3D single molecule tracking with multifocal plane microscopy reveals rapid intercellular transferrin transport at epithelial cell barriers

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Supplementary table and figures

SUPPLEMENTARY TABLE 1: Classification of exocytic and endocytic events.

	Orthogonal mode	Sliding mode
Endocytosis – short residence time	17	11
Endocytosis – long residence time	23	24
Total # of endocytosis events	40	35
Exocytosis – short residence time	15	13
Exocytosis – long residence time	7	3
Total # of exocytosis events	22	16

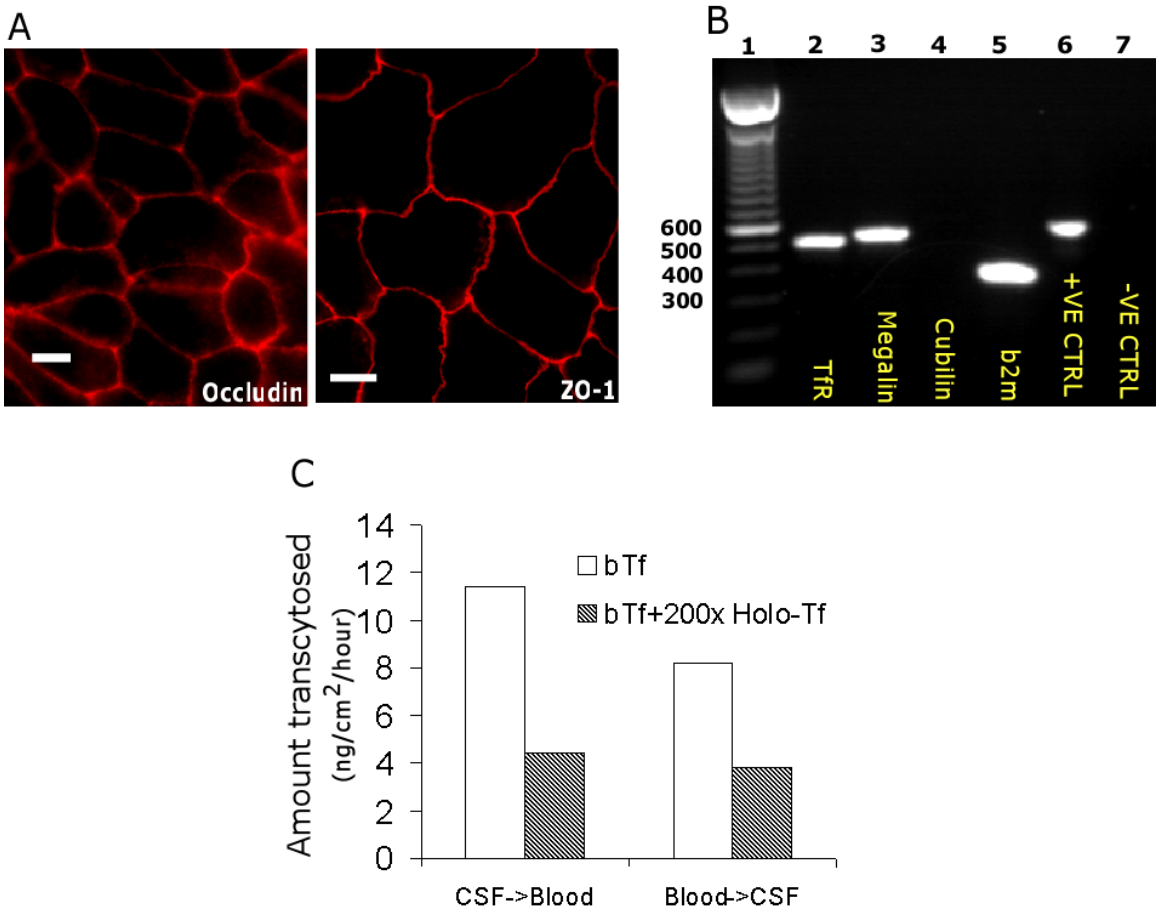
Table S1: Classification of exocytic and endocytic events. The table lists the number of different types of endocytic and exocytic events observed in this study. Endo/exocytic events with short residence times are part of the intercellular transfer events.

Supplementary Figure 1: In vitro BCSFB model.

A) Tight junction staining. Z310 cells were grown on glass cover slips as a monolayer and stained for tight junction proteins occludin and ZO-1. Scale bar = 10 μm.

