Dynamin Action at the Final Stage of Clathrin-Mediated Endocytosis

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Abstract

Dynamin plays an important role in clathrin-mediated endocytosis by cutting the neck of nascent vesicles from the cell membrane. Gold nanorods were used as imaging probes to observe dynamin action on cargo vesicles during live endocytosis events. Invariant is that at the peak of dynamin accumulation, the cargo-containing vesicle always gives abrupt, right-handed rotations that finishes in a short time (~ 0.28 s). The large and quick twist, herein named the super twist, is the result of the coordinated dynamin helix action upon GTP hydrolysis. After the super twist, the rotational freedom of the vesicle drastically increases, accompanied with simultaneous or delayed translational movement, indicating that it detaches from the cell membrane. These observations suggest that dynamin-mediated scission at the final stage involves a large torque generated by coordinated actions of multiple dynamins in the helix, which is the main driving force for scission. The super twist presumably results in membrane tube hemifission and partial destruction of the dynamin helix, followed by vesicle fission.

Introduction

Dynamin is a member of a family of GTP-binding proteins that participate in a variety of membrane fission and fusion processes, including the formation of endocytic and secretory vesicles, and mitochondrial and peroxisomal dynamics^{1–4}. Best studied is dynamin's role in clathrin-mediated endocytosis (CME), which is a major endocytic pathway in eukaryotic cells⁵. Dynamin is recruited to clathrin-coated pits (CCPs) at relatively early stages of CME to regulate their lifetime and maturation^{6,7}. At a later stage, dynamin assembles and acts as a mechanochemical scaffold that constricts and deforms the membrane at the neck of invaginated CCPs and cuts the nascent vesicles from the cell membrane^{8–10}. There is a growing consensus as to the roles that dynamin plays during the fission process¹¹: (i) dynamin self-assembles into helices around a lipid membrane tube; (ii) GTP-bound dynamin favors more constricted states of the helix; (iii) fission of the vesicle is induced by the dynamin helix in a manner dependent on GTP-hydrolysis. However, how dynamin breaks the neck of the CCPs at the final stage of endocytosis is still under debate.

Currently, there are two prevailing models proposed based on different experimental evidence. **(1) The Two-Stage Model**^{12–17}. It suggests that in the first stage, dynamin in a nucleotide-bound conformation assembles to a helical structure, leading to a constricted membrane tube inside. At the second stage, coordinated GTP hydrolysis by multiple dynamin molecules facilitates the formation of a hemi-fission intermediate of the membrane tube and destabilizes the dynamin assembly, followed by the rupture of the membrane tube. While these events normally occur too rapidly to decouple them experimentally, this could be accomplished by trapping dynamin in its high energy transition state using chemical bonds, which produces the hemi-fission intermediate without progression to fission¹⁶. Structural studies had shown that during GTP hydrolysis, the relative orientation between the GTPase and bundle signaling element (BSE) portions in dynamin changes by $\sim 70^\circ$ ^{18,19}. This conformational change is also transmitted through the stalk leading to a tilted Pleckstrin homology domain (PHD) and the wedging of

the lipid membrane underneath^{16,20}. With this strong interaction, the energy barrier for hemi-fission to occur diminishes or even disappears^{13,21}. The PHD tilting may also lead to an axial force that helps to rupture the hemi-fission intermediate.

(2) The Ratchet/Constriction Model^{10,22}. In this model, dynamin can act as a molecular motor similar to myosin. The model suggests that when assembled, adjacent rungs slide on each other progressively upon cycles of stochastic GTP hydrolysis, leading to the twisting and constriction of the helix until the membrane tube reaches a state that the hemi-fission intermediate can form spontaneously or through thermal fluctuation. This model is consistent with *in vitro* observation that dynamin helix generates twisting motion upon GTP hydrolysis¹⁰. The observed 70° rotation between the GTPase and the BSE portions is also consistent with this model, as it may cause a power-stroke that slides dynamin by one unit along the helix^{18,20}. Repeated cycles of GTP hydrolysis and sliding could tighten the helix like a ratchet. Finally, disassembly of the dynamin helix is proposed to be caused either by conformational stress as the transition state conformation cannot be docked in any of the dynamin helical structures¹⁸, or by loss of the underlying membrane tube template due to fission. A recent *in vitro* high-speed AFM observation²³ shows evidence supporting this model: dynamin helix gave localized, progressive constrictions through several cycles of GTP hydrolysis, rather than a single-step conformational change, in breaking the membrane tube.

Both models include a mechanochemical step in which GTP hydrolysis drives a large conformation change in assembled dynamin that is crucial for membrane scission. However, in the Ratchet model, stochastic GTP hydrolysis leads to stepping of dynamin along adjacent turns of the helix to progressively tighten the helix, while in the Two-Stage model, the energy from coordinated GTP hydrolysis drives a concerted conformational change of multiple dynamin molecules that, in concert with PH domain insertion and tilting ruptures the tube while destabilizing the dynamin assembly¹¹. Note that the Two-Stage model does not include but also not exclude the twisting action of the dynamin helix. A recent theoretical study suggests that the two models may be reconciled: an effective rotation of the dynamin helix around its longitudinal axis may happen simultaneously with the tilting of dynamin PH domains, which better facilitates severing of the membrane energetically²⁴.

