Endocytic Clathrin Coats Develop Curvature at Early Stages of Their Formation

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Summary: Endocytic clathrin coats acquire curvature without a major flat-to-curved transition that requires an extensive reorganization of the clathrin lattice.

Sculpting a flat patch of membrane into an endocytic vesicle requires curvature generation on the cell surface, which is the primary function of endocytic protein complexes. The mechanism through which membrane curvature is imposed during formation of clathrin-coated vesicles is an ongoing controversy. Using super-resolved live cell fluorescence imaging, we demonstrate that curvature generation by clathrin-coated pits can be detected in real time within cultured cells and tissues of developing metazoan organisms. We found that the footprint of clathrin coats increase monotonically during formation of curved pits at different levels of plasma membrane tension. These findings are only compatible with models that predict curvature generation at early stages of endocytic clathrin-coated pit formation. Therefore, curvature generation by clathrin coats does not necessitate a dynamically unstable clathrin lattice that would allow an abrupt flat-to-curved transition.

Clathrin-mediated endocytosis is the most extensively studied internalization mechanism of membrane lipids and proteins from the cell surface [1,2]. Over the past decades, a multitude of biophysical and biochemical methodologies have been employed to elucidate structural and dynamic properties of endocytic clathrin coats [3]. However, fundamental aspects of clathrin-mediated endocytosis remain controversial due to the lack of experimental approaches that allow correlation of ultra-structural and dynamic properties of clathrin-coated structures. Clathrin triskelions can assemble into coats (polyhedral cages and lattices) in seemingly infinite numbers of geometries upon their recruitment to the plasma membrane by a range of adaptor proteins including AP2 [4,5]. Based on their structural and dynamic properties, clathrin coats are categorized into pits and plaques [6,7]. Clathrin-coated pits are highly curved structures that are internalized within few minutes of their initiation at the plasma membrane [8–10]. Due to their small size (100-200 nm in diameter), they appear as diffraction-limited spots under conventional fluorescence

imaging[11]. Plaques are large and flat hexagonal lattices of clathrin that are longer-lived than pits [7,12,13].

Regardless of shape and size, formation of endocytic vesicles requires curvature generation during the lifespan of clathrin coats. However, how and at what point of its growth a clathrin coat develops curvature has been a matter of debate for almost four decades [14–16]. Currently, there are two competing models as to how clathrin and its accessory proteins generate curvature that leads to formation of clathrin-coated pits. Based on electron micrographs, it was originally proposed that clathrin initially grows into a flat hexagonal array on the plasma membrane prior to transitioning into a curved coat [4,17] (Fig. 1A, top). This flat growth is postulated to reach 70-100% of the final surface area before remodeling into a clathrin-coated pit through transformation of the hexagonal clathrin lattice into a curved polyhedron with pentagonal and hexagonal faces [4,18,19]. Eventually, 12 pentagonal faces must be introduced to generate a closed clathrin cage [20,21] (Fig. 1B & D). This model was rejected by others because insertion of even a single pentagonal face into a hexagonal lattice requires a substantial structural rearrangement (see Figure 13 of [4]), which is energetically unfavorable [14,15,22]. As an alternative, it was suggested that clathrin-coated pits can form gradually without a major structural rearrangement. According to this model, as clathrin triskelions are integrated into the coat, they organize into pentagonal as well as hexagonal faces to generate a constant amount of curvature (Fig. 1A, bottom). The debate regarding curvature formation by clathrin pits has been recently rekindled by studies employing correlative fluorescence and electron microscopy of clathrin-coated structures [16,18,19,23].

One of the major observables that distinguish the two models is the change in the projected area (i.e., footprint) of the clathrin coat as it gains curvature: While the constant curvature model predicts a steady rise in the projected area of the coat, the flat-to-curve transition requires an abrupt

~3-4 fold reduction in the same measure (see Figure 6 of [4] and Figure 1 of [19]). To demonstrate this, we adapted a previously described self-assembly model to simulate growth of clathrin polyhedra with different triangulation (T) numbers (Figure 1B-F; Movies S1 & S2) [24]. We chose to work with these specific geometries as their sizes are in good agreement with our experimental findings (Figure 3), and the previously reported clathrin-coated pits [4,18,19,25,26]. In Figure 1B, 140 triskelions are assembled into a T=7 polyhedron (60 hexagons and 12 pentagons; ~137 nm diameter) via a flat-to-curved transition taking place at 70% of completion (upper panel), and as predicted by the self-assembly (constant curvature) model (lower panel). Similarly, Figure 1D shows assembly of a larger polyhedron (T=9) using 180 triskelions (80 hexagons and 12 pentagons; ~156 nm diameter) using both models (Fig. 1D). In both scenarios, flat-to-curved transition (marked with green arrows) result in significant decrease in the footprint of the clathrin coat. Note that the change in the footprint would be more pronounced if the transition took place after the full assembly (100% completion) of the coat (i.e., constant area model), as proposed earlier [18].

Electron microscopy provides high-resolution snapshots of clathrin-coated structures with different curvature levels [4,18,19,26]. However, due to the lack of temporal dimension, electron micrographs fail to provide direct evidence for the mechanism of clathrin-driven curvature generation. Conventional live cell fluorescence imaging allowed researchers to elucidate the formation and dissolution dynamics of endocytic clathrin coats in live cells [9,11,27]. In these studies structural properties of clathrin coats are obscured by diffraction of fluorescence, which limits spatial resolution. Nevertheless, diffraction limit in fluorescence imaging can be circumvented by recently developed superresolution microscopy techniques. In these assays, curved clathrin coats (i.e. pits) appear as rings, which correspond to two-dimensional projection of a spheroid clathrin coat [28–30]. Using the triskelion coordinates in our self-assembly models, we simulated high-NA

TIRF-SIM (structured illumination microscopy in the total internal reflection fluorescence mode) images of T=7 and T=9 polyhedra at different completion levels (Figs. 1C & E). Indeed, ring patterns developed in the TIRF-SIM simulations upon formation of pits, and major reduction in the projected area could be detected on the *x-y* plane upon the simulated flat-to-curved transitions (Fig. 1F).

We employed TIRF-SIM to image formation of clathrin-coated pits in cultured cells (in vitro) and tissues of developing *Drosophila* melanogaster embryos (in vivo) with high spatial and temporal resolution and monitor curvature generation by endocytic clathrin coats in real time [30] (Movie S3). In these assays, the projected area of clathrin-coated pit images increased monotonically until the ring pattern arises, which is followed by a relatively fast disappearance of fluorescence due to uncoating (Fig. 2). To perform a bulk analysis, we developed a custom tracking software for manual selection of individual clathrin-coated pit traces that appear independent of other structures from their formation to dissolution, and contain a single phase of fluorescence intensity growth followed by a decline. We created the time average of clathrin-coated pit traces extracted from BSC-1 and COS-7 cells imaged with high-NA TIRF-SIM, and calculated the temporal evolution of the projected area (see methods; Movies S4 & S5; Figs. 3A & S1). Contrary to the predictions of the flat-to-curved transition models, we detected no decrease in the footprint of the coat prior to appearance of the ring pattern. Instead, we found that the ring pattern is observable as the projected area makes a plateau and reaches the maximum (Fig. 3A, B), as predicted by the constant curvature model (Fig. 1F).

As an alternative to manual selection, we developed an algorithm for automated detection of individual clathrin-coated pit traces that appear independent of other clathrin structures in TIRF-SIM acquisitions. To ensure detection of clathrin-coated pit formation, these automatically

selected traces had to have the ring pattern appear in at least two consecutive frames. The first appearance of the ring pattern in the trace is marked as the "ring frame", which is used for synchronization of traces and sorting them into three groups based on area (i.e., small, medium and large). Figure 4A shows the average of synchronized traces (extending to 5 frames before and after the ring frame) detected in BSC-1 cells imaged at 0.5 frames/second. In good agreement with the manually selected traces, we found that the footprint of the structures increase and reach a plateau with the appearance of the ring pattern in all three groups (Figs. 4B, C). The results of the same analysis are shown in Figures S2-4 for COS-7 cells and BSC-1 cells imaged at different frame rates. Overall, contrary to the predictions of the flat-to-curved transition models, we found that the projected area of coats increases throughout maturation of clathrin-coated pits (Fig. 4D).

