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Summary

In many vertebrate cell types, the proximal part of the primary cilium is positioned within an invagination of the plasma membrane known as the ciliary pocket. Recent evidence points to the conclusion that the ciliary pocket comprises a unique site for exo- and endocytosis of ciliary proteins, thereby regulating the spatiotemporal trafficking of receptors into and out of the cilium to control its sensory function. In this chapter, we provide methods based on electron microscopy, 3D reconstruction of fluorescence images as well as live cell imaging suitable for investigating processes associated with endocytosis at the ciliary pocket.

1. INTRODUCTION

Primary cilia are 9+0 microtubule (MT)-based, membrane-enclosed projections that emanate as solitary organelles on the surface of most quiescent cell types in vertebrates [1]. The ciliary axoneme is nucleated from the centrosomal mother centriole (basal body), which docks to the plasma membrane via its distal appendage proteins also known as transition fibers [2]. The membrane surrounding the axoneme is continuous with the plasma membrane, but it has a unique lipid composition and a complement of membrane receptors and ion channels that enable the cilium to function as a sensory organelle that relay signals from the extracellular environment to control developmental processes and tissue homeostasis. The sensory capacity of the cilium is maintained through structural and functional barriers above the basal body that set up a transition zone or molecular filter that gates the selective passage of receptors and ion channels into and out of the ciliary compartment. Cilium defects give rise to a growing number of genetic disorders known as ciliopathies [3,4].

In many cell types, the region between the plasma membrane and the ciliary membrane is infolded to produce a ciliary pocket, which comprises an interphase for the actin cytoskeleton and may function as a major site for exo- and endocytic events for the sorting and targeting of proteins to and from the ciliary base [5,6]. Indeed, the infolded plasma membrane, also known as the periciliary membrane, comprises a unique site for clathrin-dependent endocytosis, which critically regulates a series of signaling events associated with ligand-induced receptor internalization and subsequent recycling or degradation in the late endosomes/lysosomes compartment [7]. This raises the possibility that endocytosis at the ciliary pocket regulate the level of many different ciliary signaling systems, such as those operated by G protein-coupled receptors [8], Receptor Tyrosine Kinases [9], TRP ion channels [10], and receptors for extracellular matrix proteins [11]. Indeed, we previously showed that Transforming Growth Factor beta (TGFβ) signaling and activation of Smad transcription factors is associated with clathrin-dependent endocytosis of TGFβ receptors at the ciliary pocket in fibroblasts and in stem cells undergoing cardiomyogenesis [12].

Here, we provide different protocols for investigating the morphology and function of the ciliary pocket in RPE1 and NT2 cells, which are cell lines commonly used in laboratories worldwide.

Specifically, we present methods based on electron microscopy, 3D reconstruction of fluorescence images as well as live cell imaging suitable for investigating cellular and signaling processes associated with endocytosis at the ciliary pocket.

2. MATERIALS

2.1. Cells and cell culture media

1. RPE1, a human retinal pigment epithelial cell line that stably expresses human telomerase reverse transcriptase (hTERT-RPE1; CLONTECH Laboratories, Inc.; ATCC CRL4000^{TM}).
2. NT2, a human pluripotent embryonal carcinoma stem cell line (NTERA-2 cl.D1; ATCC CRL1973^{TM}).
3. Dulbecco's Modified Eagle's Medium DMEM-F12 1:1 GlutaMAX supplement (Thermofischer scientific, 31331-028) supplemented by 10% fetal bovine serum (FBS, Thermofischer scientific, 10270-106) for basic RPE1 cell culture conditions and supplemented with 0.5% FBS for low serum containing media.
4. OPTI-MEM (Thermofischer scientific, 31985-047) was used for transfections in RPE1 cells.
5. DMEM (Thermofischer scientific, 41965-039) was used for Transferrin uptake experiments in RPE1 cells and NT2 cells.
6. DMEM (ATCC, 30-2002) supplemented by 10% FBS (Sigma Aldrich, F9665) and 1% pen/strep for basic cell culture conditions in NT2 cells.
7. DMEM supplemented by 10% FBS without pen/strep was used for transfections in NT2 cells (*see **note 1)***.

2.2 Buffers and solutions for fluorescence microscopy

1. 1X Phosphate-buffered saline (PBS) (pH 7.4);
2. 1X Phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS(Ca/Mg)) (pH 7.4); PBS with
3. Transferrin internalization buffer (TIB): DMEM with 1mg/mL bovine serum albumin (BSA).
4. Fixation solution: 4% Paraformaldehyde (PFA).

5. Permeabilization buffer: Triton X-100 (0.1 or 0.2%) and BSA (1 mg/mL) in PBS. Buffer should be passed through a sterile filter to avoid cellular contamination for long-term storage. Permeabilization buffer should be stored at -20°C in aliquots and thawed and kept at 4°C upon use.
6. Blocking buffer: PBS with BSA (1 or 2 mg/mL).
7. Quenching solution: PBS-NH_4Cl (50 mM).
8. Mounting media for NT2 cells: Add 4 mL glycerol (5g) in a falcon tube and add 500µL 10 X PBS. Fill up to 5 mL with double distilled water (ddH_2O). Weigh out and add 0.1 g n-Propyl Gallate and dissolve over night at 4°C while rotating. Store covered in tin foil at 4°C.
9. Mounting media for RPE1 cells: PBS-glycerol mix (50/50) using the SlowFade Light Antifade Kit containing DAPI from Molecular Probes (Thermofischer scientific, S36938).

2.3 Equipment

1. Cells are grown in an incubator under standard growth conditions (37°C and 5% CO_2).
2. Cells are seeded on glass coverslips, 12 mm (NeuVitro, GG1212).
3. Coverslips are mounted on plain glass microscope slides (1mm, Pearl, 7101).
4. Immunofluorescence staining is performed in a humidity chamber to avoid evaporation of antibody solutions. Humidity chamber is made by placing damp filter paper in the lid of a petri dish, under a layer of Parafilm®. Coverslips can be placed on top of the Parafilm® layer, and the bottom of the petri dish can be used as the lid of the chamber.
5. 8 wells µ-slides (Ibidi, catalog #80821).
6. Ultramicrotome (Reichert ultracut S).

