Chapter 13

Design and Construction of a Cost-Effective Spinning Disk System for Live Imaging of Inner Ear Tissue

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Abstract

Confocal imaging of fluorescent probes offers a powerful, non-invasive tool which enables data collection from vast population of cells at high spatial and temporal resolution. Spinning disk confocal microscopy parallelizes the imaging process permitting the study of dynamic events in populations of living cells on the millisecond time scale. Several spinning disk microscopy solutions are commercially available, however these are often poorly configurable and relatively expensive. This chapter describes a procedure to assemble a cost-effective homemade spinning disk system for fluorescence microscopy, which is highly flexible and easily configurable. We finally illustrate a reliable protocol to obtain high-quality Ca^{2+} and voltage imaging data from cochlear preparations.

Key words Confocal spinning disk microscopy, Calcium imaging, Voltage imaging, Spontaneous activity, Calcium action potentials, Calcium signaling, Inner hair cells, Non-sensory cells, Connexins

1 Introduction

Virtually all living cells use Ca^{2+} ions as a signal for a variety of physiological processes. Due to its importance as a second messenger, signaling mechanisms which involve Ca^{2+} are probably the best characterized in cell biology. Ca^{2+} in the inner ear is fundamental for hearing and balance functions, due to its critical involvement in hair cell mechanotransduction and synaptic activity. Ca^{2+} signaling is also important in the developing cochlea, where cochlear non-sensory cells display spontaneous and ATP-dependent Ca^{2+} transients that spread from cell to cell in a wave-like fashion [1], while sensory inner hair cells fire spontaneous Ca^{2+}-dependent action potentials [2].

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Optical imaging offers an ideal tool to study Ca^{2+} dynamics as it allows simultaneous recording of signals from several cells in an intact tissue, such as the organ of Corti, using fluorescent probes that are well characterized and commercially available. Confocal microscopy is the best choice to visualize thin optical sections of the fluorescence signal arising from a given cell. In conventional laser-scanning confocal microscopy, the sample is illuminated point-by-point by a finely focused laser beam while spatial filtering through a pinhole located close to the detection system (typically, a photomultiplier) enables rejection of out-of-focus fluorescence background resulting in a dramatically improved contrast compared to wide field microscopy [3]. This is particularly important for imaging through thick samples, such as organs or organ slices.

A spinning disk confocal microscope parallelizes the point-by-point illumination and detection processes, as light from the excitation source is distributed over multiple foci and the image is formed over a 2D sensor instead of a single photomultiplier. Therefore, this technique is intrinsically faster and less prone to photo bleaching than point-wise laser scanning. A spinning disk confocal microscope typically uses a rotating element (disk) located in a plane that is optically conjugated with that of the specimen (object plane). Disk transmittance is non-uniform, resulting in the projection of an intensity-modulated light pattern onto the specimen. In the Nipkow disk configuration, thousands of pinholes are arranged in an Archimedes spiral so that light traversing pinholes traces concentric arcs across the sample as the disk revolves [4]. Transmittance patterns other than pinhole arrays have also been used, such as striped disks, in which pinholes are replaced by two orthogonal Ronchi ruling patterns [5].

When the sample contains fluorescent molecules, the emitted fluorescence light returns along the same path of excitation, passing through the objective lens and the disk, and is projected through a relay lens onto a CCD or a sCMOS camera sensor, which also sits in a plane conjugated with the object plane. Fast rotation of the disk permits one to uniformly scan the whole field of view in 10 ms or less to form a (confocal) image onto the detector. Thus, the overall speed of acquisition is normally limited by camera sensitivity and maximal frame rate.

Here, we provide a detailed description of a cost-effective, highly configurable spinning disk confocal microscope (Figs. 1, 2, 3, 4, and 5), which is suitable to perform simultaneous (two-color) imaging and electrophysiological recordings. We show how to test microscope performance (Fig. 6) and provide some illustrative examples (Figs. 7, 8, and 9) of intracellular and intercellular signaling mechanisms in mice cochlear epithelia [6–10].

We hope that the skilled reader will benefit from the information we provide here, which should be sufficient to duplicate the architecture we developed.

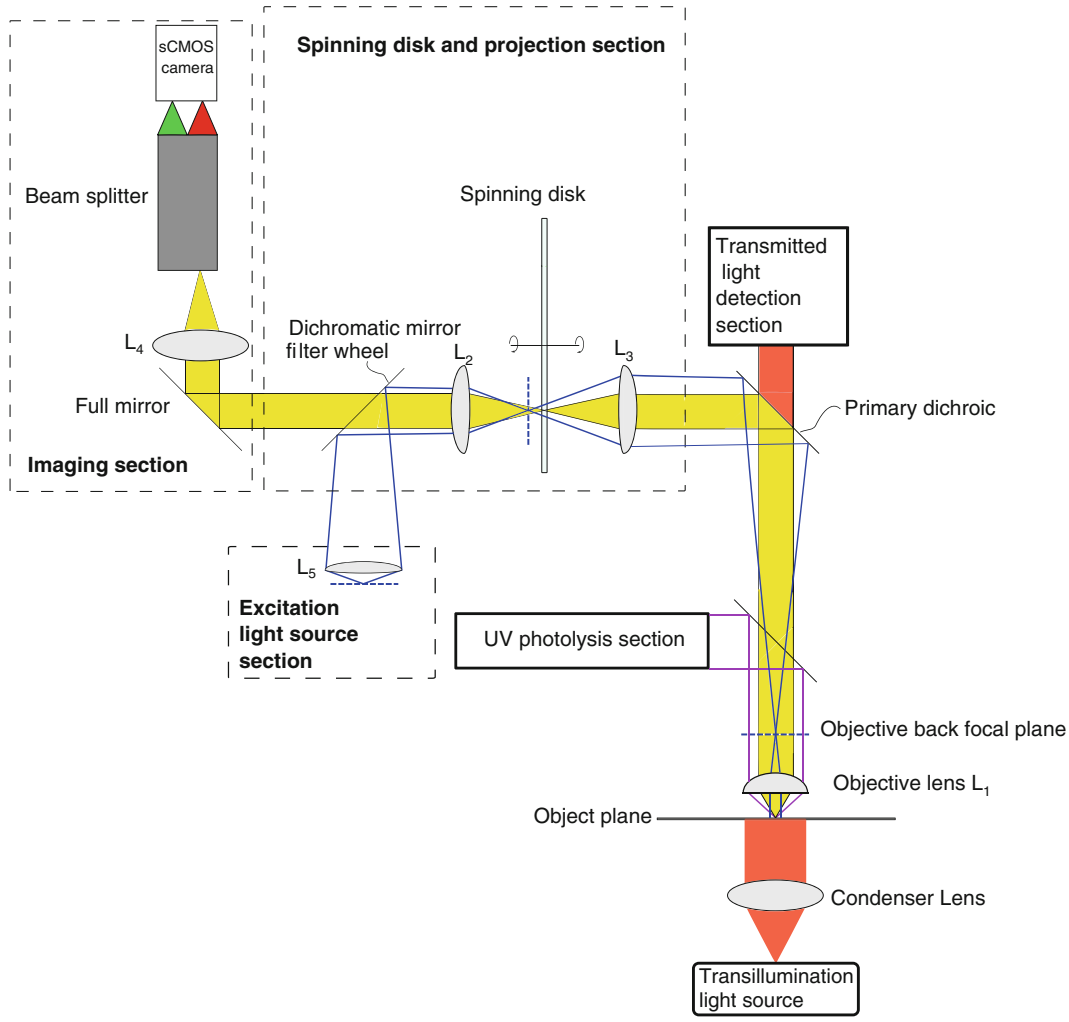

Fig. 1 Scheme of the confocal microscope system developed in our laboratory (*see* Subheading 2.1 for parts list). L4 sets the overall magnification of the system (as both telescope and the image duplicator have unit magnification), therefore its focal length must be carefully chosen depending on the objective and the final magnification of the image projected by an apochromatic lens onto the camera sensor