Plasma membrane tension is one of the most important determinants of endocytic clathrin coat structure and dynamics [19,31–34]. Our automated analysis on genome edited SUM-159 cells expressing AP2-EGFP [35] revealed that average size of clathrin-coated pits increase when plasma membrane tension is elevated by hypo-osmotic shock (Fig. 5), in good agreement with previous reports [33]. Aside from the size increase, we detected no difference in the temporal evolution of the clathrin coat footprint during formation of pits (Fig. 5B, C). These results show that increased plasma membrane tension does not induce a late stage flat-to-curved transition that leads to formation of clathrin-coated pits.

Flat-to-curved transition of the clathrin coat requires a major structural reorganization that disrupts the hexagonal arrangement of the flat clathrin lattice [14]. It is proposed that a dynamically unstable clathrin coat can go through such transitions by allowing rapid exchange of clathrin triskelions [18]. This assumption is supported by fast fluorescence recovery rates detected after photobleaching of fluorescent clathrin spots in live cells. It is important to note that a majority of the fluorescent spots

that appear as individual clathrin-coated structures under diffraction-limited imaging actually contain multiple independent structures that are positioned in close proximity (Fig. S5, Movie S6). Therefore, it is worthwhile to consider that the detected fluorescence recovery may actually correspond to initiation and growth of adjacent clathrin-coated structures.

In this study, we analyzed clathrin-coated pit formation with high spatiotemporal resolution in a wide range of cell types as well as, for the first time, tissues of a developing organism. Our findings clearly demonstrate that curvature generation by clathrin-coated pits takes place through a mechanism that is independent of a flat-to-curved transition as proposed previously. The resolving power of TIRF-SIM (~90 nm; [30]) is sufficient to detect the proposed 3-4 fold area reduction that results in a ~100 nm diameter clathrin-coated pit (Fig. 1B). Even though our findings are more compatible with the constant curvature model, we do not rule out the possibility of a flat-to-curved transition at very early stages of clathrin coat formation [36], i.e. while the flat hexagonal lattice is smaller than the resolution limit of high-NA TIRF-SIM. Theoretically, such a transition can take place with limited rearrangement of the precursor hexagonal lattice by incorporating pentagonal faces asymmetrically, i.e., predominantly at later stages of clathrin-coated pit formation [37]. It is also important to note that flat-to-curved transition and constant curvature models are proposed to explain formation of individual clathrin pits that are independent of other clathrin-coated structures. We therefore do not extend our conclusions to endocytic clathrin pits detected at the peripheral regions of plaques [16,37]. Indeed, it is possible to observe clathrin pits that appear to disassociate from larger clathrin assemblies in TIRF-SIM acquisitions. However, in these assays, it is not possible to ascertain whether these structures are independent clathrin pits that happen to originate in the vicinity of a plaque [15]. It is important to note that clathrin plaques are predominantly observed at the adherent surfaces of cells and their abundance is dependent on adhesive properties

of the substrate on which cells are cultured [5,38]. It is likely that, rather than endocytic, their primary function is related to cell-substrate adhesion[6,16] and subsequent mechanotransduction[13]. Altogether, our results signify the importance of employing methodologies comprising high resolution in both spatial and temporal dimensions for constructing dynamic models.

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Figure 1.

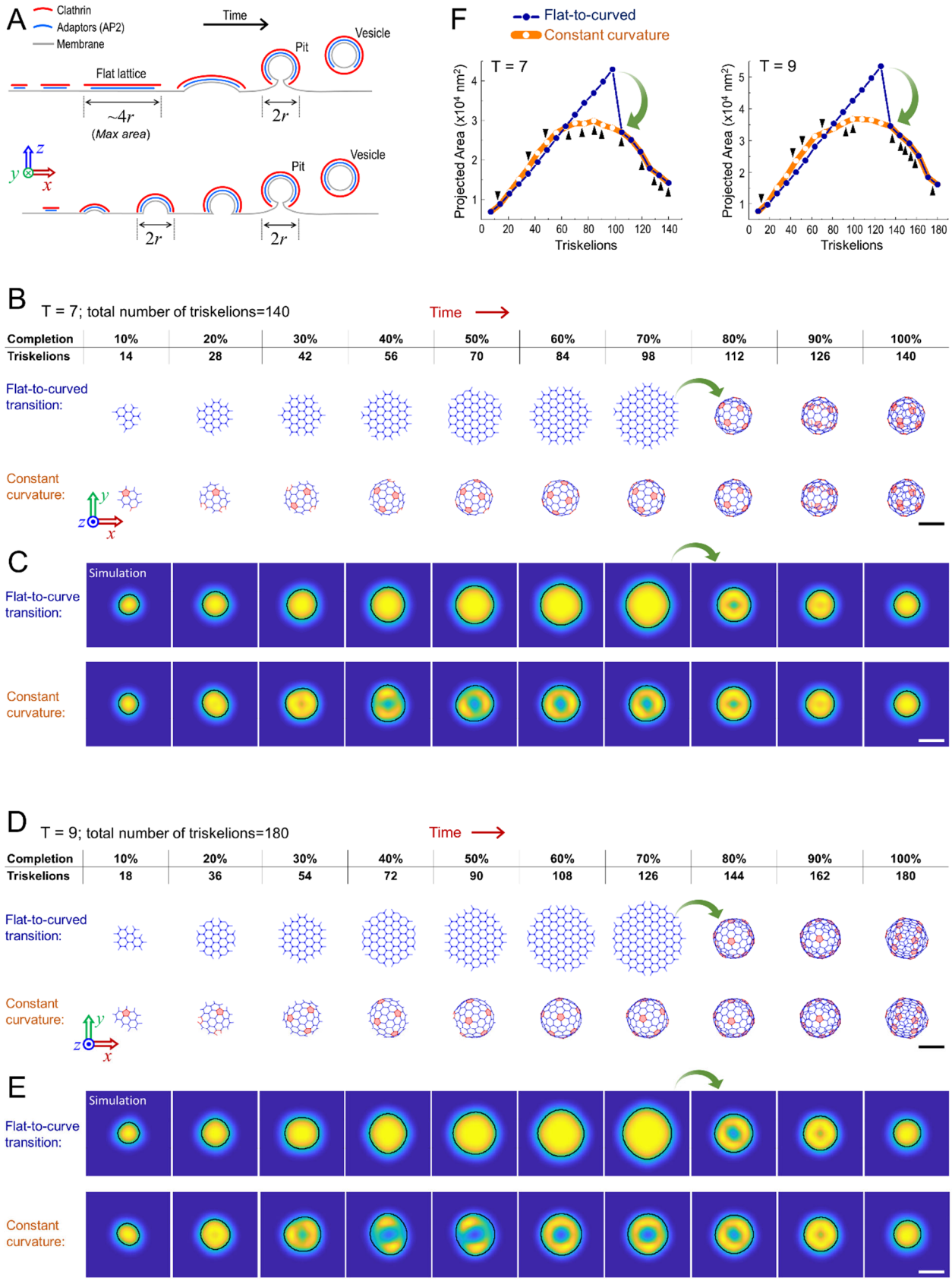
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Figure 1. Flat-to-curved transition necessitates a major change in the projected area of clathrin coats. A. Schematics depicting the cross-sections of clathrin coats as suggested by prevalent curvature generation models. According to the flat-to-curved transition models (upper), the clathrin coat initially grows into a flat hexagonal lattice constituting 70-100% of the final surface area of the coated vesicle ($4\pi r^2$; r is the radius of the pit/vesicle). A disc-shaped, flat clathrin lattice is anticipated to reach a diameter of $3r$-$4r$ prior to the transition into a pit [4,18,19]. Upon this transition, the projected area reduces to πr^2. The constant curvature model (lower) predicts a gradual increase in the projected area until the diameter reaches $2r$, which is also the diameter of the clathrin pit/vesicle. **B.** 140 triskelions are assembled into a T=7 polyhedron through a flat-to-curved transition (marked by green arrow) taking place at 70% of growth (upper panel) and as predicted by the constant curvature model (lower panel; movie S1). **C.** Snapshots of high-NA TIRF-SIM simulations correspond to the distinct completion levels of T=7 clathrin coats in (B). Detected boundaries are shown by the black demarcations for both flat-to-curved transition and constant curvature models. **D.** 180 triskelions are assembled into a T=9 polyhedron through a flat-to-curved transition (marked by green arrow) taking place at 70% of growth (upper panel) and as predicted by the constant curvature model (lower panel; movie S2). **E.** Snapshots of high-NA TIRF-SIM simulations correspond to the distinct completion levels of T=9 clathrin coats in (D). Detected boundaries are shown by the black demarcations for both flat-to-curved transition and constant curvature models. **F.** Projected area of the structures obtained from high-NA TIRF-SIM simulations (C & E) are plotted with respect to number of triskelions for both models. Green arrows mark the significant reduction in projected area upon the proposed flat-to-curved transition at 70% of coat growth. Black arrowheads mark completion of pentagonal faces predicted in the constant curvature model. Scale bars, 100 nm.

