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Visualization of Live Cochlear Stereocilia at a Nanoscale Resolution Using Hopping Probe Ion Conductance Microscopy

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Abstract

The mechanosensory apparatus that detects sound-induced vibrations in the cochlea is located on the apex of the auditory sensory hair cells and it is made up of actin-filled projections, called stereocilia. In young rodents, stereocilia bundles of auditory hair cells consist of 3 to 4 rows of stereocilia of decreasing height and varying thickness. Morphological studies of the auditory stereocilia bundles in live hair cells have been challenging because the diameter of each stereocilium is near or below the resolution limit of optical microscopy. In theory, scanning probe microscopy techniques, such as atomic force microscopy, could visualize the surface of a living cell at a nanoscale resolution. However, their implementations for hair cell imaging have been largely unsuccessful because the probe usually damages the bundle and disrupts the bundle cohesiveness during imaging. We overcome these limitations by using hopping probe ion conductance microscopy (HPICM), a non-contact scanning probe technique that is ideally suited for the imaging of live cells with a complex topography. Organ of Corti explants are placed in a physiological solution and then a glass nanopipette –which is connected to a 3D-positioning piezoelectric system and to a patch clamp amplifier– is used to scan the surface of the live hair cells at nanometer resolution without ever touching the cell surface.

Here we provide a detailed protocol for the imaging of mouse or rat stereocilia bundles in live auditory hair cells using HPICM. We provide information about the fabrication of the nanopipettes, the calibration of the HPICM setup, the parameters we have optimized for the imaging of live stereocilia bundles and, lastly, a few basic image post-processing manipulations.

Keywords

Scanning ion conductance microscopy; organ of Corti; hair cell; stereocilia; live cell imaging; nanopipette

1. Introduction

The sensory hair cells of the inner ear have mechano-sensitive actin-filled projections, termed stereocilia, on their apical surface (1, 2). In the mammalian auditory inner and outer hair cells, stereocilia are arranged in bundles composed of three rows of decreasing height.

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4. Notes

In the inner hair cells, stereocilia also vary in diameter between the rows (**1**). The tallest (first) or second row stereocilia are ~300-500 nm in diameter in live, unfixed and non-dehydrated mouse or rat inner hair cells. Therefore, individual stereocilia in these rows are visualized easily with optical microscopy. However, the shorter rows of inner hair cell stereocilia and all rows of outer hair cell stereocilia have diameters of ~100-200 nm, which is below the resolution of optical microscopy. In bright field imaging, the resolution is proportional to $\sim\lambda/\text{NA}$, where λ is the wavelength of the light source, and NA is the numerical aperture of the objective lens. In the best possible scenario, the resolution cannot be better than ~200 nm for bright field imaging. It can be somewhat improved in fluorescent microscopy, using confocal (**3**) or super-resolution (**4**) techniques. However, most of these techniques face substantial challenges and significant resolution degradation when imaging live cells, especially in thick tissue (**4, 5**). As of today, the best-known example of optical imaging of live functional auditory stereocilia is the imaging of Ca^{2+} entry into individual stereocilia through mechanotransduction channels (**6, 7**). Obviously, the optical techniques do resolve some individual stereocilia in the auditory hair cells, but they are still far from imaging any fine details of the stereocilia bundle structure, such as the links between stereocilia.

Images of the complex architecture of stereocilia bundles at the resolution of single nanometers can be obtained using scanning or transmission electron microscopy (SEM or TEM, correspondingly) (**8-10**). However, the samples for electron microscopy are “dead”, since they typically undergo chemical fixation and dehydration processes. Even freeze-substitution and cryo-electron microscopy techniques (**11, 12**), which can bypass chemical fixation, still result in static preparations that cannot be used to study any dynamic changes in the ultrastructure of the hair cell bundle. Therefore, our knowledge of such potential dynamic changes in the morphology of hair cell stereocilia bundles is largely limited.