2 Materials

2.1 Optical Components

2.1.1 Microscope and Fluorescence Imaging Components (Fig. 1)

1. Fixed stage microscope (e.g. Olympus BX51WI, Olympus) mounted on an anti-vibration table (e.g. 63–560, TMC).
2. Epifluorescence illuminator (e.g. BX-URA2, Olympus).
3. Full mirror (e.g. CM1-G01, Thorlabs, Newton, NJ).
4. Infinity-corrected imaging lens (L4, e.g. ITL200, Thorlabs).
5. Digital scientific camera, preferably with a sCMOS sensor (e.g. PCO.EDGE 5.5, PCO).

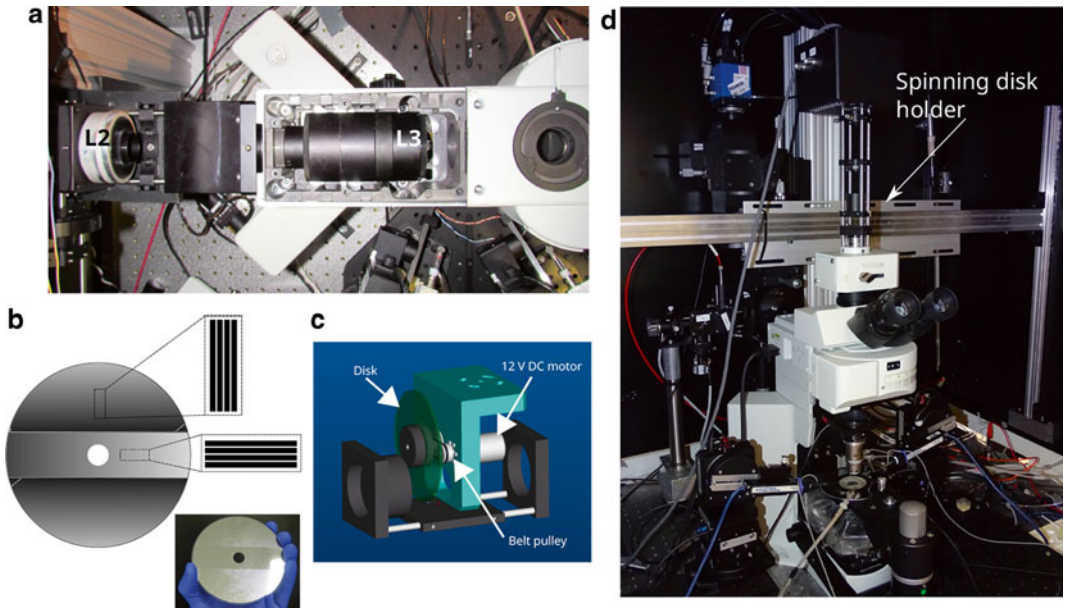

Fig. 2 Spinning disk section. (**a**) The epifluorescence illuminator was customized to allow the insertion of additional optics in the optical path. The two achromatic doublets forming the confocal telescope (L2 and L3) share the same focal plane (*dashed line*) where the spinning disk is positioned. (**b**) Confocal disk obtained by laser ablation of chromium deposition on a round glass window; the pattern consists of two orthogonal sets of stripes (38 μm width, 400 μm pitch) that permit to scan the field of view once the disk rotates. (**c**) Home-made rotary disk mount that connects the DC motor to the disk. (**d**) To avoid direct transmission of mechanical vibrations to the microscope due to disk rotation, the rotary disk mount is connected to a transversal bar anchored to the Faraday cage, which is mechanically isolated from the optical table

2.1.2 Spinning Disk and Projection Section (Fig. 1)

1. L2, L3: identical achromatic doublets, 50 mm diameter (∅), 100 mm effective focal length (efl), Ultraviolet-Visible coating (e.g. 85–878, Edmund Optics, Barrington, NJ).
2. Spinning disk (Fig. 2b): Use a round glass window (e.g. WFS-1001, UQG Ltd., UK), to form the spinning disk by drilling a precision hole in its center. One face of the window carries a light-modulating pattern (holes or stripes) which can be obtained by laser ablation of a chromium deposition (e.g. Altechna, Vilnius, Lithuania).
3. Rotary disk mount (Fig. 2c): This custom-built component can be requested from Tecnomotive snc (http://www.tecnomotive.it, Padova, Italy).
4. DC motor (e.g. A-max 121394, Maxon Motor, Fall River, MA).
5. *x*–*y*–*z* Micromanipulator (e.g. PT3, Thorlabs): This part is used to connect the rotary disk mount (Fig. 2c) to the transversal support bar (Fig. 2d) and to precisely position the disk in the light path between L2 and L3 (Figs. 1 and 2a).