To resolve these issues and define the mechanism of dynamin-catalyzed fission, it is important to directly observe dynamin action during the live endocytic process. Live-cell observation is especially crucial because dynamin may behave differently depending on its environment. For example, in the absence of the GTP, dynamin assembles into “non-constricted”, long helical coils around membrane tubes *in vitro*^{8,25–28}. The addition of GTP generates torsion and constriction but may not necessarily cut the tube. In contrast, in the constant presence of GTP, which is more relevant to *in vivo* environment, dynamin will generate highly constricted but short helices that lead to scission¹⁴. In addition, the “non-constricted” helix of dynamin with no nucleotide has a one-start helical structure, while helices of dynamin assembled with nucleotides or analogs can have a one-start (i.e., the “constricted”)²⁹, or a two-start (i.e., the “super-

other through sliding or simple reorganization^{20,30}, which brings into question which of these intermediate structures are relevant to dynamin action *in vivo*. All these indicate that *in vitro* models may not apply to *in vivo* conditions, and hence live-cell observation is crucial in decoding how dynamin works.

Herein, we used gold nanorods (AuNRs)³¹ as cargos and *in vivo* probes to directly report dynamin actions, especially the twisting behavior, if there is any, during the complete endocytic process. AuNRs strongly scatter light at their localized surface plasmon resonance (LSPR) wavelengths, which is polarized along their axes, allowing them to be detected in the crowded cellular environment. The challenge for observing the rotational movement of these probes is that they are too small to resolve their orientation using conventional optical microscopy due to the optical diffraction limit. With our newly developed three-dimensional single particle orientation and rotational tracking (3D-SPORT) system, it becomes possible to not only track AuNR's translational movement in 3D space (Focused channel, Fig. 1A) but also resolve its orientation changes (Defocused channel, Fig. 1A). Moreover, we designed the instrument to simultaneously collect two additional channels of fluorescence images, which provide dynamic molecular information at locations near the cargo. The multi-dimensional observations shed new light on how dynamin works in the scission of endocytic vesicles.

Results

3D-SPORT. The new and essential aspect of this study is to use AuNR cargos to track changes in the orientation of cargo-laden CCPs during endocytosis *in vivo* and thus measure the hypothesized twisting motions involved in dynamin-induced fission. To facilitate the CME of AuNRs, transferrin molecules were conjugated to the surface of 40 nm × 80 nm AuNRs. Gold nanoparticles with similar sizes modified this way have been repeatedly shown to be taken up by cells predominately through the CME pathway^{32,33}. Well-dispersed nanoparticles in this size are endocytosed individually. They are tightly wrapped by the lipid membrane and their motions reflect the movement of the whole vesicle^{33,34}. Given the size of these particles, we anticipate that each CCP and nascent clathrin-coated vesicle (CCV) will carry only a single transferrin-conjugated AuNR and thus, their dynamics will report those of the cargo-laden CCP/CCV³⁵.

To track the AuNR's orientation over the entire 3D space (0-360° for azimuth and 0–90° for polar angles), defocused dark-field imaging was used to collect the image pattern of AuNRs³⁶. It is worth noting that due to its D_2 symmetry, the 3D orientations of a AuNR (ϕ , θ) and ($\phi + 180$, $180 - \theta$) are degenerate. However, when the rotation of the AuNR is not too fast ($< 45^\circ/\text{frame}$ for both azimuth and polar angles), the continuous rotation and the orientation of the AuNR in the full 3D space, i.e., 0-360° for azimuth and 0-180° for polar angles, can be recovered with only one exception that the AuNR has a polar angle of exact 90°. 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. In our setup, we employed a feedback loop that automatically locks the probe axially to overcome this problem (Fig. 1A and Figure S1, refer to Methods, and Supplemental Information for

details) and enable accurate image patterns to be obtained for precise and robust determination of the probe's orientation angles.

Experimentally, the instrument can resolve the 3D orientation (defined in Fig. 1B) of the AuNRs with a time resolution of ~ 20 ms (Supplemental Information). Figure 1C shows example images of a AuNR immobilized in agarose gel with a fixed polar angle of 60° and varying azimuth angles (more experimental images are shown in Figure S2). To recover their 3D orientation, a correlation coefficient mapping method was used, which compares an experimental image with all simulated images at different orientations, followed by a weighting procedure to report the most probable azimuth and polar angles of the probe. The simulated image patterns were from the theoretical point spread functions (PSF) of an emitting dipole, which were a function of its azimuth and polar angles and can be generated using the six basic functions of dipole emission ($I_x^2, I_y^2, I_z^2, I_{xy}^2, I_{xz}^2, I_{yz}^2$) (Supplemental Information 1.3 and Figure S3). These basic image patterns are dependent on system-specific parameters including the numerical aperture and magnification of the objective, and the defocusing distance. Importantly, the defocused image patterns are unique at different azimuth/polar angles, confirming that the defocused imaging overcomes angular degeneracy in the entire 3D space (with exceptions at the polar angle of $\sim 90^\circ$, at which the azimuth angle shows a 2-fold degeneracy) and provides true rotational direction information. The analysis of the orientation-dependent uncertainties associated with the recovered azimuth and polar angles is provided in Figure S4 and S5. Under our typical live-cell imaging conditions, a precision of $< 2^\circ$ can be accomplished for most polar and azimuth angles with a signal-to-noise ratio of ~ 10 . At the same time, the AuNR's position in the 3D space can be recovered with a precision of $4 \sim 6$ nm laterally and 14 nm axially (Figure S6).

Multi-dimensional tracking. 3D SPORT can collect the translational and rotational motion of AuNR probes in the 3D space in focused channel and defocused channel, respectively. Our customized instrument (Fig. 1A) also allows two additional fluorescence color channels to be simultaneously collected with a second EMCCD camera, which provides dynamic molecular information (i.e., dynamin and clathrin in this study) around the AuNR probe with an signal integration time of 0.5 s or 1.5 s and a waiting time between frames of 0.5 s. Sparsely distributed frames over time were used to minimize photobleaching.

