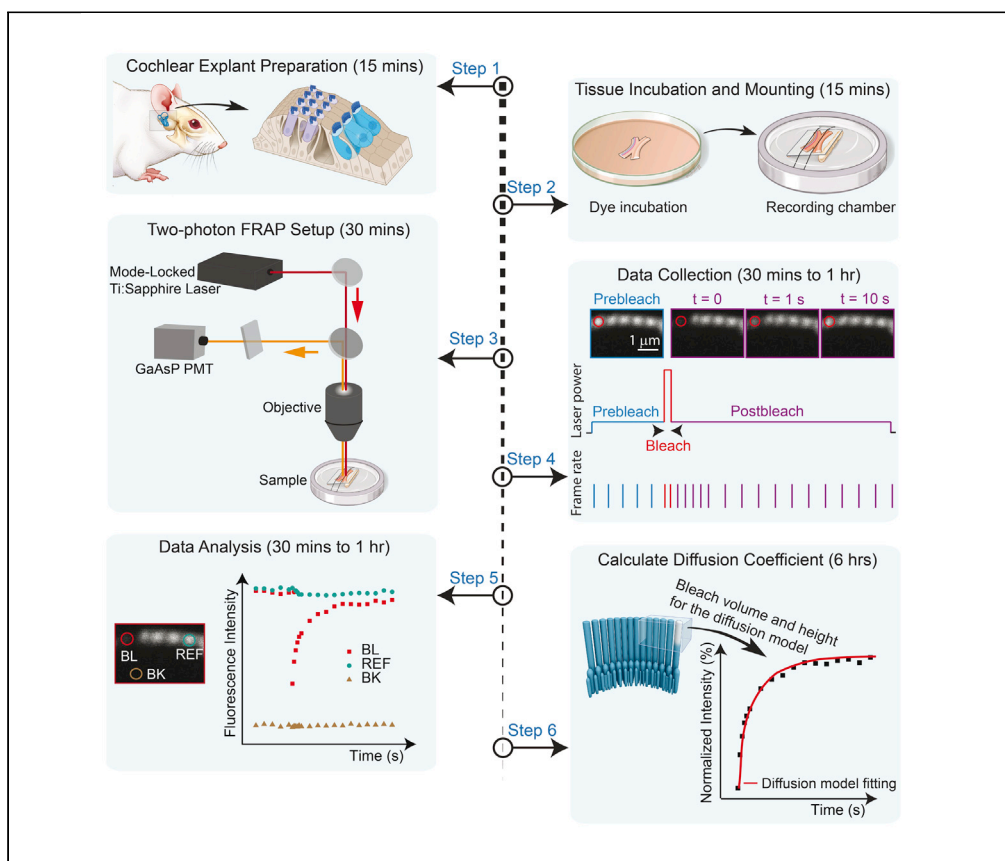# STAR Protocols

Protocol

A two-photon FRAP protocol to measure the stereociliary membrane diffusivity in rat cochlear hair cells

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Highlights

Two-photon FRAP to assess stereocilia membrane diffusivity in rat cochlear hair cell

Characterize lipophilic dye di-3-ANEPPDHQ to perform two-photon FRAP

Determine bleach volume and stereocilia length for diffusion coefficient calculation

Fluorescence recovery after photobleaching (FRAP) has been widely used to monitor membrane properties by measuring the lateral diffusion of fluorescent particles. This protocol describes how to perform two-photon FRAP on the stereocilia of live cochlear inner hair cells using a lipophilic dye, di-3-ANEPPDHQ, to assess the stereociliary membrane diffusivity. We also detail two-photon FRAP microscope setup and calibration, as well as FRAP parameter setting and data analysis.

Protocol

A two-photon FRAP protocol to measure the stereociliary membrane diffusivity in rat cochlear hair cells

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SUMMARY

Fluorescence recovery after photobleaching (FRAP) has been widely used to monitor membrane properties by measuring the lateral diffusion of fluorescent particles. This protocol describes how to perform two-photon FRAP on the stereocilia of live cochlear inner hair cells using a lipophilic dye, di-3-ANEPPDHQ, to assess the stereociliary membrane diffusivity. We also detail two-photon FRAP microscope setup and calibration, as well as FRAP parameter setting and data analysis.
For complete details on the use and execution of this protocol, please refer to George et al. (2020).

BEFORE YOU BEGIN

The protocol describes the experimental steps required for preparing postnatal day 8–9 (P8-9) rat cochlear organ of Corti to perform FRAP using two-photon microscopy to manipulate the fluorescence of di-3-ANEPPDHQ, and to quantitatively analyze the FRAP data collected from the stereocilia to extract diffusion coefficients. Two-photon microscopy provides reduced phototoxicity and photobleaching during the prolonged recovery cycle of FRAP imaging. This protocol is equally applicable to any cellular or subcellular system using other lipophilic dyes. Di-3-ANEPPDHQ is a potentiometric lipophilic dye that specifically labels cell membranes including the stereociliary membrane. The dye stock should be prepared and the FRAP parameters must be characterized in advance of data collection.

Prepare di-3-ANEPPDHQ stock solution

⊙ Timing: ~30 min

1. Prepare 30 mM di-3-ANEPPDHQ stock solution in 100% ethanol.
   a. Centrifuge the 1 mg vial of di-3-ANEPPDHQ at 1000 rpm to make sure the dye has settled down at the bottom.
   b. Add 52 µl of 100% ethanol to 1 mg of di-3-ANEPPDHQ resulting in the final concentration of 19,230 µg/mL.
   c. Sonicate the mixture for 5 min at 37 kHz followed by 5 min at 80 kHz using the sweep setting.
2. Dispense 2 µl aliquots of the stock solution into 1 mL eppendorf tubes.
3. Store the aliquots at −20°C protected from light for a maximum of 9 months.

Table 1. Glossary table with key FRAP parameters and their brief description.

Term	Description
$P_{photobleaching}$	Laser power required for photobleaching the region of interest during the photobleach cycle of FRAP
$P_{imaging}$	Laser power used for imaging during the pre and postbleach cycles of FRAP
$T_{photobleaching}$	Duration of photobleaching cycle
$T_{half\ recovery}$	Time taken for the fluorescence intensity to recover to half of the plateau value after photobleaching
Frame rate	Speed at which the images are taken
GaAsP PMT gain	Gain of the gallium arsenide phosphide photomultiplier tube detector

Determine FRAP parameters

⏱ Timing: ~3 h

In this section, we describe in detail how to determine the laser power ($P_{photobleaching}$) and duration ($T_{photobleaching}$) for photobleaching and the laser power for imaging ($P_{imaging}$) to perform the FRAP protocol (see Table 1 for FRAP parameters) using the membrane dye, di-3-ANEPPDHQ. Di-3-ANEPPDHQ has a two-photon excitation peak at 860 nm and an emission peak at 680 nm.