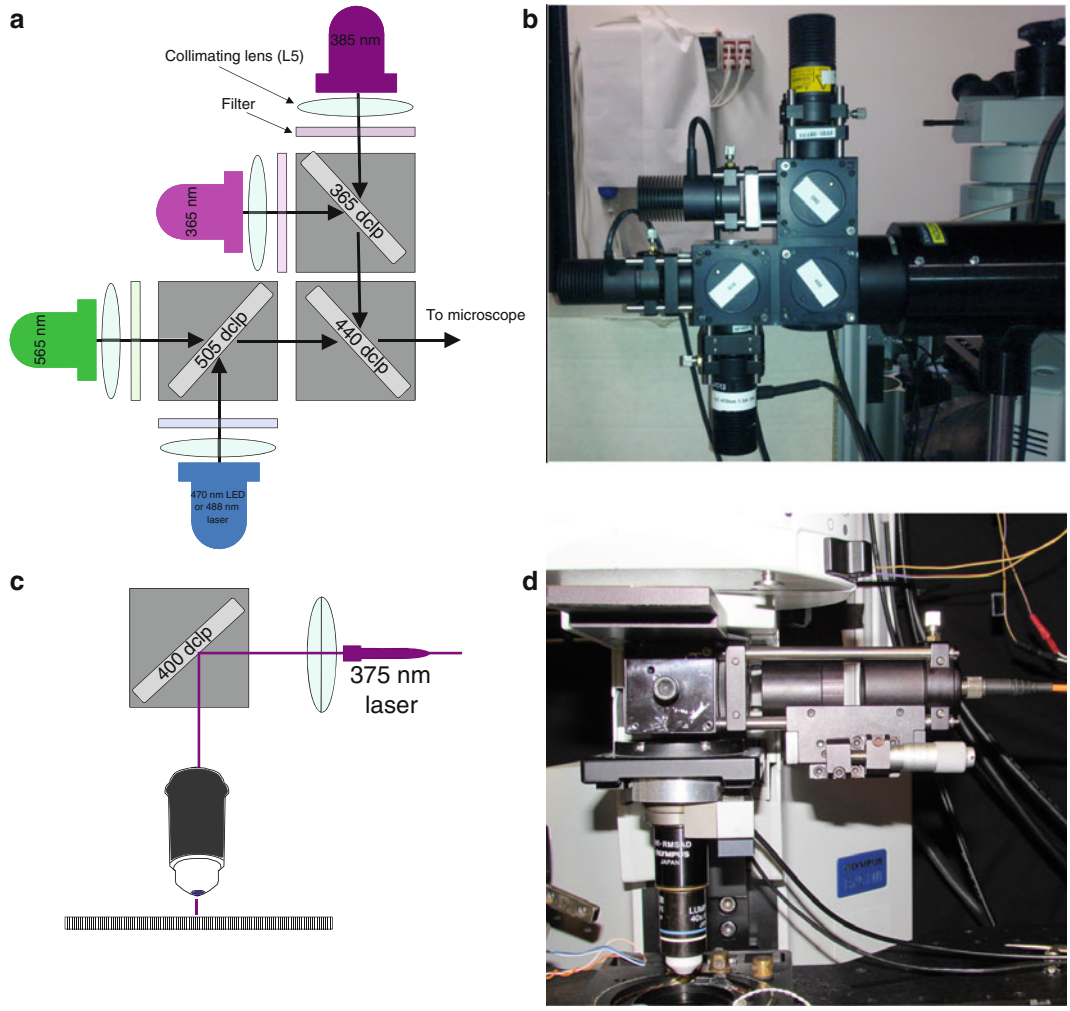

Fig. 3 Fluorescence excitation and photoactivation of caged compounds. (**a**, **b**) Fluorescence illumination uses up to four high-powered LEDs at pre-selected wavelengths. This arrangement, which consists of dichroic mirrors and filters, enables the selection of one or more wavelengths simultaneously (under TTL control) without altering the optical path. (**c**, **d**) UV photolysis of caged compounds (e.g. NP-EGTA): delivery of light from a UV laser (379 nm) is TTL-controlled by the same microcontroller board that triggers the excitation LEDs and the acquisition camera. The UV collimated beam is focused onto a small spot by the imaging objective. The optical fiber is mounted on an *x*–*y*–*z* micromanipulator, which permits precise positioning and focusing of the uncaging spot in the field of view

2.1.3 Fluorescence Excitation (Fig. 3a, b)

1. 365 nm LED (e.g. M365L2, Thorlabs).
2. 365 nm LED filter (e.g. Hg01-365-25, Semrock Inc., Rochester, NY).
3. 385 LED (e.g. M385L2, Thorlabs).
4. 385 nm LED filter (e.g. D390/70X, Chroma Technology, Bellows Falls, VT).

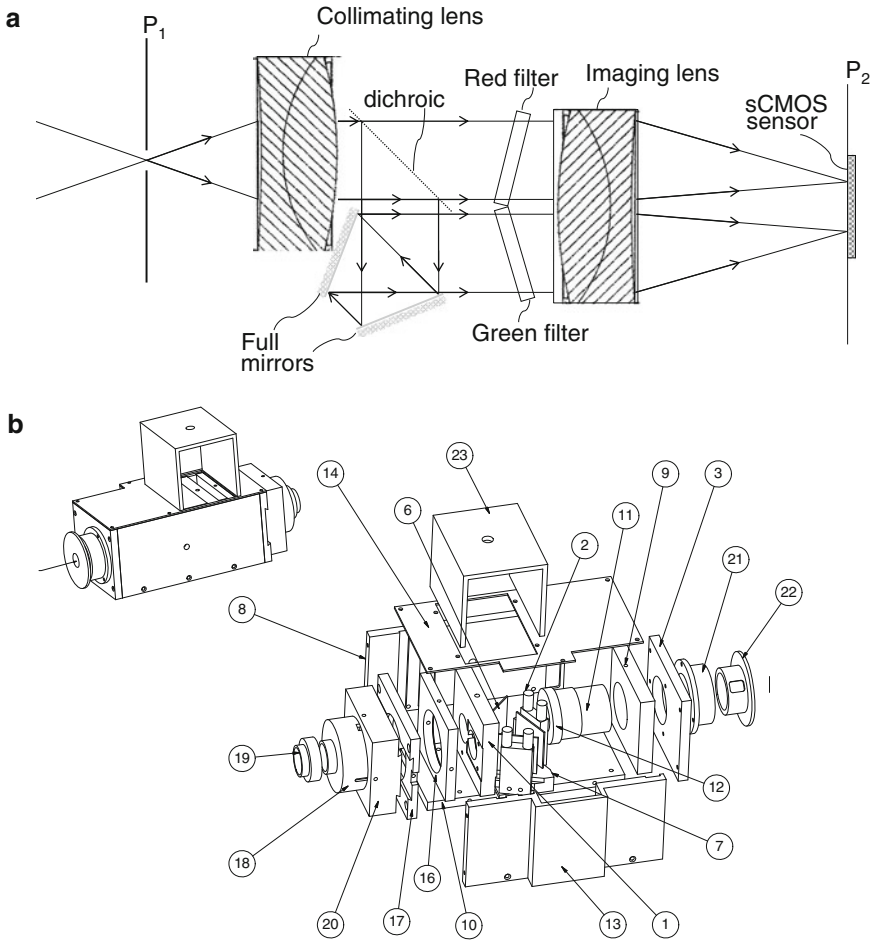

Fig. 4 Custom-made optical image duplicator. (**a**) This optical device is intercalated between the microscope output port and the 2D solid state sensor of the camera, and is used to acquire two simultaneous and spectrally distinct images of the same field of view; P1 the plane of variable slit aperture and P2 the image plane. (**b**) Exploded-view diagram: (1) Filter holder, (2) adjustable mirror holder, (3) enclosure, (6) dichromatic mirror, (7) mirror holder, (8) left wall, (9) internal threaded separation, (10) rail, (11) external threaded lens holder, (12) diaphragm, (13) right wall, (14) top enclosure, (16) projection lens holder, (17) back enclosure, (18) telescopic mount, (19) camera mount, (20) rail, (21) slit holder, (22) slit, (23) enclosure

5. 470 nm LED (e.g. M470L3, Thorlabs).
6. 470 nm LED filter (e.g. BP460-480, Olympus).
7. 565 nm LED (e.g. M565L3, Thorlabs).
8. 565 nm LED filter (e.g. 67-019, Edmund Optics).
9. Set of dichroic mirrors to combine light from different sources (e.g. 380DCLP, 440DCLP and 505DCLP, Chroma).
10. LED Collimating Lenses (L5, e.g. AC2520, Thorlabs; one lens per LED).

