University of Kentucky
Department of Physiology
College of Medicine

November 30, 2020

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Bajaj:

Enclosed please find the revised manuscript JoVE62104, *"Stereocilia Bundle Imaging with Nanoscale Resolution in Live Mammalian Auditory Hair Cells"*. We appreciate the positive and very constructive comments of the reviewers and believe that we have made all necessary changes. Below we provide a point-by-point response to each one of the issues raised by the Editor and the Reviewers. We also uploaded a version of the manuscript with all changes highlighted in blue.

We thank you for your consideration of our manuscript.

Sincerely,

A. Catalina Vélez-Ortega, PhD
Assistant Professor of Physiology

Gregory I. Frolenkov, Ph.D.
Professor of Physiology

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Reply: Done.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here we present a protocol …”

Reply: Done.

3. Please ensure for in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Reply: Done.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Ionscope, UK (ionscope.com), NX12-Bio and NX10 SICM, 137 Park Systems, Korea; and SICM modules from ICAPPIC Limited, UK (icappic.com), (World Precision Instruments, Inc., Saratosa, FL), 3M, St. Paul, MN, (The Dow Chemical Company, Alberta, Canada), AFM (Ted Pella), Sylgard, etc.

Reply: We have removed mentioning of the companies and their products from the text of the manuscript.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Reply: Done.

6. We cannot have paragraphs of text in the protocol section. Please move lines 133-166 to the introduction section instead.

Reply: Done.

7. Please include volume and concentrations throughout the protocol.

Reply: Done.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly.

Reply: Done.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

Reply: Done.

10. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please

highlight 3 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.

Reply: Essential steps in the protocol for the video are highlighted in yellow – they occupy less than two pages total.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Reply: N/A - None of the figures of this manuscript were previously published.

12. Please do not abbreviate the journal titles in the reference section.

Reply: Corrected.

13. Figure 2: Please hide or remove the commercial term.

Reply: Done.

14. Please sort the materials table in alphabetical order.

Reply: Done.

Reviewer #1

Major Concerns:

1. Please give more general information on the technique in the protocol's introduction (lines 147-159). For example, indicate who defines the setpoint and how it is predefined (line 152). Please describe what is considered as an "imaging point" and indicate how big it is (lines 154-156). Please indicate the speed at which the pipette retracts from the sample (line 154). Please explain why the sample is moved instead of the pipette. Is this relevant to the protocol? How is the sample's movement controlled? (line 155). Please indicate how the sample size is determined.

Reply: We have now indicated that the setpoint is defined by the user (line 136). We have also described acquisition of the imaging point as follows: “…the system saves Z value … as the height of the sample, together with X and Y coordinates…” (lines 136-138). We are confused by the reviewer’s question about the size of the imaging point. Since piezo actuators can move the nanopipette with a single nanometer precision or even better, the user may set a sub-nanometer pixel size during acquisition of HPICM images. Yet, this “instrumentation” pixel size will have nothing to do with the actual resolution. Throughout the paper, we discuss in detail all the factors that affect the resolution and, therefore, determine a reasonable pixel size that the operator has to choose. Practical values are shown in Table 1.

We have also specified that the speed at which the pipette retracts from the sample is also user-defined and provided the typical range of this speed (lines 138-139). Finally, we described the benefits of pipette versus sample movement in X-Y direction (lines 142-147) and indicated that “the HPICM probe and the specimen stage are moved in Z and X-Y axes by the calibrated feedback-controlled piezo actuators that have an accuracy of a single nanometer or better” (lines 157-158).

2. Please indicate how the calibration sample is attached to the chamber (line 229. Step 3.1).

Reply: Following the Reviewer's comment, we have explained that the calibration sample is attached to the chamber with silicon glue in Step 3.1.

3. Please indicate how the ground electrode is secured to the chamber (line 231. Step 3.4).

Reply: We have clarified in Step 3.3 that the ground electrode has its own magnetic holder that can be position close to the stage to allow the immersion of the electrode in the bath solution. We also labeled it on the Figure 1B.

