its figure legend.

We now have just one final Figure 1 image. The scale bar is defined in the figure and legend.  
5. Figure 4: Please change the time unit “sec” to “s”.

The graphs now use “s”.  
6. Please shorten the title if possible.

We have shortened the title.  
7. Please provide an email address for each author.

We have provided an email address for each author (lines 6-7).  
8. Keywords: Please provide at least 6 keywords or phrases.

We have provided keywords (lines 17-18).  
9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have done our best to convert lines in the protocol to imperative tense.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:  
1.2 and 1.3: Listing an approximate volume to prepare would be helpful.

We have added more details and listed volumes in these parts of the protocol.  
6.1: Please use 6.1.1, 6.1.2, 6.1.3, etc., instead of a, b, c, etc. Please write the text in the imperative tense in complete sentences.

We have rewritten section 6 to make these changes.  
Steps 8, 9, 12 (including sub-steps): Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

We have added more details and explanations to software steps.  
10.1.1: Please specify incubation temperature.

We have added incubation temperatures.  
10.1.2: What volume of NB is used to wash?

We have added a volume to this step.  
11. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have made sure to include a space between all Step headings and paragraphs.  
12. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There is a 10 page limit for the Protocol. Please revise the protocol section to meet this page limit.

We have double checked that the protocol is not more than 10 pages long.  
13. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted full sentences for a total of less than 2.75 pages of highlighted text.  
14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

We have done this.  
15. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

We have done our best to include as many relevant details in the highlighted text as possible without exceeding the page limit.  
  
**REVIEWERS' COMMENTS:**  
  
**Reviewer #1:**  
  
Minor Concerns:  
One thing that is missing from this paper is a discussion of why they chose the particular calcium indicators for their imaging. It would be nice to add a paragraph discussing the relative advantages and disadvantages of using membrane-targeting calcium indicators instead of soluble indicators –

The membrane localization of GCaMP6s is an important point to highlight and ensure is clear. We have added to our statement in the introduction to expand and clarify why this indicator is important to use:

INTRODUCTION (Lines 138-144)

*“*This membrane localization positions GCaMP6s in a location to detect *calcium influx through ion channels located in the plasma membrane that are critical for hair-cell function. For example, membrane-localized GCaMP6s can* detect calcium influx through MET channels in apical hair bundles and through CaV1.3 channels near synaptic ribbons at the base of the cell. *This is in contrast to using GECIs localized in the cytosol; cytosolic GECIs detect calcium signals that are a combination of MET and CaV1.3 channel activity, as well as calcium contributions from other sources (eg.*, *store release).”*

or other activity dependent indicators (e.g. glusnfr or pHluorins).

We have also added information on other indicators that could be used in this protocol. Based on our experience with other indicators we emphasize why we chose to highlight GCaMP6s-based activity:

DISCUSSION (Lines 1901-1936)

*“While this protocol can be adapted and used on many imaging systems, parts of this protocol can also be adapted and used 1) with other indicators besides GCaMP6s and 2) to image activity in other sensory cells and neurons within larval zebrafish. For example, in a previous study, we used this protocol to image activity using multiple genetically encoded indicators within lateral-line hair cells to detect: cytosolic calcium (RGECO1), vesicle fusion (SypHy), membrane voltage (Bongwoori), membrane calcium (jRCaMP1a-caax and GCaMP6s-caax) and within lateral-line afferent processes to detect: membrane calcium (GCaMP6s)5. Based on our experience using all of these indicators the transgenic line Tg[myo6b:GCaMP6s-caax] described in this protocol offers an excellent start for imaging activity in lateral-line neuromasts. Of all indicators listed above we have found that GCaMP6s is the most sensitive and photostable. In addition to these features, we highlight the Tg[myo6b:GCaMP6s-caax] transgenic line because it can be used to make two distinct measurements: hair-cell mechanosensation and presynaptic calcium within a single transgenic line.”*

It also might be helpful to discuss other potential pitfalls and trouble-shooting advice and additional tips and tricks. For example, I could imagine the bungarotoxin injections and fish pinning might be tricky and they may have some wisdom to pass down to novices about these procedures.

In the discussion we have added some alternatives to pinning and alpha-bungarotoxin/heart injections for immobilization and paralysis. It is our hope that the images in the figures help demonstrate our method. Ultimately the challenges with pinning and heart injection are what motivated this article. We feel that the video recordings of these procedures will be the best way to demonstrate our wisdom and how we actually do these procedures.

