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## Supplemental Information

# Revealing Nanoscale Morphology of the Primary Cilium Using Super-Resolution Fluorescence Microscopy

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## 1. Cell Culture

Mouse embryonic fibroblast (MEF) cells are used for all our samples, which stably express Smoothened proteins with a SNAP tag (SNAP-SMO) and Pericentrin-YFP (PACT-YFP). Cell lines used: (1) **wt**: SMO-/-, SNAP-SMO, PACT-YFP; (2) ***IFT25***: IFT25-/-, SNAP-SMO, PACT-YFP. Cells are cultured in DMEM/High Glucose (Hyclone, SH30243.01) with 10% Fetal Bovine Serum (FBS) (Hyclone, SH30070.03), in 25 $cm^2$ surface area cell culture flasks (Falcon) maintained in an incubator at 37°C and 5% $CO_2$ (ThermoFisher, Heracell 160i). When cells reach 80-90% confluency, cells are detached from the surface using 0.25% Trypsin (Hyclone, SH30042.01) and are pipetted into a 4-well chambered borosilicate coverglass (Fisher Scientific, 12-565-401). Immediately after they are plated, 200 *nm* diameter red fluorescent beads, ex: 580 *nm*, em: 605 *nm* (Invitrogen, F8801) are added to the sample. Cells are cultured in 10% FBS media for 24-48 *hrs* before they are serum starved in 0.5% FBS media for an additional 20-24 *hrs*. (1) **wt** cells are treated with 100 *nM* Smoothened Agonist (SAG) for 4 *hr* at the 20-*hr* mark of serum starvation. A separate chamber containing **wt** cells are treated with 10 *uM* ciliobrevin D for 1 *hr* before the 24-*hr* mark. (2) ***IFT25*** cells are either (a) treated with SAG or (b) none at all (NoAg).

## 2. Sample Preparation (3D SM Microscopy)

Cell samples are first labeled with BG-Alexa647 (NEB, S9136S) at 3 *uM* concentration for 20 *min* at 37°C and 5% $CO_2$. Each chamber of cells is washed with 0.5% FBS three times at 5-*min* intervals. Cells are fixed using 4% paraformaldehyde (PFA) (Alfa Aesar, 43368) for 15 *min* and then treated with a quenching solution of 10 *mM* $NH_4Cl$ for 10 *min*, both steps at 25°C. The samples are washed with PBS, pH 7.4 (1X) (Gibco, 1789842) at 5-*min* intervals and then stored at 4°C up to 1-week before being discarded.

## 3. 3D SM Microscopy Setup

Experiments are performed on a customized inverted microscope (Olympus, IX71) where the sample is mounted on a piezo-electric stage (PI-Nano) and is in contact with an oil-immersion objective (Olympus, 100x, 1.4 NA, UPLANSAPO) applied with a small drop of oil (Immersol, 12-624-66A) before mounting. New imaging buffer is added for each primary cilium imaged (1-2 *hrs*), which consists of glucose oxidase (Sigma-Aldrich, G2133), catalase from bovine serum (Sigma-Aldrich, C100), 100 mM Tris-HCl, pH 8.0 (ThermoFisher Scientific, 15568025), 10% (w/v) glucose solution (Sigma-Aldrich, 49139), 140 *mM* beta-mercaptoethanol (Sigma-Aldrich, M6250), and $H_2O$ (Nanopure). We locate one primary cilium and image SNAP-SMO-Alexa647 and PACT-YFP using the 641 *nm* (Coherent Cube, 100 *mW*) and 514 *nm* (Coherent Sapphire, 50 *mW*) laser, respectively. Fluorescence (emission) is collected through the objective, a dichroic filter (Semrock, FF425/532/656-Di01), and two bandpass filters (Chroma, 680-60; Chroma, 655LP). When the primary cilium is placed at the center of the field-of-view (FOV) and is in focus, the double-helix (DH) phase mask is carefully placed at the Fourier Plane (FP) in our *4f* system, with our lenses each having a focal length, $f = 90\,mm$. We increase the intensity of the 641 *nm* laser (1-5 $kW/cm^2$) and allow the fluorescent dye to bleach down to the single-molecule regime. Over the next hour, we gradually increase the 405 *nm* (Obis, 100 *mW*) laser intensity until either 1 *hr* has passed or single-molecule blinking becomes extremely sparse. Red fluorescent beads are also imaged simultaneously several microns away from the PC. Detected fluorescence is recorded using a silicon EMCCD camera (Andor Xion, DU-897U-CS0-#BV) at a speed of 14-20 frames/second (50-70 *ms*/frame) with an electron-multiplying gain of 200.