2.4 Ligands, antibodies, staining reagents, plasmids, conjugates and transfection reagents

1. Ligand: Transforming growth factor beta-1 (TGFβ-1) (R&D Systems, 240-B-010).)
2. Primary antibodies (the species, reference number and dilution are indicated):

Acetylated-α-tubulin (AcTub), mouse monoclonal clone 6-11B-1 (Sigma, T7451) (1:10,000)

ADP-ribosylation factor-like 13b (ARL13b), rabbit polyclonal (Proteintech 17711-1-AP) (1:600)

Clathrin, rabbit polyclonal (AbCam Ab21679) (1:500)

Clathrin assembly lymphoid myeloid leukemia (CALM), goat polyclonal (Santa Cruz, sc-6463) (1:300)

Transforming growth factor beta receptor I (TGFβ-RI, V22) rabbit polyclonal (Santa Cruz sc398) (1:200)

3. Secondary antibodies and conjugates:

Alexa^{350}-conjugated donkey anti-mouse IgG (Invitrogen, A10035) (1:600)

Alexa^{350}-conjugated donkey anti-rabbit IgG (Invitrogen, A10039) (1:600)

Alexa^{488}-conjugated donkey anti-mouse IgG (Invitrogen, A21202) (1:600)

Alexa^{488}-conjugated donkey anti-rabbit IgG (Invitrogen, A21206) (1:600)

Alexa^{488}-conjugated donkey anti-goat IgG (Invitrogen, A11055) (1:600)

Alexa^{568}-conjugated donkey anti-goat IgG (Invitrogen, A11057) (1:600)

Alexa^{568}-conjugated donkey anti-mouse IgG (Invitrogen, A10037) (1:600)

Alexa^{555}-conjugated Transferrin from Molecular Probes (Thermofischer scientific, T-35352)

Texas Red-conjugated Transferrin from Molecular Probes (Thermofischer scientific, T-2875)

4. Expression plasmids (available from our laboratories upon request):

Rab8 fused to GFP (Rab8-GFP) and Clathrin light chain fused to DsRed were kindly provided by Arnaud Echard (Institut Pasteur, Paris, France) and Thomas Kirchhausen (Immune Disease Institute, Boston, USA), and were described previously (ref!!!!!!).

pEGFP-F plasmid (Clontech, 6074-1) expressing farnesylated green fluorescent protein (GFP-F).

GFP-2xFYVE (kindly provided by Harald Stenmark (Institute for Cancer Research, Oslo, Norway)).

Cherry-2xFYVE (kindly provided by Harald Stenmark (Institute for Cancer Research, Oslo, Norway)).

5. Transfection reagents: Fugene 6 (Promega, E2691)
6. Nuclear staining: 4',6-diamidino-2-phenylindole dihydrochlorid (DAPI) (Invitrogen, D1306)
7. Nail polish (electron microscopy science, ref 72180)

2.5 Electron microscopy

1. Fixation: H2O is double-distilled apyrogenic sterile water rinsing and irrigation (Dominique Dutscher Products : ref 069802A). Phosphate buffer is Sorensen's Sodium-Potassium Phosphate Buffer, prepared as followed:
   Solution A: 35.61g Na2HPO4.2H2O – make up to 1000 mL with distilled H2O, stir until dissolved.
   Solution B: 27.6g NaH2PO4.H2O – make up to 1000 mL with distilled H2O, stir until dissolved.
   Add 40.5 mL of solution A to 9.5 mL of solution B to give 50 mL 0.2M phosphate buffer pH should be 7.4.
   Glutaraldehyde EM grade 25% (Electron Microscopy Science, EMS ref 16210) should be diluted in PBS
   Osmium tetroxide 4% (EMS ref 19180) stock should be diluted 1:1 in H2O then diluted 1:1 in phosphate buffer 0.2M to to obtain Osmium 1% in phosphate buffer 0.1M perform post fixation
   Phosphate buffer 0.1M is used for washing coverslips after post fixation
2. Embedding: Epon is prepared with Mollenhauer's Kit with Epon-812 (EMS ref 13940). Embedding was done using gelatin capsule type 04 (EMS ref 70105).

3. Sectionning: Ultrathin sections are performed on a reichert ultracut S (Leica Microsystem) with a Diatome Diamond Knife 2mm 45°.
10. Specimen grids: Gilder 200 mesh Standard Square Mesh Nickel Grids (EMS ref G200Ni).
11. Miscelleanous: 7% Uranyl acetate (EMS ref 22400) in water. Lead citrate (EMS ref 17800).
12. Observation/acquisition: JEOL 1011 transmision electron microscope with a GATAN CCD camera erlangshen 1000 with GATAN Digital Micrograph Software. Generated images were recorded in DM4 format then converted in TIFF.

2.6 Preparation of coverslips for immunofluorescence

For NT2 cell growth, 12 mm coverslips for immunofluorescence analysis are treated with acid prior to growing cells on them (*see **note 2)***.

1. Incubate coverslips in 32% HCl for 60 minutes, in a blue cap bottle. Mix frequently by swirling bottle around. Should be performed in the fume hood.
2. Discard HCl (can be saved and re-used) and add water, continue working in the fume hood. Swirl and discard. You can now move out of the hood for a total of 15 washes in ddH_2O.
3. Subsequently, coverslips are washed 15 times in 96% ethanol.
4. Store in 70% ethanol.