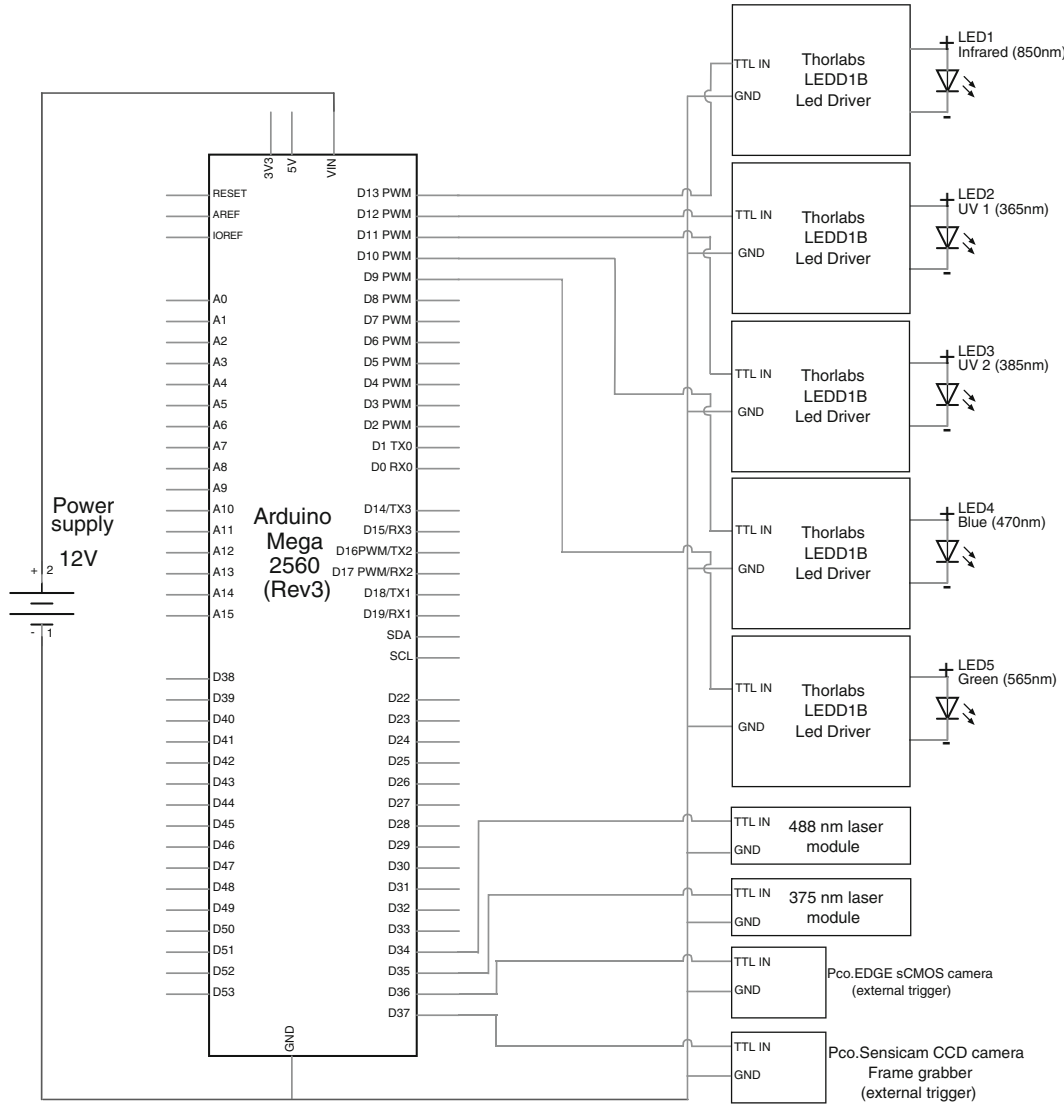

Fig. 5 Control electronics. All light sources are rapidly switched between the ON and OFF states by a home-made control board with a microcontroller (Arduino Mega 2560 Rev3) as main component. The same control board also triggers frame acquisition in both cameras. LEDs are driven by adjustable TTL-controlled LED drivers (LEDD1B, Thorlabs)

11. *x*–*y* translators: Used to center LED image in the back focal plane of the objective lens (e.g. CXY1, Thorlabs; one translator per LED).
12. Mechanical assembly: This custom-built part can be requested from Step Engineering Snc (Treviso, Italy; http://www.stepconsulting.eu/) (Fig. 3b).
13. Dichroic filter wheel (e.g. CDFW5/M, Thorlabs).

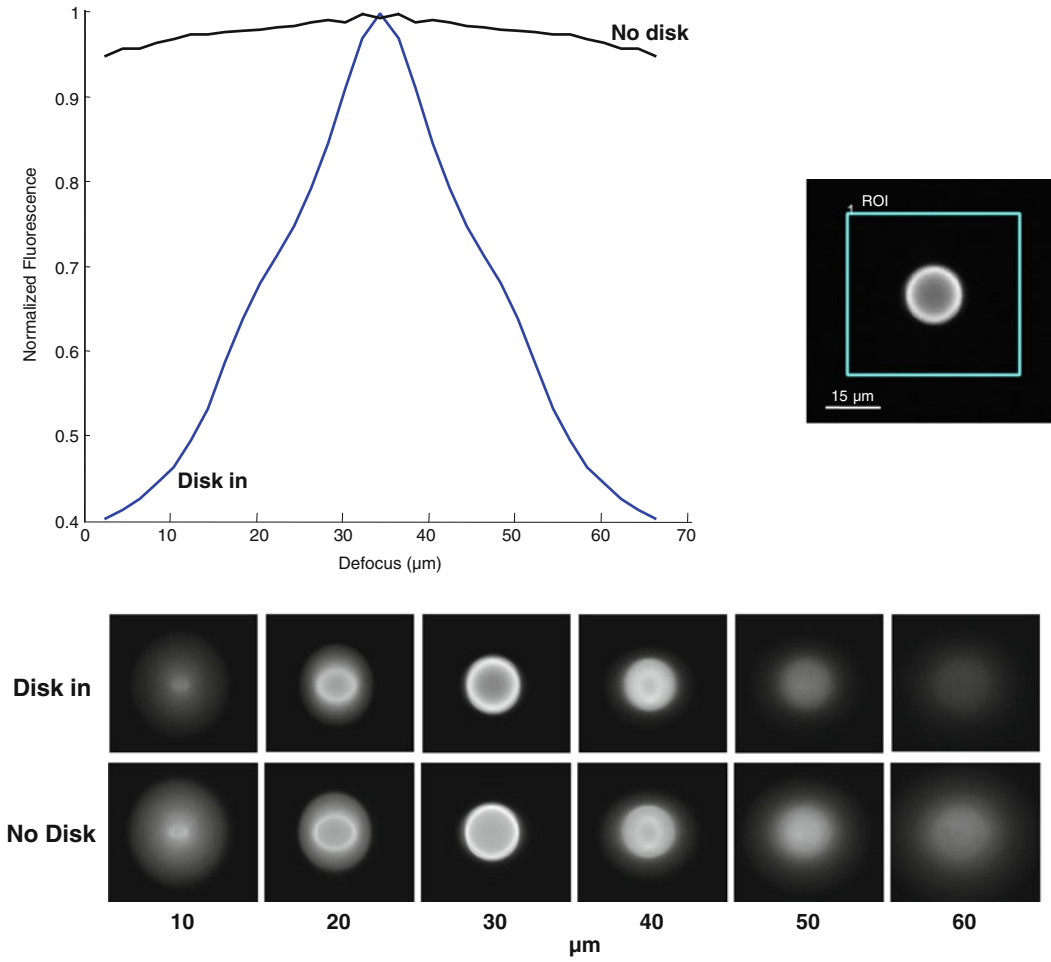

Fig. 6 Testing the optical sectioning capabilities of the confocal apparatus. (**a**) A 15 μm diameter fluorescent bead was imaged by a *z*-stack with and without insertion of the spinning disk in the optical path. (**b**) A square region of interest (ROI) around the bead was used to quantify the emitted fluorescence as shown in the graph (**c**, normalized for comparison)

14. Dichroic mirror (e.g. 515DCXR, Chroma) to separate excitation and emission light.

2.1.4 Transmitted Light Equipment (Fig. 1)

1. Infrared LED: Used to visualize the preparation with infrared (IR) differential interference contrast (DIC) (e.g., M850 Thorlabs).
2. Widefield/transmitted light camera (e.g. Sensicam, PCO, Kelheim, Germany).
3. Infinity-corrected water/oil immersion objective (L1), magnification 20–60×, with the highest practicable numerical aperture (NA) (e.g. LUMPLANFL 60×, 0.9NA, Olympus).