In theory, high-resolution imaging of live cells in physiological conditions can be obtained with scanning probe microscopy techniques such as atomic force microscopy (AFM) (**13, 14**). However, the AFM probe, a cantilever with a sharp tip, often makes physical contact with the scanned sample. Since hair cell stereocilia are tall structures that protrude from a relatively flat surface, the AFM cantilever tends to crash against them (**15, 16**) and typically disrupts the bundle cohesiveness, causing irreparable damage to the hair cell. In contrast to AFM, scanning ion conductance microscopy (SICM) is a non-contact scanning probe technique that uses a glass pipette with a nanometer-scale tip (**17-19**). An ionic current (I_p) flows through this nano-pipette into the bath solution that contains live cells or tissue in a near-physiological environment (**Fig. 1A, left**). When the tip of the nano-pipette approaches the surface of the sample, I_p decreases. A feedback system monitors the changes of the I_p and moves the pipette up or down to keep I_p at a certain “setpoint” level, thereby maintaining the constant probe-to-sample distance, while the surface is scanned in X-Y directions. To prevent the pipette from hitting the hair cell stereocilia bundles, we developed a modified SICM technique, which is known as hopping probe ion conductance microscopy (HPCIM) (**20**). In each step of HPCIM, the “reference” current through the pipette (I_{REF}) is measured, while the pipette is far from the sample. Then, the scanning pipette approaches the sample until I_p decreases from I_{REF} to a predefined threshold or “setpoint” (**Fig. 1A**).

This position is recorded as the sample height at this point. Then, the pipette is retracted from the sample before moving to a new X-Y position and repeating the approach. Using this technique, it was possible to obtain topographic images of mouse inner and outer hair cell stereocilia bundles, and to even resolve the small links (~5 nm in diameter) that connect stereocilia (20).

Here we describe the steps to perform the successful imaging of live inner hair cell stereocilia bundles in early-postnatal mouse or rat cochlea explants. We describe in detail (i) the fabrication of the nanopipettes, (ii) the calibration of the HPICM set up, (iii) the preparation of the cochlear explant cultures, (iv) the parameters of HPICM imaging that allow resolving stereocilia bundles and (v) the most common imaging post-processing manipulations.

2. Materials

2.1 SICM imaging setup

We have successfully imaged stereocilia bundles in live auditory hair cells using a custom-built SICM setup with the controller and the software from Ionscope Ltd. (United Kingdom). The other SICM systems commercially available are the Park NX-Bio and Park XE-Bio setups (Park Systems, Korea) (see **Note 1**). Users can decide to try any of these commercially-available SICM setups but, to date, the successful HPICM imaging of auditory stereocilia bundles has been performed only using Ionscope software and custom-built setups (20). In addition, keep in mind that some hardware components mentioned below may or may not be integrated in the commercial SICM systems. Finally, it might be needed also to modify the software code that controls the movements of the SICM probe.

1. SICM setup, e.g., ICnano-S2 (Ionscope Ltd.).
2. Inverted research optical microscope, e.g., Eclipse Ti-U (Nikon, Japan) or IX73 (Olympus, Japan).
3. Vibration isolation system, e.g., 63-500 series lab table (Technical Manufacturing Corporation, Peabody, MA) or BM-1 bench top platform (Minus K Technology, Inc., Inglewood, CA).
4. Low-noise patch clamp amplifier, e.g., Axopatch 200B (Molecular Devices, Sunnyvale, CA) or EPC 800 USB (HEKA Elektronik, Germany).
5. Multi-channel low-noise high-resolution digitizer, e.g., Digidata 1322A (Molecular Devices) or BNC-2090A (National Instruments Corporation, Austin, TX).

¹Although we have only used SICM controller and software from Ionscope, we are aware that other commercially available SICM setups possess the capabilities required to perform high-resolution imaging of live cell samples. We are also aware of developments of an open source code for SICM control. Unfortunately, as of today, an open source solution is not yet available. The most critical feature, of the SICM setup for imaging auditory hair cell stereocilia bundles, is a control algorithm with the hopping-probe capability. In addition, the fine pipette movement should be controlled by piezoelectric systems with a nanometer precision and with a range of at least 10 μm in the Z axis and at least $20 \times 20 \mu\text{m}$ in the X-Y axes. Lastly, the entire SICM setup should exhibit minimal drifts.

6. AFM calibration standards, e.g., HS-20MG and HS-100MG (Ted Pella, Inc., Redding, CA).
7. Non-conductive transparent and thin double-sided tape, e.g., Scotch 666 (Electron Microscopy Sciences, Hatfield, PA).

2.2 SICM software

We optimized the hopping probe parameters for the imaging of stereocilia bundles in live auditory hair cells using the HPICM Control software (Imperial College London, United Kingdom). If different software is used, users may need to adjust the approach curve parameters described in **Figure 1**.