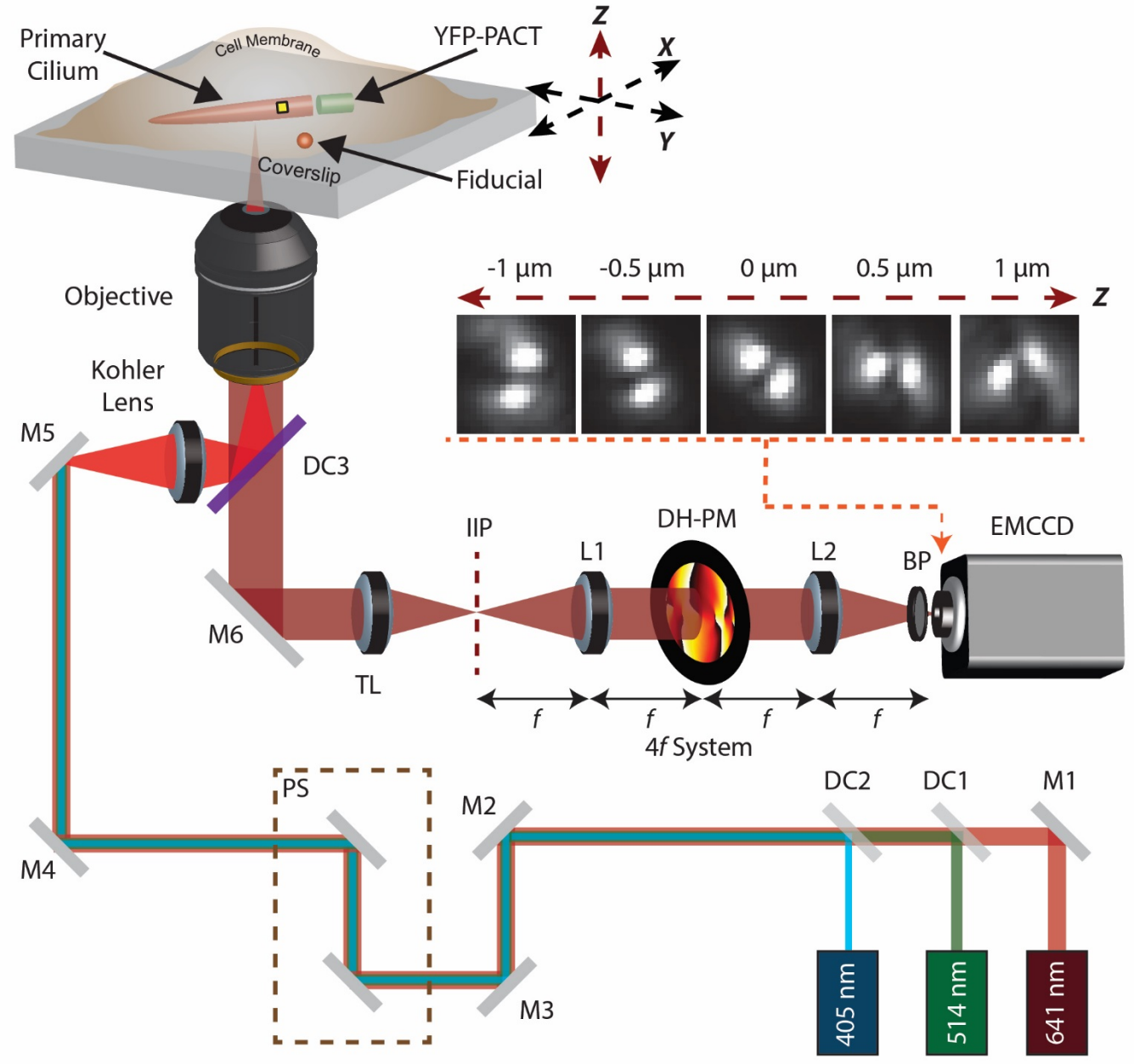


**Figure S1:** 3D SR Microscopy setup with the Double-Helix Phase Mask (DH-PM). Lasers go through a series of mirrors (M), dichroic mirrors (DC), and a periscope (PS), before it passes through the Kohler lens and the objective. The sample consists of a primary cilium that is sandwiched between the attached cell and the coverslip surface, and a nearby stationary fiducial. Emission is collected with the objective which then goes through the tube lens (TL) and rather than placing the camera at the intermediate image plane (IIP), the *4f* system is implemented, where the double-helix phase-mask (DH-PM) is placed equidistant between two *4f* lenses (L). A bandpass filter (BP) is placed right in front of the camera (EMCCD) which is used to detect the emitted fluorescence.

## 4. Z Calibration with Fiducials

DHPSF calibrations are done using two different beads: (1) 625/645 *nm*, which is for the SNAP-SMO, and (2) 580/605 *nm*, which is for fiducials in our sample, correcting for sample drift. Calibration imaging samples are prepared by spin-coating the beads in 1% polyvinyl alcohol (PVA) onto a glass coverslip. Samples are then mounted onto the 3D SR microscopy imaging setup with the DH-PM placed at the FP. Using our piezo-electric stage, we scan over a 3 $\mu m$ range along the *z* axis with a 50 *nm* step-size with 30 frames measured at each *z*-height. We perform a forward and backward scan to account for hysteresis during the *z*-scan. This calibration step also produces template images of the DH-PSF which are used for the identification of single-molecule signals during post-processing of the raw data. All imaging is done at 25°C.

## 5. 3D Localization of SNAP-SMO Molecules

Using the *easyDHPSF* MATLAB program (1), a *z*-axis calibration over a 3-$\mu m$ range is obtained via a 2D Double-Gaussian fit, which provides us with *xy* positions, width, amplitudes, and offset levels of each lobe of the fluorescent bead. Collected raw data is used to calculate the phase-correlation to locate single-molecules in the FOV and is ultimately fitted using the same model. An array of 3D positions is obtained, including information on photon counts, background counts, and other parameters relevant to our fits.