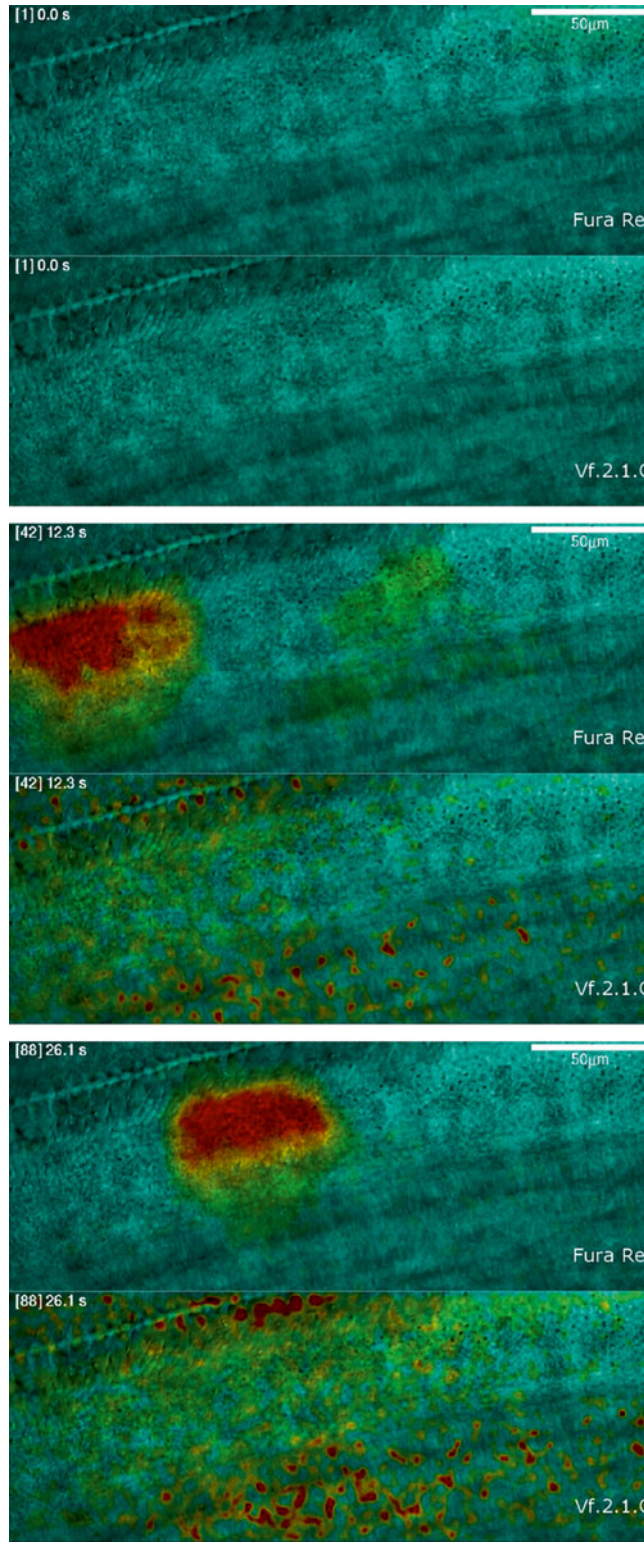

Fig. 7 Simultaneous Ca^{2+} and voltage imaging. Spontaneous Ca^{2+} activity and membrane depolarizations were recorded simultaneously using Fura-Red and Vf2.1.Cl, respectively, to show Ca^{2+} and voltage waves propagating through non-sensory cells coupled by gap-junctions in the greater epithelial ridge of the mouse postnatal cochlea (P5). *See* also Supplementary Video 1

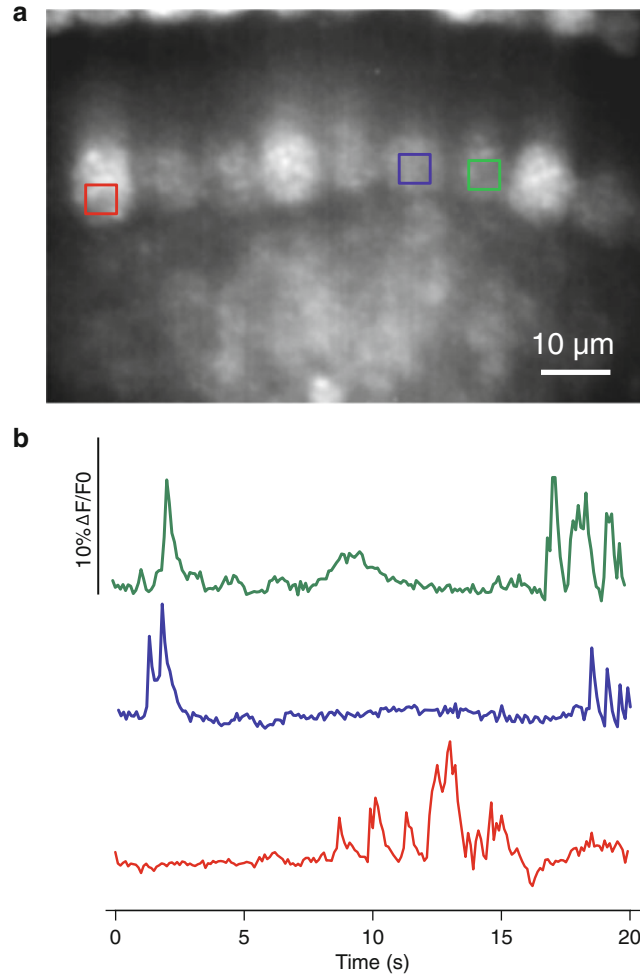

Fig. 8 Recording of spontaneous Ca^{2+} action potentials in immature cochlear inner hair cells. (**a**) Ca^{2+} dynamics during synaptic activity recorded in inner hair cells of the postnatal cochlea loaded with Fluo-4AM. (**b**) Local Ca^{2+} transients ("hotspots") due to spontaneous action potentials (*see* Supplementary Video 2) were clearly detectable by the confocal apparatus. Signals were post-processed by subtracting the Fluo-4 bleaching component and the slow fluorescence modulation due to out-of-focus non-sensory cells encasing hair cells

4. Piezoelectric objective lens positioning system (e.g. MIPOS 100 SG, Piezojena and related driver, NV 40/1 CLE) (Fig. 3d).
5. Primary dichroic mirror (e.g., 790DCXXR Chroma).