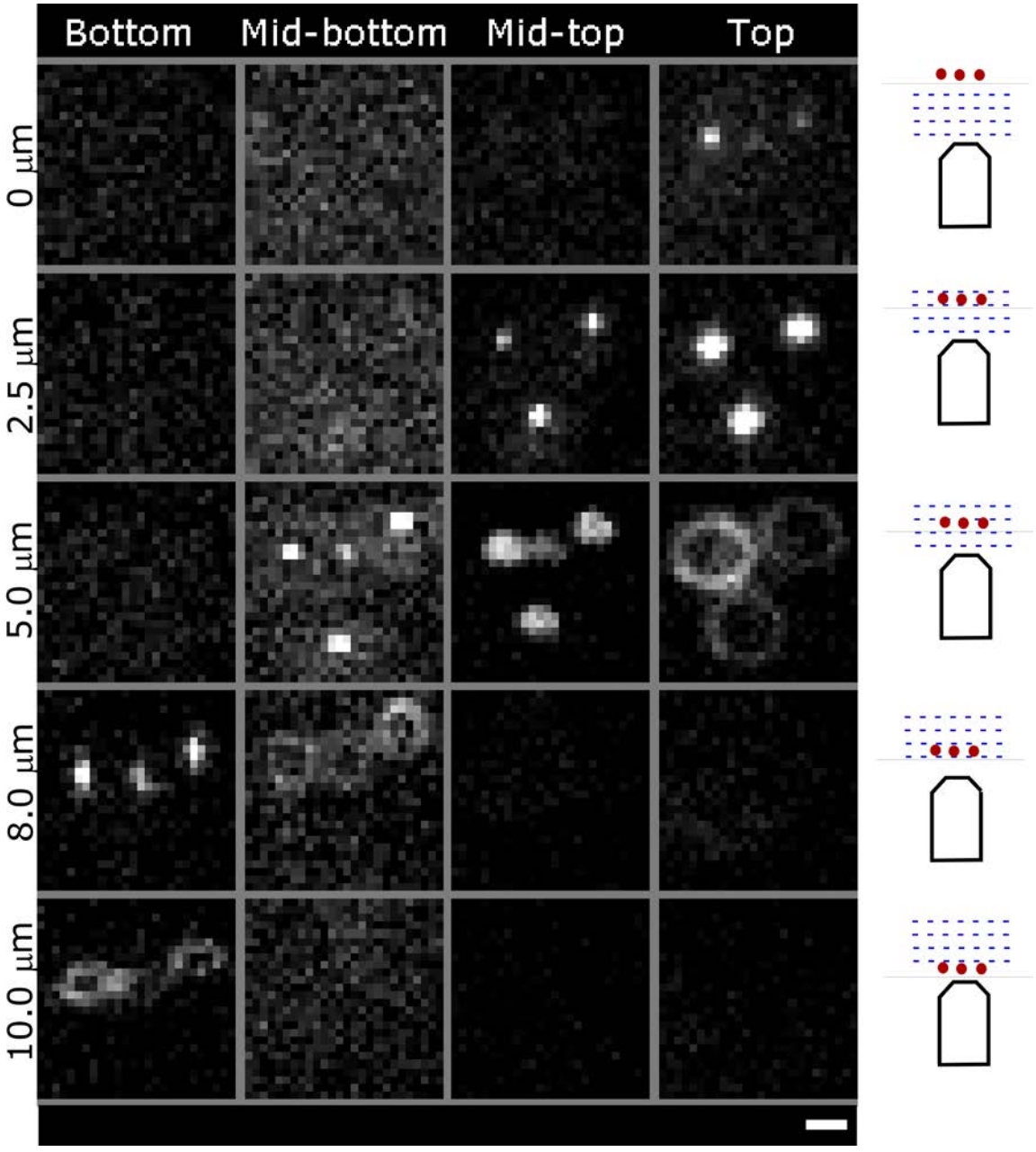
B) Results of RT-PCR experiment with Z310 mRNA. Transcripts encoding rat TfR (lane 2), rat megalin (lane 3), rat $\beta_2$microglobulin (lane 5 – RT positive control), but not rat cubilin (lane 4) were detected. Lane 1 is the 100 bp DNA ladder, and lanes 6 and 7 indicate positive control and negative control, respectively, for the PCR step.