Figure 2.

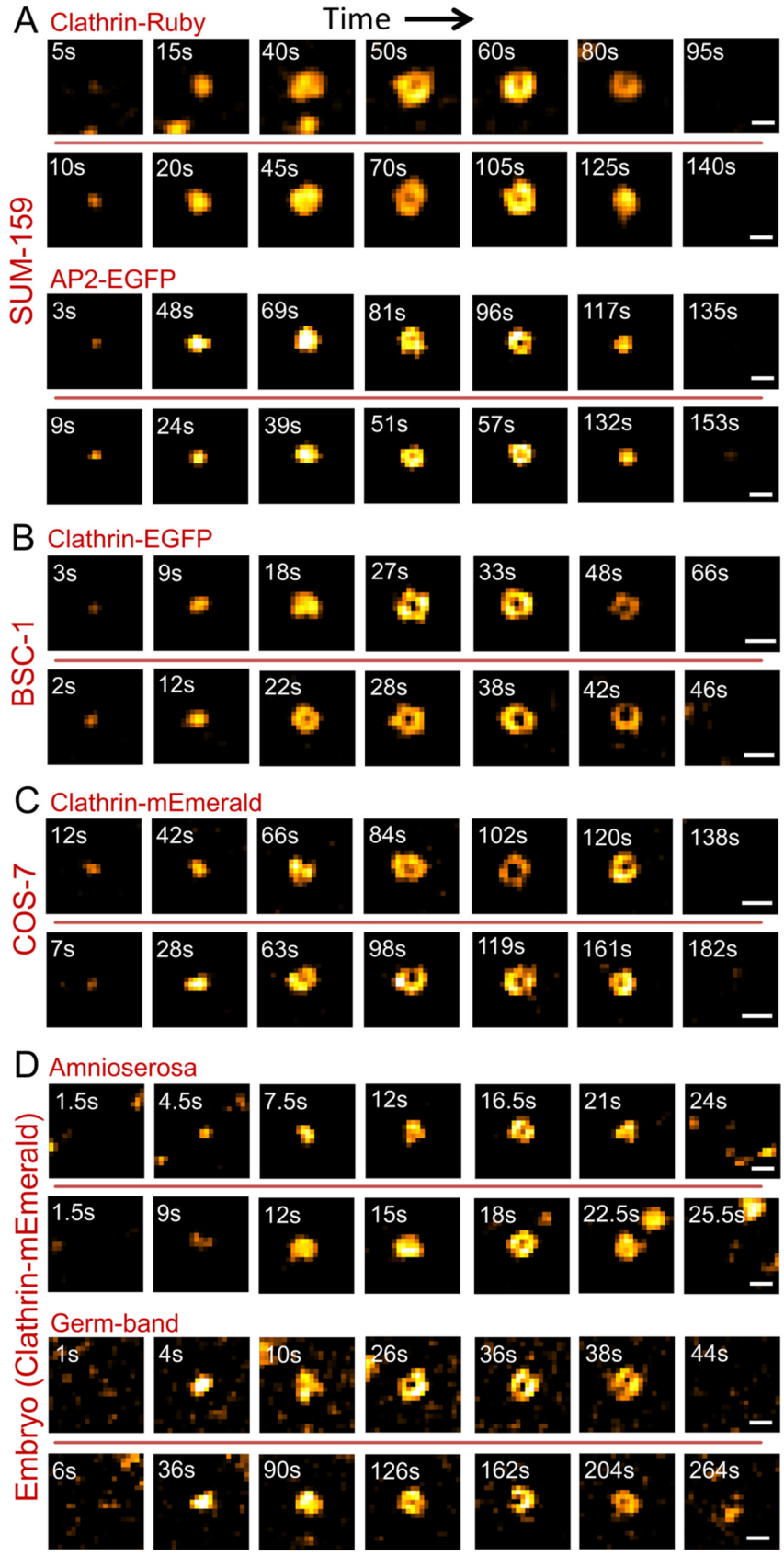
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Figure 2. TIRF-SIM allows analysis of clathrin-coated pit formation in cultured cells and in tissues of Drosophila embryos with high spatiotemporal resolution. Each row shows snapshots from individual clathrin-coated pit traces. Formation of pits (marked by a ring pattern) can be observed under various *in vitro* and *in vivo* conditions including SUM-159 cells expressing clathrin-Ruby and AP2-EGFP as endocytic markers (A), BSC-1 cells expressing clathrin-EGFP (B), COS-7 cells expressing clathrin-mEmerald (C), and amnioresora and germ-band tissues of Drosophila embryos expressing clathrin-mEmerald (D). Two traces are shown for each condition. Scale bars, 200 nm.

Figure 3.