## 6. *In-situ* Localization Precision Calculations

For one 3D localization dataset from one primary cilium, we choose one localization of interest and pool in any other localizations that are both (a) within the next 10 frames and (b) within a ~74 *nm* 3D radius ($\sqrt{(30\,nm)^2 + (30\,nm)^2 + (60\,nm)^2}$). Each cluster of localizations has a minimum of 5 frames that meet our criteria. For calculating the localization precision, we calculate the standard deviation for each set of x, y, and z positions. Average signal and background photons is calculated by evaluating the mean over all localizations within each cluster.

## 7. 2D Surface Meshing

Scatterplots are prepared in the following way. We first select a few points near the base of the cilium, perform an elliptic fit, which also defines a plane of rotation, and then produce a direct copy of the original scatter plot which is rotated 180°. This step is important for creating a closed surface and to minimize artifacts near the base when performing our final curvature analysis. This new scatterplot is then used to extract the two-dimensional surface using MeshLab. MeshLab uses surface reconstruction methods to approximate the surface of an input point cloud. This program first calculates the normal vector of each point using a range of 30-200 neighboring points, and the actual number of neighbors varies depending on how many localizations there are in the point cloud and user-selected parameters. Then, a Poisson Surface Reconstruction algorithm is applied to the processed data in order to create a

triangulated surface (Octree Depth = 13; Solver Divide = 6; Samples per Node = 5; Surface Offsetting = None). The Octree Depth defines the number of times the voxel space is divided by 2 during the estimation process and the Samples per Node defines the minimum number of samples needed for each node, which may be viewed as a parameter which limits the mesh resolution. Gaussian curvature is calculated to highlight areas of both positive and negative curvature. This data is then exported in the standard '*.ply' file format which is used to plot our 3D mesh and further curvature analysis.

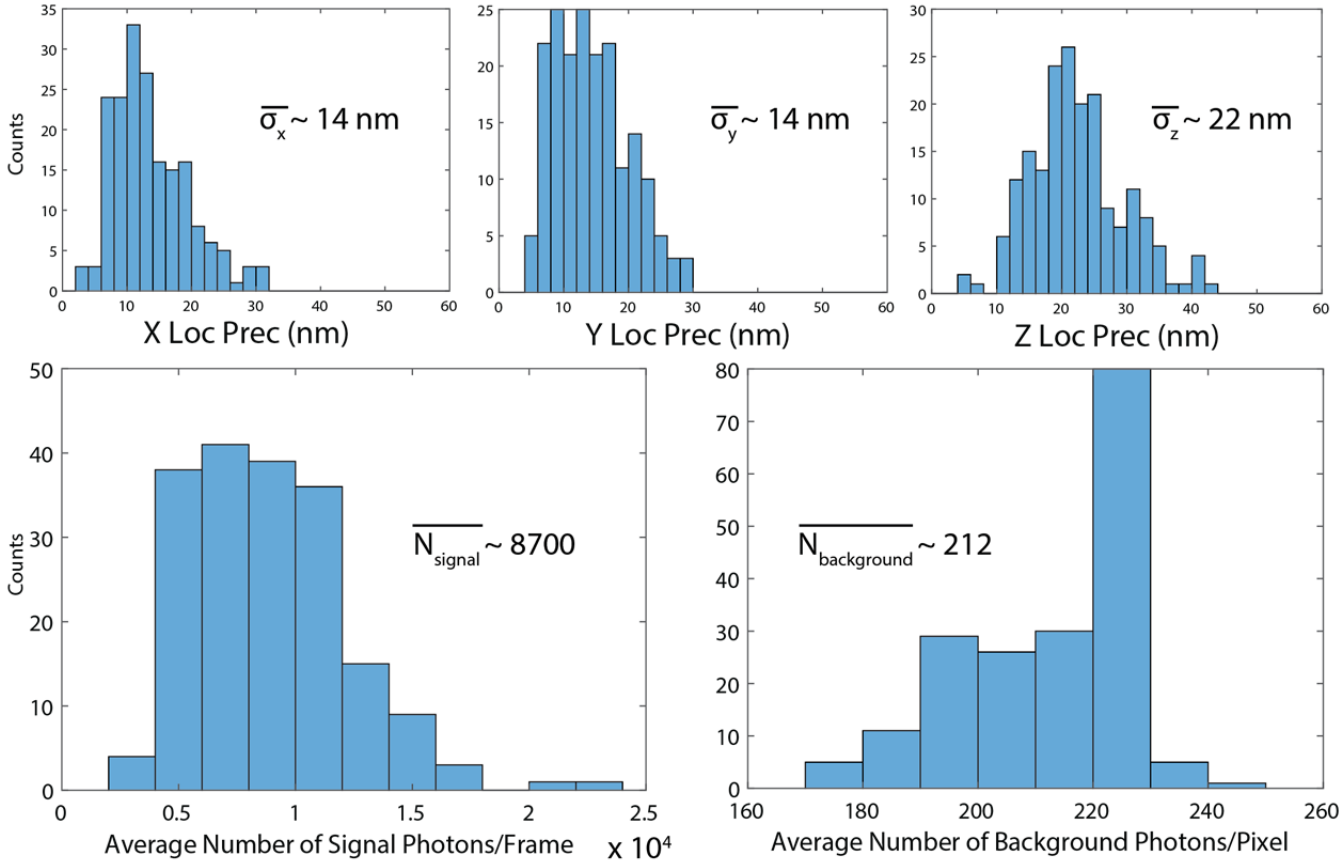


**Figure S2**: Localization precision measurements for x, y, and z with average number of signal and background photons per frame.