1. SICM software with hopping probe capability, e.g., ICnano2000 Software (Ionscope Ltd.) or HPICM Control (Imperial College London) (*see Note 1*).
2. Software for signal monitoring and recording, e.g., ICnano2000 Controller (Ionscope, Ltd.), WinWCP software (University of Strathclyde, United Kingdom) or pClamp (Molecular Devices, LLC.).
3. Software for image post-processing, e.g., ScanIC Image or ICnano2000 (Ionscope Ltd.), or HPICM Control (Imperial College London) (*see Note 2*).

2.3 Glass nanopipettes

1. Borosilicate glass capillaries with an inner filament (World Precision Instruments, Inc., Sarasota, FL) (*see Notes 3, 4*). We typically use fire polished capillaries that are 100 mm in length with a ratio of outer/inner diameters of 1/0.58 mm.
2. Micropipette puller model P-1000 or P-2000 (Sutter Instrument, Novato, CA).
3. Disposable 1 mL syringes.
4. Patch pipette microloaders (World Precision Instruments, Inc.).
5. Ca^{2+} -containing Hank's balanced salt solution (HBSS).

²SICM images represent matrix data with information from a 3D surface. Users are welcome to use any post-processing software that is able to handle these types of data. For example, the MATLAB® software (MathWorks, Natick, MA) can be used to visualize the SICM images. In addition, custom-made scripts can be created to perform image corrections and analyses.

³Other types of glass can be used for the fabrication of nanopipettes. We obtained similar tip sizes (~50–70 nm inner diameters, ~200–400 MΩ pipette resistances) and similar HPICM imaging qualities using borosilicate and aluminosilicate glass capillaries. In addition, we have obtained smaller nanopipette tips (~30 nm inner diameter, ~1 GΩ pipette resistance) with quartz capillaries. To fabricate nanopipettes with aluminosilicate glass capillaries, users would need to set up and optimize a new program in either the P-1000 or P-2000 Sutter pipette pullers. When using the P-1000 puller, use the *Sutter Cookbook* manual (available for download at http://www.sutter.com/PDFs/pipette_cookbook.pdf) for instructions. If quartz nanopipettes are desired, users need to use the laser-based Sutter P-2000 pipette puller, which has also an *Operation Manual* (http://www.sutter.com/manuals/P-2000_OpMan.pdf) for general guidelines in selecting program values.

⁴Users should always make sure that the capillaries contain an inner filament. Otherwise, due to the very small diameter of the tip, the pulled nanopipettes are nearly impossible to backfill with HBSS (or L-15 medium), without leaving small air bubbles at the pipette tip or taper region.

6. Small observation microscope equipped with a 10X objective lens, e.g., LCD Digital Microscope Model 44340 (Celestron, LLC., Torrance, CA) (*see Note 5*).

2.4 Dissecting and culturing organ of Corti explants

1. Early postnatal mouse or rat pups. To establish organotypic cultures, we typically dissect the organs of Corti from animals not older than postnatal day 4 (P4).
2. Stereo microscope for tissue dissection equipped with a light source and fiber optic light guides.
3. Dissecting tools: Dumont tweezers #5.
4. Standard 60 × 15 mm polystyrene tissue culture dishes.
5. Leibovitz's L-15 medium without phenol red.
6. Disposable borosilicate glass 14.6 cm Pasteur pipettes (Fisher Scientific).
7. Uncoated 35 mm glass-bottom dishes. .
8. Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4500 mg/L), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 4 mM L-glutamine and phenol red.
9. Ampicillin.
10. Heat inactivated fetal bovine serum (FBS).
11. Parafilm.
12. Temperature- and CO₂-controlled incubator for cell culture.

3. Methods

The following protocol assumes the user's general experience with patch clamp techniques and the relevant equipment. Users who have no experience in patch clamping are advised to learn the principles of operation of a patch clamp amplifier (*21*) and the basic techniques of pipette manufacturing (e.g., download the Pipette Cookbook from Sutter Instrument at http://www.sutter.com/PDFs/pipette_cookbook.pdf).

3.1. Pulling and filling glass nanopipettes

1. Place a capillary glass in the micropipette puller (*see Note 6*) and run a "Ramp test" following the puller's *operation manual* to find a safe heat value for the glass. This step is only required the first time the micropipette puller is used for a specific capillary glass.

⁵A critical step, before starting any HPICM imaging, is to ensure a lack of air bubbles in the nanopipette after backfilling with HBSS or L-15 medium. Therefore, we recommend inspecting the pipette taper region using a low magnification LCD microscope equipped with a 10X objective lens.