4. Turn on the two-photon acquisition system including the laser.
5. Set the microscope to operate as a laser scanning system and align the optics of the Ultima two-photon system with BX 61WI microscope.
    a. The key equipment for two-photon FRAP experiment include: a mode-locked Ti-Sapphire laser source, Pockels cell (for laser power modulation), pick-off window (to send ~5% of laser beam to the laser power meter), laser power meter, primary dichroic, objective lens, emission filter, photomultiplier tube (GaAsP), and a data recording system (see schematic in Figure 1).
    b. Tune the laser to 860 nm which is the excitation wavelength of di-3-ANEPPDHQ.
    c. Set a 600/50 nm bandpass filter in the emission pathway to redirect the emission of Di-3-ANEPPDHQ to the GaAsP PMT.
6. Adjust the laser power using the Pockels cell controller and measure the laser power at the focal plane of the objective lens using a portable laser power meter.
    a. In our study, *PrairieView* software is used to control the Pockels cell and the Ultima two-photon acquisition system.
    b. Also, making note of the reading on the laser power meter placed on the laser pathway for the corresponding Pockels cell control values can be helpful for easy monitoring of the laser power when performing FRAP experiments.
7. Place the sample of interest, adjust the PMT gain setting to $\leq$ 800 V and the laser power to the lowest possible level that can generate fluorescence within the sample. Focus within the region of interest (ROI).
    a. We used the dissected rat organ of Corti incubated in 6 μM of di-3-ANEPPDHQ for 5 min as the sample (see Step-by-Step method below for details).
    b. Within the organ of Corti, we focused within the hair bundles.
    c. We used 1 mW as the lowest laser power.
8. Take a series of intensity images of the ROI by increasing the laser power by an increment of ~ 1 mW at the focal plane of the objective lens i.e., at the ROI.
    a. Perform this step until the increase in fluorescence intensity appears to plateau or diminishes with respect to the laser power increase.
    b. Make sure the PMT is not saturated at higher laser powers. If saturation is detected, turn down the PMT gain setting and repeat step 8.
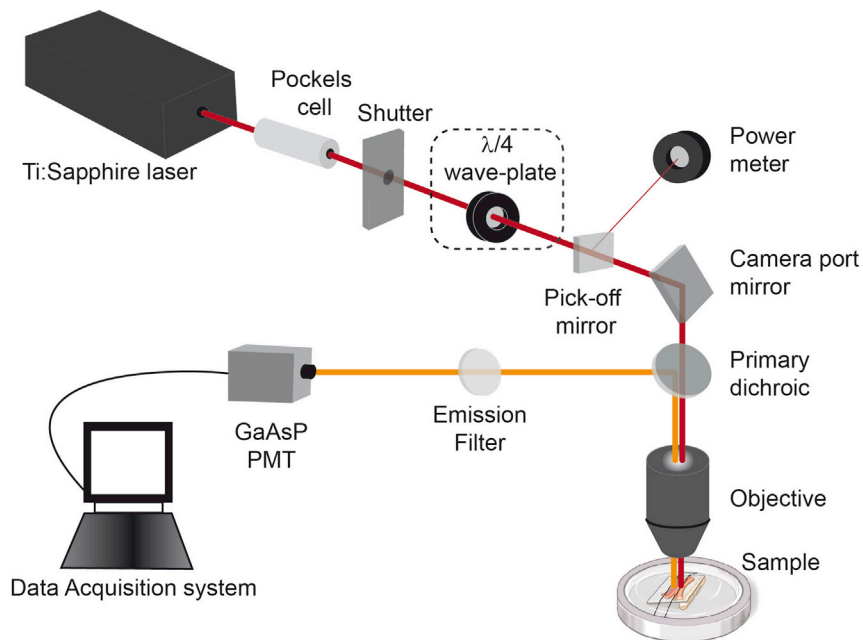9. Measure the fluorescence intensity of the ROI using *Image J*.

Figure 1. Schematic of microscope setup used to illustrate the different optical components

10. Plot normalized fluorescence intensity as a function of laser power (Figure 2A).
11. Select the maximum laser power just before saturation as the photobleaching laser power ($P_{photobleaching}$) to avoid excitation saturation (Braeckmans et al., 2006, Mazza et al., 2008, Loren et al., 2015).
12. Next, to determine the bleach duration ($T_{photobleaching}$), set the laser power to $P_{photobleaching}$ and bleach a spot at the ROI at different bleach durations.
    a. In our experiments, we increased the bleach duration from 25 ms to 1 s at $P_{photobleaching}$ of 7 mW.
    b. We used the Mark Point functionality available on the *PrairieView* to perform point bleaching using the imaging laser.
13. Measure the prebleach and postbleach fluorescence intensity of the bleach spot for each bleach duration using *Image J*.
14. Calculate the bleach level (%):

$$Bleach\ level = \frac{Prebleach\ intensity - Postbleach\ intensity}{Prebleach\ intensity}$$

15. Plot the bleach level as a function of bleach duration (Figure 2B).
16. Select the shortest bleach duration which results in > 60 % bleaching as the temporary $T_{photobleaching}$ (Sullivan and Brown, 2010).
17. Run the FRAP protocol using $P_{photobleaching}$ and $T_{photobleaching}$ as the bleach parameters (Figures 3A and 3B). Detailed description on performing FRAP is given below under "Two-photon FRAP acquisition" section.
18. Generate the FRAP curve by following the steps 33–37 (Figures 4B–4D).
19. Determine the half recovery time ($T_{half\ recovery}$) of the fluorescent dye which is the time taken for the fluorescence to recover to half of the final plateau after photobleaching (Figure 4D).
20. Confirm that the selected $T_{photobleaching}$ is ~ 10 times shorter than the $T_{half\ recovery}$ of the fluorescent dye so that there is minimal diffusion during the photobleaching time.
    a. For our experiments, we picked $T_{photobleaching}$ of 100 ms which was shorter than the $T_{half\ recovery}$ observed in the stereocilia. This bleach duration also allowed us > 60% photobleaching.
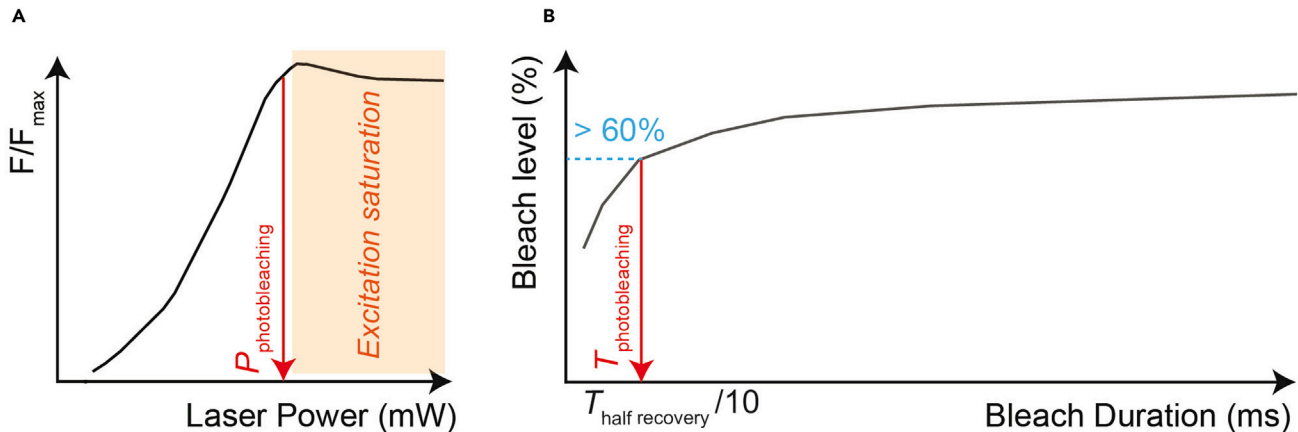
Figure 2. Characterization of FRAP parameters

(A) Laser power dependence of the normalized average fluorescence intensity for di-3-ANEPPDHQ excited at 860 nm. The laser power which marks the apparent onset of excitation saturation is selected as $P_{\text{photobleaching}}$ (highlighted in red).