C) Results of Tf transcytosis with filter grown Z310 cell-monolayers. Transport of biotinylated Tf (bTf) is observed in both directions across the Z310 cell monolayer, which is reduced in the presence of excess unlabeled holo-Tf thereby indicating that Tf transcytosis is TfR-mediated. This is consistent with prior reports on Tf transport at the BBB/BCSFB [28,29]. The results are representative of three independent experiments.



Supplementary Figure 2: QD imaging across a depth of 10 microns.

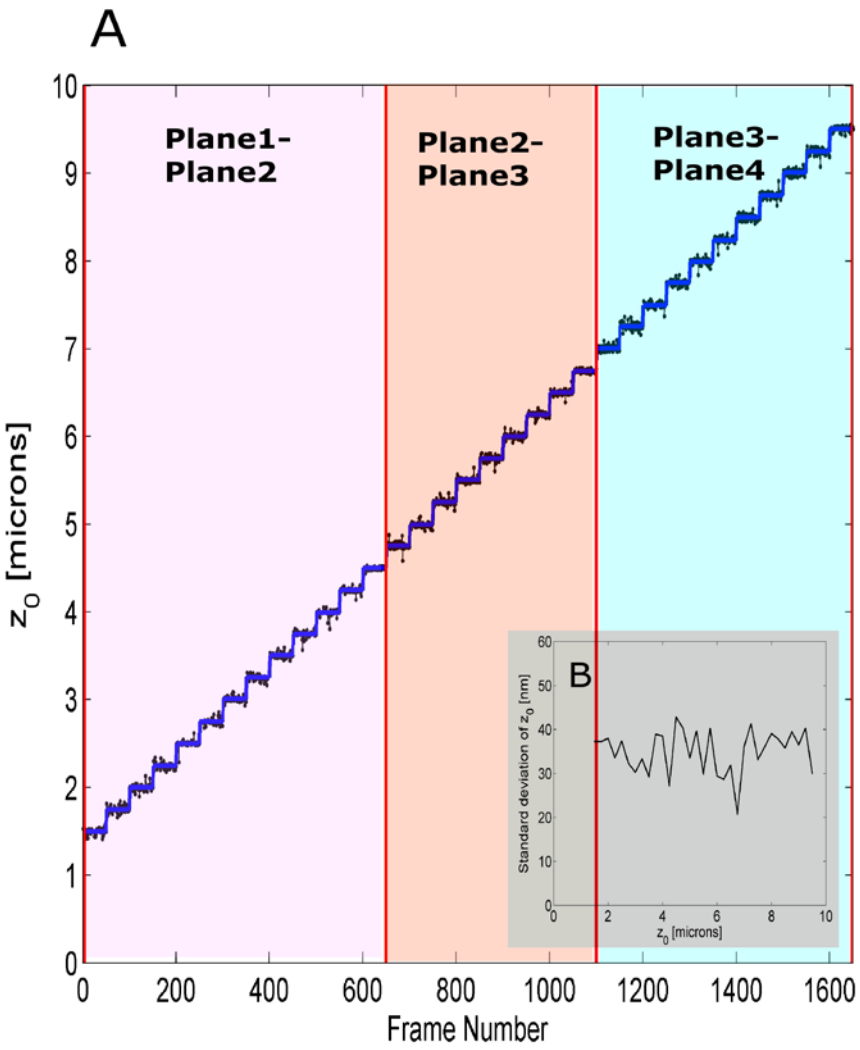
The figure-montage illustrates the effective imaging depth of the 4-plane MUM setup, which was determined by carrying out a z-stack imaging experiment. The individual images are taken from movie 1. The schematic that is shown to the right of each montage indicates the approximate location of the four focal planes relative to the sample for that montage. Scale bar = 2 μm.



Supplementary Figure 3: Experimental demonstration of MUMLA at extended depths.

Panel A shows experimental verification of MUMLA for depths of up to 10 microns. Images of a point source for a four plane MUM setup with a 40x, 1.4 NA objective lens were generated as described previously[15] with some modifications. Specifically we have used the Gibson and Lanni model[25] of the 3D point spread function and have considered non-design imaging conditions. The X-Y location of the point source was fixed to be at the center of the image array and the z-location was varied in steps of 250 nm. For each z-position, 50 images were generated and the position of the point source was then determined using MUMLA. The numerical values used to generate the images were as follows: A = 1500 photons/s per plane, t = 1 s, λ = 0.655 μm, n_s = 1.38, $t_g = t_{gd}$ = 0.17 mm, $n_g = n_{gd}$ = 1.515, t_{oil} = 0.208 mm, t_{oild} = 0.210 mm, z_{fd} = 160 mm, z_f = 152.5, 155 or 157.5 mm. image array size = [13,13], pixel size = 12.9 x 12.9 μm, background = 100 photons/pixel/s, readout noise mean = 0 e^-/pixel and readout noise variance = 64 e^-/pixel. The inset (panel B) shows the standard deviation of the z-estimates obtained using MUMLA.