In the experiments, we used the SK-MEL-2 cell line (a gift from Drubin's group at the University of California, Berkeley) that were gene-edited to express RFP-tagged clathrin (CLTA-RFP) and EGFP-tagged dynamin2 (DNM2-EGFP)³⁷. For imaging, the surface-modified AuNR solution was added to cells plated on a glass substrate ready for microscopic observation. Once a AuNR landed on the curved cell membrane surface, the recording was started and continued until convincing evidence was detected that the AuNR was inside the cell: usually linear translocation of the cargo over a long distance in the cell. All channels including AuNR scattering, dynamin and clathrin fluorescence were synchronized and recorded simultaneously. Only one AuNR was monitored during each experiment. To exclude any unambiguity, we focused on endocytosis events where both dynamin and clathrin were observable (Movies S1 show an endocytosis example. For clarification, only the dynamin channel and the focused AuNR scattering

Figure 2 shows an example of an endocytic event with the AuNR probe's translational displacements and two fluorescence channels. The colocalization of the scattering image of a AuNR cargo with clathrin and dynamin fluorescence gave direct evidence that CME happens for the observed endocytic event (Fig. 2A-D). The detachment of the nascent vesicle from the cell membrane occurred at 12.2 s, which was indicated in this case by a sudden large xy-displacement, accompanied with a 260 nm inward z-directional change from the original entry spot (Fig. 2F, bottom two panels). This indicates that fission happened at, or slightly before the point at 12.2 s (i.e. the detachment point). Before this point, fluctuation and drifting of the AuNR in the range of several hundred nm both laterally and axially were observed, possibly caused by thermal fluctuation of the cell membrane and live cellular activities.

The accumulation of dynamin at the CCPs showed large variations from case to case but usually peaked around the detachment point. In this specific case, dynamin fluorescence on the vesicle quickly dropped to the baseline after fission; whereas the opposite was observed for clathrin fluorescence, which was lost from the entry spot, but remained on the vesicle for several seconds and dropped quickly to the baseline (Fig. 2E and 2F, top two panels). At the entry spot on the membrane, although the dynamin fluorescence persisted, the intensity dropped, possibly reflecting partial disassembly of the dynamin helix. It is worth noting that the focal plane moved away from the entry spot along with the vesicle, which also contributed, but to a lesser extent, to the fluorescence intensity drop at the entry spot (Fig. 2F). This example shows that these simultaneous fluorescence observations give detailed dynamics of the recruitment of these two proteins on the vesicles and the entry spot. These observations are of crucial importance in elucidating their functions as discussed later.

Characteristic rotational motions of AuNR cargos during endocytosis. When the rotational motion of the cargo is considered, new information of endocytosis can be obtained. Figure 3A shows as a typical example of, from top to bottom: the lateral displacement of the particle with respect to the initial contact point, the azimuth and polar angles, and the z-displacement. These movements can be interpreted in the context of dynamin fluorescence intensity, which is shown in shaded light green in the background. (Figure S7 shows the cell images, clathrin, dynamin information for this example.) Note that the azimuth and polar angles are cumulative with respect to the AuNR's initial orientation so that they may exceed their nominal ranges (0-360° and 0-180°, respectively).

At first glance, the AuNRs showed complicated motional patterns, experiencing multiple rotation-immobilization cycles. However, when combined with molecular information from the fluorescence observation, these motions can be classified into characteristic "stages". All of the endocytosed AuNR cargos (45/45) showed a sequential combination of these characteristic stages throughout the process, although in some cases individual stages were missing or repeated. These stages of characteristic rotational motions, which are delineated in Fig. 3 (more examples of complete endocytosis events are shown in Figure S8 and S9) are: (a) the initial active rotation – immobilization cycles, (b) the dynamin accumulation stage toward its peak with high variability in rotations, (c) a static period before the super twist, (d) the right-handed, super twist at dynamin peak, (e) a short, relatively slow random rotation period,

diffusion, or linear transport with little rotation inside the cell. Of these, stages a-c are more variable, and thus will be discussed only briefly. Invariant, however, is the super twist observed prior to vesicle departure.

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 ($65 \pm 92^\circ/\text{frame}$ for azimuth angle, $13 \pm 16^\circ/\text{frame}$ for polar angle, Mean $\pm$ SD, $n = 2000$, Figure S10 and Figure S11 A and 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^{38,39}.

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 duration⁵. 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 $\sim 2^\circ/\text{frame}$, 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 $< 10^\circ/\text{s}$ (equivalent to $0.2^\circ/\text{frame}$) 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, S8 and S9) and in rotational freedoms. In 5 out of 45 cases, the AuNR cargos would maintain static during the whole period (e.g. Figure S9 A and D). In other cases, the AuNR cargos would restore slow or fast random rotations intermittently (e.g., 147.0-165.8 s in Fig. 3A). The histograms of the steps in Fig. 3A show an average step size of $5 \pm 8^\circ/\text{frame}$ in the azimuth plane and $3 \pm 6^\circ/\text{frame}$ in the polar direction (Mean $\pm$ SD, $n = 990$, Figure S11 C and D).

This stage is defined by the recruitment of dynamin to the underlying CCP (shaded light green fluorescence in the background of Fig. 3A and B 147.0-165.8 s) toward its peak, although the kinetics and the extent of dynamin recruitment varied considerably (Fig. 3, S8 and S9), as has been reported by other groups^{7,40}. This period also corresponds to the increasing curvature of the underlying CCP⁵. It is thus

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, S8 and S9B) 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^{37,41}.

Stage c. Static period before the super twist. Stage b is invariably followed by a brief static period, or waiting time (Stage c, Fig. 3C, 165.8-166.7 s), as if the vesicle went into a deadlock, or was waiting for a signal to restart motion. To accurately calculate the duration time of stage c, only 40 cases with AuNR rotation in stage b were taken into account. Therefore, the duration was 1.9 ± 1.5 s (Mean $\pm$ SD, $n = 40$, Fig. 4C). This stage occurs at or near the peak of dynamin recruitment and could correspond to a constricted coated pit, which has been identified biochemically and linked to dynamin assembly¹².