⁶Always make sure the puller bars are moved all the way to the center of the stage, when the knobs are tightened, and avoid over-tightening the knobs.

2. Create a program in the micropipette puller using the parameters given in **Table 1**. These programs have been designed to create pipettes with a resistance of 200-400 M Ω and estimated inner tip diameters of 50-70 nm (*see* **Notes 7, 8**).
3. Place a new capillary glass in the micropipette puller bar grooves and center its position to obtain two nanopipettes of ~50 mm length. To fabricate shorter nanopipettes *see* **Note 9**.
4. Run the program previously created in the micropipette puller.
5. Use a disposable syringe connected to a flexible filament needle to backfill the freshly pulled nanopipette using Ca²⁺-containing HBSS (*see* **Note 10**). Make sure not to overfill the pipette (*see* **Note 11**).
6. Use a small LCD digital microscope to ensure no bubbles are present in the HBSS-filled nanopipette.

3.2. Checking pipette resistance and signal noise

The successful imaging of live auditory stereocilia bundles using HPICM relies on the use of pipettes with small tip diameters (~50 nm) and, importantly, low noise (~5 pA). Therefore, each time a new nanopipette is used, it is necessary to check whether it meets the acceptable diameter and noise criteria.

1. Mount a freshly pulled HBSS-filled nanopipette into the SICM head (*see* **Note 12**).
2. Place the ground electrode in the bath solution where the sample is located (i.e. into the dish where either a calibration sample or organ of Corti explant is present, as described below in **Sections 3.3** and **3.5**).
3. Zero the voltage applied to the pipette by the patch clamp amplifier.
4. Immerse the pipette tip into the bath solution.
5. Adjust the pipette offset to minimize the ion current flowing through the pipette to zero.

⁷Users may need to perform small adjustments to the pipette puller programs provided in **Table 1** to get nanopipettes with the correct size and resistance. To do so, please refer to the puller's *Operation Manual* for detailed information about program parameters.

⁸The lateral resolution of SICM ultimately relies on the inner tip diameter of the pipette. The first high-resolution HPICM images of mouse hair cell stereocilia bundles were obtained using borosilicate pipettes with an ~30 nm inner tip diameter (i.e. ~1 G Ω resistance) (**20**). However, we can successfully visualize individual stereocilia from rat and mouse inner hair cell bundles using borosilicate pipettes with an ~50-70 nm inner tip diameter. We recommend that users start imaging stereocilia bundles using the latter pipettes, since they are easier to manufacture and typically provide lower electrical noise.

⁹We recommend using the shortest pipettes the SICM pipette holder can utilize, to minimize the mechanical resonance in the lateral direction at the pipette tip. When 100 mm glass capillaries are centered in the middle of the pipette puller, two nanopipettes are produced of ~50 mm in length. Therefore, to obtain shorter pipettes (~30 mm), either shorter glass capillaries are used (e.g., 70 mm) or a 100 mm glass capillary is placed in the pipette puller with an alignment offset (i.e. not centered).

¹⁰Pulled nanopipettes are backfilled with L-15 cell culture medium. However, we seem to obtain longer recordings when we use HBSS as the pipette solution instead of L-15 medium.

¹¹Depending on the length of the electrode and the nanopipette length, we recommend backfilling pipettes with HBSS (or L-15 medium) to the point where only ~1-2 mm of the electrode tip are immersed in the solution.

¹²We recommend pulling nanopipettes on the same day of use, since pipette tips become more hydrophobic with time and backfilling with HBSS (or L-15 medium) becomes more difficult.

6. Apply +100 mV to the pipette and measure the ion current. The value of the current allows calculating the pipette resistance and estimating the tip diameter (*see* **Notes 13, 14**).
7. Increase the voltage applied to the pipette until the current reaches a value of 1 to 2 nA (*see* **Note 15**).
8. Start with a high setpoint value (e.g., 1-5 %) to obtain a stable hopping-probe approach behavior (**Fig. 1B**).
9. Slowly decrease the setpoint to the lowest value that maintains the stability of the hopping-probe approach (the “optimal setpoint”). The system is unstable if the pipette position sensor demonstrates a “jumping” behavior (**Fig. 1C**). In this case, the setpoint is too low and it needs to be increased slightly. From our experience, smaller setpoints produce hair bundle images of better quality. Typically, we use setpoint values lower than 0.6 % to successfully image live hair cell bundles.
10. If the optimal setpoint value is slightly higher than desired, the user can modify one or more of the following parameters from the HPICM approach curve (*see* **Fig. 1A** for details) to obtain a more stable system. For these parameters, we provide a range of values in **Table 2** that we typically use during the HPICM imaging of live hair cell bundles and calibration samples.
 - a. Decrease the speed of approach (i.e. fall rate).
 - b. Decrease the retraction speed (i.e. hop rise rate).
 - c. Decrease the cutoff value of the current filters (*see* **Note 16**).