Figure S3 contd…

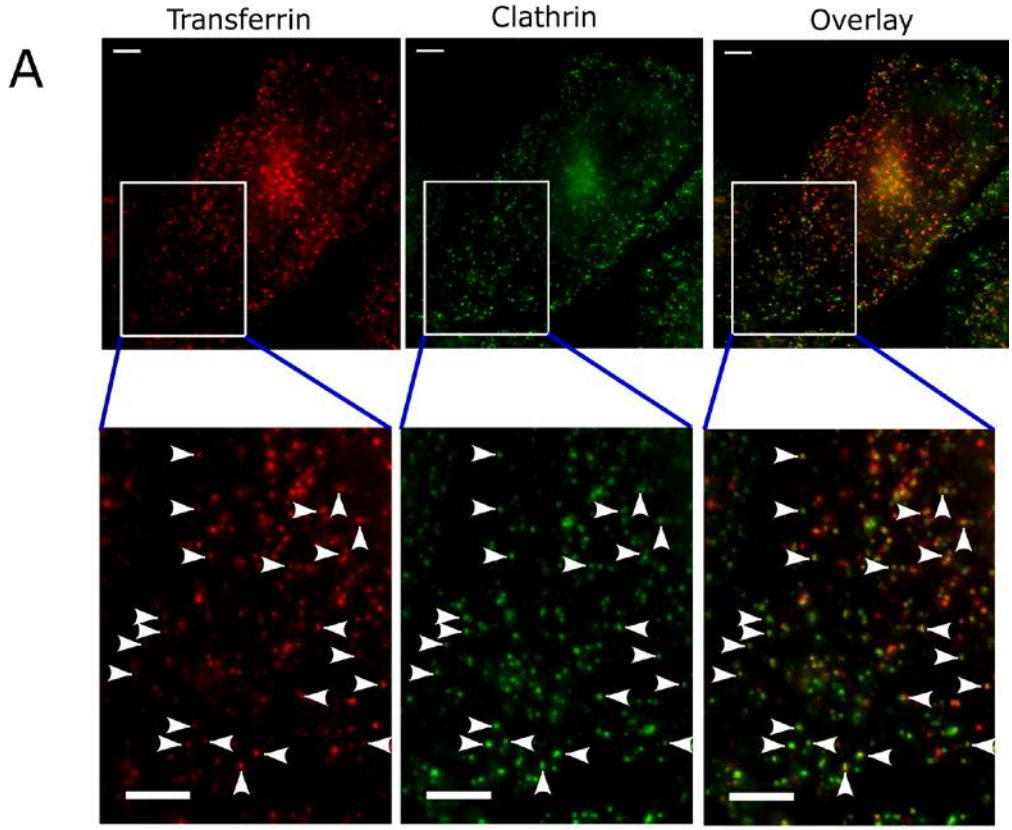


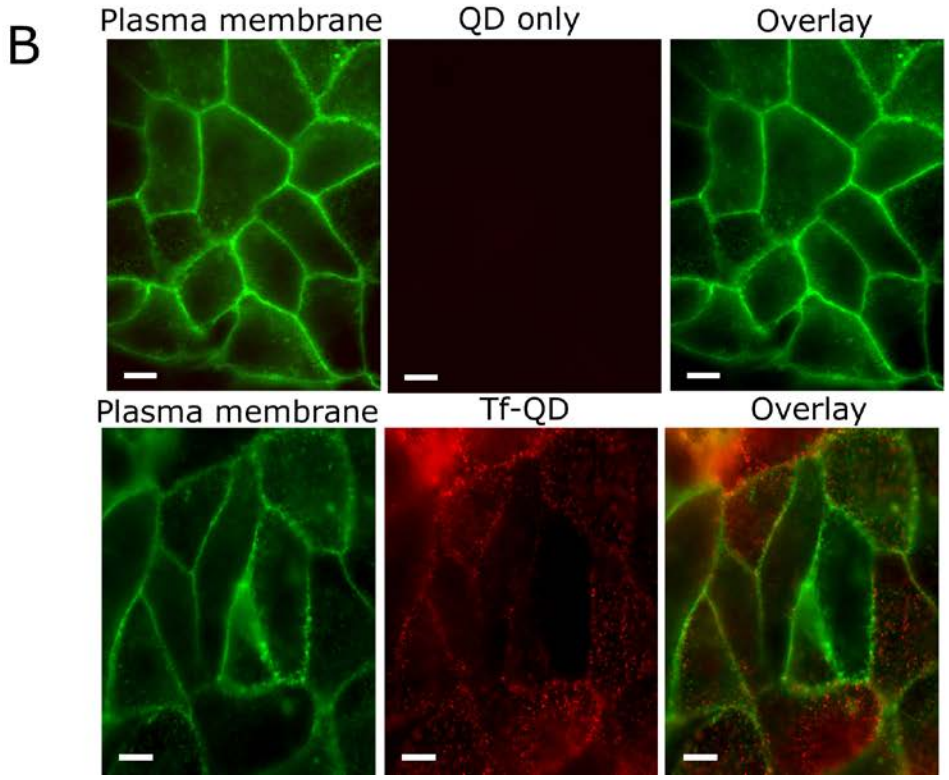
Supplementary Figure 4

A. Colocalization between Tf and Clathrin in Z310 cells: The panel shows the image of a Z310 cell that was labeled with 20 μg/ml human holoTf-Alexa555 (5 minute pulse at room temperature) and stained with antibody against clathrin heavy chain. Top row shows the individual channels along with the overlay and the bottom row shows the boxed region in each channel. The arrow heads in the bottom row images indicate structures that contain both Tf and clathrin. Scale bar = 5 μm.

B. Pinocytic activity in a Z310 cell monolayer: The top row shows the image of a Z310 cell monolayer that was labeled with 0.5 μg/ml cholera toxin B (green) on ice for 10 minutes and then incubated with 1 nM QD at 37^{o}C for 15 minutes. The monolayer was then fixed and imaged. Under these conditions there is no detectable level of QD uptake. The bottom row shows the image of a Z310 cell monolayer that was labeled with 0.5 μg/ml cholera toxin B (green) and 10 nM biotinylated Tf for 10 minutes on ice and then incubated with 1 nM QD655 streptavidin conjugate for 10 minutes on ice. The monolayer was then fixed and imaged. In the presence of biotinylated Tf, there is a significant amount of QD bound to the cell surface. This shows that the QD signal is due to receptor-mediated uptake of Tf-QD molecules. In preparing these images, the same intensity settings were applied for both QD-channel images. Scale bar = 5 μm.