(B) Bleach level plotted as a function of bleach duration. The bleach duration which results in > 60% photobleaching is selected as $T_{\text{photobleaching}}$ (highlighted in red).

  b. If the $T_{\text{photobleaching}}$ is not significantly shorter than the $T_{\text{half recovery}}$, increase the $P_{\text{photobleaching}}$ to the maximum possible value below excitation saturation. Otherwise, use another membrane dye such as RH795 or di-4-ANEPPDHQ.

21. To determine the laser power for imaging (P_{imaging}), adjust the laser power to the lowest possible level that can produce fluorescence within the sample.
  a. We used ∼1 mW as the lowest laser power at the sample.

22. Run the prebleach and postbleach cycles of the FRAP protocol as shown in Figures 3B and 3C. For the prebleach cycle, collect the images at a rate (frame rate) slow enough to avoid any photobleaching. For the postbleach cycle, start with a high frame rate in order to collect more data
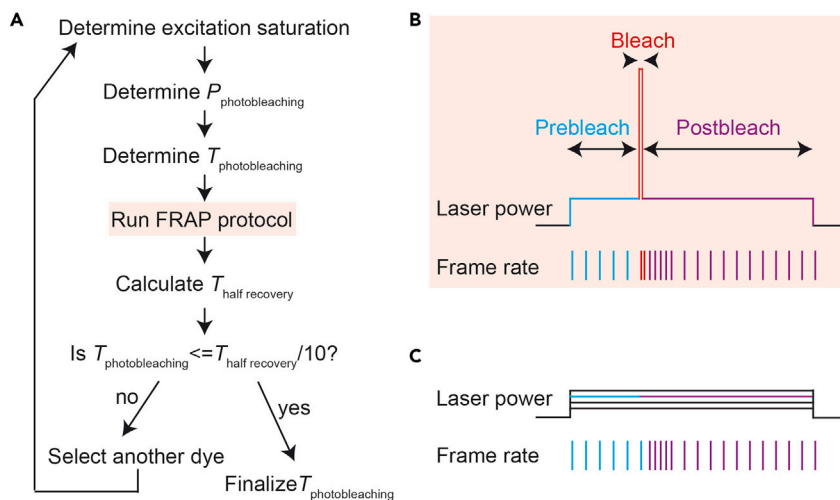

Figure 3. Illustration of FRAP parameters and FRAP cycles

(A) Flowchart showing the process of determining the photobleaching parameters $P_{\text{photobleaching}}$ and $T_{\text{photobleaching}}$.

(B) Schematic representation of FRAP protocol with the prebleach cycle in blue, photobleaching cycle in red and postbleach cycle in purple. The laser power and frame rate for each cycle is shown is corresponding colors.

(C) Schematic showing how P_{imaging} for prebleach and postbleach cycles (blue and purple traces in the top panel) is determined.
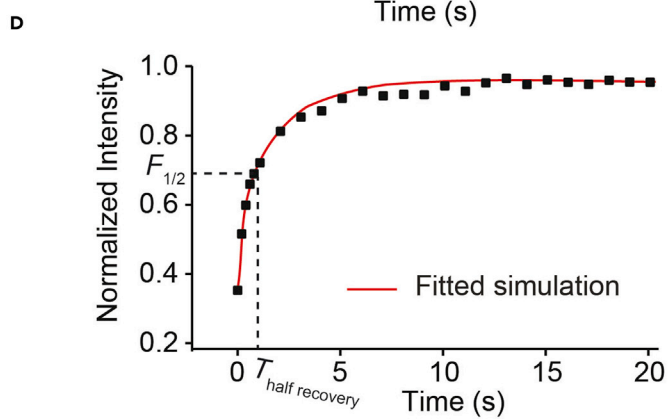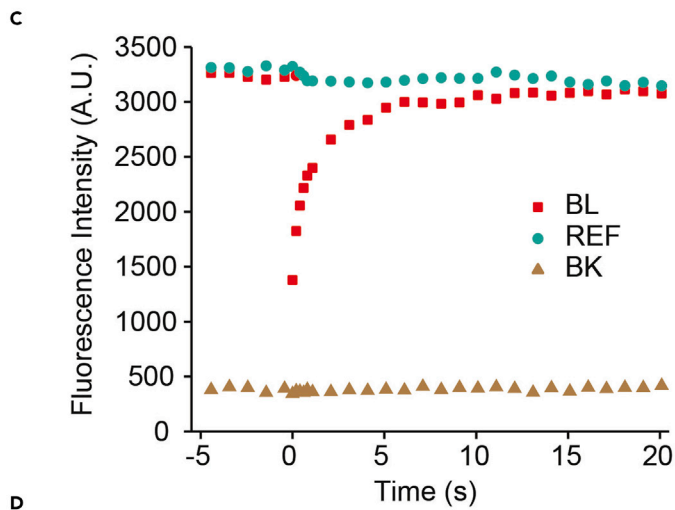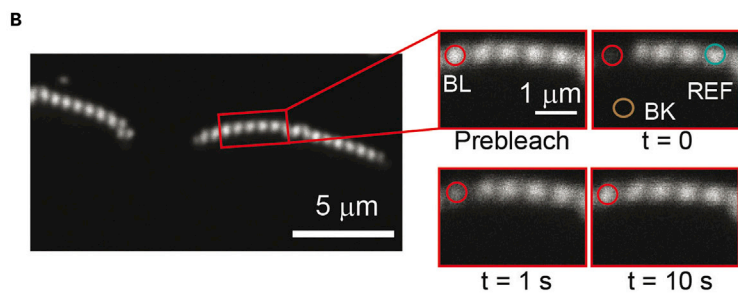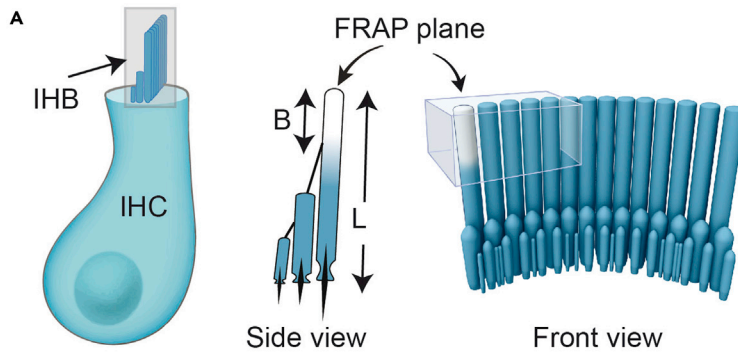
Figure 4. Example of outcomes of two-photon FRAP experiments

(A) Depiction of an IHC bundle (IHB) highlighting the approximate position of the focal plane at the tip of the row 1 stereocilia for FRAP experiments. Only one stereocilia is bleached at a time. The gradient filling indicates the degree of bleaching with white representing the highest degree of bleaching. The bleach volume is indicated as "B" and the stereocilia length is indicated as "L".