4. Please define "hop amplitude" in line 239. This seems like a critical concept that is not explained nor defined. In line 245, the authors state that "the hop amplitude can be decreased significantly after obtaining the low-resolution images and determining the highest features within the imaging region." Please explain the relationship between hop amplitude and image resolution.

Reply: As suggested by the Reviewer, in Step 3.8, we have defined the hop amplitude as the amplitude of nanopipette retraction during scanning of the sample. We have also explained that "a smaller hop amplitude allows faster scanning, which is preferred for high resolution imaging due to diminished effects of the drifts and decreased vibration".

5. Please explain how the center of the calibration standard is identified (step 3.5).

Reply: We have explained in Step 3.5 that the nanopipette is positioned over with the center of the calibration sample with patch clamp micromanipulator under visual inspection. We also indicated in this step that, in case of the organ of Corti explants, it is possible navigate the pipette more precisely by observing the tissue and the pipette with an inverted microscope.

6. Please indicate how the chambers are stored and whether they are kept sterile or sterilized before used (step 4).

Reply: We have added the Step 4.3.4 as following, "Place the chamber upside down on a filter paper to let it dry until next experiment. The chambers do not need to be sterilized, unless culturing of the organ of Corti is planned after the imaging."

7. Please indicate how the organ of Corti explants are attached to the custom-build chambers (line 285, Step 5).

Reply: We have explained in Step 5, that the organ of Corti explants are secured under either two flexible glass pipettes or two dental floss strands. This is also shown in Figure 4A and 4B, correspondingly.

8. Please indicate the volume of bath solution added the tissue-containing chambers for image acquisition (line 288, step 5)

Reply: Done. The volume is 4 mL.

9. In line 292, the authors recommend securing the chamber to the HPICM stage to minimize sample drifts. And in line 315, the authors recommend spending less than 15 minutes when imaging a whole hair bundle to minimize the effect of the drift due to the constant movement of living cells over time. A distinction between stage and sample drift would help understand the drift' importance and how to minimize it. Please explain and discuss the differences between the drifts indicated in line 292 and line 315.

Reply: We clarified our terminology in step 6.1 and indicated that the chamber is firmly secured to minimize its drift in X and Y axes. In step 6.9, we are now using a different terminology, "Keep in mind that the hair cell bundles in the live tissue are not still but may change their orientation, for example, due to shape changes in the underlying supporting cells. Therefore, the images may exhibit movement artefacts if the image acquisition is too slow". Usually, "chamber drift" and "cell movement" artefacts

produce different distortions in HPICM images, since chamber drift affects the whole image while cell movements occur only in a group of the cells.

10. Please explain how to check and assure stability in the system (line 295. Step 6.3).

Reply: We have re-phrased this step (now 6.4) as follows, “Check if the system is stable with a setpoint of 0.5% or lower by recording the real-time current and Z positioning signal on the oscilloscope (as in Figure 1C). If the Z signal is not stable, replace the nanopipette.”

11. Please explain why the noise appears as white dots in the images acquired at low setpoint or high hop amplitude. Is there a way to differentiate between noise coming from debris and noise coming from a low setpoint or a high hop amplitude?

Reply: We have now described it in the step 7.3: “While obtaining images with HPICM, small fluctuations in the nanopipette current can lead to the nanopipette stopping far from the surface of the sample, especially with low setpoints. It results in the appearance of small white dots in the image as seen in Figure 8A, left.” We also re-phrased this explanation in the Representative Results: “with a very low setpoint, the system might interpret small fluctuations in the current as encountering the cell surface and this will lead to the “white dot” noise in the image (Figure 8A).“ This noise is indistinguishable from the one occurring when the nanopipette encounter a floating piece of debris during its approach to the sample and, therefore, they both filtered out in the same way during post-processing (see new section 7).

12. Please define "sensing volume" in line 461.

Reply: Following Reviewer’s comment, we have explained it as follows, “The current generated by the nanopipette in the solution attenuates quickly (inverse proportional to the cubic distance from the pipette tip), thereby establishing a “sensing volume”, beyond which the nanopipette cannot “sense” the surface. We have previously developed a model of this phenomenon and showed that the lateral resolution of the SICM probe is determined by the cross-section of this “sensing volume” with the cell surface, which could be extremely small at low setpoints[25].”