3. METHODS

3.1 Induction of ciliogenesis in RPE1 cells

1. Set down one coverslip per well in a regular 24 wells plate under the hood. Coverslips are previously autoclaved to avoid any contamination.
2. To optimize cell culture and transfection steps, fix your coverslips to the bottom of the wells by filling each well with 0.5 mL of PBS and then aspirate it with a 200µL tip pipet to create a vacuum between the coverslip and the plate.
3. Seed 250.000 cells per well and grow them in basic cell culture conditions for 24 hours.
4. Wash the cells twice in PBS. To avoid cell detachment, bend the plate and use a 200µL tip pipet to aspirate the medium without touching the cells.
5. Add low serum containing media for an additional 24 to 48 hours period to allow primary cilia formation.
6. Monitor the efficiency of primary cilia formation by staining with acetylated α-tubulin or ARL13b antibodies (Tipically ~80% of ciliated cells; see Immunofluorescence section below).

3.2 Induction of ciliogenesis in NT2 cells

Seed cells as described for RPE1 cells and grow under basic cell culture conditions. Primary cilia will form when cells reach confluence of approximately 80% or above, and can be visualized using specific antibodies against structural proteins and/or ciliary receptors, e.g. ARL13b. NT2 cells form rather long primary cilia, which may at times connect to cilia from adjacent cells, as shown in Figure 1a.

3.3 Transmission Electron Microscopy (TEM) protocol for flat-embedding

1. Seed RPE1 cells on coverslips and grow them in 24 well plates to induce ciliogenesis as described above.

2. Wash cells twice with PBS(Ca/Mg) to avoid cell detachment.
3. Fix the cells on coverslips with 3% glutaraldehyde (200µL/well) for 1 hour at room temperature, RT.
4. Wash cells twice with PBS(Ca/Mg).
5. Postfix your samples in 1% osmium tetroxide in 0.1 M phosphate buffer.
6. Dehydrate your samples in successive baths of 70, 90 and 100 % ethanol (3 baths for 100% Ethanol).
7. Incubate the cells in 50% ethanol and 50% Epon for 20 minutes.
8. Incubate the cells in 25% ethanol and 75% Epon for 20 minutes.
9. Incubate the cells in 100% Epon for 20 minutes.
10. Fill gelatin capsules with freshly prepared epoxy resin and polymerized at 60°C for 24 hours.
11. Gently remove the coverslip from the wells, pay attention to not break them.
12. Remove the Epon excess on the coverslip by gently holding the coverslip with the forceps and letting it flow into the well, only leaving a thin layer of Epon on the cells.
13. Place the coverslip on a glass slide, with the cells facing up.
14. Cover the coverslip with the Epon-filled capsule. One capsule is usually enough; putting two or more could lead to difficulties later to separate them. It can be also useful to place the capsule where the cells are more concentrated (this can be simply found by placing the slide/coverslip under a binocular).
15. Polymerize at 60°C for 24 hours.
16. Remove the Gelatin capsule from the coverslip by heating the glass slide at 70°C for 5 minutes.
17. Trim the block using a clean razor blade and cut your samples in 90 nm thin sections with an ultramicrotome. Ultrathin sections are put on specimen grids. Do not try to find region of interest by using 5 µm thin sections; the cell monolayer is too thin.

18. Perform serial cutting in the orientation showed in Figure 2a. The better way is to keep the grids in the cutting order for observation.
19. Stain your sections with uranyl acetate (10 minutes, followed by H_2O 3x5 minutes) and Reynold's lead citrate (5s, followed by H_2O 3x5 minutes).
20. Perform observation beginning with the region corresponding to the adherent surface of the cells, i.e. the first section, then the following, to be sure to not miss the level where the region where cilia are. They are usually found in the same region close to the nucleus (on the "side") as indicated by our analysis of cilia positioning in RPE1 cells (PMCID: PMC2972276) and as shown in Figure 2.

3.4. Transfection of cells with plasmid DNA in RPE1 cells

1. Seed 100,000 cells per well in a 24 well plates containing a coverslip and grow them as indicated above for 24 hours.
2. Prepare the transfection mix. For 4 wells, mix 1µg of DNA with 100µL of Opti-MEM and 3µL of Fugene 6.
3. Wash the cells twice with PBS(Ca/Mg) as described above.
4. Aspirate the PBS and add 500 µl of low serum containing Opti-MEM (0.5% FBS) to each well.
5. Add 25µl of transfection mix per well. Move gently your plate in perpendicular directions in order to disperse the transfection mix in the wells. Avoid circular movements.
6. Incubate the cells with the transfection mix for 24 hours before fixation and/or life imaging analysis.

3.5. Transfection of cells with plasmid DNA in NT2 cells

1. Grow cells to approximately 80% density under basic culture conditions.
2. Prepare the transfection mix as described for RPE1 cells.

3. Wash cells in PBS and add 100 µl of DMEM (10% FBS) without antibiotics to each well. Proceed as above and transfect cells using Fugene 6 and DNA in a 3:1 dilution. Dilute reagents in DMEM without serum or antibiotics.

An example of transient plasmid transfection is shown in Figure 1, where NT2 cells express Cherry-FYVE (Figure 1a) and 2xGFP-FYVE (Figure 1b) that mark early endosomes (EEs) in close proximity to the pocket region of the primary cilium stained with either ARL13b or acetylated α-tubulin. EEs are enriched in phosphatidylinositol-3 phosphate, which anchors FYVE zinc finger domain-containing proteins, such as SMAD anchor for receptor activation (SARA) that conveys SMAD2/3 to the TGF-β-Rs and promotes TGF-β-RI-mediated SMAD2/3 activation (PMID: 20515759).

3.6. Transferrin internalization in RPE1 cells

1. Incubate RPE1 cells grown on coverslips (previously transfected or not, see above) for 20 minutes at 37°C in serum free DMEM (*see **note 3)***.
2. Wash the cells twice in TIB previously warmed up at 37°C.
3. Incubate the cells for 1 minute at 37°C in TIB containing 6 µg/mL Alexa^{555}-conjugated transferrin (Tf) to allow accumulation of receptor-bound Tf into clathrin-coated pits and vesicles (CCP/CCV).
4. Wash rapidly your cells in cold PBS (4°C) to stop membrane trafficking.
5. Fix rapidly the cells with 4% PFA (10 minutes at 4°C, then 20 min at RT).
6. Process for immunofluorescence as described above.