(B) Two-photon section of the IHC bundle stained with di-3-ANEPPDHQ and oriented vertically, focused at the tip of the row 1 stereocilia (left panel). Representative FRAP experiment (right panels) on the row 1 stereocilia with t = 0 indicating the first image taken post-bleaching. The red, blue, and brown circles define the bleached (BL), reference (REF), and background (BK) regions, respectively.

(C) The average fluorescence intensity measured from the BL, REF and BK regions during the FRAP experiment.

(D) The generated FRAP curve (black square) is fitted with a one-dimensional diffusion model to estimate the diffusion coefficient D, yielding a value of 4.6 μm^2/s with a fitting error of 1.2% in this example. Half recovery time ($T_{half\ recovery}$) is the time required for the fluorescence to reach half of the steady state.

points during the initial steep recovery in the fluorescence (see Figures 3B, 3C, 4C, and 4D) and slow down the frame rate after the initial 1 s.

    a. In our study, we used a frame rate of 1 fps for the prebleach cycle and postbleach cycle after 1 s and 5 fps for the initial 1 s of the postbleach cycle.

23. Increment the laser power by 0.1 mW and repeat step# 22 (Figure 3C) until the laser power is high enough to cause significant photobleaching.
24. Measure the prebleach and postbleach fluorescence intensity using *Image J*.
25. Calculate the bleach level during the postbleach cycle using the equation in step 14.
26. Select the highest power that results in < 5% bleach level during the postbleach cycle, especially during the final 5 secs, as the imaging power ($P_{imaging}$).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
di-3-ANEPPDHQ	Thermo Fisher Scientific	Cat#D36801
Ethanol	Fisher Scientific	Cat#22-032
NaCl	Fisher Scientific	Cat#S271
KCl	Fisher Scientific	Cat#BP366
CaCl$_2$	Alfa Aesar	J63122; CAS:10043-52-4
MgCl$_2$	Fisher Scientific	Cat#BP214
HEPES	Spectrum Chemical	Cat#H1084
Glucose	Fisher Scientific	Cat#BP350
L-Ascorbic acid	Fisher Scientific	Cat#A61-100
Creatine monohydrate	Acros Organics	AC226790250; CAS: 6020-87-7
DOPC	Avanti Polar Lipids	SKU: 850375C
Deposited data		
Mathematica code for the diffusion model	Mendeley Data	Mendeley Data: https://doi.org/10.17632/3wrd9xp4gc.1
Experimental models: Organisms/strains		
Sprague-Dawley P8-P9 rats	Charles River	Crl:CD(SD)
Software and algorithms		
Prairie View	Bruker Technologies	https://eliceirilab.org/sites/default/files/PrairieViewManual_5_2013_0.pdf
ImageJ	Fiji	https://imagej.net/Fiji
Igor Pro	WaveMetrics	https://www.wavemetrics.com/products/igorpro
Mathematica	Wolfram	https://www.wolfram.com/mathematica/resources/
Imaris	Oxford Instruments	https://imaris.oxinst.com/support/imaris-release-notes/9-3-0

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Microcentrifuge	USA Scientific	Model#1 R
Sonicator	Elma	Model#Elmasonic P
T-tanium:Sapphire tunable laser	Coherent	Model#Chameleon Ultra II
Two-photon system	Bruker	Model#Ultima
Upright microscope with a 100× 1.0 NA water dipping objective	Olympus	Model# BX61WI
GaAsP photo multiplier tube	Hamamatsu	Model#7422PA-40
Bandpass filter, 600/50 nm	Chroma Technology	Model#ET600/50m-2p
Z-Axis Piezo Drive for Ultima	Bruker	Model#519600
Confocal microscope with 40× 1.0 NA objective	Zeiss	LSM 880
Milli-Q reverse osmosis water purification system	Millipore	Cat#ZR0Q008WW
Osmometer, freezing point depression	Advanced Instruments	Model#3320
pH meter	METTLER TOLEDO	Model#MP220
Mini-Extruder	Avanti Polar Lipids	SKU 610000

MATERIALS AND EQUIPMENT

The equipment and software for two-photon FRAP are listed in the key resource table above. This list is based on the setup we have in our lab.

Alternatives: Di-4-ANEPPDHQ, RH 795 or di-8-ANEPPS can be used as alternatives to di-3-ANEPPDHQ. Other upright microscopes with a 100× water dipping objective available from Olympus, Zeiss or Leica can be used. We used a custom-built light protection cage for the microscope.

10× extracellular stock solution

Reagent	Final concentration	Amount
NaCl	145 mM	42.37 g
KCl	2 mM	0.75 g
CaCl$_2$ (1M solution)	2 mM	10 mL
MgCl$_2$ (1M solution)	0.5 mM	2.5 mL
HEPES	10 mM	11.92 g
Milli-Q H$_2$O	n/a	Complete to 500 mL
Total	n/a	500 mL

Note: The expected osmolality of this solution is ∼290 mOsm. pH to 7.4 by adding NaOH pellets before making the volume to 500 mL.

⚠ CRITICAL: 10× stock solution can be used for up to 3 months. The solution should be stored at 4°C and should be discarded if any discoloration or precipitate form.

1× extracellular solution (freshly made)

Reagent	Final concentration	Amount
10× stock solution	1×	25 mL
Glucose	6 mM	0.27 g
Ascorbate/Pyruvate (1M solution)	2 mM/2 mM	0.5 mL
Creatine	2 mM	0.74 g
Milli-Q H$_2$O	n/a	Complete to 250 mL
Total	n/a	250 mL

Note: The expected osmolality of this solution is 300–310 mOsm. pH to 7.4 by adding 1M NaOH before making the volume to 250 mL. If needed, raise the osmolarity with NaCl.

⚠ CRITICAL: The extracellular solution should be made freshly daily and can be kept at room temperature (19°C–22°C) during the duration of the experiment.

30 mM di-3-ANEPPDHQ stock solution

Reagent	Final Concentration	Amount
di-3-ANEPPDHQ	30 mM	1 mg
Ethanol	n/a	52 µL
Total	n/a	52 µL

⚠ CRITICAL: The 30 mM di-3-ANEPPDHQ stock solution can be used for up to 9 months. The solution should be stored at −20°C protected from light.

60 µM di-3-ANEPPDHQ stock solution

Reagent	Final Concentration	Amount
30 mM di-3-ANEPPDHQ stock	60 µM	2 µL
Extracellular solution	n/a	1 mL
Total	n/a	1 mL
6 µM di-3-ANEPPDHQ stock solution		

⚠ CRITICAL: The 60 µM di-3-ANEPPDHQ stock solution can be used for up to 3 months. The solution should be stored at −20°C protected from light.

6 µM di-3-ANEPPDHQ stock solution

Reagent	Final Concentration	Amount
60 µM di-3-ANEPPDHQ stock	6 µM	100 µL
Extracellular solution	n/a	900 µL
Total	n/a	1 mL

⚠ CRITICAL: The 6 µM di-3-ANEPPDHQ stock solution is made freshly daily and can be kept at room temperature (19°C–22°C) protected from light.