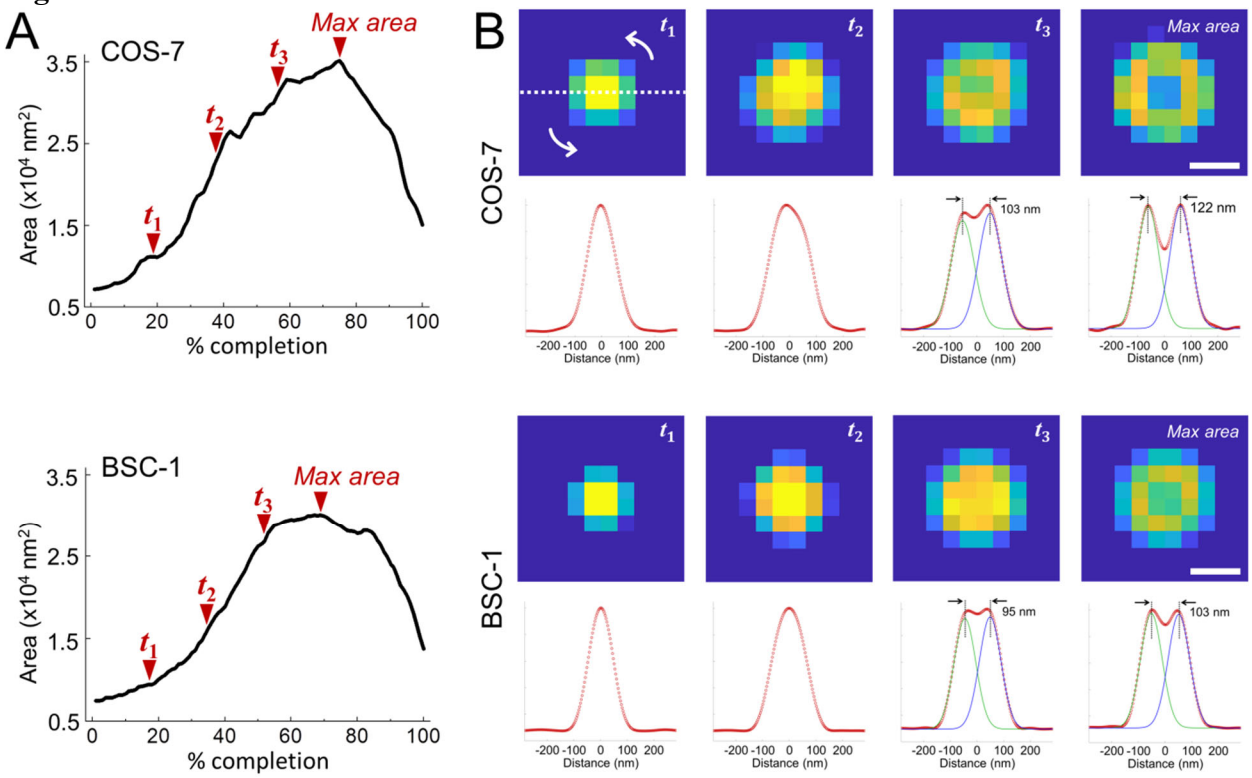
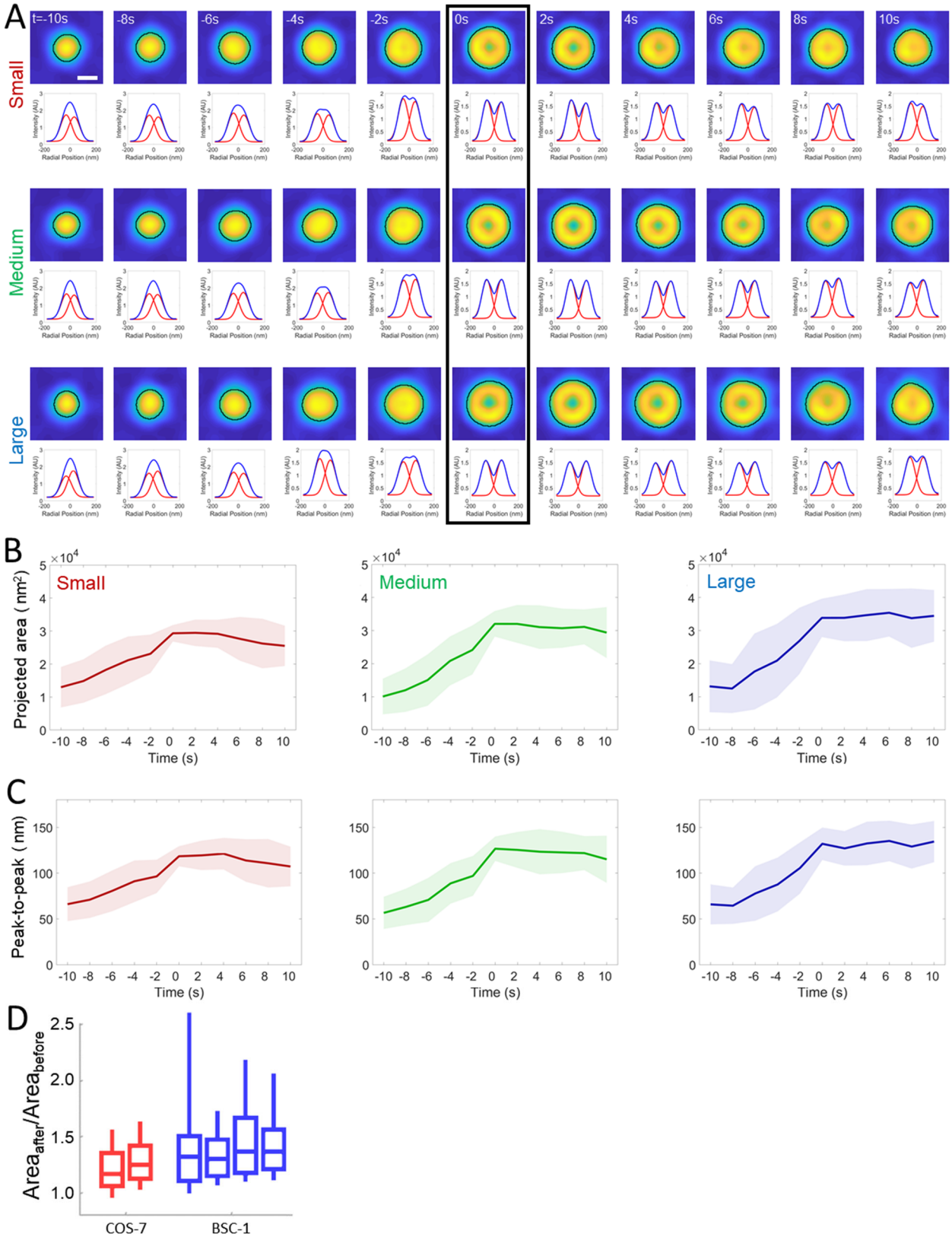


Figure 3. Clathrin coats reach maximum projected area by formation of pits. A. Average clathrin-coated pit formation and dissolution images using 34 and 81 individual traces are obtained from COS-7 cells expressing clathrin-mEmerald and BSC-1 cells expressing clathrin-EGFP, respectively (Movies S4 & S5). In both cases, the area of the average image increases monotonically until a plateau is reached, which is followed by a relatively sharp decrease. **B.** For both cell types, average images of clathrin coats are shown for four equally spaced % completion points, last of which is the maximum area point (denoted as t_1, t_2, t_3 and *max area* in A). Note that the ring pattern becomes apparent as the area converges to the maximum. Below each image is the radial average (red circles), which is obtained along a cross-section rotated 180° around the center. We used two Gaussian fits to the radial average (green and blue) to calculate the peak-to-peak separation as an estimate of the ring size. Scale bars, 90 nm.

Figure 4.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COS-7
BSC-1

Figure 4. Automated analysis of the projected area during formation of clathrin pits. A. 187 traces detected from two BSC-1 cells (imaged at 0.5 frames/second) are grouped and synchronized using the first detected ring pattern (black rectangle). Average images (top) and corresponding radial averages (bottom) are shown for each group (small, medium and large). Detected boundaries are shown by the black demarcations. The temporal evolution of the average area **(B)** and peak-to-peak separation of the Gaussian fits to the radial averages **(C)** are plotted for the three groups. Shaded areas represent the standard deviation. **D.** Boxplots show the ratio of the maximum projected areas detected after and before the ring frame ($\text{Area}_{\text{after}}/\text{Area}_{\text{before}}$) in clathrin traces automatically detected from two COS-7 (red; $N_{\text{traces}} = 694$) and four BSC-1 cells ($N_{\text{traces}} = 397$) imaged with high-NA TIRF-SIM. Overwhelming majority of the clathrin structures reach maximum area upon formation of pits, in good agreement with the manual analysis (Figure 3). Boxes extend to the quartiles, with a line at the median. Whiskers extend from the 10th to 90th percentiles. Scale bar, 100 nm.

Figure 5.