An example of RPE1 cells transfected with pEGFP-F, treated to follow transferrin internalization at the ciliary pocket, fixed and stained for acetylated α-tubulin is shown in Figure 3a.

3.7. Transferrin internalization in NT2 cells

1. Wash NT2 cells grown on coverslips (previously transfected or not, see above) twice in serum free DMEM, previously warmed to 37°C.

2. Incubate cells for 20 min at 37°C in serum free DMEM.
3. Wash the cells twice in TIB previously warmed up at 37°C.
4. Incubate the cells for 10 minutes in TIB pre-heated to 37°C containing 15mg/mL Texas Red-conjugated transferrin (Tf), to allow receptor internalization (*see **note 4)***.
5. Wash rapidly your cells in TIB pre-heated to 37°C. Let cells sit in the incubator for 10 minutes to allow surface-bound receptor to be internalized.
6. Wash your cells in cold PBS (4°C) to stop membrane trafficking.
7. Fix the cells in 4% PFA (15 minutes at 4°C). Proceed with immunofluorescence as described below.

An example of NT2 cells pulsed for 10 minutes with Tf is depicted in Figure 4, where Tf is largely confined to EEs marked by 2xGFP-FYVE. Localization of Tf to EEs is shown both in an interphase cell (Figure 4a) and in a growth-arrested cells (Figure 4b), where Tf and 2xGFP-FYVE partly co-localizes in multiple endosomes around the pocket region of the primary cilium.

3.8. Preparation of cells for immunofluorescence microscopy in RPE1 cells

1. Wash the cells twice with PBS as described above (room temperature, RT).
2. Fix the cells in 4% PFA for 20 minutes at 4°C (by placing the plate on ice) followed by a 10 minutes incubation in quenching solution at RT.
3. Wash the cells twice with PBS.
4. Incubate the cells with primary antibodies in permeabilization buffer for 30-45 minutes at RT. Incubation can be made directly in the well, the minimal mix volume is then 200µL per well to avoid cells to get dry. To save precious antibodies, you can prepare a 40µL drop containing the antibody/permeabilization mix on Parafilm® attached to the bench, and then gently reverse your coverslip to put the cells in contact with the liquid.
5. Wash the cells twice with blocking buffer at RT avoiding cell detachment. For cells incubated on a drop, put back the coverslips into the wells properly reversed. In case of

doubt about the cells' orientation, cells can be seen on the coverslip by holding it with the forceps and by exposing it to a source of light.

6. Incubate mixed secondary antibodies in blocking buffer for 30 minutes at RT in the dark to preserve the fluorescence of secondary antibodies.
7. Wash once with blocking buffer and twice with PBS.
8. Lay down your cells on microscope slides in mounting medium.
9. Add nail polish by putting small drops around the coverslip and then complete the circle once drops dried. Avoid small holes that will favor oil contact with mounting media and the cells.
10. Store at 4°C, keep in the dark.

3.9. Preparation of cells for immunofluorescence microscopy in NT2 cells

1. Wash the cells twice with PBS as described above (room temperature, RT).
2. Fix the cells in 4% PFA for 15 minutes at RT (*see **note 5)***.
3. Wash the cells twice with PBS.
4. Incubate your cells in permeabilization buffer for 12 minutes at RT.
5. Incubate your cells in blocking buffer for 30 minutes RT or at 4°C overnight (ON)
6. Incubate your cells in primary antibodies in blocking buffer for 90 minutes at RT or 4°C ON (*see **note 6)***.
7. Wash the cells 3 times, each 5 minutes, with blocking buffer at RT avoiding cell detachment. Transfected NT2 cells are easily detached upon mechanical stress.
8. Incubate for 45 minutes at RT in blocking buffer containing mixed secondary antibodies.
9. Wash once (5 minutes) with blocking buffer and twice (2x5 minutes) with PBS.
10. Lay down your cells on microscope slides in mounting media. Apply light pressure, getting rid of excess mounting medium.
11. Add nail polish around the edge of the coverslip. Allow 20 minutes to dry.

12. Store at 4°C, keep in the dark.

An example of immunofluorescence staining with antibodies directed against CALM and clathrin, which marks clathrin-coated pits (CCPs) and vesicles (CCVs) at the ciliary pocket, is shown in Figure 4c-e. As expected, CCVs display little co-localization with EEs as evidenced by co-staining with either 10 minutes Tf pulsing (Figure 4d) or expression of 2xGFP-FYVE (Figure 4e). This gives a good overview on the spatiotemporal machinery of endoctosis at the primary cilium, which can be elaborated by live-cell imaging, e.g. Figure 6.

3.10. Fluorescence microscopy and 3D image reconstruction in RPE1 cells

1. Acquire Z-stacks epifluorescence images every 200 nm along the Z axis using a piezo-electric (PIFOC, E662-LR controller, Physik Instrumente) 100x objective (plan-apo; Axiovert 100M, Zeiss) with a micromax camera (Princeton Instruments).
2. Three methods of deconvolution of Z-stacks can be recommended including MetaMorph (3D deconvolution option), Image J plugin "Deconvolutionlab – EPFL » http://bigwww.epfl.ch/algorithms/deconvolutionlab/ or Huygens https://svi.nl/HomePage.
3. Obtain 3D reconstruction of deconvoluted images with the Imaris software (Bitplane). Images can be extracted from Imaris and then used to obtain the final pictures used in the figures.

An example of RPE1 cells transfected with pEGFP-F, treated to follow Transferrin internalization, fixed and stained for acetylated tubulin and CALM is shown in 3D reconstruction images in Figure 3b.

3.11. Fluorescence microscopy and 3D image reconstruction in NT2 cells

Capture NT2 cell images on a fully motorized Olympus BX63 upright microscope with a DP72 color, 12.8-megapixel, 4,140 × 3,096-resolution camera and differential interference contrast (DIC). The software used include Olympus CellSens dimension, which is able to perform 3D blending projections on captured z stacks, 3D animation videos, and slice views (see Figures 1, 4 and 6).