¹³Based on Ohm's law, pipette resistance is easily calculated as follows:

$$R = \frac{V}{I}$$

where R is the resistance of the nanopipette in megaohms, V the potential applied to the pipette in millivolts and I the ion current flowing through the pipette in nanoamperes.

¹⁴The resistance value of the nanopipette, which is measured in the bath solution, is inversely correlated with the area of the tip opening. Therefore, if the inner diameter of the pipette is halved, the area at the tip opening is reduced to a quarter and the pipette resistance quadruples. Given that a 1 MΩ pipette has a tip inner diameter of ~1 μm and assuming that the inner/outer diameter ratio of the glass is maintained throughout the entire length of the pulled nanopipette, one can quickly estimate the tip inner diameter using the following equation:

$$ID_{Tip} = \frac{1000}{\sqrt{R}}$$

where R is the resistance of the nanopipette in megaohms and ID_{Tip} is the inner diameter at the tip in nanometers.

¹⁵For nanopipettes with resistance values between 200 and 400 MΩ, we obtain the best results applying an ion current of 1 to 2 nA. However, for smaller nanopipettes with resistance values near 1 GΩ, we recommend an ion current of ~0.5 nA.

¹⁶Both, analog and digital ion current (I_p) filters can be modified. They are found in the patch clamp amplifier and the HPICM Control software, correspondingly. When the cutoff values of the filters are lowered, the I_p floor noise is reduced, but the reaction

- d. Increase the length of the reference ion current measurement (i.e. I_{REF}).
 - e. Increase the delay before the reference ion current is measured.
11. If the lowest setpoint value remains high, it is recommended to replace the nanopipette for a new one and repeat all previous steps.
 12. When a stable low setpoint value is achieved (i.e. $< 0.6\%$), the nanopipette can begin to approach the sample as described in **Sections 3.3** and **3.5**.

3.3. HPICM calibration

Before attempting to image live hair cells, it is recommended to use pre-fabricated calibration standards with features of known shapes and sizes to test the X-Y-Z resolution of the HPICM setup. We recommend starting with the HS-100MG calibration standard for AFM, which represents a silicon chip with pillars and holes of different shapes (i.e. circles, squares and lines). Each of these features has a height of 100 nm. Once this 100 nm calibration standard is successfully imaged, we suggest moving to the HS-20MG calibration chip to guarantee the Z resolution of the system is below 20 nm. These 5×5 mm calibration chips come glued to a 12 mm metal disk that needs to be fixed to the bottom of a dish before imaging.

1. Cut a $\sim 13 \times 13$ mm piece of double-sided tape and place it to the bottom of a glass-bottom dish.
2. Place the calibration standard disk on the tape and make sure it is firmly secured to the bottom of the dish (*see Note 17*).
3. Add Ca^{2+} -containing HBSS to the dish (~ 2 mL) and make sure the calibration chip is covered with the solution.
4. Place the dish in the SICM chamber (*see Note 18*).
5. Set the HPICM approach curve parameters as described in **Table 2**.
6. Follow the steps described in **Section 3.2** to check the pipette resistance, set the ion current, test the signal noise and, if necessary, adjust the HPICM parameters.
7. Set the scan size to the full X-Y range of the system.
8. Use the coarse X-Y movement to center the pipette position over the calibration chip.

speed of the HPICM system also decreases. That is, a fast decrease of I_p might be filtered, when the pipette tip approaches the sample. As a result, the setpoint is reached later, when the pipette is closer to the surface of the sample. To help compensate for this error, the user can decrease the speed of approach.

¹⁷Once glued to a glass-bottom dish using double-sided tape, calibration chips are not removed. Instead, after each use (i.e. HPICM imaging), they are rinsed with distilled water, air-dried and stored in a dust-free chamber.

¹⁸To minimize drifts during HPICM imaging, small pieces of double-sided tape are used to firmly secure the dishes onto the SICM stage.