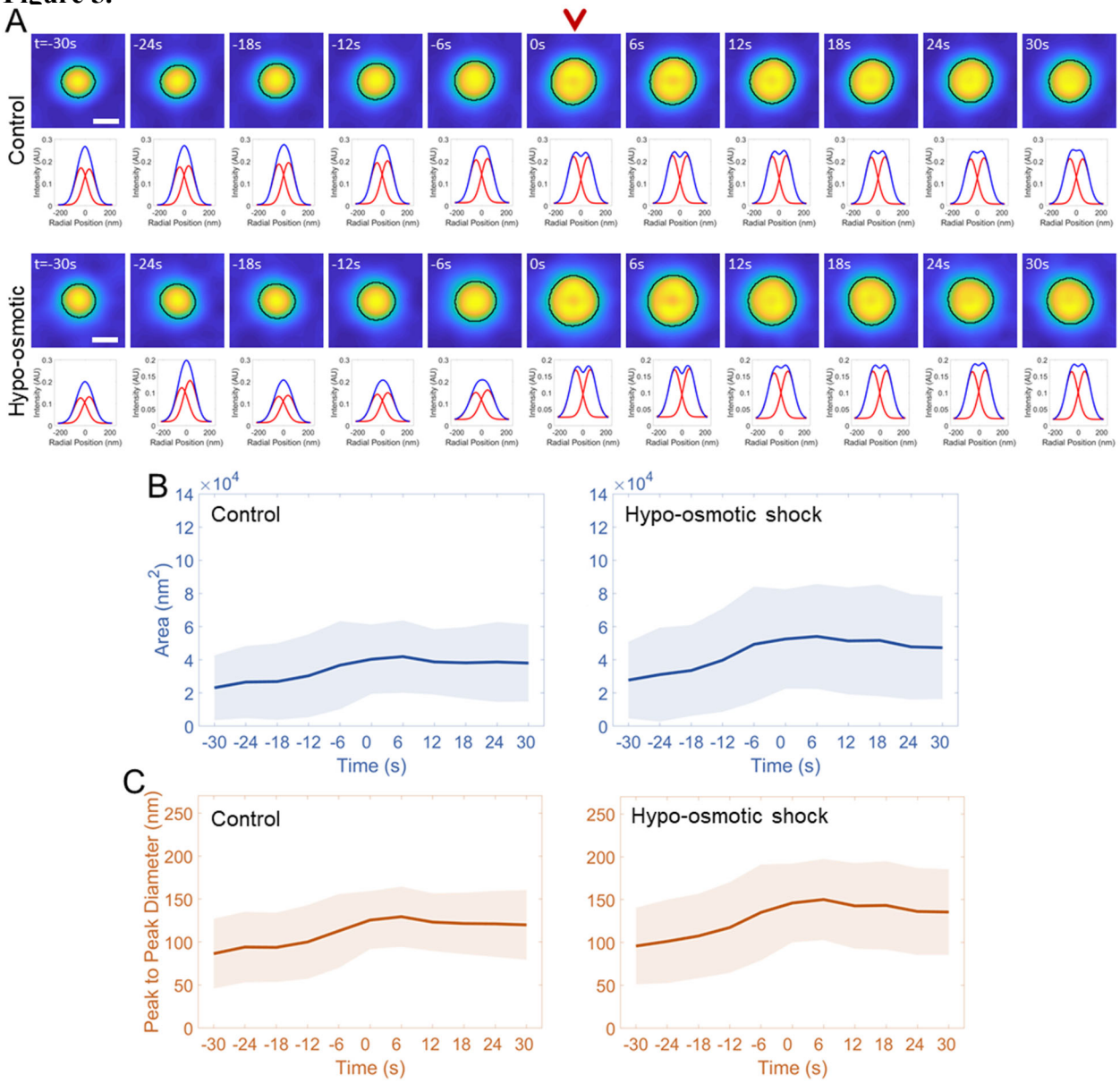

Figure 5. Curvature generation by clathrin coats under high plasma membrane tension. Membrane tension is increased in cells by hypo-osmotic shock. Clathrin traces are automatically selected in SUM-159 cells genome edited to express AP2-EGFP under isotonic (N_{cells} = 3; N_{traces} = 366) and hypotonic (N_{cells} = 9; N_{traces} = 396) conditions. **A.** The average of the synchronized traces and corresponding radial averages are shown for both conditions. The arrowhead denotes the ring frame. The temporal evolution of the average area **(B)** and peak-to-peak separation of the Gaussian fits to the radial averages **(C)** are plotted for the two groups. Shaded areas represent the standard deviation. Scale bar, 100 nm.

Materials and Methods

Cell Culture:

BSC-1 cells stably expressing EGFP-CLTA and COS-7 cells transiently expressing mEmerald-CLTB were cultured and prepared for microscopy as described in [1]. SUM-159 cells genome edited to express σ2-EGFP [2] and transiently expressing mRuby-CLTB (Addgene; Plasmid #55852) were cultured in F-12 medium with hydrocortisone, penicillin-streptomycin and 5% fetal bovine serum (FBS). Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, CA, USA) was used for transient transfection of SUM-159 cells following manufacturer's instructions. SUM-159 cells were cultured on 35mm glass bottom dishes (MatTek) and imaged 24-48 hours after transfection in phenol-red-free L15 (Thermo Fisher Sci.) supplemented with 5% FBS at 37°C ambient temperature.

Drosophila Embryos:

To construct pUAST-mEmerald-Clc, the in-frame GFP fusion from pUAST-GFP-Clc [3] was excised with an EcoRI/BglII digest and replaced with mEmerald (Addgene), PCR amplified with primers GGAATTCCACCATGGTGAGCAAGGGCGAGG and CGAGATCTGAGTCCGGACTTGTACAGCTCGTCCATG (the EcoRI and BglII sites in the primers are underlined). After verifying the resulting plasmid by sequencing (Purdue Genomics Core Facility), UAS-mEmerald-Clc transgenic flies were generated by P element-mediated transformation [4].

UAS-CLC-mEmerald flies were mated with a GAL4-Arm (Bloomington Drosophila Stock Center) line to produce embryos expressing CLC-mEmerald in the amnioserosa and surrounding germ-band tissue. The embryos were collected and aged for 10-12 hours at 25°C. After

dechorionation, embryos were mounted on coverslips and immersed in voltalef oil for imaging at 22°C.

Microscopy

High-NA TIRF-SIM images of COS-7 and BSC-1 cells were acquired with 1.7-NA 100x objective (Olympus Life Science, CA, USA) as described in [1]. SUM-159 cells and Drosophila embryos were imaged using the TIRF-SIM system at the Advanced Imaging Center (AIC) of the Janelia Research Campus. This system comprises a 1.49-NA 100x objective lens (Olympus Life Science, CA, USA) fitted on an inverted microscope (Axio Observer; ZEISS) equipped with a sCMOS camera (ORCA-Flash4.0; Hamamatsu). Structured illumination was provided by a spatial light modulator as described in [1] and movies were acquired using 20 ms exposure time and with frame rates ranging from 0.15 to 0.5 Hz. To image clathrin activity in tissues of Drosophila embryos, the apical surface of amnioserosa and germ-band cells were positioned within the evanescent illumination by gently pressing the dechorionated embryos against the coverslip.

Image Analysis

a. Manual analysis:

Clathrin-coated structures in the TIRF (diffraction-limited) channel were tracked using two-dimensional cmeAnalysis software [5]. The traces extracted this way were filtered based on their intensity profiles as described in [6]. Briefly, to distinguish complete clathrin coat formation events, only the traces that contain both positive and negative intensity slopes (corresponding to coat growth and dissolution, respectively) were selected.

To analyze the images in the TIRF-SIM (superresolved) channel, a program was written in MATLAB 2017b (Mathworks) for the user to manually locate acceptable traces based on the following rejection criteria: 1) The structure must not come into contact with any other structure

at any point during its lifetime (to exclude pair spots). 2) The structure must perform a single phase of intensity increase followed by a single phase of intensity decrease (to exclude hotspots). Once a structure was selected, the user defined the first and last frames of the structure's existence. The *x-y* position of the structure was taken as the intensity-weighted center of the image. The area was calculated using the MATLAB built-in function, edge, set to calculate the Canny edge of a small window containing only the structure of interest. When the edge finder failed to close the edge, the algorithm allowed the user to close the edge manually (Fig. S6). The area within the closed boundary is calculated as the sum of the pixels enclosed.

Time averages of clathrin-coated pit traces are determined using TIRF-SIM images centered on the intensity-weighted center of the structure at each time point. These image sequences are of varying lengths, so each is extended to a length of 100 frames by linearly interpolating respective pixels at each time point (Fig. S7). Images corresponding to each time/completion point are averaged pixel-by-pixel to obtain the time averages shown in Movies 4 & 5. In each frame, the area is calculated using the Canny edge-detection algorithm as described above.

To get the radial averages shown in Figure 3, the images were upscaled by a factor of 10 with bicubic interpolation. For each upscaled image, 36 radial kymographs (separated by 5° increments and concentric with the intensity-weighted center of the structure; Fig. S8A) are averaged and fit to the sum of two Gaussian functions (Fig. S8B):

$$F = B + A_1 \mathrm{e}^{-\frac{(\mathrm{x}-\mathrm{x}_1)^2}{2\sigma^2}} + A_2 \mathrm{e}^{-\frac{(\mathrm{x}-\mathrm{x}_2)^2}{2\sigma^2}}$$

where B is the background, A_1 and A_2 are the amplitudes, x_1 and x_2 are the center points, and σ is the standard deviation. σ values for the largest area deciles are 40.6 nm for COS-7 Clathrin-mEmerald, 37.3 nm for BSC-1 Clathrin-EGFP, 68.7 nm for SUM-159 Clathrin-Ruby, 45.4 nm for

SUM-159 AP2-EGFP and 51 nm for embryo Clathrin-mEmerald. As expected, the standard deviations are the smallest for the data sets obtained by high-NA TIRF-SIM (i.e. COS-7 and BSC-1) and the largest for the SUM-159 Clathrin-Ruby dataset due to longer fluorescence wavelength.

b. Automated analysis:

Particle tracking. We used TraCKer [7] for automated tracking of clathrin-coated structures in TIRF-SIM (superresolved) channel. Since TraCKer is originally developed to trace diffraction-limited spots in fluorescence movies, curved clathrin structures that appeared as rings were frequently not detected. To circumvent this, as an initial step, holes (in rings) were closed using an averaging convolution followed by an erosion. After obtaining the traces of clathrin coats (*x-y* coordinated in multiple frames) all further analysis utilized the unmodified TIRF-SIM images.