3.12. TGFβ-1 stimulation and localization of TGFβ-RI to the ciliary pocket

1. Grow NT2 cells to a density of approximately 80%.
2. Wash cells twice in PBS and add serum-depleted DMEM (37°C) for 30 minutes.
3. Add TGFβ-1 to the media at a final concentration of 2ng/mL for 10-30 minutes.
4. Proceed with immunofluorescence microscopy as described in section 3.9 with antibodies against Transforming growth factor beta receptor I (TGFβ-RI), clathrin to mark CVVs, and acetylated α-tubulin to mark the primary cilium.

Figure 5 shows an example of the co-localization of TGFβ-R to CCPs and CCVs at the pocket region of the primary cilium and in conjunction with microtubules that sprays out from the ciliary base *(see **note 7**).* After TGFβ-1 stimulation, the receptor is preferentially internalized in CCVs at the pocket region, and localization along the cytosolic microtubules may indicate trafficking of receptor-positive CCVs along these microtubules for later fusion into EEs, where activation of SMAD2/3 takes place.

3.13 Live cell imaging for dynamic analysis of clathrin or actin at the ciliary pocket

1. Seed 60,000 RPE1 cells in wells of uncoated 8 wells µ-slides.
2. Carry out transfection as described above.
3. Culture cells in the transfection media for at least 24 hours before analysis.
4. Acquire images successively every 3 seconds for 3 to 20 minutes using an Apo 100X NA 1.43 objective with an inverted epi-illumination microscope (Axiovert 200M, Zeiss) with CCD camera (CoolSNAP HQ; Photometrics) placed within a temperature-controlled enclosure set at 37°C. Shutters, filters, camera and acquisition were controlled by MetaMorph. Generate the final movies and derived pictures using ImageJ (http://rsbweb.nih.gov/ij/index.html).

An example of RPE1 cells transfected with Rab8-GFP and Clathrin-DsRed endocytic dynamic activity associated with a primary cilium is shown in Figure 6.

4. NOTES

1. NT2 cells may be difficult to transfect, however, we find increased efficiency when transfecting in media without antibiotics. Cell density does not appear to be very important for these particular constructs; however, we see a clear positive effect when using 1:3 DNA concentration to Fugene amount.

2. Preparation of coverslips using acid wash is an optional step, often used to increase cell attachment to surfaces. We find that this helps our growth rate and cell dispersion when culturing NT2 cells for immunofluorescence experiments. Further, we find an increase in transfection efficiency, possibly due to a decrease in cell loss during IFM preparation steps.

3. This step eliminates endogenous receptor-bound transferrin, and therefore increases binding and endocytosis of fluorophore-conjugated transferrin.

4. For NT2 cell experiments, we have used Texas Red-conjugated transferrin. This can be replaced by Alexa^{555}-conjugated transferrin used in our RPE1 experiments. Concentration may be adjusted, and lower amounts may be used. Further, chasing with non-labelled Tf may be included to optimize the distinct visualization of CCPs, CCVs and EEs at the ciliary pocket.

5. Variations in protocol for preparation of IFM slides between RPE1 and NT2 cells are not a reflection of an optimization in protocol. Rather, it is a result of different lab techniques, and one may very likely be interchanged with another. For instance, fixation for 15 minutes RT followed by 12 minutes in permeabilization buffer and 30 minutes in blocking buffer, as we here describe for NT2 cells, is also successfully used for RPE1 cells.

6. 90 minutes or shorter is often sufficient for visualization using a fluorescence microscope, however, some antibodies bind more weakly than others, and optimization steps often includes longer incubation times. Therefore, incubating overnight may increase signal strength.

7. When using antibodies directed against microtubules (MTs) we will inevitably obtain staining of other MTs than those constituting the primary cilium. For instance, using AcTub allows us to visualize the MTs of dividing cells as they organize the DNA before cell division, midbodies, as well as cytosolic MTs, the latter is seen in figure 5.

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Figure Legends

Fig. 1. Fluorescence microscopy analysis on the localization of early endosomes (EEs) around the ciliary pocket region in NT2 cells. (a) The localization of endosomes was observed in cells transiently transfected with plasmids expressing either Cherry-FYVE (a) or 2xGFP-FYVE (b) and primary cilia were stained with either anti-ARL13b or anti-acetylated α-tubulin (AcTub). In some cases, the nucleus was stained with DAPI (b). In some cases, the tip of primary cilia from adjacent cells connected to each other as shown in (a). Endosome localization is shown both with normal epifluorescence (b, upper panel), and with 3D reconstruction (lower panels in a and b).

Fig. 2. Transmission electron microscopy analysis on the ciliary pocket in RPE1 cells, which were grown on coverslips to confluence, then serum-starved, fixed and treated for microscopy. (a) Sections were realized parallel to the adherent surface as depicted in the scheme and explained in the methods. (b) Examples of cilia from cells starved for 24 (left) or 72 hours (right) in which both the basal body (BB) and the axoneme (Ax) are clearly visible. All observed cilia in RPE1 cells present a similar organization, with the basal body not at the same plane as the plasma membrane but inside the cytoplasm and a membrane domain around the proximal region of the axoneme. (c) A representative TEM image is showed to highlight the shape and organization of the ciliary pocket (CiPo) relative to the axoneme (Ax) and the basal body (BB). The extracellular distal part of the axoneme is stressed by arrowheads. Scale bars: 500 nm in (b), 100 nm in (c). Adapted from figures previously published in (PMID: 20427320).