**Reviewer #2:**  
  
Minor Concerns:  
  
I've included a list of comments referencing line numbers. These are minor comments and a few corrections that copy editors may not catch.  
  
  
The wording is funny here. You could say, "Deflection puts tension on the linkages" rather than "tensions the linkages".  
We have made this change.  
You may want to state that you are talking about field recording here. It is possible of course to patch onto invididual cells in the hair bundle, which gives you the "resolution" to look at individual cells, but not a collection of individual cells simultaneously.  
We have made a note of the differences between these techniques more clearly the manuscript.

INTRODUCTION(Lines 84-119)

For many years, electrophysiological techniques such as whole-cell patch clamping have been used to probe the functional properties of hair cells *in many species, including zebrafish15–20*. These electrophysiological recordings have been particularly valuable to the field of hearing and balance because they can be used to make extremely sensitive measurements from individual sensory cells whose purpose is to encode extremely fast stimuli over a wide range of frequencies and intensities21, 22. *Unfortunately, whole-cell recording are unable measure the activity of populations of hair cells.* *To measure the activity from populations of cells in the zebrafish lateral-line, microphonic potentials and afferent action potentials have been used to detect measure the summed mechanosensitive and postsynaptic response properties of individual neuromasts23, 24.* *Unfortunately, neither whole-cell recordings nor local field potential measurements have the spatial resolution to pinpoint where activity is occurring within an individual cell or measure the activity of each cell within a population.* More recently, calcium dyes and GECIs have been employed to bypass these challenges25,26.

You many want to briefly discuss why you are using the membrane targeted GCaMP rather than just a cytosolic version, and why you are specifically using the GCaMP6 version.  
I think you have good reason to use this specific indicator, and the community should be clear on why it matters.  
Great point. We do think that it is important to use the membrane targeted version of GCaMP for these measurements, and we also currently, based on our experience with indicators thus far prefer GCaMP6s for calcium measurements. Please see the parts above that we added for Reviewer #1 suggestions- he/she has made the same recommendation.

You might suggest centrifuging the phenol red solution to clear any crystals and prevent them from clogging the heart injection needle.  
*5.1. Centrifuge the alpha-bungarotoxin* *aliquot briefly prior to use to prevent clogging of the heart injection needle.* (Lines 415-416)

I'm not really clear on why you need the embryo to be in a special neuronal buffer rather than just E3. Can you discuss that?

Sometime E3 media is not made as accurately and is generally not stored at 4°C or filter sterilized. In addition, from our results we have found that the calcium responses in E3 are smaller as well.

We have added the following note:

*Note: 1X E3 can also be used for functional imaging, but responses are more robust and reliable in NB.* (Lines 202-203)

A needle polisher could be useful here. Maybe it's worth mentioning.  
Great idea.

We have mentioned this in the protocol.

*NOTE: A needle polisher can be used to fix jagged breaks.* (Line 344)

Add added a pipette polisher to the Table of Materials

You mean 0.35mm (as described above), not nm.  
Yes! Thank you, we have made this change.

Possibly labs should always confirm what deflection results in near-saturation of the calcium signal. I don't know what the variability of the calcium signal is if you repeatedly deflect a bundle. If it's relatively low, maybe it's fairly easy to find the saturation point that in your example occurs around a 5um deflection. I wonder if the deflection needed to reach saturation varies among zebrafish strains, since kinocilia length does.  
Good point. We have made it more clear that saturation will not always be a 5 µm deflection for every age and indicator. We have added to our original “note”.

“NOTE: *Using GCaMP6s in larvae 3 – 7 dpf*, a 5 µm deflection should achieve near saturating GCaMP6s calcium signals and should not damage the apical hair-bundle structures (Figure 3A3’’). Smaller displacement distances can be used to deliver non-saturating stimuli (Figure 3A3’). Displacement distances > 10 µm are hard to estimate (Figure 3A3’’’) and can be damaging over time. *Signal saturation is dependent on age of neuromast (and kinocilial height) as well as the indicator used.”* (Lines 835-840)

It seems like binning 2X2 would generally be a good idea since the noise contribution will be less and you can run at lower laser power. Does not using binning help isolate the separate cells?