## 8. 3D-Printed Meshes

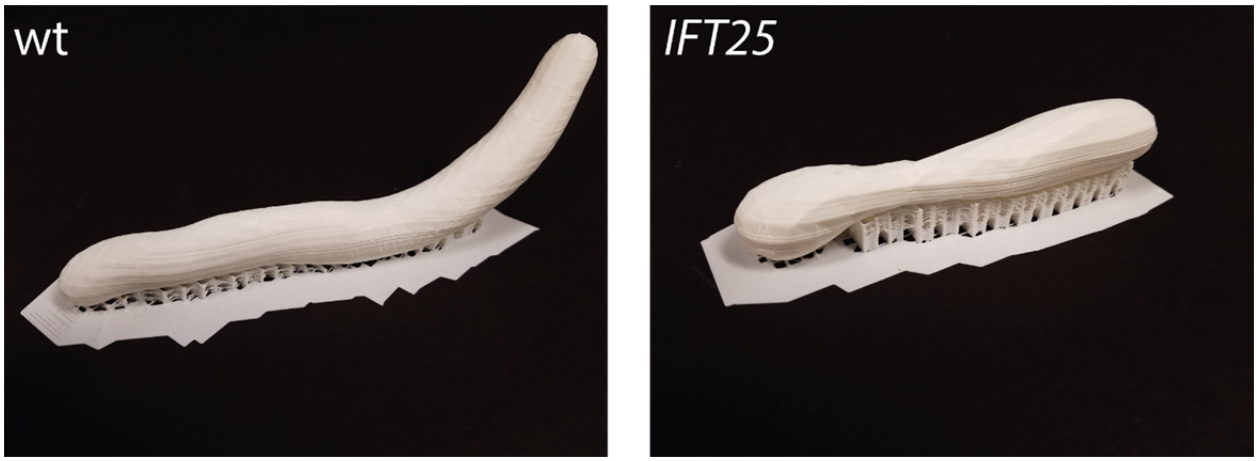


**Figure S3**: 3D printed cilia using a MeshLab-created .STL file which lists the *x*,*y*,*z* coordinates of the vertices on the mesh. An Ultimaker-2 printer using PLA filament and an infill setting of 25% printed the model. Printing was performed at the Atherton public library in San Mateo County.

## 9. Curvature Analysis

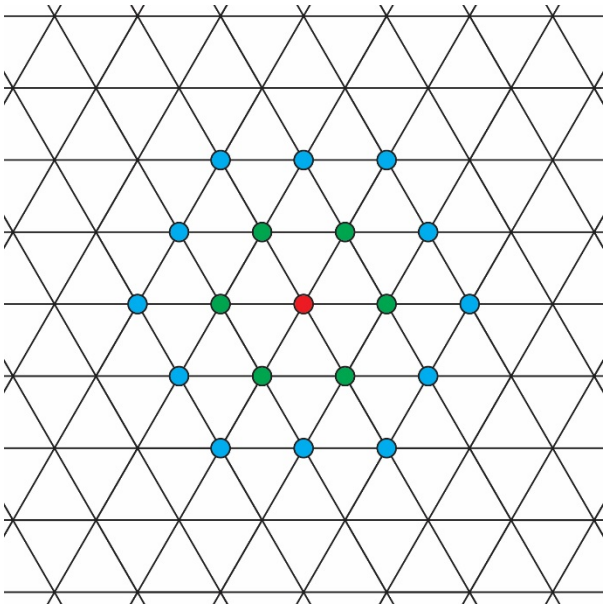

**Figure S4**: Illustration of triangular mesh with 1st (green) and 2nd (blue) order neighboring points to the red point.

We first choose a vertex along the mesh and search for its 1st & 2nd order nearest neighbors, which all make up a "patch" of triangles. Within each patch, we calculate the normal vector for every triangle and the angle at the vertex of interest. We determine the overall weighted normal vector of the patch and is used to rotate all points such that this vector is oriented along the *z*-axis. We then fit a surface to our points of the following form:

$$z = f(x, y) = ax^2 + bxy + cy^2 + dx + ey$$

If we further define $p = [a\ b\ c\ d\ e]$, $A = [x^2\ xy\ y^2\ x\ y]$, we solve for $p$ by evaluating the following:

$$Ap = z \rightarrow p = (A^T A)^{-1} A^T z$$

Using the coefficients in $p$, we then calculate the Mean Curvature (H) and Gaussian Curvature (K) using the following expressions [46]:

$$H = \frac{a + c + ae^2 + cd^2 - bde}{(1 + d^2 + e^2)^{\frac{3}{2}}}, \qquad K = \frac{4ac - b^2}{(1 + d^2 + e^2)^2}$$

This was done for every vertex and are represented as heat maps, interpolated along the mesh. We calculate the Willmore Energy by calculating the following

$$W_E = \sum_i a_i ({H_i}^2 - K_i)$$

where $H_i$, $K_i$, and $a_i$ are the mean curvature, Gaussian curvature, and surface area of the $i^{th}$ triangle of the mesh respectively.

