Supplemental Information

for

Dynamin-dependent Vesicle Twist at the Final Stage of Clathrin-mediated Endocytosis

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I. Supplemental Discussion

I.1. Calibration of Parallax Microscopy.

In parallax microscopy, two half-plane images are formed and arranged vertically (upper and lower images in Fig. 1). When the target probe is in the focal plane, the separation distance between the two images is measured as Δy_0. The separation distance between the two images (Δy) changes when the target probe moves along the z-axis.

To establish the relationship between the movement in the z-axis (Δz) and Δy, a set of half-plane images were obtained by scanning a nanoparticle probe along the z-direction. The lateral positions of the two half-plane images were superlocalized using non-linear least squares (NLLS) fitting. Practically, the two half-plane scattering images are ellipse-shaped and were fitted by 2D Gaussian function (Extended Data Fig. 1B):

$$I(x,y) = A + B \cdot exp(-(\frac{(x-x_0)^2}{2S_x^2} + \frac{(y-y_0)^2}{2S_y^2})) \quad \text{(S1)}$$

where (x_0, y_0) is the center position, A is the background level, B is the peak intensity at (x_0, y_0), S_x and S_y are the standard deviations of the Gaussian distribution along the x- and y-axis, respectively.

To generate a calibration curve of Δy as a function of Δz, 20 AuNRs immobilized on a glass slide surface with various orientations were scanned along the z-axis from -1000 nm to 1000 nm with 10 nm scanning steps with an objective scanner. The averaged Δy from the 20 AuNRs were used to establish the calibration curve with Δz (Extended Data Fig. 1A). The center portion of the curve (within the z range of -750 nm to 750 nm) can be satisfactorily fitted with a linear curve, which can be used to recover the z-position in the sample images.

I.2. Auto-Chasing in Axial Direction.

A major hurdle for defocused imaging in practical biological imaging is that the axial position of the probe is constantly changing, resulting in image pattern changes that interfere with the orientation recovery. Based on the established calibration curve, an auto-feedback z adjustment system has been implemented with a closed-loop nanometer-precision piezo stage and a tracking program in ImageJ μManger[1,2]. When the target probe is in focus, the separation distance between the upper and lower images is measured and assigned as the reference (Δy_0). When the automatic feedback loop system is enabled, the axial movement of the probe would contribute to the change in measured Δy, and the tracking program would respond by moving the objective scanner accordingly so that the probe is back in focus again. The response time of the stage is usually less than 1 ms. The axial movement of the probe is obtained from the physical output of the objective scanner.

I.3. Simulation of Defocused Scattering Imaging

In dark field microscopy mode, AuNRs were illuminated by monochromatic, unpolarized light through an oil-immersion dark field condenser and the scattering images were collected by a NA 1.0 objective. The anisotropic object can be defined as a multi-oscillation dipole, and the far-field intensity distribution of a dipole is characterized by a spatial position $x_{ff} = (x_{ff}, y_{ff}, z_{ff})$ and orientation angle which can be described as a unit vector $\hat{r}$ or an azimuth angle φ and polar angle θ, where $\hat{r} = (\sin\theta_{ff}\cos\varphi_{ff}, \sin\theta_{ff}\sin\varphi_{ff}, \cos\theta_{ff})$. The measured scattering spot can be written as[3]:

$$h_{\theta_{ff},\varphi_{ff}}\left(x; x_{ff}, \tau\right) = |\varepsilon|^2$$

$$
\begin{aligned}
&= \sin^2\theta_{ff}\left(|I_0|^2 + |I_2|^2 + 2\cos\left(2\varphi_{ff} - 2\varphi_d\right)\Re\left\{I_0^* I_2\right\}\right) \\
&-2\sin(2\theta_{ff})\cos(\varphi_{ff} - \varphi_d)\Im\left\{I_0 + I_2\right\} + 4|I_1|^2\cos_2\theta_{ff} \\
&= \hat{r}^T M_{ff}
\end{aligned}
\tag{S4}
$$

where $\varphi_d = \tan^{-1}((y - y_{ff})/(x - x_{ff}))$. An asterisk represents the adjoint operator. $\hat{r}^T$ denotes the conjugate transpose of $\hat{r}$, $\Re\{\nu\}$ and $\Im\{\nu\}$ stand for the real and imaginary components of ν, and τ is the parameters of the optical setup. M_{ff} is a symmetric matrix which contains six non-orthogonal templates given by:

$$
\begin{aligned}
m_{11} &= |I_0|^2 + |I_2|^2 + 2\Re\left\{I_0^* I_2\right\}\cos 2\varphi_d \\
m_{12} &= 2\Re\left\{I_0^* I_2\right\}\sin 2\varphi_d \\
m_{13} &= -2\cos\varphi_d \Im\left\{I_1^*\left(I_0 + I_2\right)\right\} \\
m_{22} &= |I_0|^2 + |I_2|^2 - 2\Re\left\{I_0^* I_2\right\}\cos 2\varphi_d \\
m_{23} &= -2\sin\varphi_d \Im\left\{I_1^*\left(I_0 + I_2\right)\right\} \\
m_{33} &= 4|I_1|^2
\end{aligned}
\tag{S5}
$$

where

$$
I_0\left(x; x_{ff}, \tau\right) = \int_0^\alpha B_0(\theta)\left(t_s^{(1)} t_s^{(2)} + t_{ff}^{(1)} t_{ff}^{(2)} \frac{1}{n_s}\sqrt{n_s^2 - n_i^2\sin^2\theta}\right) d\theta
$$

$$
I_1\left(x; x_{ff}, \tau\right) = \int_0^\alpha B_1(\theta) t_{ff}^{(1)} t_{ff}^{(2)} \frac{n_i}{n_s}\sin\theta d\theta \tag{S6}
$$

$$
I_2\left(x; x_{ff}, \tau\right) = \int_0^\alpha B_2(\theta)\left(t_s^{(1)} t_s^{(2)} - t_{ff}^{(1)} t_{ff}^{(2)} \frac{1}{n_s}\sqrt{n_s^2 - n_i^2\sin^2\theta}\right) d\theta
$$

with
$$
B_m(\theta) = \sqrt{\cos\theta}\sin\theta J_m\left(k r n_i \sin\theta\right) e^{ik\Lambda(\theta, z, z_{ff}, \tau)} \tag{S7}
$$

where the integrals for I_0, I_1 and I_2 are the vectoral PSF equation, and $\Lambda(\theta, z, z_{ff}, \tau)$ denotes the system's aberrations affected by defocusing.

Using a linear combination of these basis templates (Extended Data Fig. 3), theoretical PSFs as a function of dipole orientation were simulated. Compared to the previous methods that extracted a simplified function between orientation (polar angle and azimuth angle) and scattering intensities by omitting higher-order polynomials, our method is advantageous by retaining all polynomials to decrease the uncertainty of angular determination in pattern matching with the experimental images.

I.4. Uncertainties of Angle Determination

The uncertainties of the azimuth and polar angle are dependent on orientation and background noise. Under conventional cell imaging conditions, the signal-to-noise (S/N) of the cellular regions with relatively high background is ~10. Simulated images with artificial noise (S/N = 10; with 10° interval for both polar and azimuth angles; 50 images per position) were generated to evaluate the angular errors. When the polar angle (θ) is between 10-80 °, the precision of the azimuth and polar angle for any combination of these two angles are ~2 ° (Extended Data Fig. 4). When the polar angle is very small (i.e., an upright nanorod), the image patterns become analogous to each other so that the azimuth angle cannot be recovered (random φ being reported). When the polar angle is very close to 90° (i.e., a nanorod lying flat), the azimuth angles become degenerated so that the azimuth angle only be recovered within the range of 0-180°, which is expected because the asymmetry in the PSF disappears when the nanorod is placed flat on a horizontal plane.

I.5. Angle Degeneration

It is worth noting that due to its D_2 symmetry, the 3D orientations of a AuNR (φ, θ) and (φ+180, 180-θ) are degenerate. However, when the rotation of the AuNR is not too fast (<45 °/frame for both azimuth and polar angles), the continuous rotation and the orientation of the AuNR in the full

3D space, i.e., 0-360 º for azimuth and 0-180 º for polar angles, can be recovered with only one exception that the AuNR has a polar angel of exact 90 º.

I.6. 3D Localization Precision

The z-coordinate for the AuNR position is obtained by the movement of the microscope objective when performing the auto-focusing tracking. On account of anti-foreshortening of the images due to the refractive index difference between the glass (n = 1.515) and cell culture medium (n = 1.33), the z-movement in bifocal parallax imaging may be magnified. The magnification effect is corrected by multiplying all z coordinate by a factor of 0.77[4]. In addition, the z rescaling issue for the refractive index mismatch is related to the distance between the sample and the interface. The relationship between the actual focal plane and the apparent focal plane for n_1 = 1.515 (glass) and n_2 = 1.34 (water) with imaging focal plane range of 0-3 μm has been established[5]. The fitting curve can be approximated as a straight line with small deviations above 1.5 μm.

As discussed above, the x and y coordinates of AuNRs were resolved by Gaussian fitting their intensity distributions (Extended Data Fig. 1B). Even with utmost care in adjusting the position of the wedge prism in the light path, the scattering intensities of the upper and lower channels are not always the same due to uneven splitting of light. To maintain higher accuracy, only the lateral positions of the brighter image is used for tracking the lateral movement.