Stage d. A right-handed super twist precedes membrane fission and vesicle release. The most dramatic and invariant (45/45) observation is the occurrence of a large and rapid right-handed, in-plane rotation(s) of the AuNR (Step d in Fig. 3AB, in which case the AuNR rotated 208° in-plane from 166.7 to 167.2 s). During the whole period, the polar angle barely changed (Fig. 3B).

The average twisting angle for all the observed cases ($n = 45$) was $130 \pm 56^\circ$ (Mean $\pm$ SD) and was finished in a very short time of 0.28 ± 0.18 s (Mean $\pm$ SD, Fig. 4A and B). Given their magnitude, we named these rotations the “super twist” in this manuscript. Movies S3 show 3 examples of these super twists imaged in the defocused channel starting from the static period. A large portion of these super twists (33/45, e.g., Fig. 3, S9 B, S9 C and D) were finished in a clear single step, with the rest (12/45, e.g. Figure S8 and S9A) showing two steps. No more than two steps were identified. The total length of time from the end of Stage b to the departure of the vesicle (Stage f, discussed below), which encompassed the peak of dynamin recruitment (Fig. 3A) was short (e.g., 2.0 s in this example).

The coincidence of the peak of dynamin accumulation and the super twist suggests that the super twist may be driven by dynamin hydrolysis of GTP and is the major factor for driving fission and dynamin disassembly. To test this hypothesis, we carried out a control study using cells that express the K44A dynamin mutant defective in GTPase activity. These cells showed significantly reduced efficiency in internalizing AuNRs (Figure S12 B). In single-particle observation experiments with these cells, no

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js sufficiently long observation time (tens of

minutes). While characteristic AuNR motions consistent with early stages up to the static stage (Stage c) were detected, no super twist was detected (for a specific example, Figure S12 A). As a result, CME was not completed. These data confirmed that the dynamin helix generates the super twist upon GTP hydrolysis.

Stage e. Constrained random rotations precede vesicle detachment. After the super twist, the vesicle had another period of relatively slow, random rotations within a limited range before the detachment from the cell membrane (Stage e). The rotation steps ($10 \pm 13^\circ/\text{frame}$ for azimuth and $7 \pm 10^\circ/\text{frame}$ for polar, Mean $\pm$ SD, $n = 900$, Figure S11 E and F) were larger than those in the first slow rotation Step b ($5 \pm 8^\circ/\text{frame}$ for azimuth and $3 \pm 6^\circ/\text{frame}$ for polar, Mean $\pm$ SD, $n = 990$, Figure S11 C and D), yet much smaller than those in the free diffusion step (Step f: $21 \pm 32^\circ/\text{frame}$ for azimuth and $12 \pm 18^\circ/\text{frame}$ for polar, Mean $\pm$ SD, $n = 1000$, Figure S11 G and H). These observations possibly indicate that the rigid scaffold that stabilizes the vesicle and the neck was broken but the vesicle was still associated with the cell membrane through the remaining protein-protein interactions. The duration of this period was highly variable (2.1 ± 1.4 s, mean $\pm$ SD, $n = 45$, Fig. 4D).

Stage f. Vesicle detachment and severing point. We will use “severing point” or “detachment point” rather than “fission point” to describe the vesicle’s status in the study. The “fission point”, by convention, marks the time at which the vesicle membrane is no longer continuous with the plasma membrane. Since we cannot exclude the possibility that mature vesicle is still connect to the cell membrane through the protein scaffold, the fission point cannot be identified using our imaging method. Here, “severing” describes a status that the vesicle is no longer connected to the cell membrane by the lipid tube, or the *rigid* protein scaffold on the CCP neck. In practice, vesicles in this state may still be tethered to the membrane through *loose* connections such as actin filaments so they do not leave the entry spot immediately.

We applied a rigorous set of criteria in finding the detachment point. First, our observations are based on dark-field imaging, which has a large depth of view and allows a more direct and complete assessment of the displacement of the *cargo* rather than associated proteins. A long-distance of diffusion with active rotation or linear traveling (with barely any rotation) provides definitive evidence that the cargo is already inside the cell. Second, the onset of the displacement, however, cannot be used as the criteria for severing because the vesicle may be still trapped in the actin mesh, or attached to the cell membrane *loosely* through other proteins such as actin filaments. Thus, the most important criterium was the restoration of active rotational motions, which can be visually identified and usually happens slightly earlier than, or at the same time as the onset of the lateral displacement. This is defined as the severing point since a relatively rigid connection by the lipid membrane tube, or the remaining of the broken protein scaffold such as dynamin or other tightly bound coat proteins, would not allow large rotational steps. For example, in Fig. 3, the mean rotation step sizes starting from Stage f are increased to $21 \pm 32^\circ/\text{frame}$ in the azimuth plane, $12 \pm 18^\circ/\text{frame}$ in the polar direction (Mean $\pm$ SD, $n = 1000$, Figure S11 G and H), as opposed to Stage b ($5 \pm 8^\circ/\text{frame}$ in azimuth plane and $3 \pm 6^\circ/\text{frame}$ in polar direction, Mean $\pm$ SD, $n = 990$, Figure S11 C and D). Third, in many events ($\sim 70\%$), the AuNR cargos were observed to have an

with the onset of the active rotation (e.g., Fig. 3A at

167.8 s, which had an inward (negative) z-movement of 300 nm). The rapid inward z-movement is possibly caused by the release of tension that is generated by the rigid protein scaffold and other endocytic protein machinery, upon the severing of the vesicle from the membrane^{42,43}. Finally, the recruitment dynamics of dynamin and clathrin on both the nascent vesicle and the entry spot facilitate the assignment of rotation stages (Figs. 2 and 3).