Detection of ring patterns in TIRF-SIM Images. After tracking, each clathrin trace is evaluated for appearance of ring pattern using an 11x11 pixel window (~361nm x 361nm) centered on the structure in each frame (Fig. S9A & C). A coordinate pair (r_i , Int_i) is defined for each pixel in the cropped image, where r_i is the distance from the center of the ith pixel to the center of the structure and Int_i is the intensity of that pixel. Plotting these coordinates gives a radial profile of the structure to which a Gaussian distribution is fit using least squares (Fig. S9B & D). If the peak of the Gaussian is determined to be at least 2 pixels (60 nm) from the center of the structure, the structure is considered to have the ring pattern (Fig. S9B). Only the traces that have the ring pattern in two consecutive frames and no ring pattern in the first frame are used in further analysis. The latter constraint is applied because of the in analyzing the frames leading up to maximum curvature.

Boundary detection. The average image cross-section is determined by averaging 36, equidistant, radial crossections (Fig. S10A & F). This 1D profile image was fit to the sum of two

Gaussians (Fig. S10D & I), and the boundary of the structure was determined by the points where the magnitude of the Gaussians' slopes were found to be a maximum (Fig. S10E & J).

Self-assembly model

Figure S11 shows the schematic representation of clathrin triskelions used in simulations, where D_{hh} (~18.5 nm) is the nearest neighbor hub-hub distance (i.e., "strut" length) and D_{ee} (= $\sqrt{3}D_{hh}$ = 32 nm; $\phi \approx 120$) is the next to nearest hub-hub distance [8, 9]. From basic topology, any closed shell will contain 12 pentagons. If we further assume that those pentagons arrange so that there is icosahedral symmetry, then all possible configurations are classified by the T number according to

$$T = h^2 + k^2 + hk$$

with h and k non-zero positive integers. Then, the clathrin coat consists of $20T$ triskelions, $30T$ "struts" of length D_{hh} and $10T$+2 polygonal faces; $10(T\text{-}1)$ of them hexagons and 12 pentagons. The radius R of the clathrin coats will therefore be defined by the distance between the hub and the center of the circumscribed sphere, which is given by

$$R = \frac{1}{4}(1 + \sqrt{5})r_c$$

where r_c is the distance between two nearest neighbor pentagons. This distance may be evaluated as $r_c = \sqrt{3T}D_{hh}$ and therefore, we have a relation between the sphere radius R and D_{hh} according to

$$R = \frac{1}{4}(1 + \sqrt{5})\sqrt{3T}D_{hh}$$

We adapt a previous model that successfully described the self-assembly of capsid subunits as assisted by scaffolding proteins [10]. Here, the role played by the latter are the AP2 adapters and the former are clathrin triskelions. The model is defined by

$$F_d = E_s + E_b = \sum_i \frac{1}{2} k_s (b_i - b_0)^2 + \sum_{i,j} k_b [1 - \cos(\theta_{ij} - \theta_0)]$$

with θ_0 a preferred angle, related to the spontaneous curvature H_0. The stretching energy sums over all bonds i with b_0 the equilibrium bond length and the bending energy is between all neighboring triskelions indexed with i. We further assume that there is an attractive force between the triskelions and the preformed scaffolding layer (AP2 subunits), which involves a simple LJ-potential $E_{LJ} = \sum_i 4\epsilon[\left(\frac{\sigma}{r_i}\right)^{12} - 2\left(\frac{\sigma}{r_i}\right)^{6}]$ with ϵ the depth of the potential and σ the position of minimum energy corresponding to optimal distance between the center of the core and the subunits. The mechanical properties of the subunits (the ratio of stretching to bending moduli) are described by the Foppl von-Karman (FvK) number that is defined by

$$\gamma = \frac{b_0^2 k_s}{k_b}.$$

In our simulations (Figs. 1B&D, Movies S1&2), FvK number γ =100 is selected based on the previous work [10]. It is important to remark that the pentagons are dynamically generated as the simulation proceeds and therefore, their location is an unambiguous prediction by the geometry and theory (Fig. 1F). It should be emphasized that, in contrast with the situations for viruses [11], clathrin shells may not exhibit icosahedral order. Still, the mechanism of defect growth is *universal* (independent of FvK number) and the absence of icosahedral order does not qualitatively modify any of the conclusions.

Movies displaying the geometry of clathrin coat assembly (Movie S1&S2) were rendered in MATLAB using an orthographic projection. In the case of curved structures, the relative positions of clathrin triskelia are given by the results of the self assembly model. After the addition of each new triskelion the entire structure is rotated so that the position vector of the center of mass moves

towards vertical. For flat structures, triskelia are added in any position in the hexagonal lattice such that the distance from the new triskelion to the center of mass is minimized. When new triskelia are added, the structure translates in order to move the center of mass towards the origin. The strut length is consistent in all structures.

High-NA TIRF-SIM simulations in Figure 1 and Movies S1&S2 were created by modeling the fluorescence of each labeled triskelion as a 3D Gaussian. The amplitude of the each Gaussian is proportional to the intensity of the incident light at its position within evanescent TIR field, in which the intensity profile used is an exponential decay along the z-axis. The image is generated by calculating the sum of all emitted intensities at the central position of each image pixel at the focal plane. All numeric parameters were implemented as reported in [1]: TIRF decay length = 50 nm; XY Gaussian SD = 84 nm; Z Gaussian SD = 500 nm; image pixel size = 30 nm.

References:

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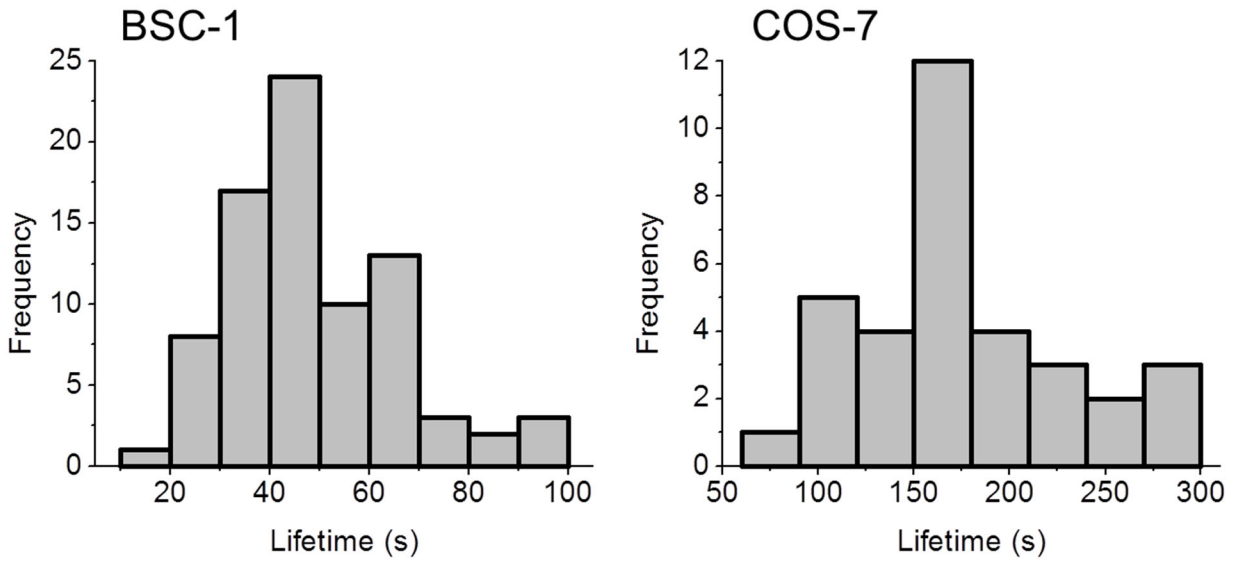
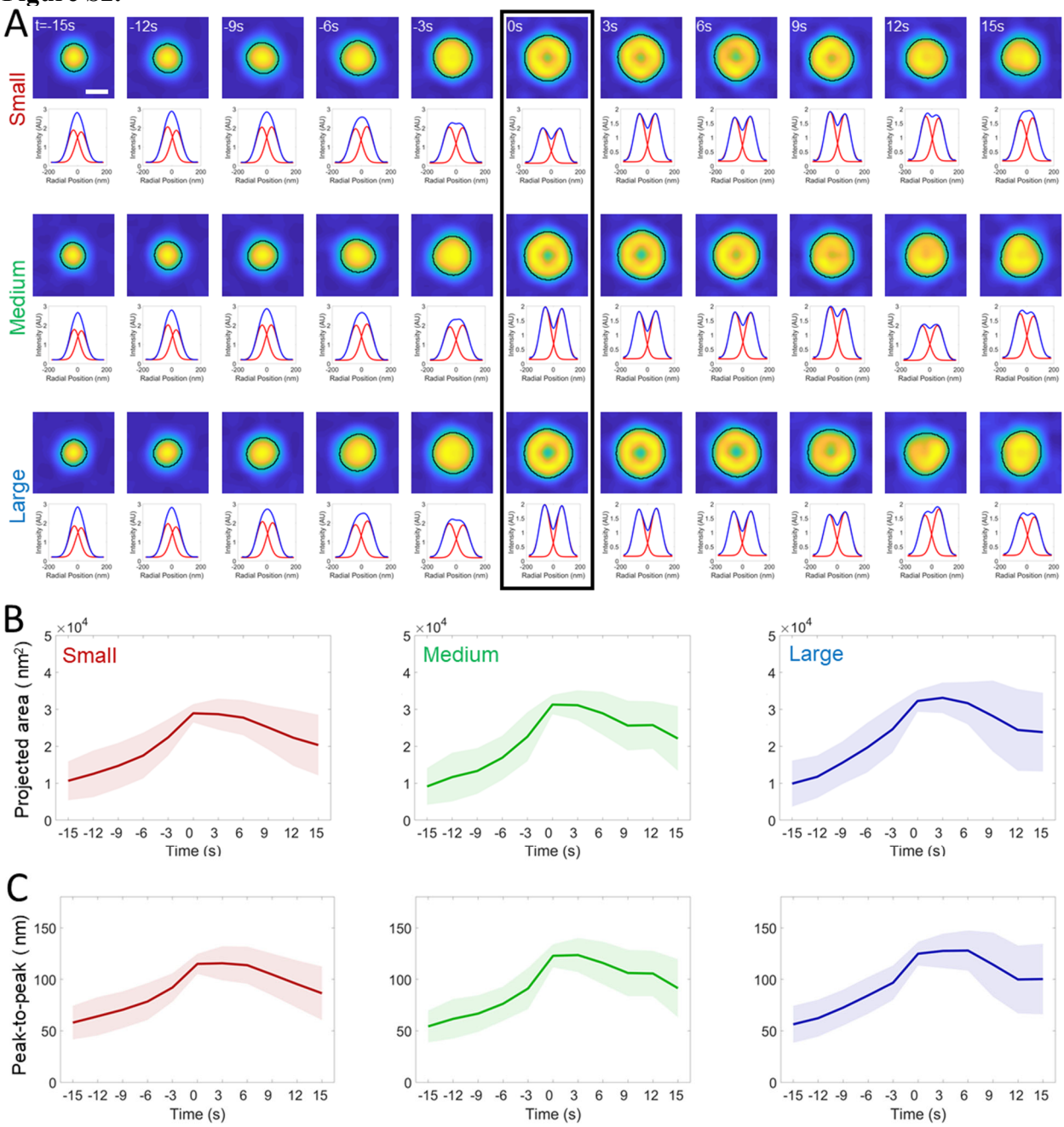
11. J. Wagner, R. Zandi, Biophys. J. 109, 956–965 (2015).

Figure S1.



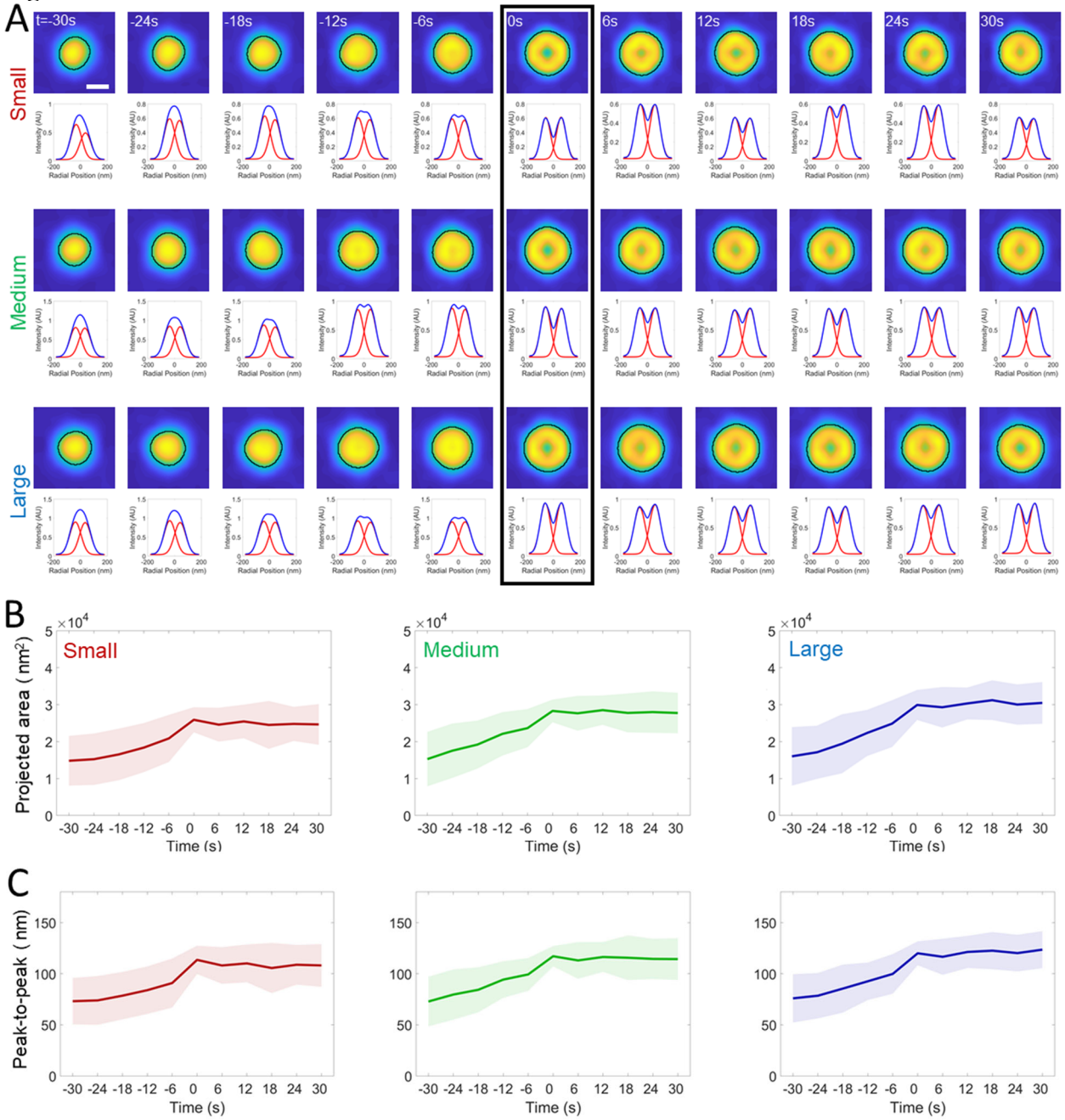
Lifetime distributions of manually selected clathrin-coated pit traces extracted from high-NA TIRF-SIM acquisitions of BSC-1 cells expressing clathrin-EGFP (left) and COS-7 cells expressing clathrin-mEmerald (right).

Figure S2.