To fully evaluate the localization precision of the system in 3D, we collected 500 images of one AuNR when auto-focusing system was engaged. A 3D cluster was obtained (Extended Data Fig. 1C), showing how much the localized position spread out. Gaussian fitting of the histogram distribution of AuNRs positions in x, y, and z was used to determine the localization precisions in 3D, giving σ_x = 4.9 nm (Extended Data Fig. 1D), σ_y = 6.3 nm (Extended Data Fig. 1E) and σ_z =

14.0 nm (Extended Data Fig. 1F). Note that the worse localization precision in y than that in x is caused by the elongated half PSF in y in our imaging system.

I.7. Detailed discussion of Stage a-b

Stage a. Initial active rotation – immobilization cycles and clathrin accumulation. When AuNRs first attached onto cell membrane, they showed active rotation accompanied by lateral diffusion. This stage, which usually lasts for minutes (~2 minutes for the example in Fig. 3), is not plotted in full in Fig. 3 for clarity. Movie S2 shows a typical example of active rotation of a AuNR on the cell membrane surface at this stage. The initial rotation is characterized by large rotation steps (65 ± 92 °/frame for azimuth angle, 13 ± 16 °/frame for polar angle, Mean ± SD, n = 2000, Fig. 6A- B). The active rotation indicates that the AuNRs were loosely attached to the membrane surface, possibly through a single point of attachment that allowed the AuNRs to wave in the cell medium. The translational and rotational freedoms of the AuNRs were lost gradually, and they could become immobilized on the cell membrane through multi-point attachments. The attachments were often weak, with the AuNRs occasionally going back to the active rotation mode[6,7].

The accumulation, and presumably assembly of clathrin is often deemed as an indication that endocytosis has started, which can be observed in Stage a with large variations in duration[8]. The AuNR became completely immobilized with the initiation of clathrin assembly, although immobilization of a AuNR does not always require clathrin assembly. This can be attributed to the curvature of the CCP forming on the membrane, which could spatially constrain the AuNR and restrict its rotational freedom due to more contact points. In practice, the "completely immobilized" AuNRs at this stage was always observed to fluctuate due to thermal activity, with a rotation speed of ~2 °/frame, slightly larger than the angle recovery precision. In addition, drifting of the AuNRs can be observed possibly because of the translational and rotational drifting of the whole patch of the membrane supporting the AuNRs. This kind of drifting was usually very slow, with an accumulative rotation speed of several degrees per second. An arbitrarily chosen accumulative rotation speed of < 10 °/s (equivalent to 0.2 °/frame) was used as a criterion to differentiate drifting from dynamin-induced rotation that occurred later.

Stage b. Dynamin accumulation stage showing high rotation variability. This stage is highly variable in length of time (Fig. 3, Extended Data Fig. 6 and 7) and in rotational freedoms. In 5 out of 45 cases, the AuNR cargos would maintain static during the whole period (e.g. Extended Data Fig. 7A and D). In other cases, the AuNR cargos would restore slow or fast random rotations intermittently (e.g., 147.0-166.0 s in Fig. 3A). The histograms of the steps in Fig. 3A show an average step size of 5 ± 8 º/frame in the azimuth plane and 3 ± 6 º/frame in the polar direction (Mean $\pm$ SD, n = 990, Fig. 6C and D).

This stage is defined by the recruitment of dynamin to the underlying CCP (shaded light green fluorescence in the background of Fig. 3 A and B 147.0-166.0 s) toward its peak, although the kinetics and the extent of dynamin recruitment varied considerably (Fig. 3, Extended Data Fig. 6 and 7), as has been reported by other groups[9,10]. This period also corresponds to the increasing curvature of the underlying CCP[8]. It is thus reasonable to assume that as the CCP invaginates and the newly recruited dynamin begins to reshape the lipid membrane on the neck of the vesicle, the cargo inside may, but not necessarily always, undergo constrained rotations with random directions.

Toward the end of this period, the rotations, in some cases, may occur in a fast and abrupt manner. This behavior was more pronounced at pits where dynamin recruitment was greater (Fig. 3, Extended Data Fig. 6 and 7) and thus may be an indication that the membrane tension is high at the end of dynamin recruitment. While it is unclear what events cause these rotations and movements, they may reflect cycles of dynamin assembly and disassembly, twisting and squeezing the vesicle neck so that the AuNR cargo rotates along with the invaginated pit.

At the end of this stage, which coincided with the peak of dynamin recruitment/assembly, the cargo vesicle would pause rotation for those showed rotation earlier. This whole Stage b took 18.8 s for this specific example in Fig. 3, which is consistent with the literature reports of the time for dynamin recruitment[11,12].

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