## 10. Ciliary Length and Surface Area

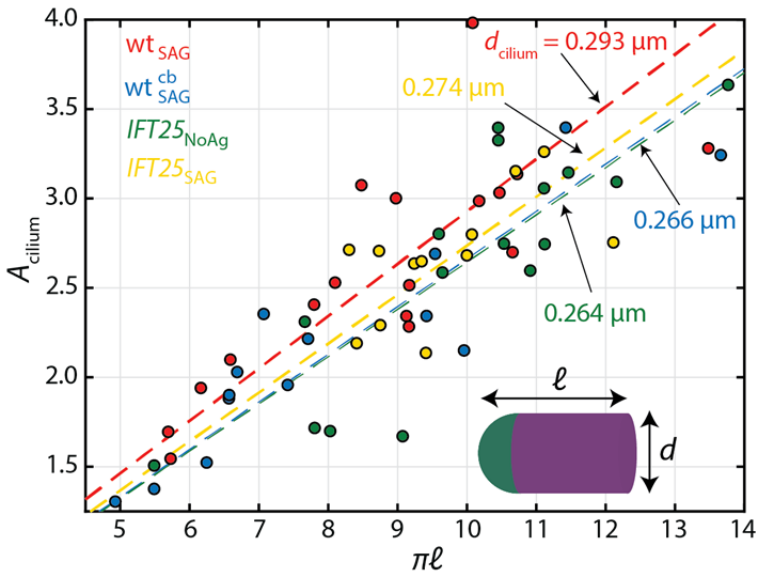


**Figure S5**: **Ciliary length and surface area for each condition, cell by cell**. The total surface area of the cilium, $A_{cilium}$, is calculated by adding up the areas of all the triangles of the ciliary mesh. The ciliary length, $l$, is calculated as the length of the ciliary axis from the base to the tip. The population diameter, $d_{cilium}$, is estimated by the output slope of a linear regression performed for each data set. Here we are making an assumption that the cilium takes on the approximate shape of a cylinder with a hemispheric cap, as shown in the figure inset on the bottom-right corner.

For values of the diameter for each cell, $d$, in Table 1, the following derivation and calculation was utilized:

$$\begin{aligned} A_{total\ surface} &= A_{hemispheric\ cap} + A_{cylinder} \\ &= 2\pi r^2 + 2\pi r(l - r) \\ &= 2\pi rl = \pi dl \\ \rightarrow d &= A_{total\ surface}/\pi l \end{aligned}$$