STEP-BY-STEP METHOD DETAILS
Prepare the system for FRAP acquisition

⏱ Timing: 20 min

1. On the day of the experiment, turn on the two-photon acquisition system.
   a. Tune the laser to 860 nm.
2. Test the laser power for $P_{photobleaching}$ and $P_{imaging}$.
   a. In our study, the Pockels cell values are set for the $P_{photobleaching}$ and $P_{imaging}$ and the laser power reading on the power meter installed along the laser pathway is confirmed to read the previously determined values.
3. Test the photobleaching.
   a. Align the optics to focus on a chroma slide.
   b. Test the Mark Point functionality to perform point bleaching. Refer *PrairieView* manual for details, [https://eliceirilab.org/sites/default/files/PrairieViewManual_5_2013_0.pdf].

Prepare extracellular solution

🕐 Timing: 30 min

The aim of this part is to prepare fresh extracellular solution in which the tissue is placed throughout the experiment.

4. Measure 25 mL of 10× extracellular stock solution prepared previously with volumetric flask.
5. Add to 250 mL beaker with 200 mL milli-Q H_2O.
6. Add 74.5 mg creatine monohydrate, 270 mg glucose and 0.5 mL of 1M/1M of pyruvate/ascorbate.
7. Adjust pH to 7.4 with 1M NaOH.
8. Use volumetric flask to raise volume of solution to 250 mL with milli-Q H_2O.
9. Check osmolality. Raise to 304–307 with NaCl if needed.

Prepare working dye solution of di-3-ANEPPDHQ

🕐 Timing: 5 min

10. Thaw a vial of 2 μl aliquot of 30 mM di-3-ANEPPDHQ stock solution.
11. Add 1 mL extracellular solution to the stock to make di-3-ANEPPDHQ concentration of 60 μM.
12. Pipette 100 μl of the 60 μM solution, transfer it to 1 mL vial and add 900 μl of extracellular solution and vortex it.
    a. This will result in a final di-3-ANEPPDHQ concentration of 6 μM.
13. Keep it in the dark at room temperature (19°C–22°C) until used.
14. Store the remaining 60 μM stock solution in the freezer at −20°C until the next experiment for up to 3 months.

Cochlear explant preparation, incubation, and mounting

🕐 Timing: 30 min

15. Dissect the apical turn of isolated organ of Corti from Sprague Dawley rat pups at postnatal day 8 (P8) to P9 of either sex as described in Ricci et al. (2005) and (Beurg et al., 2009). It is not necessary to take off the tectorial membrane to expose the stereocilia.
16. Incubate the dissected tissue in the 6 μM di-3-ANEPPDHQ working solution for 5 min at room temperature (19°C–22°C), while protecting from light.
17. Transfer the tissue into the recording chamber with dye-free extracellular solution.
18. Place the tissue above the angled cover slip and orient the tissue so that the stria vascularis is at the top and the organ of Corti at the bottom (as shown in step 2 of the graphical abstract).
19. Place the top dental floss on the stria vascularis and the bottom dental floss near the epithelial ridge area, ideally close to the IHC row.
20. Once both dental flosses are on the tissue, pull the bottom one even lower to stretch the tissue to ensure that the IHC bundles are oriented vertically.

⚠ CRITICAL: It is important to orient the hair cell bundles vertically to perform FRAP experiments at the stereocilia tip. Angled coverslip on the recording chamber and placing the dental floss as close as possible to the IHC row are critical in achieving the vertical orientation of the hair bundles (Figure 4).

Two-photon FRAP acquisition

🕐 Timing: 1 h

In this section, we describe in detail how to perform two-photon FRAP experiments on cochlear IHC stereocilia using a Bruker Ultima two-photon system coupled with an Olympus BX 61WI microscope, with a light-shielded cage. Di-3-ANEPPDHQ is excited at 860 nm. The fluorescence emission is collected through a 100× 1.0 NA water dipping objective lens and redirected to a 700 nm primary dichroic and then to a GaAsP photo multiplier tube using a 645/65 nm bandpass filter. The data acquisition is driven by the software *PrairieView*.

21. Position the recording chamber with the mounted tissue under the microscope.
22. Align the optics and focus the tissue with 100× objective lens.
23. Pick the hair bundle to image in the standard bright field illumination mode.
24. Set the Ultima system to operate as a laser scanner system.
25. Adjust the Pockels cell control value corresponding to $P_{imaging}$ and set the GaAsP PMT gain to 800 V.
26. Focus just above the tip of the IHC bundle and take intensity images in the live scan mode while moving down in z-axis slowly along the bundle tip using the remote focus control (Figure 4A).
27. Pick the focal plane at the bundle tip where the fluorescence intensity of the stereocilia is at least 3 times that of the background (Figures 4A and 4B).
28. Select a small image frame that incorporates the ROI to perform FRAP. The frame should be small enough to perform fast image acquisition and large enough to view the ROI.
    a. In our study, we selected a 2.3 μm by 3.3 μm region that enabled a frame period of 72 msec as well as image 5–6 stereocilia at the same time.
29. Set the following FRAP protocol sequences (Figure 3B) using the T-series functionality:
    a. Pre-bleach cycle of duration = 5 s and frame rate = 1 fps
    b. Photobleaching pulse of duration = 100 ms
    c. Post-bleach cycle of duration = 1 s and frame rate = 5 fps
    d. Post-bleach cycle of duration = 11 s and frame rate = 1 fps
30. To set up the photobleaching cycle, use the Mark Point functionality.
    a. Mark a point at the tip of the stereocilia to be bleached (see *PrairieView* manual for more details).
    b. Set the Pockels cell control value corresponding to $P_{photobleaching}$ as the laser power.
31. Make sure the Pockels cell control value on the main window is set to $P_{imaging}$.
32. Run the T-series.

⚠ CRITICAL: Appropriate laser safety warning signs and "laser in use" sign for Class 4 laser should be used before and during the two-photon FRAP experiment to indicate the laser system is in use. The experiment should be performed in a light controlled cage to protect the highly sensitive GaAsP PMTs from excessive light exposure.

Data analysis

⏱ Timing: 30 min

In order to obtain quantitative information from the FRAP images acquired, further analysis of the images is necessary.

33. Import all the images from one FRAP sequence together using *ImageJ*.
    a. Go to File -> Import -> Image Sequence. Select the first image in the sequence. Enter the number of images in the sequence in the pop-up window and click OK.
34. Run the StackReg Plugin in *ImageJ* [https://imagej.net/StackReg] to align the images in case the images are not aligned in x and y axis due to motion artefact.
35. Mark all the ROIs for the average fluorescence intensity to be measured: the bleached region (BL), a reference region (REF) which is a neighboring unbleached stereocilia and a background region (BK) as illustrated in Figure 4B.

36. Measure the average fluorescence intensity of all the regions: the average fluorescence intensity within the bleached spot (I_{BL}), the reference region (I_{REF}) and the background (I_{BK}). An example is shown in Figure 4C.