Fig. 3. Fluorescence microscopy analysis on the localization of clathrin-coated pits (CCPs) at the ciliary pocket in RPE1 cells, which were transiently transfected with the farnesylated green fluorescent protein (GFP-F) encoding plasmid for 24 hours and then incubated 2 minutes at 37°C with Alexa^{555}-conjugated transferrin (Tf). Cells were immediately fixed and processed for immunofluorescence using antibodies against acetylated-α-tubulin (AcTub) to stain the axoneme, and CALM to stain clathrin-coated pits (CCPs). Epifluorescence (a) and 3D reconstruction (b) images of a representative cilium are shown. CiPo-associated CCPs present along the axoneme

(arrows) are stained for both CALM and Tf. GFP-F is prominently targeted to the membrane systems around the cilium and the ciliary pocket. Adapted from figures previously published in (PMID: 20427320).

Fig. 4. Fluorescence microscopy analysis on clathrin-coated pits (CCPs), clathrin-coated vesicles (CCVs) and early endosomes (EEs) at the ciliary pocket region in NT2 cells. NT2 cells were transiently transfected with the plasmid expressing 2xGFP-FYVE followed by pulsing with Texas red-conjugated transferrin (Tf) for 10 minutes. Cells were quickly fixed and prepared for IFM. Following Tf incubation, the endocytic pathway can be visualized. (a) Epifluorescence and 3D reconstruction of Tf localization to 2xGFP-FYVE positive EEs in an interphase cell. (b) 3D reconstruction of accumulation of Tf at EEs around the pocket region of the primary cilium. (c) 3D reconstructions of co-staining with CALM shows that CCVs are localized around the pocket region of the primary cilium. (d) 3D reconstruction shows little or no co-localization between CCVs (CALM) and Tf, which after a 10 minutes pulse primarily localizes to EEs. Similarly, CCPs/CCVs show no or little colocalization with EEs marked 2xGFP-FYVE. Primary cilia were stained with either ARL13b or anti-acetylated α-tubulin (AcTub) antibodies.

Fig. 5. Fluorescence microscopy and 3D reconstruction analysis of the localization of Transforming growth factor beta receptor I (TGFβ-RI) to clathrin-coated vesicles (CCVs) at the pocket region of the primary cilium (stained with anti-acetylated α-tubulin, AcTub) in NT2 cells. Stimulation with TGFβ-1 results in accumulation of the receptor around the base of the cilium as well as along microtubules (MTs) that project from the ciliary base into the cytosol. The right panel shows a merged image exclusively with co-localization between the receptor and Clathrin.

Fig. 6. Live-cell imaging of RPE1 cells were transiently co-transfected with plasmids encoding for Rab8-GFP (green) and clathrin-DsRed (red) fusions and analyzed by fluorescence microscopy. Live cells were imaged at 37°C (one picture for each color every 3 seconds for 4 minutes). The first taken picture is shown (left). An image stream of a clathrin spot (red, arrow) which disappeared during the acquisition is shown (right).The initial position of the followed

clathrin spot is stressed by an arrow. Adapted from figures previously published in (PMID: 20427320).

Figure 1

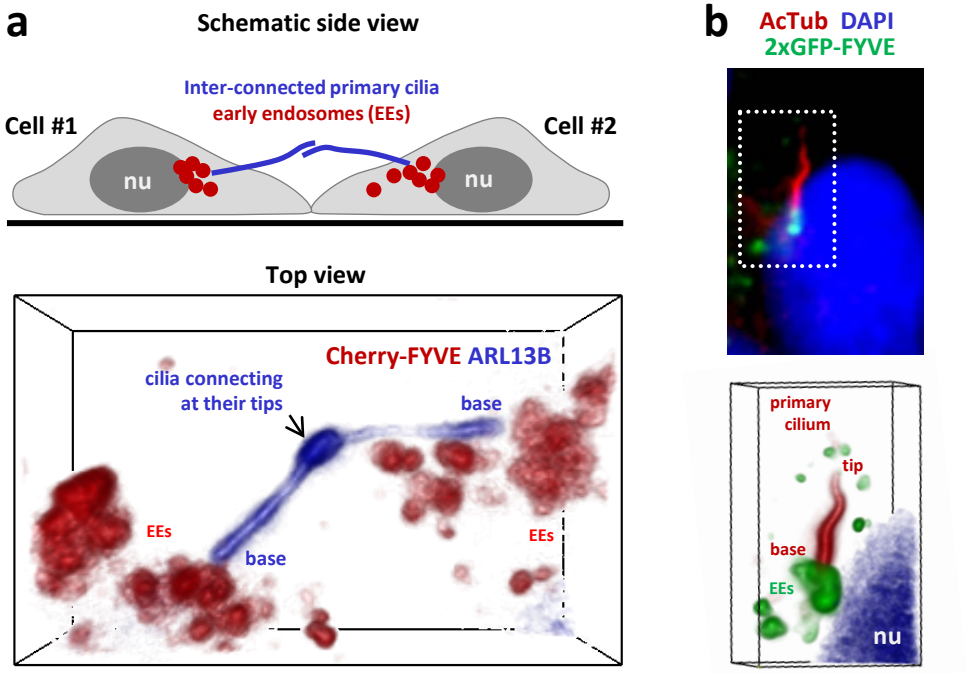
a
Schematic side view
Inter-connected primary cilia
early endosomes (EEs)
Cell #1
Cell #2
nu
nu
Top view
Cherry-FYVE ARL13B
cilia connecting
at their tips
base
EEs
base
EEs
b
AcTub DAPI
2xGFP-FYVE
primary
cilium
tip
base
EEs
nu