Figure S4 contd…

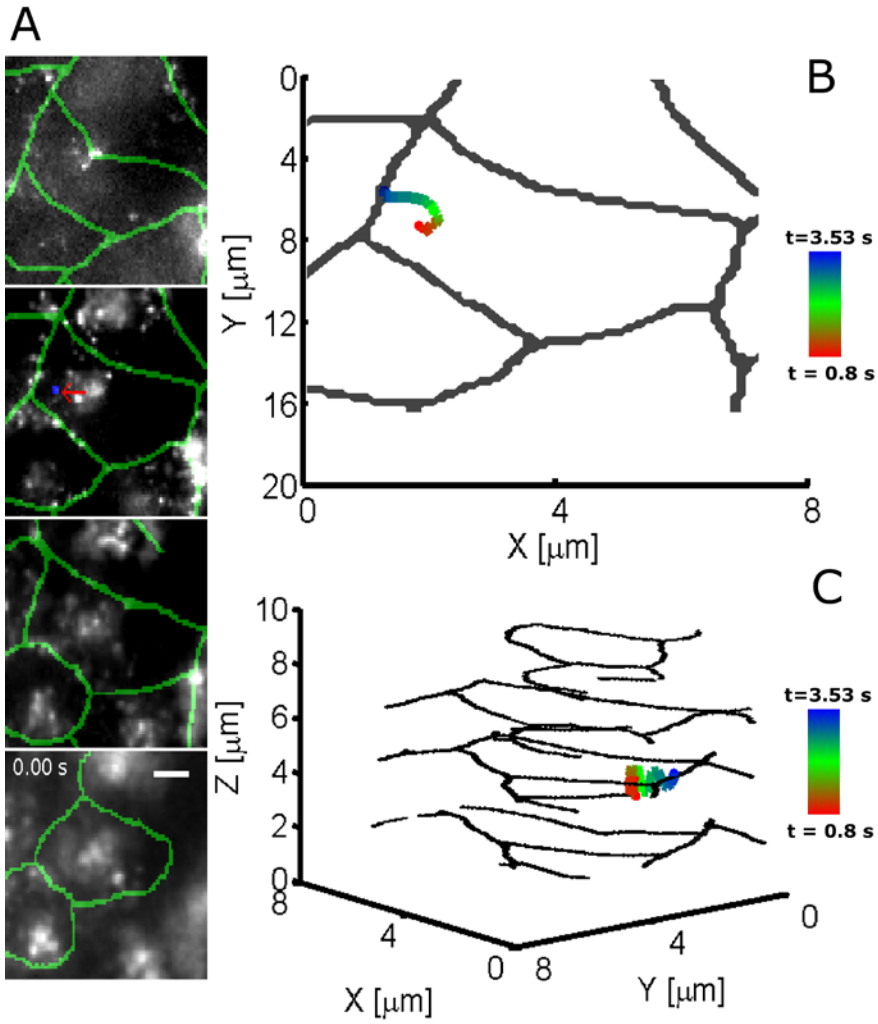




Supplementary Figure 5: Orthogonal mode of exocytosis at the lateral plasma membrane.

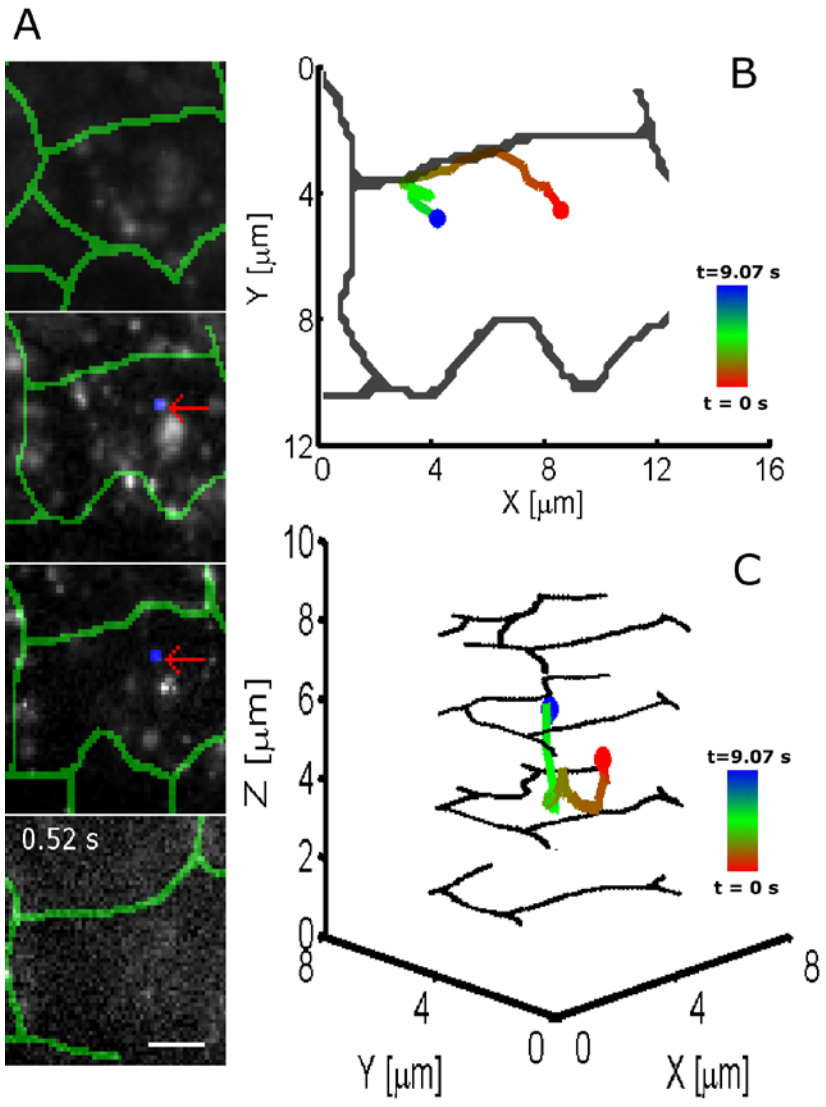
Panel A shows the overlay of the segmented plasma membrane channel (green) and the Tf-QD channel (grey scale) that was acquired from a live cell monolayer using a 4-plane MUM setup. Each image corresponds to a distinct focal plane within the Z310 monolayer. The Tf-QD molecule of interest (red arrow) is pseudo-colored in blue for visualization. Scale bar = 5 µm.