37. Generate a FRAP curve with normalized fluorescence intensity on the y-axis and time on the x-axis, where t = 0 is the first post bleach measurement (Figure 4D). This can be done with custom code implemented in Igor Pro (available through the authors).
    a. First, subtract I_{BK} from I_{BL} and I_{REF}.
    b. Next, normalize the background-subtracted intensity values at each time point to the pre-bleached intensity averaged from the first 5 images just preceding the photobleaching, such that average pre-bleach intensity is set to 1.

$$I_{BL_{norm}}(t) = \frac{I_{BL}(t) - I_{BK}(t)}{I_{BL\_Prebleach}}$$

$$I_{REF_{norm}}(t) = \frac{I_{REF}(t) - I_{BK}(t)}{I_{REF\_Prebleach}}$$

    c. Finally, to account for the limited bleaching that occurs during recovery, normalize the normalized intensity from the bleached ROI ($I_{BL_{norm}}$) to the intensity from the reference ROI ($I_{REF_{norm}}$) at each time point to generate the FRAP curve as given below:

$$I_{FRAP\ curve}(t) = \frac{I_{BL_{norm}}(t)}{I_{REF_{norm}}(t)}$$

Confirm that the photobleaching of di-3-ANEPPDHQ is irreversible

⊙ Timing: 3 h

Following Axelrod et al. (1976), it is presumed the photobleaching is a simple irreversible reaction. The fluorescence recovery that we observe with FRAP should be due to the diffusion of the unbleached fluorophore into the bleached region instead of the bleached fluorophore recovering from the photobleached state. Therefore, it is important to test if the photobleaching of the dye that is being used is irreversible and does not contribute to the fluorescence recovery. Here, we describe how to test if the photobleaching of di-3-ANEPPDHQ is irreversible using a simple membrane model system of pure DOPC vesicles. We select vesicles of size < 0.5 μm, which is the smallest bleach size possible with our optics, so that we can achieve complete bleaching of the vesicle and any observed fluorescence recovery after photobleaching would be contributed only by the reversible photobleaching of the fluorophore.

38. Aliquot 0.1 mL of 10 mg/mL pure DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in chloroform to a glass vial.
39. Evaporate the lipid stock solution by vacuum for 30 min to leave a dried lipid film.
40. Hydrate the dried lipids in 0.25 mL of 100 mM KCl, 10 mM HEPES, pH 7.4 for an hour.
41. Once the sample is fully hydrated, extrude the solution using the Mini extruder through a 200 nm polycarbonate membrane 11 times.
42. Add 41.6 μL of 60 μM di-3-ANEPPDHQ stock to the lipid solution to make [dye: lipid] ratio of 1:1000.
43. Keep the lipid dye solution at 4°C, protected from light.
44. Mount the DOPC vesicles on a slide in 0.5% agarose gel at 1:1 ratio to keep them mechanically stable.
45. Tune the laser to 860 nm and align the optics to focus within the vesicles.
46. Pick a vesicle of size < 0.5 μm. This is to make sure that complete bleaching of the vesicle can be achieved.
47. Run the FRAP protocol sequences as described earlier (Figure 3B) which includes the prebleach, bleach and postbleach cycles using the FRAP parameters determined previously (steps 4–26).
48. Analyze the FRAP data to generate FRAP curve using *Image J.*

49. Irreversible bleaching of di-3-ANEPPDHQ should show no recovery in the fluorescence signal after photobleaching (see Figures S1C and S1D in George et al. (2020)).

Determine parameters for the FRAP diffusion model

⏱ Timing: 6 h

In this section, we describe the important parameters needed for an accurate estimation of the diffusion coefficient D from the FRAP curve extracted from the IHC stereocilia. For the detailed description of how to calculate the diffusion coefficient D using *Mathematica*, please refer to George et al. (2020) and the code associated to the implementation of the model is available through Mendeley Data [Mendeley Data: https://doi.org/10.17632/3wrd9xp4gc.1]. The model is based on the pure-diffusion model from Axelrod et al. (1976).

The time course of FRAP recovery can be greatly influenced by the initial bleach volume. It is also important to develop a morphologically accurate model to correct for the geometry of the investigated system. We describe here how to calculate the initial axial extent of photobleaching at the tip of the stereocilium and the length of the stereocilium.

50. Mount a 250 μm Z-axis Piezo Drive on the microscope.
51. Dissect and mount the organ of Corti incubated in 6 μM of di-3-ANEPPDHQ for 5 min as described in Ricci et al. (2005) and Beurg et al. (2009).
52. Select the ROI at the tip of one stereocilium.
53. Bleach the tip with the bleaching parameters determined previously in steps 4–20, followed by taking a fast z-stack image series with step size of 0.5 μm.
54. Measure the degree of bleaching at each z-plane.
55. Normalize the degree of bleaching at each z-plane to that at the plane of bleaching i.e., the stereocilium tip and plot against the position along the z-axis relative to the bleach spot.
56. Use Gaussian fitting to measure the effective axial extent of bleaching.

Note: For an accurate estimation of the bleach volume, it is important to acquire the z-stack at a fast rate to minimize the recovery of fluorescence during the data acquisition. Adjust the imaging parameters such as image size, pixel size, dwell time etc to get a short frame period. In our setup, the frame period was 15 ms enabling us to take the z-stack of the stereocilium within 60 to 75 ms.

57. Incubate the organ of Corti tissue in 6 μM di-3-ANEPPDHQ and mounted in a recording chamber such that the stereocilia is oriented to stand up.
58. Take live-cell z-stack images of the hair bundle using a high-resolution microscope.
    a. We used LSM 880 confocal microscope (Zeiss) in Airy-scan mode with a water immersion 40× 1.0 NA objective (Zeiss).
59. Create 3D reconstruction of the hair bundle and measure the stereocilia length (Figure 5).
    a. We used the image processing software Imaris 9.3.1 (Oxford Instruments; https://imaris.oxinst.com/support/imaris-release-notes/9-3-0).