## 11. 2-color 2D STED Microscopy

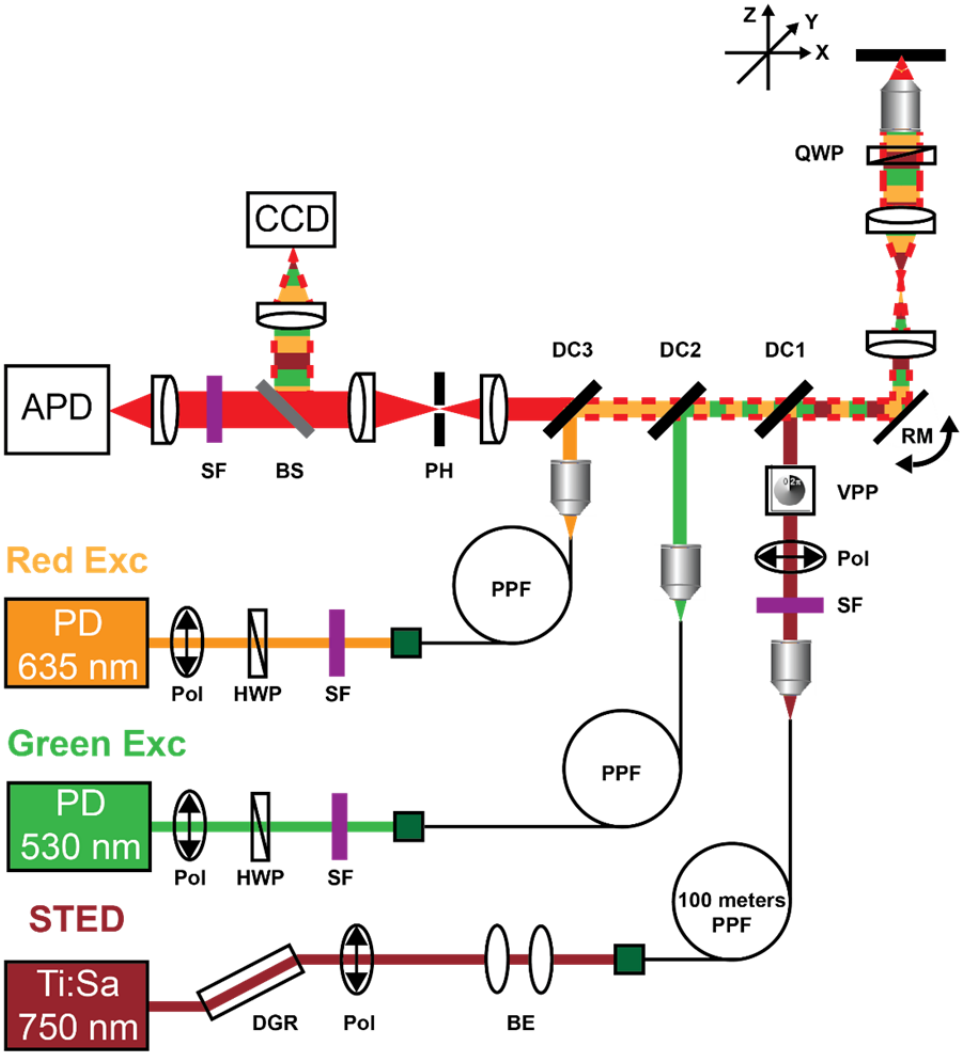


**Figure S6: 2D STED Microscopy Schematic**. Lasers are sent through a cleanup polarizer (Pol) and spectral filter (SF). The two excitation lasers' polarization are rotated by half-wave plates (HWP) prior to coupling into a polarization preserving fiber (PPF). The depletion laser pulse is temporally stretched by a dispersive glass rod (DGR) before the beam is expanded by a beam expander (BE) and is further temporally stretched by 100 meters of PPF. The fiber outputs are collimated using objective lenses. The depletion beam is sent through another Pol and the donut shape is imparted by a vortex phase plate (VPP). All three beams are coupled by a series of dichroics (DC), and are scanned by a resonant mirror (RM), made conjugate to the back focal plane of the imaging objective by a telescope lens pair. The beams are made circularly polarized by a quarter-wave plate (QWP). Emission is collected with the

imaging objective, is de-scanned by the same RM, and passes through the DCs. It passes through a confocal pinhole (PH), is filtered by a SF, which changes depending on the color channel, and is detected on an avalanche photodiode (APD). A beam splitter (BS) is used to image a small fraction of reflected/emitted fluorescence onto a camera (CCD) for alignment purposes.

STED images were collected on a bespoke 2-color fast scanning STED microscope (Figure S6). The 750 *nm* depletion laser is provided by a titanium-sapphire mode-locked oscillator operating at 80 MHz (Mira 900D, Coherent). The pulses are dispersed to ~200 *ps* in duration using 30 *cm* of SF2 glass, 10 *cm* of SF6 glass, and 100 *m* of polarization maintaining optical fiber (OZ Optics). The pulses are spectrally filtered (FF01-715/LP, Semrock) and the donut shape is created using a vortex phase-plate (RPC Photonics). The excitation pulses are provided by 530 *nm* and 635 *nm* pulsed diode lasers (LDH-P-FA-530B & LDH-P-C-635B, PicoQuant) that are electronically triggered to arrive prior to the depletion pulses. The excitation beams are spatially filtered by polarization maintaining fibers (Thorlabs), and combined using a 532 *nm* longpass and 514/640 *nm* notch dichroic (ZT532RDC & ZT514/640RPC, Chroma). The excitation beams are combined with the depletion beam by a 5 *mm* thick 710 *nm* shortpass dichroic (Z710SPRDC, Chroma). The beams enter the back-port of a Nikon TE300 inverted microscope and are converted to circularly polarized light using a quarter-wave plate (767 *nm* zero-order, Tower Optical). The light is focused through an oil immersion objective (Plan Fluor 100x/1.3 NA, Nikon). At the sample plane the green and red excitation beams have an average power of 40-60 $kW/cm^2$ and 50-80 $kW/cm^2$, respectively. The depletion beam has an average power of 120-130 $MW/cm^2$. The fast axis is scanned using a 7.5 kHz resonant mirror (Electro-Optical Products) that is imaged onto the back focal plane of the objective using a Keplarian telescope. The slow axis is scanned using a piezo stage (PD1375, Mad City Labs). Fluorescence is detected through the same objective, is de-scanned by the same resonant mirror, and passes through the previously mentioned dichroics. An aperture corresponding to a ~0.7 AU and 0.8 AU (red and green channels respectively). The fluorescence is then spectrally filtered by a 715 *nm* shortpass (FF01-715/SP, Semrock) followed by either a 635 *nm* longpass (BLP01-635R, Semrock) or a bandpass (ET585/65m, Chroma) for the red and green channels respectively. Fluorescence is detected on a Si APD detector (SPCM-ARQH-13, Perkin Elmer). Scan control and image acquisition use a custom LabVIEW algorithm running on an FPGA (PCIe-7842R, National Instruments) and host computer. STED images have a pixel size of 20 *nm*. Images in the red channel are obtained as a single frame with 1000 resonant mirror scans per line or an average pixel dwell time of ~0.1 *ms*/pixel. Images in the green channel are obtained as 2-3 frames with 200 resonant mirror scans per line for each frame or an average pixel dwell time of ~20 *μs*/pixel/frame.

## 12. Sample Preparation (2D STED)

Cell samples are fixed with 4% PFA for 15 *min* at 25°C, washed with 1x PBS, then immersed in a blocking solution, consisting of 1% TritonX-100, Normal Donkey Serum (Jackson ImmunoResearch, 017-000-121), and 1x PBS, for 30 *min* at 25°C. We stain our samples with primary antibody for 1 *hr*, consisting of anti Smo-C and anti AlphaTubulin (Sigma-Aldrich, T6199) in blocking solution. Samples are then washed 3x with 1x PBS at 5-*min* intervals, then stain our samples with secondary antibody for 1 *hr*, consisting of goat anti-rabbit atto647N (Active Motif, 15048) and goat anti-mouse Star520SXP (Abberior, 2-0002-009-9) in blocking solution. Samples are then washed 3x with 1x PBS at 5-*min* intervals then are either immediately imaged or are stored at 4°C for up to 1 week.