13. Please explain the relationship between the setpoint, pipette diameter, resolution, and noise.

Reply: A special paragraph in the Discussion is dedicated to this (lines 569-581). “The noise of the nanopipette current represents another limitation, since it sets the minimal practically achievable setpoint. The current generated by the nanopipette in the solution attenuates quickly (inverse proportional to the cubic distance from the pipette tip), thereby establishing a “sensing volume”, beyond which the nanopipette cannot “sense” the surface. We have previously developed a model of this phenomenon and showed that the lateral resolution of the SICM probe is determined by the cross-section of this “sensing volume” with the cell surface, which could be extremely small at low setpoints[25]. This is particularly important for imaging hair cell tip links that have a diameter of ~5 nm[12,32]. On the first glance, the pipettes with smaller inner diameter would result in better resolution of HPICM imaging. This is indeed true for relatively large pipettes (>50 nm). However, decreasing the inner diameter of the nanopipette below ~50 nm results in unproportionally large increase of the pipette noise and, hence, the loss of resolution at low setpoints that are essential for imaging stereocilia bundles. As of today, we do not know how to solve this problem and we are working on finding the proper solution.”

14. Please indicate the band pass filter used for data acquisition and how this may affect the experimental outcome.

Reply: We have re-phrased step 6.4 as follows, “Check if the system is stable with a setpoint of 0.5% or lower by recording the real-time current and Z positioning signal on the oscilloscope (as in Figure 1C). If Z signal is not stable, try to decrease the cutoff frequency of the low-pass filter of the patch clamp amplifier. However, it cannot be lower than the response time of Z piezo actuator (to avoid pipette collision with the sample due to delayed current readings). In practice, we have found that 5 kHz setting of this filter is optimal. It is better to replace the nanopipette, if Z-signal is still unstable.”

15. Several materials and equipment were not listed in the Table of Materials. Please include the information on the puller and capillaries used to fabricate the pipettes, the material used to prepare the custom-built chambers, the calibration standards, and the software used to acquire the experimental data and perform the processing.

Reply: Following the Reviewer's comment, we have updated our Table of Materials.

Minor Concerns:

16. Please indicate the preferred direction of the HPICM probe in figure 1A.

Reply: We have indicated the preferred left-to-right direction of scanning with an arrow in Figure 1A. We have described it in the figure legend as follows, "Illustrated hop amplitude would work for left-to-right scanning (from smallest to a tallest stereocilium, indicated by an arrow). However, it is too small for right-to-left scanning when the pipette meets the tallest stereocilium first."

17. In Figure 3-7, please indicate the meaning of the grayscale (um).

Reply: We are again confused by the comment. All our HPICM images in Figure 3, Figure 5 (B and C), Figure 6 (B, D, and F) and Figure 7 do have grayscale calibration bars. However, Figure 4 show optical images, while Figure 5A, Figure 6 (A, C, and E) show scanning electron microscopy images. In all of them, grayscale cannot be meaningfully interpreted in a quantitative way. Perhaps, the Reviewer wanted us to mention that, in all HPICM images, the greyscale of a pixel indicates the height of the sample at that point. We have mentioned it now in the figure legends.

18. Please label the glass capillaries in figure 4A and wires in figure 4B.

Reply: Done

19. Please indicate the outer and inner hair cells in figure 5.

Reply: Done

20. In Figure 7 and text, please clarify if the sample was constantly imaged for 6 hours or one image was taken every hour.

Reply: We have clarified that the sample was continuously imaged for 6 hours.

Reviewer #2:

Major Concerns:

1. The authors describe the advantages of their protocol/technique, but what are the disadvantages? And drawbacks. Please discuss comprehensively.