EXPECTED OUTCOMES

In Figure 4, examples are given of possible data obtained with this protocol in cochlear IHC stereocilia with the above-mentioned optical system. The Gaussian laser beam is focused at the top of 5-6 stereocilia (Figure 4A). This allows monitoring of the average fluorescence intensity of those stereocilia over an axial length determined by the resolution of the optical system (1.9 um for the system reported here). Typically, each FRAP experiment consists of a series of time lapse scans of a 2.3 μm by 3.3 μm region
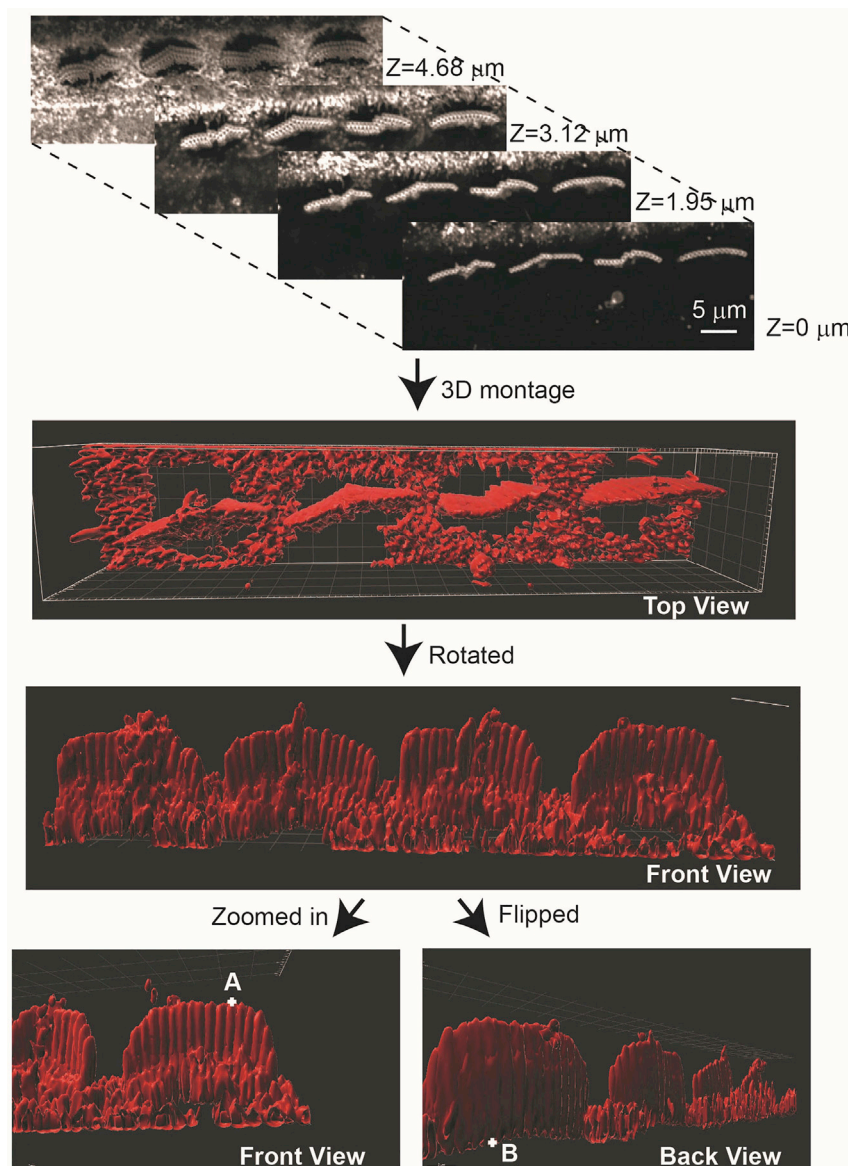
Figure 5. Confocal z-stack images of live IHBs stained with di-3-ANEPPDHQ

The z-stack images are used to create a 3D reconstruction of the hair bundle to measure the length of the stereocilia. The 3D image of the bundle is viewed from different sides to mark the top point "A" and the bottom point "B" of a particular stereocilia to estimate its length.

(Figure 4B). This region size allows for a sampling period of 72 msec using a pixel dwell time of 4 μs with image resolution of 1024 × 1024 and pixel size of 23 nm and zoom of 4×.

Figure 4B shows a set of IHC stereocilia before and after photobleaching, with the bleached (BL), reference (REF) and background (BK) regions highlighted. The average fluorescence intensity measured for all regions of interest (ROI): BL, REF and BK is illustrated in Figure 4C. Following background subtraction and photobleaching correction, a normalized fluorescence intensity vs time plot (FRAP curve) is generated with t = 0 representing the first post-bleach measurement (Figure 4D). During the prebleach cycle, the fluorescence intensity is monitored using a low laser power of ∼1.5 mW to obtain a steady baseline signal without photobleaching. During the photobleaching cycle, up to 60%–70% of photobleaching is achieved by focusing the laser power of ∼7 mW for a duration of 100 ms. During the postbleach cycle,
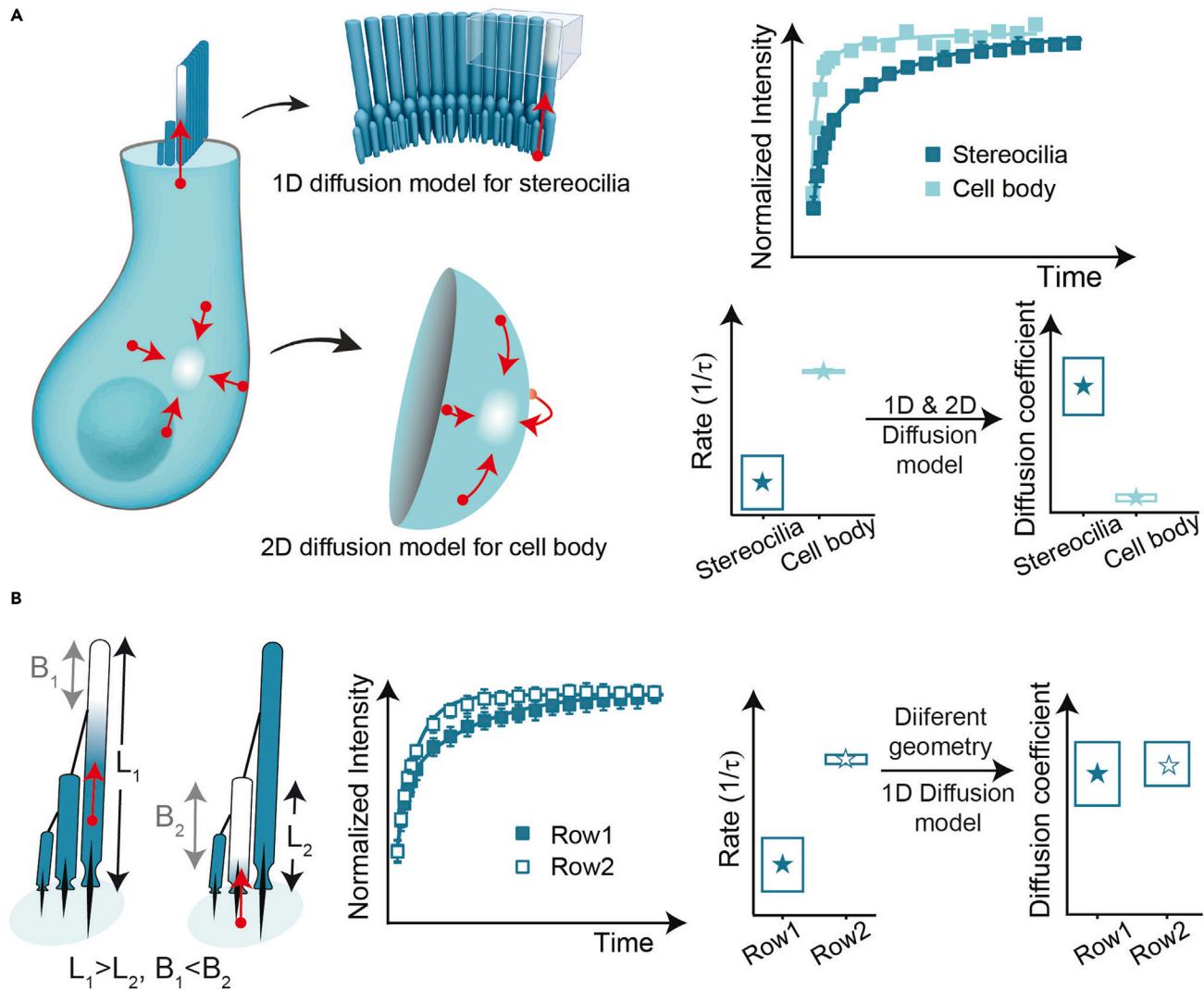
Figure 6. Illustration of diffusion model

(A) Schematic showing the need for a one-dimensional diffusion model for the stereocilia and a two-dimensional diffusion model for the cell body. Red arrows indicate the direction of the diffusion with red filled circles indicating the approximate location of the dye reservoir for the recovery. Examples of the experimental FRAP curves and the corresponding fitted curves from the stereocilia and the cell body are shown on the top right panel. Time constant τ derived from fitting the data shows significantly faster recovery for the cell body than the stereocilia. On the contrary, the diffusion coefficients extracted from the models that consider the direction of the diffusion and the dimensions of these two different structures suggest that cell body has slower diffusion than the stereocilia. Boxes represent the SD and the star symbol indicates the mean.