Panels B and C show the X-Y projection and the full 3D trajectory, respectively, of the QD-Tf molecule highlighted in A. The trajectories are color-coded to indicate time. The Tf-QD molecule traffics from the cell interior in a highly directional manner and merges with the lateral plasma membrane (see movie 5). The exocytosis step is characterized by Tf-QD movement that is perpendicular to the plasma membrane. Once on the plasma membrane, the molecule becomes stationary and exhibits very limited movement.



Supplementary Figure 6: Sliding mode of exocytosis at the lateral plasma membrane.

Panel A shows the overlay of the segmented plasma membrane channel (green) and the Tf-QD channel (grey scale) that was acquired from a live-cell monolayer using a 4-plane MUM setup. Each image corresponds to a distinct focal plane within the Z310 monolayer. The Tf-QD molecule of interest (red arrow) is pseudo-colored in blue for visualization. Scale bar = 5 µm. Panels B and C show the X-Y projection and the full 3D trajectory, respectively, of the QD-Tf molecule highlighted in A. The trajectories are color-coded to indicate time. The Tf-QD molecule traffics out of an intracellular compartment, moves parallel to and in close proximity to the lateral plasma membrane and then appears to undergo exocytosis. The molecule briefly stays on the plasma membrane and is then internalized through the orthogonal mode of endocytosis in the same cell (see movie 6).



MOVIE LEGENDS

Movie 1: Single QD imaging across a depth of 10 μm. The movie corresponds to figure S2. The movie shows images of a 2D QD sample that was acquired by moving the objective lens in steps of 500 nm and simultaneously imaging the sample at four distinct focal planes. Scale bar = 2 μm.

Movie 2: Intercellular transfer event. The movie corresponds to figure 2. The arrow indicates the event of interest (highlighted in blue). Scale bar = 5 μm. The 3D trajectory is color coded to represent time (with red to blue indicating increasing time). Movie is played at the acquisition speed.

Movie 2B: The movie shows the multi-angle view of the 3D trajectory pertaining to the intercellular transfer event that is displayed in Figure 2. Movie is played at 15 frames/s.

Movie 3: Orthogonal endocytosis. The movie corresponds to figure 4A. The arrow in the figure montage indicates the event of interest (highlighted in blue). Scale bar = 5 μm. The 3D trajectory shown is color coded to represent time (with red to blue indicating increasing time). Movie is played at the acquisition speed.

Movie 3B: The movie shows the multi-angle view of the 3D trajectory pertaining to the orthogonal endocytosis event that is displayed in Figure 4C. Movie is played at 15 frames/s.

Movie 4: Sliding endocytosis. The movie corresponds to figure 4D. The arrow in the figure montage indicates the event of interest (highlighted in blue). Scale bar = 5 μm. The 3D trajectory shown is color coded to represent time (with red to blue indicating increasing time). Movie is played at the acquisition speed.

Movie 4B: The movie shows the multi-angle view of the 3D trajectory pertaining to the sliding endocytosis event that is displayed in Figure 4F. Movie is played at 15 frames/s**.**

Movie 5: Orthogonal exocytosis. The movie corresponds to figure S5. The arrow in the figure montage indicates the event of interest (highlighted in blue). Scale bar = 5 μm. The 3D trajectory shown is color coded to represent time (with red to blue indicating increasing time). Movie is played at half the acquisition speed.

Movie 5B: The movie shows the multi-angle view of the 3D trajectory pertaining to the orthogonal exocytosis event that is displayed in Figure S5C. Movie is played at 15 frames/s**.**

Movie 6: Sliding exocytosis. The movie corresponds to figure S6. The arrow in the figure montage indicates the event of interest (highlighted in blue) as described in the figure. The 3D trajectory shown is color coded to represent time (with red to blue indicating increasing time). Movie is played at the acquisition speed. Scale bar = 5 μm.

Movie 6B: The movie shows the multi-angle view of the 3D trajectory pertaining to the sliding exocytosis event that is displayed in Figure S6C. Movie is played at 15 frames/s.