9. Approach the sample with the nanopipette at a slow speed (e.g., 5 to 25 $\mu\text{m/s}$).
10. Once the nanopipette is “in control” (i.e. in the vicinity of the sample), start HPICM imaging at low resolution (*see Table 2*).
11. If it is necessary to move to a new X-Y location (i.e. a different field of view), it is recommended to temporarily retract the pipette by at least 50 μm before the coarse X-Y movement. Once in the new position, it will be necessary to repeat steps 9 and 10 (i.e. approach to the sample and low-resolution scan).
12. When the calibration chip is successfully visualized at low resolution, choose a region of interest within the field of view and start a high-resolution scan (*see Table 2, Note 19 and Fig. 2*).

3.4. Culturing organ of Corti explants

1. Dissect cochlear epithelia from early postnatal mouse or rat pups (<P5), removing the organ of Corti from the modiolus and the stria vascularis, but leaving the tectorial membrane attached (*see Note 20*).
2. Using a disposable glass Pasteur pipette, transfer the dissected organ of Corti to a clean glass-bottom dish.
3. Add ~400 μL of culture medium (i.e. DMEM supplemented with 7% FBS and 10 $\mu\text{g/mL}$ ampicillin) to the glass-bottom dish.
4. Using a pipette tip, spread the culture medium around the glass bottom making sure it makes contact with the plastic edges surrounding the glass.
5. Remove ~200 μL of the culture medium to guarantee that the surface tension of the liquid keeps the tissue explants pressed against the glass bottom (*see Note 21*).
6. Carefully seal the dishes with a small piece ~1 $\times$ 2.5 cm of Parafilm.
7. Place the dishes in a cell culture incubator set at 37°C and 5% CO_2 .
8. After ~10-14h incubation, the organ of Corti explants should attach directly to the glass bottom. Carefully remove the Parafilm and add ~1-2 mL of culture medium to each dish and place the dishes back in the incubator (*see Notes 22, 23*).

¹⁹The HPICM Control software offers an “adaptive” compression profile to set up different lateral resolution values, depending on the complexity of the sample (**20**). Values provided in **Table 2**, should be used for regions with high complexity.

²⁰Remove the tectorial membrane immediately before HPICM imaging and not during the dissection. The tectorial membrane is still growing in young, postnatal organ of Corti explants. Its premature mechanical disruption results in secretion and accumulation of a large amount of unorganized extracellular matrix that eventually covers the stereocilia bundles and easily sticks to the nanopipette. Therefore, HPICM imaging of the hair cell bundles becomes extremely difficult.

²¹If an excess of culture medium is left in the dish, organ of Corti explants do not attach to the glass properly. However, if too much culture medium is removed, explants may dry out during the overnight incubation, causing stereocilia bundle disruptions or even death of the hair cells.

²²Be careful when adding fresh culture medium or rinsing organ of Corti explants, since they can easily detach from the glass-bottom dishes. Similarly, avoid shaking the dishes while explants are transported from the incubator to the SICM setup.

3.5. HPICM imaging of live hair cells

1. Take a dish from the incubator and remove the culture medium.
2. Carefully rinse the organ of Corti explant with L-15 medium at room temperature.
3. Add ~2-3 mL of fresh L-15 medium to the dish.
4. Remove the tectorial membrane with fine tweezers.
5. Repeat steps 2 and 3.
6. Place the dish in the SICM chamber (*see* **Note 18**).
7. Set the HPICM approach curve parameters as described in **Table 2**.
8. Follow the steps described in **Section 3.2** to check the pipette resistance, adjust the current parameters and test the signal noise.
9. Set the scan size to the full X-Y range of the system.
10. Use the coarse X-Y movement to position the pipette over the hair cell rows on the organ of Corti explant.
11. Approach the sample with the nanopipette at a slow speed (e.g., 5 to 10 $\mu\text{m/s}$).
12. Once the nanopipette is “in control” (i.e. in the vicinity of the explant), start the HPICM imaging at low resolution (*see* **Table 2**).
13. If it is necessary to move to a new X-Y location, retract the pipette by at least 200 μm before using the coarse X-Y movement to avoid hitting the tissue with the pipette. In the new position, repeat steps 9 and 10.
14. When the hair cell rows are successfully visualized, choose a region of interest (i.e. a hair cell bundle) within the field of view, and repeat the scan (*see* **Table 2**, **Note 19** and **Fig. 3A**).
15. If the stereocilia within the hair cell bundle appear abnormally thin (**Fig. 3B**), it is necessary to decrease the setpoint value. Keep in mind that few noise pixels on the HPICM image are corrected easily as described in **Section 3.6**.
16. If the thicknesses of the hair cell stereocilia appear normal, start a high-resolution HPICM imaging scan (*see* **Table 2**).