(B) Schematics showing the difference in the model parameters (length L and bleach volume B) for the different rows of stereocilia. Experimental FRAP curves (mean $\pm$ SD) and the time constant τ suggest faster recovery for the row 2 stereocilia compared to the row1 stereocilia. However, the diffusion coefficients measured from the one-dimensional diffusion model with different model parameters suggest similar diffusion for both rows of stereocilia. Boxes represent the SD and the star symbol indicates the mean.

the fluorescence intensity is measured again with the lower laser power which results in < 5% photobleaching during the recovery phase. The intensity images are collected long enough to monitor the plateauing of the recovered signal so that the FRAP curve can be fitted with appropriate diffusion model.

To estimate the diffusion coefficient D, the FRAP curve is fitted with a stereocilia-specific one-dimensional diffusion model (Figure 6). The initial bleach extent along the stereocilia and the length of the live stereocilia are critical model parameters. In our study, the initial axial extents of bleaching were measured as $1.35 \pm 0.06\ \mu m$ and $1.84 \pm 0.3\ \mu m$ and the length was estimated as $6.6 \pm 0.2\ \mu m$ and

3.0 $\pm$ 0.2 μm for row 1 and row 2 stereocilia respectively. Using these parameters, the estimated diffusion constants between stereociliary rows were not significantly different from each other, with values of 5.1 $\pm$ 1.1 μm^2/s for row 1 stereocilia and 5.6 $\pm$ 0.8 μm^2/s for row 2 stereocilia (Figure 6B). These measured values are within the range typically reported for living cells i.e., 0.01–10 μm^2/s with most cells exhibiting values from 0.1–1 μm^2/s at 18°C –22°C (Lee and Jacobson, 1994, Almeida and Vaz, 1995, Edidin, 2003).

LIMITATIONS

A main limitation for FRAP measurements is the limited temporal resolution given that the recovery times are a function of the ability to image at higher rates. The technology does not allow for monitoring dynamic changes in membrane properties. FRAP measurements are also spatially limited by the diffraction limit imposed by the microscope optics. The volume bleached is typically dictated by the radial and axial resolution of the microscope and so local changes will be filtered or averaged into the larger volume being monitored. Another concern with FRAP is related to the properties of the fluorophore that is used. The possibilities of the fluorophore binding to the membrane proteins, the fluorophore internalizing into the cytoplasm, and the diffusion of the fluorophore during the photobleaching cycle can interfere with the diffusion rate measurements.

TROUBLESHOOTING

Problem 1

Time constant derived from the FRAP curve is not a direct reflection of the diffusion coefficient; The time constants of recovery are greatly influenced by the geometry of the investigated system and the bleach volume (Figure 6) (steps 50–59).

Potential solution

To get an accurate value of the diffusion coefficient, it is critical to measure the initial bleach volume and the dimensions of the structure and develop a morphologically accurate model to correct for these parameters. Additionally, care must be taken on how the tissue is oriented with respect to the bleaching laser as this can greatly influence the initial bleach volume.

Problem 2

Point bleaching can drift; The mark point bleaching can slightly drift from the ROI with time resulting in inconsistent and variable bleaching between experiments (step 30).

Potential solution

At the start of the experiment, perform calibration of mark point bleaching to ensure bleaching at the position marked.

Problem 3

x-y drift in time lapse FRAP images; The region of interest can move during imaging. This is problematic for downstream data analysis and extraction of diffusion coefficient (step 34).

Potential solution

Use the StackReg Plugin in *ImageJ* [https://imagej.net/StackReg] to align all the images from one FRAP sequence before measuring the average fluorescence intensity of the ROIs.

Problem 4

Excitation photoselection effect of di-3-ANEPPDHQ; Due to the orientation of di-3-ANEPPDHQ in the stereociliary membrane and the polarization of the excitation laser, the two-photon images of stereociliary membrane appear as filled circles instead of unfilled circles giving the illusion that the dye is in the cytoplasm (Figure 4B) (Parasassi et al., 1997, Bagatolli, 2006) (step 26).
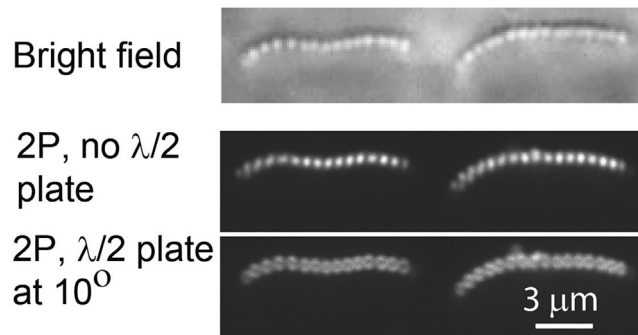
Figure 7. Example of excitation photoselection effect

Potential solution

The *excitation photoselection effect* can be corrected by rotating the laser polarization with a λ/2 (half-wave) plate, thus converting filled stereocilia to unfilled stereocilia (Figure 7) (George et al., 2020).

Problem 5

Photostability of the fluorophore; The fluorophore can undergo photoswitching (reversing from the photobleached state) resulting in fluorophores regaining the fluorescence during the recovery cycle. Additionally, the fluorophore can undergo significant photobleaching during the recovery phase. This can impact the diffusion rate measured during FRAP (steps 29–31, 38, and 49).

Potential solution

Confirm the irreversibility of photobleaching of the fluorophore of interest before selecting it to perform FRAP experiment. Pick a fluorophore that doesn't regain its fluorescence during the entire recovery cycle. Adjust the imaging rate and monitoring laser power such that the fluorophore undergoes <5% photobleaching during postbleach cycle. If not possible, pick another fluorophore.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Anthony Ricci, aricci@stanford.edu

Materials availability

This study did not generate new unique reagents.

Data and code availability

All code associated with this Protocol has been uploaded to Mendeley Data [Mendeley Data: https://doi.org/10.17632/3wrd9xp4gc.1] and are also available through the authors.

AUTHOR CONTRIBUTIONS

S.S.G. and A.J.R. designed and optimized the protocols; S.S.G. performed the experiments; C.R.S. developed and implemented the diffusion model; S.S.G. and A.J.R. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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