Ambiguity in determining fission point using imaging methods. To clearly understand the events during the late stages of vesicle formation, it is of critical importance to identify the time of fission. However, pinpointing the fission point in live cells has been a challenging problem^{41,44}. The vesicle itself can remain tightly associated with the plasma membrane, held in place by, for example, actin filaments or its movements restricted by the congested intracellular environment. These factors exclude using spatial displacement as the sole criterion in finding the fission point. Indeed, the disappearance of dynamin and/or clathrin fluorescence from the evanescent field as measured in TIRF studies was shown to imprecisely reflect the scission point, as -7 ± 22 s from the point of the disappearance of the dynamin fluorescence in the evanescent field⁴⁰.

In addition, it is unclear whether the break of the lipid connection precedes the disassembly of the protein scaffold or the opposite. In the former case, imaging methods can only disclose the point when the protein connection is broken and the vesicle is detached. It is practically impossible to disclose the fission point using imaging methods. In the latter case, the fission point coincides with the detachment point. In our data, the existence of Stage e possibly suggests that scission is more like a gradual process that involves the complete disassembling of proteins (e.g., dynamin) connecting the vesicle and cell membrane no matter which case is true.

The multi-dimensional approach used in our experiments allows us to accurately pinpoint the time of vesicle detachment as well as the onset of the super twist. Even though we are unable to determine the fission point, it must occur after the start of the super twist, and before or at the point of vesicle detachment. Thus, we are able to narrow down the time range that fission happens with a high confidence level, i.e. fission must occur within the range of 2.4 ± 1.4 s (Mean $\pm$ SD, $n = 45$) that encompass Stages d and e.

Super twist-like motion and abortive scission events. In the experiments, we also observed that dynamin accumulation/disassembly may happen without successful scission. Figure S13 shows such a case in which dynamin peaked at 75 s and disassembled in the following frames of images, without subsequent vesicle detachment. While a smaller twist action of $\sim 50^\circ$ was detected, it failed to produce a large enough torque or twisting action to drive scission. These may represent failed scission attempts and we name these actions as “super twist-like actions”. It is unclear why fission may fail but a plausible explanation is that excessive resistance stalled the super twist. After the super twist-like action, dynamin disassembled, possibly suggesting that the GTP hydrolysis leads to the disassembly of dynamin helix.

The super twist. We report that prior to scission, endocytic pits/vesicles invariably undergo a rapid super twist that coincides with the peak of dynamin recruitment and requires dynamin's GTPase activity. The twisting motion generated by pre-assembled dynamin helix upon GTP hydrolysis has been observed *in vitro*¹⁰. Here, we observed, for the first-time the twisting action generated by dynamin upon GTP hydrolysis *in vivo*. The twist unequivocally discloses that dynamin action generates a torque at the final stage of the scission.

This twisting action is partially consistent with the Ratchet model, in which the GTPase domains of dynamin work as molecular motors and slide on the helical turns by cycles of association/power stroke/dissociations, leading to the twisting and tightening of the membrane tube. However, the twisting occurs in an abrupt manner, and the whole process is finished in a very short time, $\sim 0.28 \pm 0.18$ s (Mean $\pm$ SD) for 45 cases (Fig. 4B), during which the cargo vesicle rotated by a large angle of $130 \pm 56^\circ$ (Mean $\pm$ SD, $n = 45$) (in the range of $50 \sim 225^\circ$, Fig. 4A) in the azimuth plane, with a peak rotation angle at $\sim 130^\circ$. Given that the rate of assembly-stimulated GTPase activity of dynamin is much slower⁹, our data suggest that the super twist involves a concerted conformational change rather than sequential GTPase-driven ratcheting steps.

So far, it is unclear how constricted the dynamin assembly is before the super twist. CryoEM experiments have shown that the “non-constricted” helix composed of dynamin without nucleotides may have 14–18 dynamin dimers per rung^{45,46}, the “constricted” helix containing dynamin in the presence of GTP analogues has 13.2 dimers per rung²⁹, and the “super constricted” helix has a unique two-start structure and has 11.8 pairs of dynamin dimers per rung^{20,30}. Assuming that the assembled dynamin before the super twist adopts a helical structure similar to one of the above-mentioned structures, a rotation of the vesicle by $\sim 130^\circ$ (one third of a circle) requires that the GTPase domain of dynamin slides by at least ~ 4 dynamin units along the helix. In majority of these events (33/45), the super twist was completed in a single step. This suggests that this process involves coordinated actions from multiple dynamins in the helix, rather than independent, sequential GTP hydrolysis and twisting steps. This is consistent with the Two-Stage model. Considering the stepping nature of dynamin sliding on each other in the helix upon GTP hydrolysis, it is possible that once GTP hydrolysis starts, a cascade of reactions follows such that the completion of the earlier reaction triggers the next one, until the twist is completed. Thus, the super twist may consist of several steps that appear as a single large step and the rates of GTP hydrolysis measured *in vitro* could be much slower than those accomplished *in vivo*. Note that a defective dynamin in the helix may pause or terminate the cascaded reactions, which is consistent with the observation that a small fraction of defective dynamin drastically decreases the fission activity¹².

We also speculate that the super twist may be paused or terminated in the process. A fraction of these events resumed the super twist (e.g., 12/45 of the completed endocytosis events) very quickly (< 0.1 s) and scission was completed. In many other cases ($n > 50$), we observed super twist-like actions that did not result in scission and instead were likely terminated. These observations suggest that in some cases ($> 50\%$) the GTP hydrolysis reactions failed to produce sufficient constriction to drive scission and are

consistent with the stochastic nature of dynamin-catalyzed scission observed *in vitro*^{13,14}. Regardless of whether the super twist continues to complete scission, or fails, the accumulated dynamin dissociates. This is consistent with the Two-Stage model that GTP hydrolysis destabilizes dynamin helix and leads to its disassembly.