Figure 2

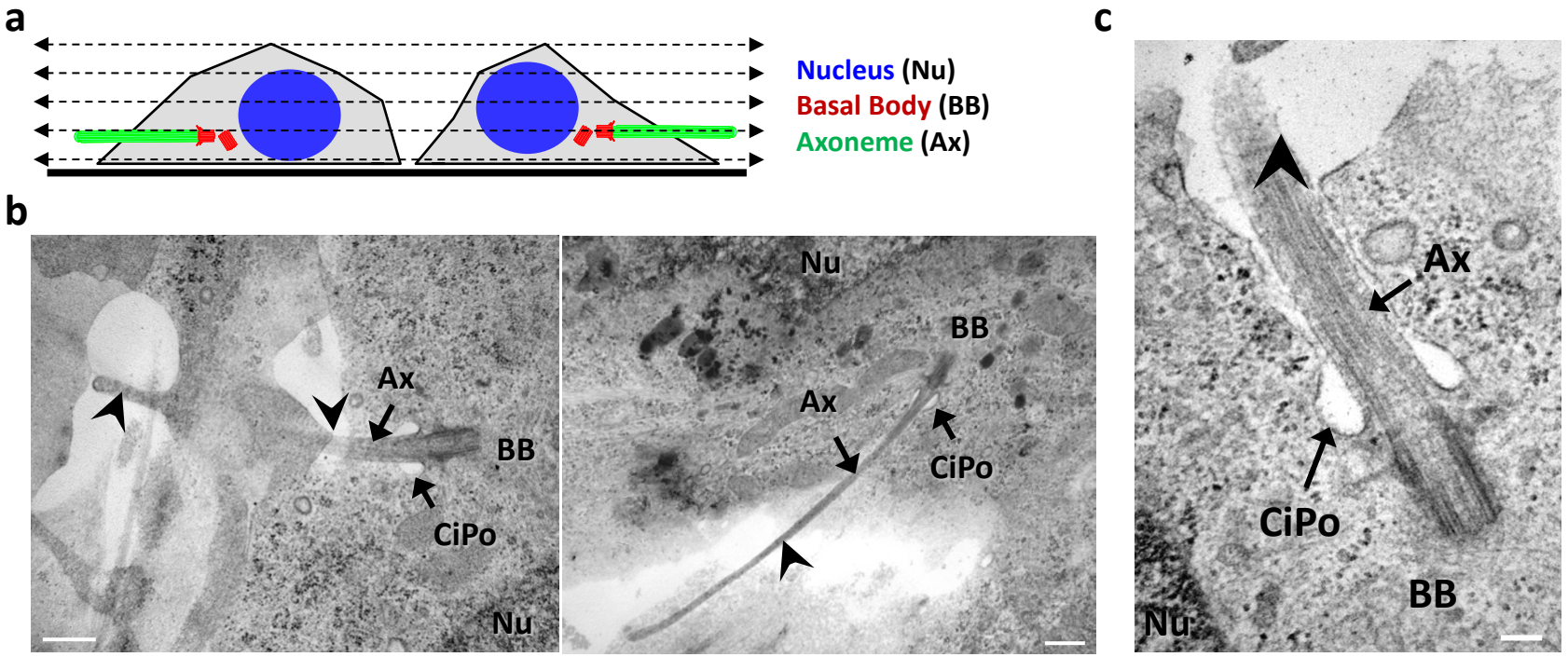
a
Nucleus (Nu)
Basal Body (BB)
Axoneme (Ax)
b
Ax
BB
CiPo
Nu
Nu
BB
Ax
CiPo
c
Ax
CiPo
BB
Nu

Figure 3

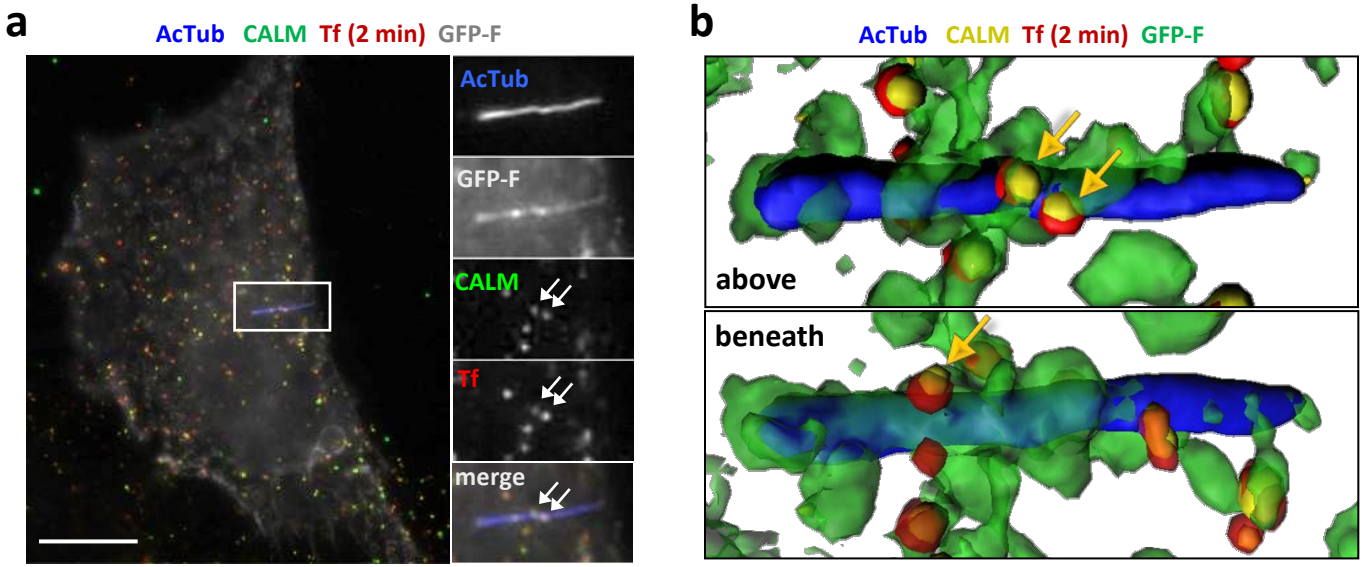
a
AcTub CALM Tf (2 min) GFP-F
AcTub
GFP-F
CALM
Tf
merge
b
AcTub CALM Tf (2 min) GFP-F
above
beneath