3.6. HPICM image processing

3.6.1. Noise filtering—Small fluctuations in I_p can sometimes stop the nanopipette approach far from the sample surface and lead to isolated bright pixels in the image (**Fig.**

²³After adding fresh cell culture medium and returning the dishes with the organ of Corti explants to the incubator, we recommend waiting for at least 2-4 hours before starting the HPICM imaging. This time allows the explants to firmly attach to the glass. Alternatively, the explants can be left in the incubator for up to one week before HPICM imaging.

4A). These imaging artifacts are particularly common when working with low setpoint values.

1. Open an image in the HPICM Control software.
2. Select the *Image Processing* tab.
3. Check the *Noise Reduction* box.
4. Set the threshold filter (in μm) for the pixels that need to be removed.

3.6.2. Line alignment—Depending on the complexity of the sample to be imaged (i.e. the “hop” amplitude and the speed of approach) and the desired image resolution (i.e. the total imaging points), HPICM images of live hair cell bundles can take a considerable amount of time (e.g., ~16 min for an $8 \times 8 \mu\text{m}$ image with a lateral resolution of 62.5 nm). Therefore, any drifts in the tissue or the HPICM setup could cause visible image artifacts. Any sources of drift in the X and Y axes need to be corrected or minimized, since they typically lead to imaging artifacts that cannot be corrected (see **Note 24**). However, small thermal drifts in the Z axis of the system result in misaligned lines of the image that are fixed easily (**Fig. 4B**):

1. Open an image in the ScanIC Image software (see **Note 25**).
2. Select the *Correct Slope* tab.
3. Choose the width of the lines that need to be aligned and press the *ButtonDestripeLineFit* button for an automated alignment.

3.6.3. Slope correction—When HPICM images are shown in 2D representations, they exhibit a better contrast if the overall slope of the sample is minimized. Given that the HPICM images are composed of a matrix of points with information in all three dimensions, the slope correction simply changes the “angle of view” (**Fig. 4C**)

1. Open an image in the ScanIC Image software (see **Note 26**).
2. Select the *Correct Slope* tab.
3. Press the *Correct Slope* button for an automated correction.

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²⁴The most common sources of drift during HPICM imaging are: tensioned or improperly fixed cables, loose mechanical connections (e.g., loose pipette holder), and even small temperature changes. Anecdotaly, an SICM setup without proper thermal insulation exhibited dramatic drifts in the Z-axis when a person entered or left the room.

²⁵Users can use the ScanIC Control Software as well. Select the *Image Processing* tab, check the *De-stripping* box, type the line width (“Reference stripe”) and the sensitivity of vertical alignment.

²⁶Alternatively, HPICM Control Software can be used to manually correct the slope of the image. Select the *Image Processing* tab, check the *Slope correction* box, and input the slope values for the X and Y axes.

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Fig. 1. HPICM approach curve and setpoint stability

(A) *Left*, in hopping probe ion conductance microscopy (HPICM), the ionic current flowing through a nanopipette (I_p) is monitored while the pipette “hops” above the sample surface. *Middle*, hopping probe approach curve steps over time. *Right*, representative decrease in I_p , when the nanopipette is in close proximity to the sample. *Middle and right*, while the pipette is in a resting position away from the sample surface, a reference baseline recording is established of the ionic current (I_{REF}). Next, the pipette is moved down toward the sample surface at a constant speed (Fall Rate). The approach is stopped when I_p decreases below a pre-defined threshold or “setpoint” (typically 0.3 to 0.6%), indicating that the pipette tip has reached the vicinity of the sample surface. The Z position of the pipette at this point is recorded as an imaging pixel. Then, the pipette is quickly retracted away from the sample (at a speed set by the “hop rise rate”) and a new I_{REF} is recorded (after a short delay, to avoid artifacts in I_p induced by the fast movement of the pipette). Finally, a new downward approach is initiated, after the pipette is moved to a new X-Y position (not shown). (B, C) Representative recordings of I_p (*top*) and the pipette Z position (*bottom*), when the pipette is in the bath solution away from the sample surface. (B) Recordings obtained with a setpoint value of 1.0%, showing a stable approach curve. The user should find the lowest setpoint value that maintains the stability of the approach. (C) Recordings obtained with a setpoint value of 0.45%, showing an unstable approach curve. If the setpoint value is too low, minor fluctuations in I_p will lead to false positive “detections” by the HPICM system and result in noisy images.