The state of dynamin assembly before and after super twist. The super twist always occurs near the peak of dynamin accumulation, which is an indication that dynamin recruitment has been completed. An important question is what are the structures of dynamin molecules before and after the twist?

It is generally accepted that assembled dynamin adopts a helical structure before scission but the structure during and after scission is still under debate. Several dynamin helical structures have been discovered by cryoEM experiments⁴⁷. Especially of interest is the “super-constricted” helix (11.8 pairs of dimers per turn) with an inner diameter of 3.7 nm, which has such a small radius that the membrane tube inside can proceed to the hemi-fission intermediate with barely any energy barrier²⁰. It is tempting to assign the dynamin helix after the super twist as the “super-constricted” helical state, and dynamin assembly before the super twist as one of the less constricted helices. However, it also has been reported that the super-constricted dynamin helix has a unique two-start structure. Recruiting of additional dynamin and elongation of the membrane tube are required to convert one-start, less constricted helices to the super-constricted state³⁰. These observations contributed to the argument that the helices captured in cryoEM experiments may not necessarily be on the same pathway leading to scission; other undiscovered intermediates may exist. Yet, it is also possible that they are on the same pathway: the conversion of one-start helix to two-start helix could happen during the cutting, and the super twist could reflect this twisting and tightening process with the insertion of pre-accumulated dynamins into the helix. All these indicate the complexity of the scission process.

While we are unable to assign any of the known constricted helices to the dynamin states before or after the twist, we speculate that after the super twist, the dynamin helix is either in a very constricted state ready for hemi-fission, or, more likely, has triggered hemi-fission and partial disassembly of the dynamin helix during the twist. It is important to note that after the super twist, a limited increase of rotational freedom (Figure S11) was observed yet the vesicle did not leave the endocytosis spot, suggesting that the stable dynamin-helix/membrane tube structure has already been broken but the vesicle is still connected to the membrane, possibly thought the remaining of the broken protein scaffold. Complete detachment of the vesicle from the membrane, which is characterized with drastic rotational freedom and sometimes a translational movement in the 3D space, usually happens at a later time shortly after the super twist (Stage e, 2.1 ± 1.4 s, mean $\pm$ SD, $n = 45$, Fig. 4D). So far, we are unable to determine the exact fission point, whether at the end of the super twist, or at the beginning point of the active rotation, or sometime in the middle. However, the delayed start of active rotation possibly suggests that scission is not an instantaneous action but a gradual process that takes some time to finish. It is likely that the super twist irreversibly destabilizes the dynamin helical structure and induces hemi-fission and partial disassembly of dynamin. Yet, the complete disassembly of dynamin and severing of the vesicle from the membrane

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but in a restrained mode, as observed in Stage e. Finally, it is unclear but also possible that more GTP hydrolysis is required in this process to completely sever the connection between the vesicle and the membrane. Other methods will be needed to identify the dynamin helical structures before and after the super twist.

The static period (Stage c) before the super twist. The static period, which lasted for 1.9 ± 1.5 s (Mean $\pm$ SD) for 40 cases, is another interesting finding (Stage c, Fig. 4C). We speculate that the dynamin collar has formed around the CCP at this stage so as to limit the fluctuation of membrane and restrict the cargo rotation. The static nature indicates that dynamin hydrolysis of GTPs did not start immediately but instead the dynamin assembly seemed to wait for a signal to trigger the hydrolysis reaction.

This unexplained phenomenon possibly suggests that GTP hydrolysis by assembled dynamin helix is regulated through an unknown mechanism. Indeed, there are many processes happening during scission that are less clear. For example, although the proline-rich domain (PRD) of dynamin is not required for fission activity *in vitro*, dynamin's GTPase and fission activities are regulated by SH3 domain proteins that bind to the PRD^{48,49}. Scission is also regulated by BAR-domain proteins such as endophilin and amphiphysin^{6,48-51}. Indeed, endophilin, which is recruited in a synchronized manner with dynamin at the late stages of scission⁵¹ and was thought to promote dynamin function⁵¹ has also been shown to inhibit dynamin function at high concentrations^{52,53}, which may be relevant to the pause of GTPase functionality after dynamin assembly. Another study showed that the stimulatory action of amphiphysin on dynamin is dependent on lipid bilayer curvature⁵⁴. These interactions may be relevant to the paused start of GTPase functionality after dynamin assembly. Or, as an alternative explanation to chemical signaling, the control of GTP hydrolysis may simply be physical: the restoring force from the squeezed membrane tube may stall GTP hydrolysis. Further study is needed to better explain this phenomenon.

Scission model based on information derived from cargo vesicle rotation. From a perspective based on cargo's rotation, there are many processes happening during scission. Combined with observations from other channels including molecular fluorescence and cargo's translational movement, these processes are summarized in Fig. 5.

The endocytosis starts with the cargo's active rotational motion slowing down to complete immobilization (Stage a). Clathrin accumulates during this stage. Then, a pulse of dynamin accumulation starts, initiating Stage b. The cargo may restore intermittent slow or fast random rotations, or stays immobilized. For those cargos restored rotational freedom, their rotation pauses (Stage c) when dynamin accumulation peaks. GTP hydrolysis by assembled dynamin is then initiated either through an external trigger or simply by the completion of dynamin assembly, which generates a $\sim 130^\circ$ right-handed super twist (Stage d). It is likely that the super twist leads to the hemi-fission of the membrane tube and the partial disassembly of dynamin helix. Then, the cargo undergoes another period of relatively slow, constrained rotation (Stage e), during which dynamin completely disassembles and scission completes. At the final stage (Stage f), the vesicle is detached from cell membrane. It either restores active random

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Our observations that membrane fission occurs during or after a twisting motion driven by dynamin are consistent with the Ratchet model. However, that the super twist happens in an abrupt manner and completes within a very short time, suggests that this process involves the coordinated actions of multiple dynamins. Moreover, our data suggest that GTP hydrolysis induces dynamin helix disassembly in both successful and failed scission events. These observations are in part consistent with the Two-Stage model.