## 13. 2D STED Resolution

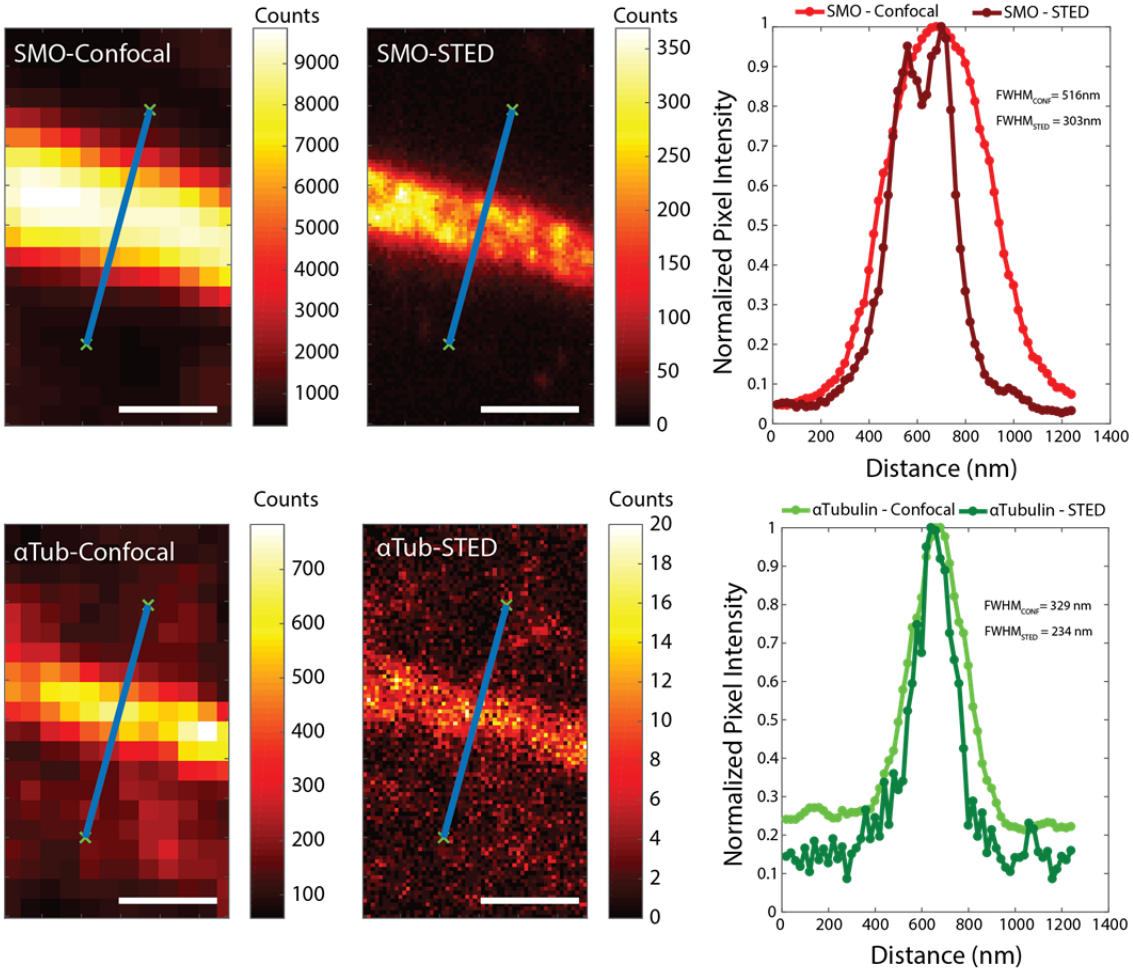


**Figure S7:** Comparing confocal and STED image line profiles for one primary cilium in both color channels for SMO (upper) and α-tubulin (lower). Line profiles were generated by extracting signal counts that reside in 20 nm bins underneath the drawn line for both channels.

## 14. Additional 2D STED Images of Unusual Primary Cilia

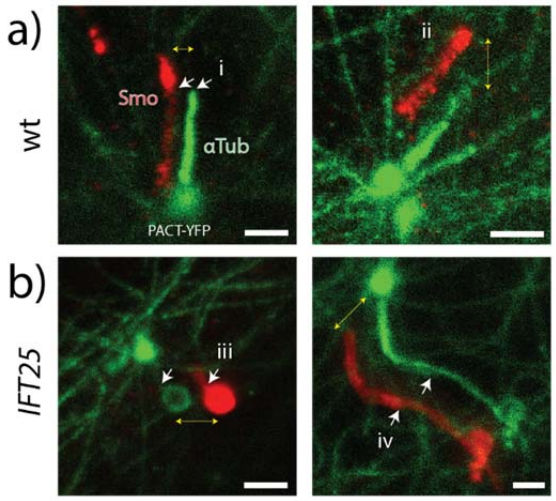


**Figure S8: Additional 2D STED images of unusual primary cilia**. (a) Control MEF cells also exhibit (i,ii) bulging at the tip at times, where the axoneme does not extend to the tip. (b) Some cases of *IFT25* mutant cells produce oddly-formed cilia which are (iii) very short with a circular tip or (iv) very long with a narrowing along the shaft of the cilium with a bulging tip