2.1.5 UV Photolysis (Fig. 3c, d)

1. TTL-controlled continuous-wave diode laser (370–380 nm center wavelength) coupled to 50–100 µm ∅ UV-permissive optical fiber, with fiber output power ≥100 mW (e.g. FC-375-050-MM1-PC-1-0, RGBLase LLC, Fremont, CA).
2. Collimating achromatic doublet (e.g. AC254-075-A, Thorlabs).
3. Dichroic mirror (e.g. 400DCLP, Chroma).

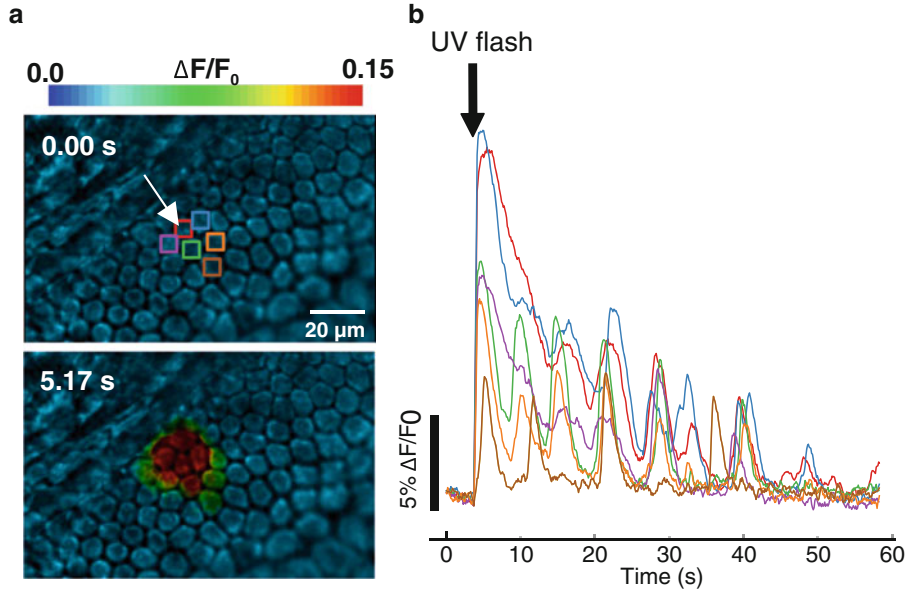

Fig. 9 Focal photoactivation of caged IP_3 in non-sensory cell of the lesser epithelial ridge of the cochlea. (**a**) Single-cell UV photoactivation of caged IP_3 (170 ms) stimulates propagation of an intercellular Ca^{2+} wave followed by spontaneous Ca^{2+} oscillations. (**b**) Traces correspond to ROIs in (**a**). Ca^{2+} concentration was measured by Fluo-4AM, co-loaded with caged IP_3 AM (*see* Subheading 3 and Supplementary Video 3). Note: the size of UV laser spots in the focal plane (~10 μm using Nikon 60×/1.0 NA Water Objective) can be estimated by measuring the dimension of the spot carved in a thin film of black ink deposited on a microscope coverslip

4. Dichroic cage cube (e.g. CM1-DCH, Thorlabs).
5. Cage translation stage: Used to position optical fiber output in the focal plane of the achromatic doublet (e.g. CT1, Thorlabs).
6. *x*–*y* translator: Used to position the focal spot in the field of view (e.g. CXY1, Thorlabs).

2.1.6 Image Duplicator Components (Fig. 4)

1. Green/yellow emission filter (e.g. ET535/30M, Chroma).
2. Red emission filter (e.g. D650/50M, Chroma).
3. Dichroic mirror (e.g. 585DCXR, Chroma).
4. Pair of identical achromatic doublets (e.g. AC508-100-A, Thorlabs). These lenses should have high (>90 %) transmittance in the visible region (400–700 nm) and suitable anti-reflection coating.
5. Adjustable mirror mount (e.g. 56–344, Edmund Optics).
6. Adjustable mechanical slit (e.g. VA100C/M, Thorlabs).
7. Mechanical assembly (Fig. 4b).

2.1.7 Optics Alignment Tools

1. Optical test target (e.g. R3L3S1N, Thorlabs).
2. Fluorescent microspheres (e.g. F-7238, ThermoFisher).
3. Fluorescent test target: Used for light source optimization (e.g. VRC2, Thorlabs).

2.1.8 Image Acquisition Hardware and Software

1. Computer workstation.
2. Programmable microcontroller (e.g. Arduino/Genuino MEGA 2560 Rev. 3, Arduino) or computer I/O board (e.g. Model 826, Sensoray) to control image exposure trigger and light switching (Fig. 5).
3. High-power TTL-controlled LED drivers (e.g. LEDD1B, Thorlabs).
4. Image acquisition software: We use software developed in our laboratory. Alternatives are Metamorph (Molecular Devices) or MicroManager (University of California, San Francisco).

2.2 Cochlear Dissection Materials

1. Sterile dissection tools.
2. Cell-Tak solution (Becton Dickinson).
3. 0.1 M sodium bicarbonate solution (pH 8.0).
4. Sterile water.
5. Ice-cold Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM Hepes Buffer (~4 mL for each cochlea).
6. Culture medium (~2 mL for each culture): DMEM/F12 supplemented with 5 % Fetal Bovine Serum (FBS, Thermo Fisher), ampicillin (0.1 mg/mL), and fungizone (0.1 mg/mL).

2.3 Fluorescent Dyes and Photoactivatable Compounds

1. VoltageFluor (Vf) 2.1.Cl voltage-sensitive dye [11], kindly provided by Professor Roger Y. Tsien (University of California, San Diego).
2. Pluronic F-127 (Thermo Fisher), 20 % w/v solution in DMSO.
3. Sulfinpyrazone (Sigma-Aldrich): Prepare a 20 mM stock solution in water. Bring pH to ~11 using NaOH and stir, then bring back to ~8.5 using HCl once dissolved.
4. Ca^{2+} dyes Fluo-4AM and Fura-Red AM (Thermo Fisher): Solubilize dyes in DMSO to make 2 mM stock solutions. Mix using a vortexer until solution is homogeneous.
5. Caged IP_3 (ENZO Life Sciences): Prepare 1 mM stock solutions in DMSO.

2.4 Solutions

1. Perfusion medium (EXM) (mM): 135 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.7 NaH_2PO_4, 0.9 MgCl, 10 HEPES-NaOH, 6 D-glucose, 2 pyruvate, amino acids and vitamins (from kit), and pH to 7.48 using NaOH. Amino acids (without L-Glutamine) and vitamins are added from concentrates (50× and 100×, respectively, Sigma-Aldrich). The final osmolarity of the solution is ~307 mOsm/L.

3 Methods

An ethics committee of the University of Padua approved all protocols, according to Italian law on animal Welfare and Experimentation and the 3R criteria.

3.1 Optical Alignment

Using Fig. 1 as a reference, the microscope is conveniently subdivided into the following categories.