Reply: We would like to thank the reviewer for this particular comment. We now discuss disadvantages in many places of the manuscript: i) an original SICM requirement of a relatively flat surface that was alleviated by HPICM (lines 110-113); ii) the requirement for personnel with the electrophysiological background (lines 170-174); iii) relatively slow imaging with HPICM (lines 561-567); and yet not optimal resolution of HPICM (lines 569-581). To summarize major advantages and disadvantages, we re-phrased the last paragraph of the Discussion as follows: "The biggest advantages of the HPICM are: i) its ability to visualize label-free nanoscale structures at the surface of living cells without touching them; and ii) to probe the function of these structures with patch clamp recordings and/or local nanoscale delivery of mechanical or chemical stimuli. To the best of our knowledge, these advantages are unique to HPICM. Of course, there are some disadvantages. First, due to limitations on the height of the structures to be imaged, HPICM may not be suitable for imaging extremely tall structures, such as stereocilia bundles of the vestibular hair cells in the mammalian ampullae. Second, HPICM is still developing and further

improvements in the speed and resolution of imaging are needed. However, the physical principles of HPICM and our own experience suggest that it is possible. We do believe that HPICM will provide unique data on the function of individual protein complexes at the stereocilia surface."

2. How difficult, demanding, time and money consuming is it to establish the procedure for a scientist with and without patch clamp experience? Please describe comprehensively in the text.

Reply: Following the reviewer's comment, we have added the approximate cost of materials/equipment in the table of materials. We also clarified in the Introduction that HPICM "integration is relatively easy for any researcher, who is proficient in patch clamping. However, a scientist without proper background would definitely need some training in electrophysiology first." According to our experience, a scientist with a proper background becomes excited very easily by HPICM capabilities, while a scientist without proper background either in electrophysiology or in electrical engineering may have hard time understanding even basic principles of HPICM.

3. I may ask the authors to include a comprehensive section about the analysis and processing of the data to provide a detailed protocol for the visualization of stereocilia bundles as claimed in line 470.

Reply: Done. See entirely new section 7.

Minor Concerns:

Introduction-Section:

4. line 61: Could the authors please describe the local mechanisms that regulate signalling at the tips of stereocilia?

Reply: We are referencing two recent papers (refs #2 and 3) that provide substantial evidence for such local mechanisms regulating mechano-electrical transduction at the tips of stereocilia. The mechanisms themselves are still subject of intense investigation and, therefore, it is hard to speculate about them in the Introduction. In fact, we are developing HPICM technique in order to study these mechanisms.

5. line 108: I am not sure whether an 11-year old publication can be described as "only recently"

Reply: Words "only recently" have been removed.

6. line 128 and lines 283/284: I was wondering about why the technique apparently is limited to maximally 8 days old rodents and hence only allows for analysis of premature hair cells? I think this is also some important information for readers interested in analyses of the inner ear.

Reply: We have clarified that "older hair cells are more susceptible to the damage during dissection and, therefore, cannot be used for hours-long time lapse HPICM imaging" (lines 329-331).

Protocol-Section:

7. The first paragraph of the protocol-section addressed the equipment and the technique as such. To increase clarity, I was wondering whether it was possible to introduce a subheading at that point.

Reply: We have moved this paragraph to the Introduction.

8. lines 139 (following): The authors describe the need of integrating several components into available recording systems. For non-expert readers, it may be beneficial to briefly describe those component, to highlight the difficulties and pitfalls of integration and maybe also to present a schematic of the setup with all components in a Figure (the setup is hard to appreciate from the presentation in Figure 1B in its present form). Is it possible to give approximate costs for the setup and also minimally required time to establish the procedure (let´s say from a working pclamp setup).

Reply: Following the reviewer's comment, we have added the cost for materials/equipment in the Table of Materials. We have also added to the Figure 1 a new panel D, which describes the most essential equipment that needs to be added to a standard patch clamp setup. As to the time required to assemble and troubleshoot the HPICM rig, it is highly variable, depending on qualification and experience of the researcher.

9. line 176: Could the authors please specify the "resonance" expected to increase with the length of pipettes.

Reply: We have clarified in the step 1.2 that "The length of the pipette is crucial because it determines the frequency of the lateral mechanical resonance of the pipette. The longer is the pipette, the lower is the resonant frequency and the harder is to avoid this resonance."

10. Figure 2C: Could you please also give a scale in this image?

Reply: Done.

11. line 180: D-glucose apparently is added to the solution to adjust the osmolarity. Please, give this information also here in the text.