Yes, 2X2 binning generally does give better signals because there is less noise and less bleaching due to lower laser setting. The main drawback is that you lose half your resolution. It just depends what you are after. Depending on the resolution of the camera either 1 X or 2 X binning can still separate individual cells. For the camera listed in this protocol 2 X is still good for isolating individual cells. In the representative results we have added the calcium signals in Figure 4 and Figure 5 were acquired at 2 X binning.

We have added to our “note” on binning.

NOTE: Apply 2 X binning if signals are too weak, *noisy*, or have excessive photobleaching. *2 X binning will enhance signal detection at the cost of spatial resolution.* (Lines 856-858 & 996-998)

I think you should present the BAPTA control as simply part of the process, not an optional control.  
We made these changes and removed “(optional)”

Just so people aren't confused about how GCaMP works, I would always say that the change in fluorescence IN RESPONSE TO DEFLECTION is gone (in the presence of BAPTA, etc.), not that the signal is gone. People may naively think that chelating calcium causes the GCaMP to be "dark". There is always weak signal from the GCaMP, even in the presence of BAPTA, right?

Good point, we have added detail to Section 10 and 11 to clarify this point.

10.4. Redo Step 8 or 9. After BAPTA treatment *there should be no change in GCaMP6s fluorescence in response to fluid-jet* stimulation in either the apical hair bundles or the synaptic plane. *If changes in GCaMP6s fluorescence persist,* these are not true calcium signals and may be motion artifacts. (Lines 1010-1115)

11.3. Without performing a wash, redo Step 8 or 9.After treatment, *there should still be GCaMP6s fluorescence changes in response to fluid-jet stimulation* in apical hair-bundles but not in the synaptic plane. *If changes in GCaMP6s fluorescence* persist in the synaptic plane, these are not true calcium signals and may be motion artifacts. (Lines 1122-1125)

This is a contentious, but I wonder if a max intensity wouldn't be better than an average intensity projection of the 5 planes. This is a more obvious issue if for example the GCaMP is targeted to an intracellular organelle, since the average projection will average in values from regions where there is no signal, and then effectively drop the dynamic range of the readouts. (Of course, the Max Intensity drops out all the data except the highest signals at each point, which is what people don't like about it.) Maybe a brief mention of the pros and cons of the different types of projections is in order here, but I'll let you decide whether you want to raise that issue. Clearly the average projection works well for this system.

This is a good point. We are going to stick with the average projection as it works best for this analysis. It is true that for our organelle measurements we do use Max intensity projections. This will have to be an adaptation of the protocol.

Similar to the comment above: The GCaMP signal is not eliminated (unless the baseline was set at 0); the response of the signal is eliminated.  
We have added more detail to the representative results to make this it more clear that it is the changes in GCaMP6s fluorescence associated with stimulation that are eliminated in the controls.

Figure 1A: This is a trivial thing, but a number of diagrams have this wrong. Are the orientations of the kinocilia correct in relation to the direction of the response? In the figure, you would expect the right and left-of-center cells to respond. I thought the kinocilia mostly face the center of the circle of HC's (central relative to stereocilia), but maybe that's not true.

There is no 100% of the time answer for this question. When there are 6 hair cells the kinocilia are the center of the neuromast and there is a clear line of polarity reversal. In older neuromasts, generally, in the center of the neuromast, the kinocilia are still more commonly central, and face the center of the circle of hair cells. But along the sides of the neuromast bundle orientations are often similar to what we depicted in Figure 1. Because our Figure 1 implies we are in the “center” of a neuromast we have changed the bundle orientation to reflect the bundle orientations that are more commonly depicted in figures in the literature.

By "splaying of the apical hair bundles" I assume you mean splaying of the kinocilia since you cannot usually see the stereocilia. And I assume this must mean that the cupula is broken down. The cupula is something the field tends to ignore, but it's worth mentioning here.

Because we do not know whether the stereocilia are also disrupted when kinocilia are splayed we have modified this statement.

*..third, when kinocilia tips splay out in different directions35*. (Lines 1882-1883)

It is unclear when kinocilia splay if it is due to disruptions in the cupula. It could be, but we are not sure. We do know that if larvae are not maintained and cleaned well that that cupula can be compromised. We added a statement to this effect in our discussion.

A clean aqueous environment is particularly important for young larvae (2 – 4 dpf) or mutants that cannot maintain an upright swimming position and primarily lie on the bottom of the petri dish. In these situations, *lateral-line hair cells and the protective cupula surrounding the hair bundles can easily become compromised*. (Lines 1872-1876)