Figure 4

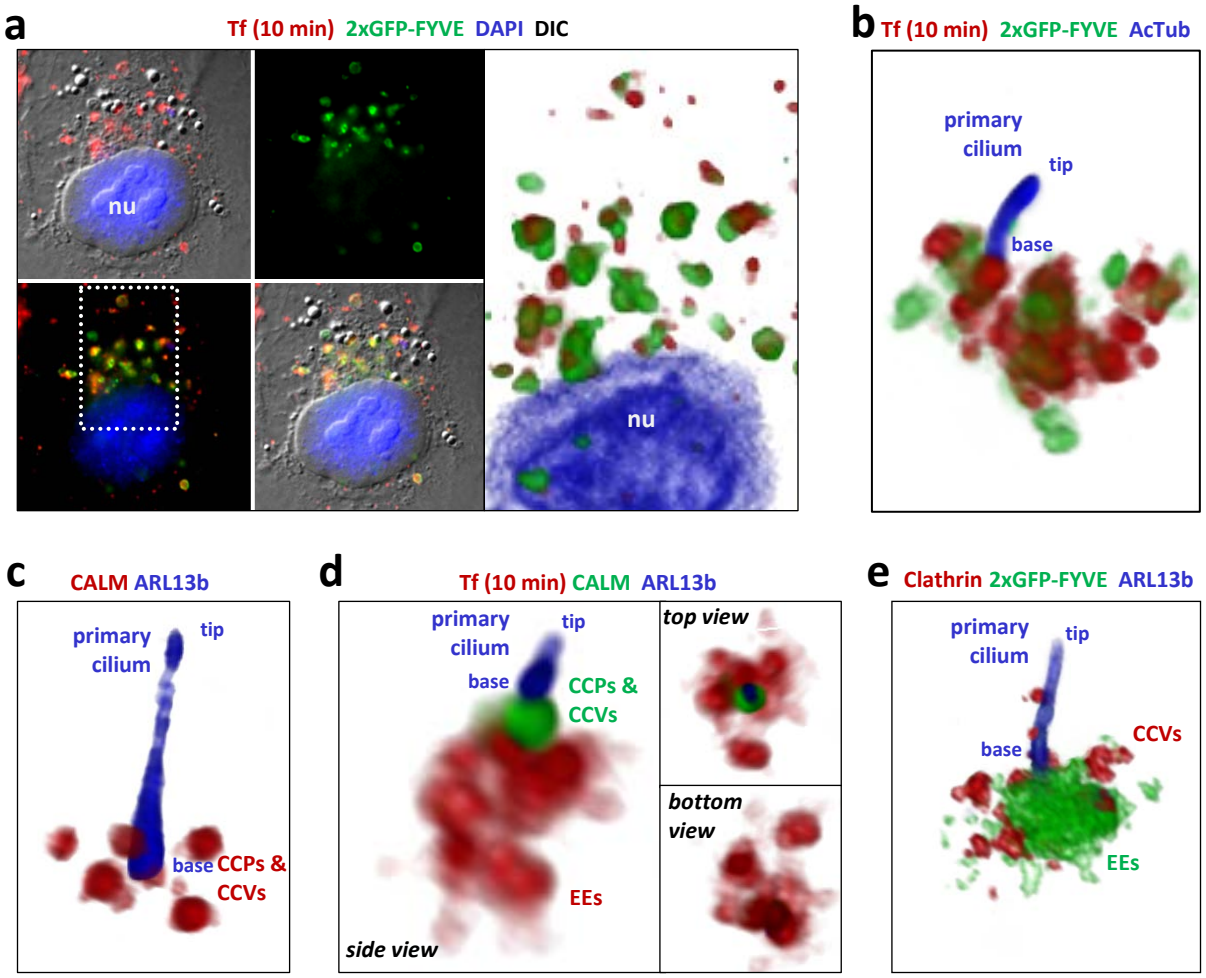
a
Tf (10 min) 2xGFP-FYVE DAPI DIC
nu
nu
b
Tf (10 min) 2xGFP-FYVE AcTub
primary cilium
tip
base
c
CALM ARL13b
primary cilium
tip
base
CCPs & CCVs
d
Tf (10 min) CALM ARL13b
primary cilium
tip
base
CCPs & CCVs
EEs
side view
top view
bottom view
e
Clathrin 2xGFP-FYVE ARL13b
primary cilium
tip
base
CCVs
EEs

Figure 5

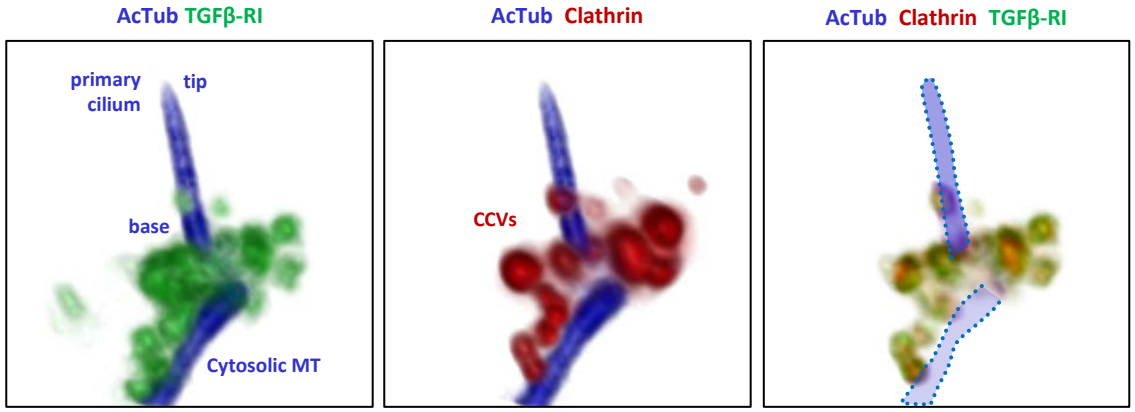
AcTub TGFβ-RI
primary cilium
tip
base
Cytosolic MT
AcTub Clathrin
CCVs
AcTub Clathrin TGFβ-RI

Figure 6