Reply: The Reviewer is correct; glucose was added to adjust osmolarity. This info has been added to the text.

12. line 185: I was wondering, why it is more difficult to remove bubbles from "older" pipettes, as I have not yet noticed this correlation.

Reply: We were also puzzled by this phenomenon since we also never noticed it in the larger pipettes used for patch clamping. Therefore, we have admitted it by saying, "For the reasons that we do not yet understand, it is harder to remove bubbles in the pipettes that have been pulled several hours before the experiment."

13. line 202: Apparently the ideal pipette resistance value range is rather large and between 200 - 400 MΩ. Can the authors maybe precise this (narrow it down) a bit, or is 200 MΩ exactly as good as 400 MΩ.

Reply: There is a compromise that we have explained as follows: "In our experience, the ideal resistance value is between 200 and 400 MΩ. Pipettes with a resistance higher than 400 MΩ might lead to an unstable current due to their small size (< 50 nm inner diameter). On the contrary, pipettes with resistances smaller than 200 MΩ are too large (> 70 nm inner diameter) and would not resolve small features. Its recommended to start imaging with the pipettes of 200 MΩ resistance, as they are easier to manufacture and tend to provide less electrical noise." The exact value depends on what the researcher wants: a stable recording with a worse resolution or a better resolution with the recordings that may be unstable.

14. line 205 "…and will be unable to..": Maybe, the authors could change this to "…impeding resolution of…" or something like that.

Reply: Corrected.

Minimizing sample drifts and vibrations + making custom-built chambers (Sections 2+4)

15. I fully understand that minimizing vibrations/movements of the tissue and the setup are crucial for these kind of recordings. As an electrophysiologist, I was wondering whether a setup optimized for standard patch clamp (as far as vibration is concerned) is suitable or whether the vibrations even need to be lower. Maybe this is also important information for other readers and should be mentioned in the next (together with highlighting the need for vibration-reduced tables, etc.)

Reply: Again, we thank the Reviewer for the comment. We have clarified that “HPICM setup represents a patch clamp rig with more stringent vibration and drift requirements” (Introduction) and that “a small drift with a speed of less than a micrometer per minute is usually not noticeable in a regular patch clamp setup. Yet, it could produce artefacts of several tens of nanometers in HPICM imaging, which is significantly larger than the resolution of HPICM” (step 7.2).

16. I find the sections on custom-built chambers (Section 2+4) quite difficult to understand and I guess that this may be more easy to understand by means of the video. Nevertheless, the authors should revise these sections (2 + 4) to increase clarity and also show a schematic representation of these custom-built devices.

Reply: We have added additional info (steps) to the Sections 2 and 4 to improve clarity. We have also explained the reason why we are using two types of chambers. “The glass pipette chamber could be sterilized and used for the cultured organs of Corti, while dental floss chamber provides a more secure holding of the sample and a control over stereocilia bundle orientation during mounting.”

17. Figure 4: The described components (coverslip, wire, floss, etc.) are difficult to appreciate and should be highlighted in the figure. The cochlear structures are very difficult to see in the image inset.

Reply: We have now labeled the components of the chambers in Figure 4. The goal of the inset, however, is to show the organ of Corti mounting rather than the cochlear structures. Higher magnification would show these structures better but would not show the mounting.

Testing the system’s resolution (Section 3)

18. Line 219: Do the authors advise calibration of the system on a daily basis or only once week, month,… (this should be mentioned in the text).

Reply: We apologize for the apparent confusion. HPICM system doesn’t need often calibrations, because every axis is driven by a piezo translation stage (or an actuator) with a build-in movement sensor (calibrated during the setup, see new Figure 1D). The rationale for imaging of the AFM standards is to test the stability and the noise of the system and, hence, the practically achievable resolution. We have clarified it now as follows. “We strongly recommend imaging AFM standards (see Materials table) before imaging live cells in order to troubleshoot the system and test its resolution in the X-Z-Y axes.”

19. Figures 5, 7B, 8: Insert blank between value and unit above scale bars

Reply: Corrected.

20. line 265: Should it be "of" rather than "off"?

Reply: Corrected

21. line 481: change "advise" to "advice"

Reply: Corrected