Conclusions

In summary, we demonstrated the characteristic translational and rotational motions of the AuNR cargos at different stages throughout the clathrin-mediated endocytosis process. We found that as dynamin accumulates, and presumably assembles, it induces the cargo to rotate slowly with uncontrolled directions. At the final stage of scission, the dynamin helix induces a large, right-handed twist through coordinated actions from multiple dynamin molecules. This unequivocally shows that a torque is generated in dynamin action *in vivo*. The super twist is the driving force for the scission to happen because: (1) it happens at the dynamin accumulation peak time; (2) after the super twist, significant enhancement of rotational freedom is observed; and (3) such a twist is absent in cells that express a dynamin mutant defective in GTPase activity. The new observations derived from the live-cell imaging give direct evidence of dynamin action during it functioning. It paves the way toward a deeper, more comprehensive understanding of how dynamin works by combining other feature studies such as structural characterization and molecular simulations.

Experimental Procedures

Multi-dimensional microscopy. A Nikon Eclipse 80i upright microscope equipped with a heating stage (Bioscience Tools) was used in this study. For dark-field microscopy, a Nikon oil immersion dark-field condenser (numerical aperture (NA): 1.20–1.43), and a Nikon 60 $\times$ Plan Fluor oil immersion objective (NA: 0.5–1.25) were used with the NA set to 1.0. The same objective was used for both excitation and emission collection. The scattering of gold nanoparticles was excited using a transmission light from a halogen lamp that is filtered with a band pass filter (FF01-650/13, Semrock). The fluorescence of GFP-Dynamin and RFP-Clathrin were excited using two collimated and coaligned 488 nm (50 mW) and 561 nm (100 mW) lasers (Oxxius) which were reflected into the collecting objective by a dual-band dichroic mirror (Di03-R488/561, Semrock). A dual-band pass filter (FF01-482/563, Semrock) in front of the dichroic mirror was used to clean up the two lasers. To reduce the fluorescence background, variable angle epifluorescence illumination configuration was used. Both illumination pathways (dark-field scattering and fluorescence) are turned on at the same time in multi-dimensional imaging experiments.

Simultaneously dark-field scattering, and fluorescence imaging were achieved using a self-design and home-made 4-channel imaging module (Fig. 1). Briefly, the fluorescence and dark-field signals were collected simultaneously through the same objective and relayed to the 4-channel imaging module for

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js background from the lasers, a combination of

two notch filters (ZET488NF and ZET561NF, Chroma) was used before entering the 4-channel imaging module. The collected signals were first split by a long pass dichroic mirror (BLP01-635R, Semrock): reflection for fluorescence (left arm) and transmission for dark-field scattering (right arm). The reflected fluorescence signals were further split into 2 channels using a long pass dichroic mirror (LP03-532RU, Semrock). Fluorescence signals of dynamin and clathrin in each channel were filtered using two band pass filters FF01-512/25 (Semrock) and FF01-598/25 (Semrock), respectively. Both channels of the fluorescence images were then focused to different portions of a same EMCCD camera chip (Andor Technology; iXonEM + Ultra 888) through the same left exit port of the imaging module. Bifocal imaging, parallax microscopy, and the auto-chasing under dark-field mode were achieved in the right arm of the 4-channel imaging module. First, the dark-field image was split into a focused channel and a defocused channel using a beam splitter (Thorlabs) where 30% of signal in focused channel for localization and auto-chasing and 70% of signal in defocused channel for orientation determination. In defocused channel, an additional focal lens ($f = 500$ mm) was inserted into the optical pathway to create a defocusing distance of $0.9 \mu\text{m}$, which was found to generate the most suitable defocused image patterns for orientation determination. The dark-field images in focused and defocused channels were focused to different portions of a second EMCCD camera (Andor Technology; iXonEM + Ultra 897) through the same right exit port of the 4-channel imaging module.

All dark-field movies were recorded at 50 frames per second (fps). The heating stage was set to 37°C to maintain the living conditions for cells. Fluorescence images were recorded at 1 or 0.5 fps. The image acquisition on two EMCCD cameras were triggered and synchronized using a mechanical shutter. The acquired images were analyzed using ImageJ and MATLAB.

3D-SPORT. Bifocal imaging, parallax microscopy, and an automatic feedback control module are the key components of the imaging system. The purpose of the design is to keep the target probe in focus continuously in the focused channel to extract the 3D spatial coordinates (x, y, z) with nanoscale precision while obtaining image patterns with a constant defocusing distance in the defocused channel to determine the probe's orientation angles accurately and robustly.

The auto-chasing was achieved using parallax imaging in the focused channel. Briefly, a custom-cut wedge prism (0.5° , Edmund) was inserted in the focused channel near the back focal plane of the objective to deviate half of the scattering light from the sample by a very small angle, effectively splitting the light to form two *half-plane* images (as opposed to the standard full-plane images using the full pupil) in only the focused channel. The two associated half-plane mirror images for a single probe are always manually aligned vertically, and they will be referred to as the *upper* and *lower* image, respectively, throughout our discussions. The x and y coordinates were resolved by Gaussian fitting. The z position of a probe is a function of the separation distance (Δy) between its upper and lower images. A calibration curve was constructed experimentally to correlate this relationship. The experimental localization precision in the x, y , and z axes (Figure S6) is 4.9, 6.3 and 14.0 nm, respectively. Finally, the automatic feedback control system, composed of a piezo objective scanner (Physik Instrumente) and a tracking probe in focus. When the target probe was in

perfect focus, the y distance between the upper and lower images in the focused channel was obtained as the reference (Δy_0). During the tracking, Δy was calculated for every other frame. The tracking program would move the objective scanner to keep the target probe in focus in the next frame.