## 15. Membrane/Axoneme Diameter Calculations

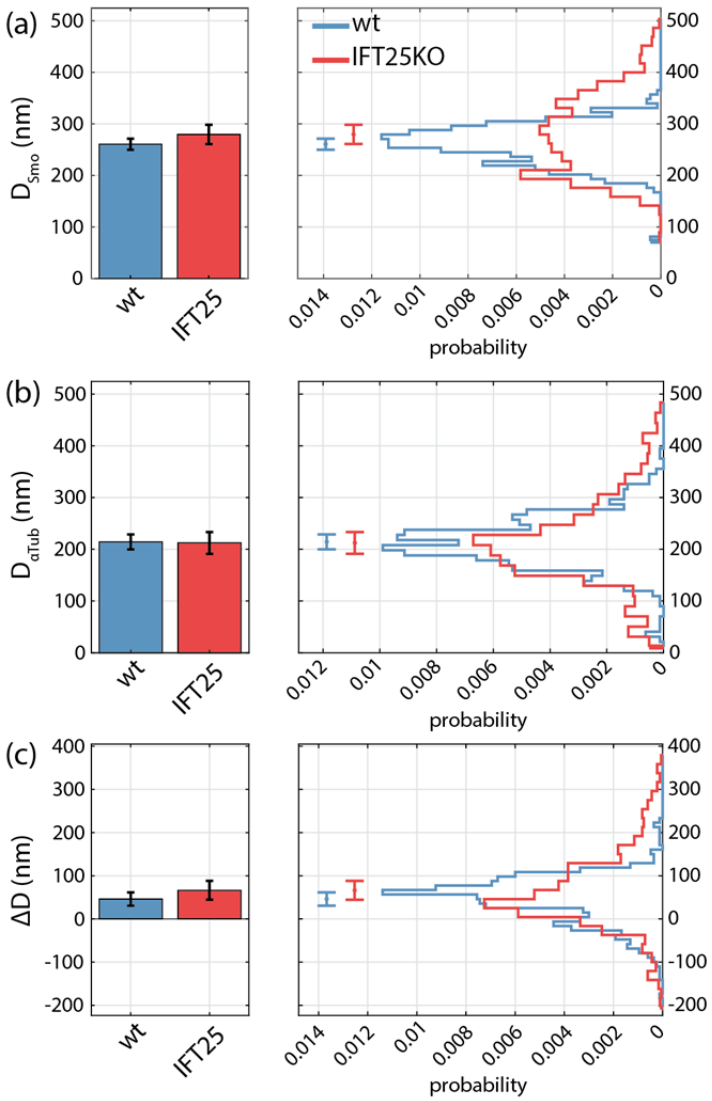


**Figure S9**: Distributions of diameter measurements using 2D STED. Histograms and bar plots of (a) SNAP-SMO ($D_{Smo}$), (b) α-tubulin ($D_{\alpha Tub}$), and (c) difference ($D_{Smo} - D_{\alpha Tub}$). There is ~18% and 24% difference between SNAP-SMO and α-tubulin for *wt* and *IFT25* mutant cells, respectively. Reporting mean ± S.E.M ($N_{wt,cilia} = 12$, $N_{wt,profiles} = 801$, $N_{IFT25,cilia} = 15$, $N_{IFT25,profiles} = 993$).

2D STED SNAP-SMO images are used to determine the ciliary axis, which is done by performing a spline fit to a set of user-selected input points chosen along the center of the cilium by eye. The intensity line profile, 600 *nm* long with a bin width of 20 nm perpendicular to the ciliary axis is determined at 50-

*nm* long steps along the ciliary axis for both channels, resulting in a representation of the lateral extent of the cilium at each position. A 1D double-Gaussian fit to each profile is performed of the following form:

$$f(x) = A * exp\left(-\frac{(x-x_{01})^2}{2\sigma_1^2}\right) + B * \exp\left(-\frac{(x-x_{02})^2}{2\sigma_2^2}\right) + C$$

where A,B are the amplitudes, C is the constant background, $x_{01}, x_{02}$ are the centroids, $\sigma_1, \sigma_2$ are the standard deviations. The full-width half-maximum (FWHM) is calculated for each profile, $FWHM = x1_{f(x1)=1/2} - x2_{f(x2)=1/2}$, which we define in this study as the diameter for the SMO-determined membrane and the axoneme.

## 16. Supplementary Movies

**Movie 1: Control wt MEF primary cilium 3D mesh**

Camera flythrough, which starts above the mesh, then follows along the ciliary axis, and finally pans away. This is the same mesh as shown in Figure 2a. White bounding box dimensions, 2750 *nm* x 1500 *nm* x 1500 *nm*; video speed, 30 *frames/sec*. The HSV color scaling represents *z* in the image over a range from -175.8 to 1194.5 *nm*.

**Movie 2**: ***IFT25* mutant MEF primary cilium 3D mesh**

Camera flythrough, which starts above the mesh, then follows along the ciliary axis, and finally pans away. This is the same mesh as shown in Figure 2b. White bounding box dimensions, 3700 *nm* x 900 *nm* x 600 *nm*; video speed, 30 *frames/sec*. The HSV color scaling represents *z* in the image over a range from -196.4 to 227.1 *nm*.

## 17. Supporting References

(1) Lew, M. D., A.R.S. von Diezmann and W.E. Moerner 2013. Easy-DHPSF open-source software for three-dimensional localization of single molecules with precision beyond the optical diffraction limit. Protocol Exchange, doi:10.1038/protex.2013.026.