1. Spinning disk and projection section, which consists of a unit gain keplerian telescope (lenses L2 and L3) and the disk itself (*see* **Note 1** and Fig. 2).
2. Fluorescence excitation section, which consists of the light sources and the collimation lenses L5 (*see* **Note 2** and Fig. 3a, b).
3. Imaging section, which consists of a projection lens (L4), the image duplicator (Fig. 4) and the camera (*see* **Note 3**).
4. UV photolysis section (*see* **Note 4** and Fig. 3c, d).
5. Transmitted light detection section, which consists of trinocular/camera (*see* **Note 5**, Fig. 2d).
6. Control electronics (*see* **Note 6** and Fig. 5).

3.1.1 Alignment Guidelines

1. Using the eyepiece of the microscope and the transmitted light source, focus a test target as a sample.
2. Remove the lenses L2, L3, the image duplicator (if present) and the spinning disk from the optical path.
3. Move the sCMOS camera along the optical axis until a sharp image of the target lines is formed.
4. Insert L3 as close as possible to the objective inside the modified epifluorescence illuminator (Fig. 2a). The exact position of this lens is not important, provided that (1) it is sufficiently close to the objective to minimize light dispersion and (2) its back focal plane lies sufficiently outside the epifluorescence illuminator to allow the insertion of the spinning disk.
5. Insert L2 in the optical path. Regulate the position of the lens until a sharp image of the test target is formed on the camera. The relative position of L2 and L4 along the optical axis is irrelevant. Relative misalignment and tip/tilt of L2 and L3 lenses should be carefully avoided.
6. Remove the test target and insert the spinning disk in the optical path at L2 and L3 focus. Finely regulate its position along the optical axis until a sharp image of the disk stripes/holes is acquired on the camera.
7. Remove the disk and L4 and insert the image duplicator in the optical path.

8. Remove the image duplicator collimating lens and open the aperture P1 completely (*see* Fig. 4).
9. Using the eyepiece of the microscope and the transmitted light source, focus the test target as a sample.
10. Adjust the position of the camera until a sharp image of the target appears.
11. Insert the collimating lens of the image duplicator. Adjust the position of the lens relative to the aperture P1 until a sharp image of the aperture borders appears on the camera sensor.
12. Insert L4 again. Maintaining the relative distance between the image duplicator and the camera, move the entire image duplicator/camera until a sharp image of the target appears.
13. Close the aperture until the image in the red channel occupies half of the camera sensor.
14. Adjust the full mirrors inside the image duplicator until the image in the green channel occupies the other half of the camera sensor.
15. The UV photolysis laser should form a focused spot on the image plane. Using a marker pen, deposit a film of ink on the surface of a glass coverslip. Focus the UV laser on the surface to carve a spot in the ink. Adjust the optical fiber by the *x*–*y*–*z* micromanipulator in order to obtain the smallest spot as possible.

3.2 Assessing Optical Sectioning

1. To test the optical sectioning capabilities of the microscope, image fluorescent beads (15 μm diameter, Fig. 6) while stepping the objective in small (<1 μm) increments along its optical axis (*z* direction) by a piezoelectric actuator.
2. Measure the average fluorescence intensity collected by the sCMOS camera sensor within a ROI containing the bead image. Graphs in Fig. 6 show normalized intensity as a function of objective position (defocus) when the disk was excluded from the optical path and when it was inserted in the path. The system's capability to reject out-of-focus fluorescence appears evident.

3.3 Preparation of Cochlear Organotypic Cultures

1. Dilute Cell-Tak 1:10 using the sodium bicarbonate solution.
2. Immediately dispense a drop of the solution on a glass coverslip (~10 μL).
3. Let the coverslip dry completely at room temperature for at least 30 min.
4. Wash the coverslip with sterile water to remove residual bicarbonate. Coated coverslips can be stored at 2–8 °C for 1 week if not used immediately.

5. Quickly dissect the cochlea in ice-cold Hepes buffered (pH 7.2) HBSS. Removal of the stria vascularis during cochlear culture preparation helps to correctly position the cochlea on the coverslip, thereby, greatly improving visualization for action potential recording.
6. Place the cochlea onto the glass coverslip coated with Cell-Tak.
7. Incubate overnight at 37 °C in DMEM/F12 supplemented with 5 % FBS.

3.4 Loading of Dyes and Caged Compounds

3.4.1 Dye Loading for Simultaneous Ca^{2+} and Voltage Imaging

1. Incubate cochlear cultures for 40 min at 37 °C in DMEM/F12 supplemented with 10 μM Fura-Red AM. The incubation medium contains pluronic F-127 (0.1 %, w/v) and sulfinpyrazone (250 μM) to maximize dye sequestration and to prevent its loss, respectively.
2. Incubate cultures for 15 min in DMEM/F12 supplemented with 200 nM Vf2.1.Cl and pluronic F-127 (0.1 % w/v).
3. Transfer the culture onto the microscope stage and perfuse it with extracellular solution for 20 min at 2 mL/min in the dark before starting the experiment in order to complete dye de-esterification.
4. Example: Fig. 7 and Supplementary Video 1 show a representative simultaneous recording of spontaneous Ca^{2+} and depolarizing transients and membrane depolarizations in cochlear non-sensory cells, due to spontaneous ATP release from connexin hemichannels [10]. The cochlear culture was co-loaded with Fura-Red AM and VF2.1.Cl and the image duplicator beam splitter was inserted in the optical path for simultaneous two-color imaging.

3.4.2 Dye Loading for Detection of Calcium Action Potentials in Inner Hair Cells

1. For detection of Ca^{2+} action potentials in inner hair cells, incubate cochlear cultures for 40 min at 37 °C in DMEM/F12, supplemented with Fluo-4AM (16 μM), pluronic F-127 (0.1 % w/v), and 250 μM sulfinpyrazone.
2. Transfer the culture onto the microscope stage and perfuse with extracellular solution for 20 min at 2 mL/min in the dark before starting the experiment in order to complete dye de-esterification.
3. Example: Fig. 8 and Supplementary Video 2 display a representative recording of spontaneous action potential activity in cochlear inner hair cells. Single action potential detection was prevented during high frequency bursts due to the relatively long fluorescence decay time constant of Fluo-4 (300 ± 11 ms, Mean ± S.E.) [12].

3.5 Focal Photostimulation with Caged IP_3

1. Incubate cochlear cultures for 60 min at 37 °C in DMEM/F12 supplemented with Fluo-4AM (16 μM), caged IP_3 AM (5 μM), pluronic F–127 (0.1 %, w/v), and sulfinpyrazone (250 μM).

2. Transfer the culture on the microscope stage and perfuse in extracellular solution for 20 min at 2 mL/min in the dark to allow for de-esterification.
3. In a typical experiment, baseline (pre-stimulus) fluorescence emission (F_0) is recorded for a few seconds, thereafter a UV laser pulse of 100–200 ms is applied to release IP_3 and fluorescence emission is monitored for 1 min or more.
4. Example: Fig. 9 and Supplementary Video 3 show a representative recording of Ca^{2+} transients evoked by focal UV photostimulation by caged IP_3.