Preparation of surface-modified gold nanorods for cell experiments. Sodium citrate-capped AuNRs (40 nm $\times$ 80 nm) were purchased from Nanopartz. The average aspect ratio of the AuNRs is 2.0. The electrostatic adsorption method establishing transferrin-coated AuNRs was used by following a published method⁵⁵. Briefly, 100 μ L of the AuNRs solution (3.7×10^{10} mL⁻¹) was mixed with 4 μ L of 50 mM borate buffer (pH 8.5). Then 5 μ L of 3 mg/mL transferrin (CAS: 11096-37-0, Sigma-Aldrich) was added to the gold nanorod solution and reacted for 3 hours. Finally, the mixture was centrifuged at 5000 rpm for 5 min twice and resuspended in 100 μ L of 2 mM borate buffer (pH 8.5).

Cell culture. A549 human lung cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA; catalog number: CCL-185). SK-MEL-2 cell line was a gift from the Drubin group (UC Berkeley). The cells were cultured in a T25 cell culture flask (Corning) and grown in a cell culture medium supplemented with 10% fetal bovine serum (FBS). When subculturing, 150 μ L of cell suspension solution was transferred to a 22 $\times$ 22 mm coated coverslip, housed in a 35-mm Petri dish (Corning). After that 1.5 mL of the cell culture medium with 10% FBS supplement was added to immerse the coverslip, the Petri dish was kept in the incubator for 48 hours.

Transfection of A549 cells to express GFP-Dynamin 2- K44A. *E. Coli* bacteria with GFP-Dynamin 2-K44A plasmid was purchased from Addgene (Plasmid 22301). The plasmid was purified from the bacteria with the plasmid extraction kit (cat. 27104). The high density A549 cells were subcultured on a coverslip in a Petri dish in the incubator. After 24 hours, the GFP-Dynamin 2- K44A was mixed with lipofectamine 3000 reagent in the Opti-EME medium (ThermoFisher) first and then add to the Petri dish. After 48 hours of incubation, the cells were ready for imaging.

Quantification of AuNRs Internalization. The wild type A549 cells and A549 cells expressing GFP-Dynamin 2-K44A were cultured on a coverslip in Petri dishes overnight in the incubator. Then, AuNRs functionalized with transferrin were injected into the Petri dish and the final concentration of AuNRs was 3.7×10^9 mL⁻¹. After two-hour of incubation in the incubator, the cells were ready to be used. The uptake of AuNRs was visualized with a DIC microscope at 650 nm wavelength and z-stacks were obtained using an objective scanner and reconstructed with ImageJ. Then the endocytosed AuNRs in individual cells were identified and counted. The counted numbers of AuNRs per cell in A549 cells expressing GFP-Dynamin 2- K44A and wild type A549 cells were 2.0 ± 1.0 (Mean $\pm$ SD, $n = 20$), 11.4 ± 4.4 (Mean $\pm$ SD, $n = 20$), respectively.

Live-cell imaging of endocytosis. Two pieces of double-sided taps were adhered to the clean glass side. A cover glass with adherent cells was placed on the top of the double-sided taps with cells facing to the glass slide, to form a chamber. Then 5 μ L of the transferrin@AuNRs solution mixed with 45 μ L of the cell

culture medium was injected into the chamber. To prevent evaporation and leakage of the medium, the cover glass was sealed by nail polish.

Declarations

Author Contributions

X.C., K.C., B.D. contributed equally to this work. G.W. and N.F. conceived the idea. X.C., K.C., B.D., G.W. and N.F. designed the research. X.C., K.C. and B.D. built the imaging setup. All authors performed the experiments and wrote the manuscript.

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Figures

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Figure 1

Scheme of 3D SPORT. (A) Schematic of the instrument. (B) Definition of the 3D orientation: the polar angle θ and the azimuth angle φ . (C) Experimental and simulated defocused images of a AuNR with fixed polar angle at different azimuth angles. The scale bar in C is 1 μm .

Figure 2

Multi-dimensional imaging of a AuNR and surrounding molecular fluorescence. (A) Clathrin channel. (B) Dynamin channel. (C) Focused scattering channel for AuNRs. (D) Overlaid images. (E) Zoomed-in views of the squared region in Figure (D) of the dynamin channel (top row), clathrin channel (middle row) and focused scattering channel (bottom row) at different times. (F) Time evolution of clathrin and dynamin fluorescence on the entry spot, clathrin and dynamin fluorescence on the vesicle, the lateral (xy), and axial (z) displacements of the particle from the entry spot during an endocytosis event. Z-axis direction is defined as pointing toward the outside of the cell. The dashed line indicates the time of fission.

Figure 3

A typical example of an endocytic event with vesicle rotational information. (A) The overlay of the time evolution of the cargo's xy- displacements, rotational azimuth, and polar angles, z-displacement from top to bottom, and dynamin fluorescence in shaded light green in the background, respectively. Labels a, b, c, d, e, and f represent various stages during endocytosis. (B) Expanded time window near the fission point (orange frame in A). (C) Cargo's defocused image patterns showing the "super twist" at Stage d, $t = 20$ ms. (D) Schematic diagram of the AuNR's right-handed rotation during the "super twist".

Figure 4

Statistics of major events before fission. (A) Distribution of twisting angles. (B) Duration of the super twist averaged at 0.28 ± 0.18 s (Mean $\pm$ SD, $n = 45$) (Stage d). (C) Waiting time before the super twist (Stage c, $n=40$). (D) Duration of the slow non-directional rotation time after the super twist and before fission (Stage e).

Figure 5

Scheme of the dynamin scission model.

Supplementary Files

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