Fig. 2. Determining the resolution of the HPICM system

(A) Representative HPICM image (with a lateral step size of 221 nm) of the HS-20MG calibration chip, from an area filled with squared pillars. (B) Z-axis topography of the sample along the dashed line in A. Note that the heights of the pillars are approximately 20 nm.

Fig. 3. Selecting an area of interest and adjusting the setpoint

(A) Low-resolution HPICM images of a live rat organ of Corti (*left*) and a stereocilia bundle of an inner hair cell (*right*) within the region highlighted on the left panel. The lateral resolutions of the images are 313 nm (*left*) and 148 nm (*right*). (B) HPICM images of the same inner hair cell bundle obtained with a low setpoint (*left*) or with a setpoint that was too high (*right*). The lateral resolution of the images is 62.3 nm.

Fig. 4. Imaging post-processing

(A) Raw (*left*) and noise-filtered (*right*) HPICM images (lateral resolution of 110 nm) of a stereocilia bundle in a live rat inner hair cell. The noise was filtered using the *Noise Reduction* feature of the HPICM Control software to exclude pixel values higher than 6 μm.

(B) Raw (*left*) and line-aligned (*right*) HPICM images (lateral resolution of 70 nm) of another stereocilia bundle of a live rat inner hair cell. The lines were aligned using the *ButtonDestripeLineFit* feature of the ScanIC Image software. The arrows highlight some of the lines that are not perfectly aligned due to potential thermal drifts along the Z-axis.

(C) Raw (*left*) and slope-corrected (*right*) HPICM images of the HS-20MG calibration chip. The *Correct Slope* feature of the ScanIC Image software was used. The images have a lateral resolution of 221 nm and were both line-aligned as described in B.

Table 1

Pipette puller settings to obtain ~200-400 M Ω borosilicate nanopipettes

Sutter P-1000 puller					
	Heat	Pull	Velocity	Time	Pressure
Line 1	Ramp + 7	0	33	200	500
Line 2	Ramp + 7	250	50	200	
Sutter P-2000 puller					
	Heat	Filament	Velocity	Delay	Pull
Line 1	Ramp	4	40	170	10
Line 2	Ramp + 20	2	16	155	250

Table 2 Reference HPICM parameters for the imaging of calibration samples and live hair cell stereocilia bundles

~20 or 100 nm Calibration Samples						
Resolution	Scan Size (μm) ²	Lateral Resolution (nm)	Hop Amplitude (μm)	Fall Rate (nm/ms)	Min. Scan Hop (nm) ³	
Low	20 × 20	~300 - 400	~0.5 - 1	~90 - 110	100 - 200	
High		~100 - 200	~0.1 - 0.5		50 - 100	
Live Organ of Corti Explant						
Resolution	Scan Size (μm) ²	Lateral Resolution (nm)	Hop Amplitude (μm)	Fall Rate (nm/ms)	Min. Scan Hop (nm) ³	
Low	20 × 20	~300 - 400	~6 - 8	~60 - 100	500 - 1000	
Live Rat Inner Hair Cell Stereocilia Bundle ⁴						
Resolution	Scan Size (μm)	Lateral Resolution (nm)	Hop Amplitude (μm)	Fall Rate (nm/ms)	Min. Scan Hop (nm) ³	
Low	~8 × 8	~150 - 200	~4 - 5	~60 - 100	500 - 1000	
High		~50 - 100	~3 - 4		300 - 800	

¹For all of these samples, the following parameters can be used: Delay of 1-5 ms; IREF measurement for 3-10 ms; Ip filters 5 kHz; and Hop Rise Rate of 700-1000 nm/ms.

²The full X and Y range of the SICM setup can be used.

³These values are needed when using the “adaptive” HPICM compression algorithm (20).

⁴HPICM imaging parameters for mouse and rat inner hair cell stereocilia bundles are very similar. After locating a mouse inner hair cell stereocilia bundle, the scan size area to be used can be smaller (~6 × 6 μm) and the hop amplitude can be reduced by ~1-2 μm , while the other parameters remain the same.