3.6 Fluorescence Excitation and Acquisition

1. Appropriate dichroic, excitation, and emission filters should be selected for the particular application (*see* **Note 7**). Fluo-4, Vf2.1.Cl, and Fura-Red are efficiently excited with blue light (470 nm LED). Emission spectra of Vf2.1.Cl (green) and Fura-Red (red) are suitable for simultaneous recording without the need of spectral cross-talk correction.
2. Image exposure and frame rate should be selected depending on the phenomena under study (*see* **Note 8**).
3. To minimize photobleaching and phototoxic effects, it is important that excitation light and image acquisition are carefully synchronized (*see* **Note 9**) in order to illuminate the sample only during frame exposure.

3.7 Image Analysis

1. Calcium or voltage vsignals are quantified pixel-by-pixel as relative changes of fluorescence emission intensity ($\Delta F/F_0$), where F_0 is fluorescence at the beginning of the recording, F is fluorescence at time t during the experiment, and $\Delta F = F - F_0$ (*see* **Note 10**).
2. Signal extraction and data analysis can be performed using software for numerical analysis (e.g. ImageJ and ImageJ-based software or Matlab).

4 Notes

1. Spinning disk—After reflection off a dichromatic mirror, excitation light is fed to a unit-gain keplerian telescope formed by two identical UV permissible achromatic doublets (L2 and L3; to position this critical pair of lenses, we removed all intermediate optics from our epifluorescence microscope and replaced them with L3). An *x*–*y*–*z* precision manipulator is used to place the striped disk in the focal plane shared by these two doublets, which is also a primary image plane for the entire microscope. The two achromatic doublets replace the standard lens in the BX-URA2 Olympus epifluorescence illuminator (Fig. 2a) and

are mounted using a combination of 30 and 50 mm lens tubes and holders available from Thorlabs GmbH (Dachau, Germany). The custom-made disk has a pattern on its face of chromium stripes obtained by laser ablation (Fig. 2b) and is spun by a DC motor up to a speed of 7200 rpm (Fig. 2c). To avoid transmission of mechanical vibrations to the microscope and the optical table due to the disk rotation, the disk *x*–*y*–*z* manipulator is coupled to a suspended horizontal beam attached to the frame of the Faraday cage that encases the entire system (Fig. 2d).

2. Fluorescence excitation—The rotating disk acts as a spatial filter, blocking a substantial amount (~90 %) of the excitation light. Thus, spinning disk confocal microscopy requires a light source with substantial (>200 mW) power at the relevant excitation wavelengths. High-power LEDs are the preferred option, and their combination permits fast switching between different wavelengths. Each LED is used in combination with a bandpass filter (Fig. 3a, b). Light from each LED is collimated by an aspheric lens (AC2520, Thorlabs, L5 in Figs. 1 and 3a). Note: the 470 nm LED can be replaced with a 488 nm diode laser (e.g. COMPACT-150G-488-SM, World Star Tech) that is more efficient, although considerably more expensive to excite fluorophores such as Fluo-4.
3. Fluorescence imaging—Emitted fluorescence light collected by the objective is spatially filtered through the striped spinning disk, traverses the illumination dichroic and emission filter and is finally focused onto a sCMOS detector (PCO. edge, PCO, Germany) by a projection lens (L4 in Fig. 1, ITL200, Thorlabs). The detector must be located in a plane optically conjugated with the disk as well as the object plane. For two-color acquisition, light emitted by different fluorophores is separated by the optical image duplicator (Fig. 4) and imaged onto separated halves of the sCMOS sensor (Fig. 1).
4. UV photolysis section—The output of a TTL-controlled semiconductor laser module (150 mW, 379 nm, RGBLase LLC, CA, USA) is injected into a UV permissive optical fiber (multimode step index 0.22 NA, 105 μm core, part number AFS105/125YCUSTOM, Thorlabs). Fiber output is projected onto the specimen plane by an achromatic doublet (75 mm effective focal length, part number AC-254-75-A, Thorlabs) and the re-collimated beam is directed onto a dichromatic mirror (400 DCLP, Chroma) placed at 45° just above the objective lens of the microscope (Fig. 3c, d). The image of the fiber core is projected as a circular spot in the focal plane by the (infinity corrected) objective. To obtain a sharp spot profile, an *x*–*y*–*z* manipulator is used to adjust the fiber

position with respect to the aspheric lens for light re-collimation. Under these conditions, the optical fiber diameter accurately sets the laser-irradiated area, which encompasses from one to a few cells (depending on the objective).

5. Transmitted light detection section—Light from a substage infrared LED passes through the primary dichroic mirror (Fig. 1) and forms differential interference contrast (DIC) images on a scientific grade CCD camera.
6. Control electronics—LEDs (or lasers) are activated by TTL-controlled drivers in sync with the acquisition camera using a programmable microcontroller (Arduino Mega 2560 Rev3, Arduino, Italy) connected to a computer (Fig. 5). Reprogramming the microcontroller on the fly enables a local scheduler to run commands with extreme precision and flexibility. In fact, the microcontroller device eliminates the latency and jitter intrinsic in a system under direct computer (CPU) control. Combined with an electronically simulated digital ON/OFF switch, this approach achieves a temporal precision on the order of a few microseconds.
7. In our experience, Fluo-4, Vf2.1.Cl, and Fura-Red fluorescence are all efficiently excited by 470 nm LED light (M470L2, Thorlabs) passing through a BP460–480 bandpass filter (Olympus) and directed onto the sample through a 515 DCXR dichromatic mirror (Chroma). For standard calcium imaging, Fluo-4 emission is filtered through a 535/43M bandpass filter (Edmund). For simultaneous voltage and calcium imaging, Vf2.1.Cl and Fura-Red emission are separated by a dichromatic mirror (585DCXR, Chroma) located within the optical image duplicator (Fig. 4), followed by an ET535/30M filter (Chroma) for Vf2.1.Cl and a D650/50M filter (Chroma) for Fura-Red. With or without an image duplicator, fluorescence images are collected by a water immersion objective (typically 60×, 1.0 NA, Fluor, Nikon) and projected onto a scientific-grade camera (PCO.Edge 5.5; PCO AG) controlled by software developed in our laboratory.
8. Some guidelines for deciding the correct frame rate and exposure are:
    - Deliver excitation light to the sample only during frame exposure, in order to minimize photobleaching, by switching on the excitation LED source a few milliseconds before exposure starts and switching off a few milliseconds after.
    - The duration of frame exposure should be long enough to obtain a fluorescence signal-to-noise ratio (SNR) higher than 1. The goal is to achieve the minimal SNR that permits discrimination between noise and fluorescence variations related to the biological phenomenon under study.

- The duration of frame exposure should be short enough to avoid camera pixel saturation during the experiment, which results in loss of biological information.
- The frame rate should be fast enough to track the biological phenomenon under study. For periodic phenomena, a lower bound to the acquisition frequency is set by the Nyquist criterion.

9. Miller et al. [11] reported that Vf2.1.Cl and other PeT-based voltage indicators have a slower rate of photobleaching and are less toxic than FRET-based dyes. We did not perform direct comparisons between these two classes of indicators. However, in our hands, patch clamp recordings from cochlear non-sensory cells in Vf2.1.Cl-loaded cultures were stable for tens of minutes during continuous illumination by the excitation LED. In addition, we did not notice any visible sign of cellular degeneration.
10. When excited at 470 nm, Fura-Red fluorescence emission decreases upon binding Ca^{2+}.

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