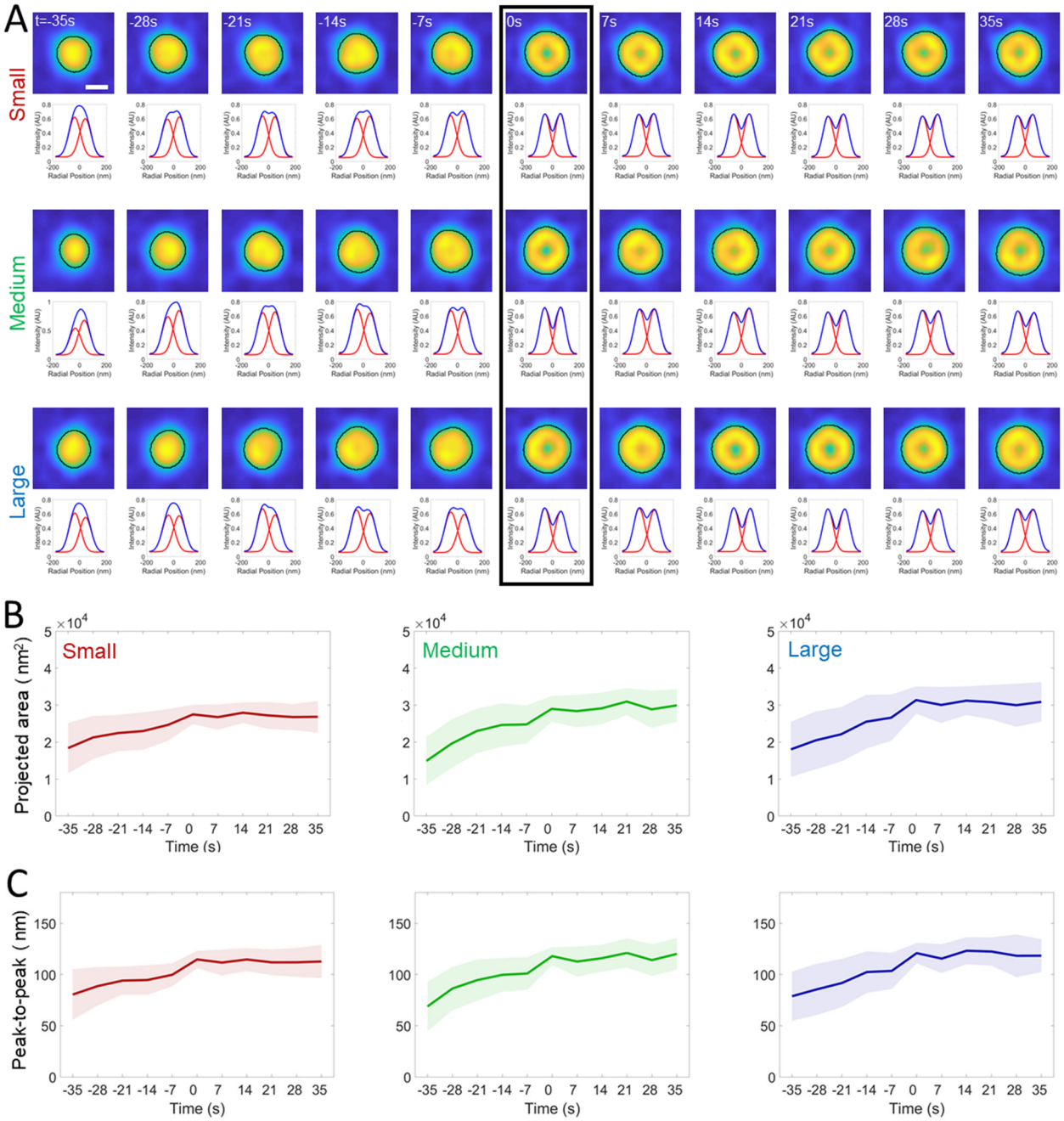
Automated analysis of the projected area during formation of clathrin pits in BSC-1 cells. A. 210 traces detected from two BSC-1 cells (imaged at 0.33 frames/second) are grouped and synchronized using the first detected ring pattern (black rectangle). Average images (top) and corresponding radial averages (bottom) are shown for each group (small, medium and large). Detected boundaries are shown by the black demarcations. The temporal evolution of the average area **(B)** and peak-to-peak separation of the Gaussian fits to the radial averages **(C)** are plotted for the three groups. Shaded areas represent the standard deviation. Scale bar, 100 nm.

Figure S3.



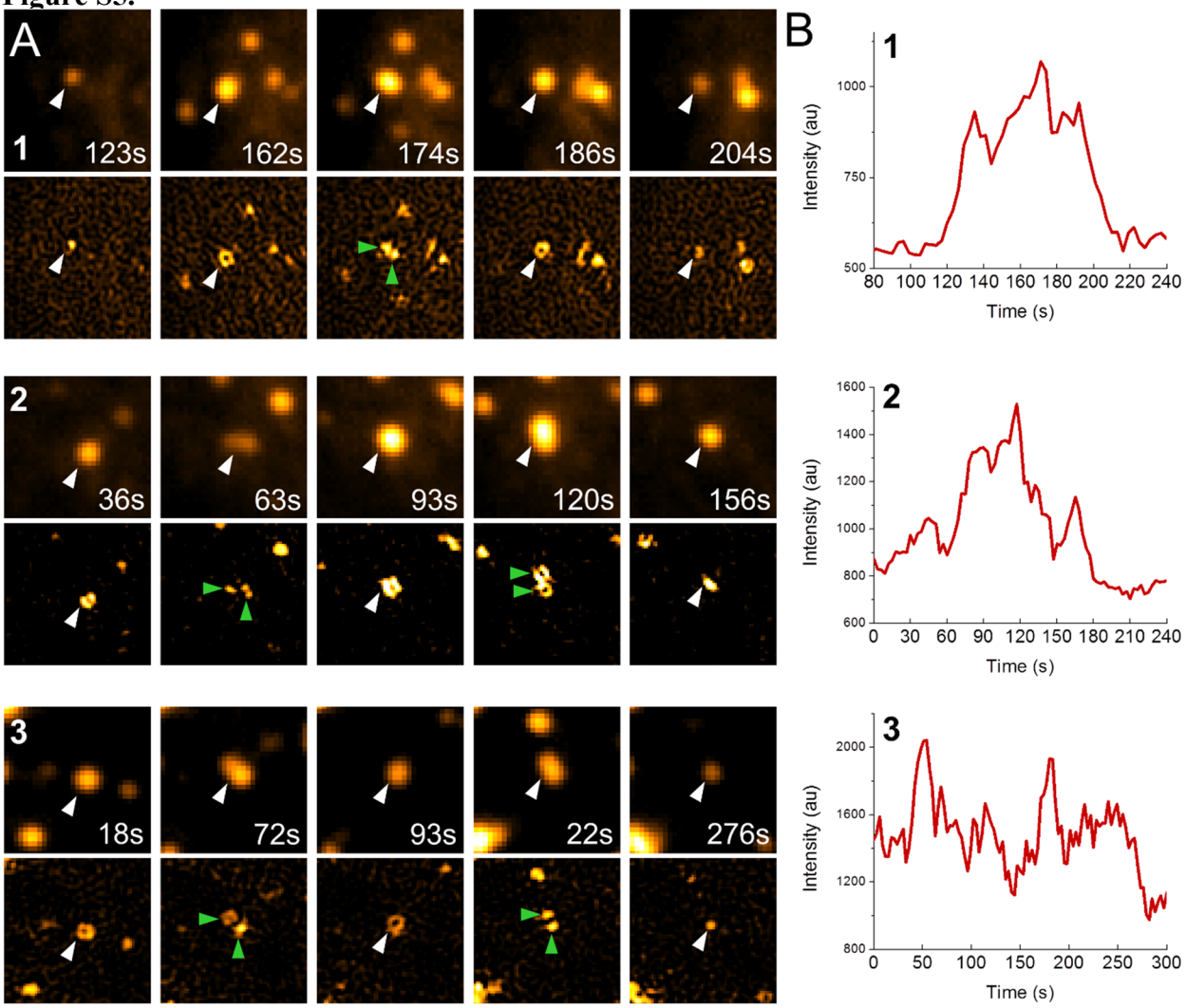
Automated analysis of the projected area during formation of clathrin pits in COS-7 cells. A. 519 traces detected from one COS-7 cell (imaged at 0.167 frames/second) are grouped and synchronized using the first detected ring pattern (black rectangle). Average images (top) and corresponding radial averages (bottom) are shown for each group (small, medium and large). Detected boundaries are shown by the black demarcations. The temporal evolution of the average area **(B)** and peak-to-peak separation of the Gaussian fits to the radial averages **(C)** are plotted for the three groups. Shaded areas represent the standard deviation. Scale bar, 100 nm.

Figure S4.



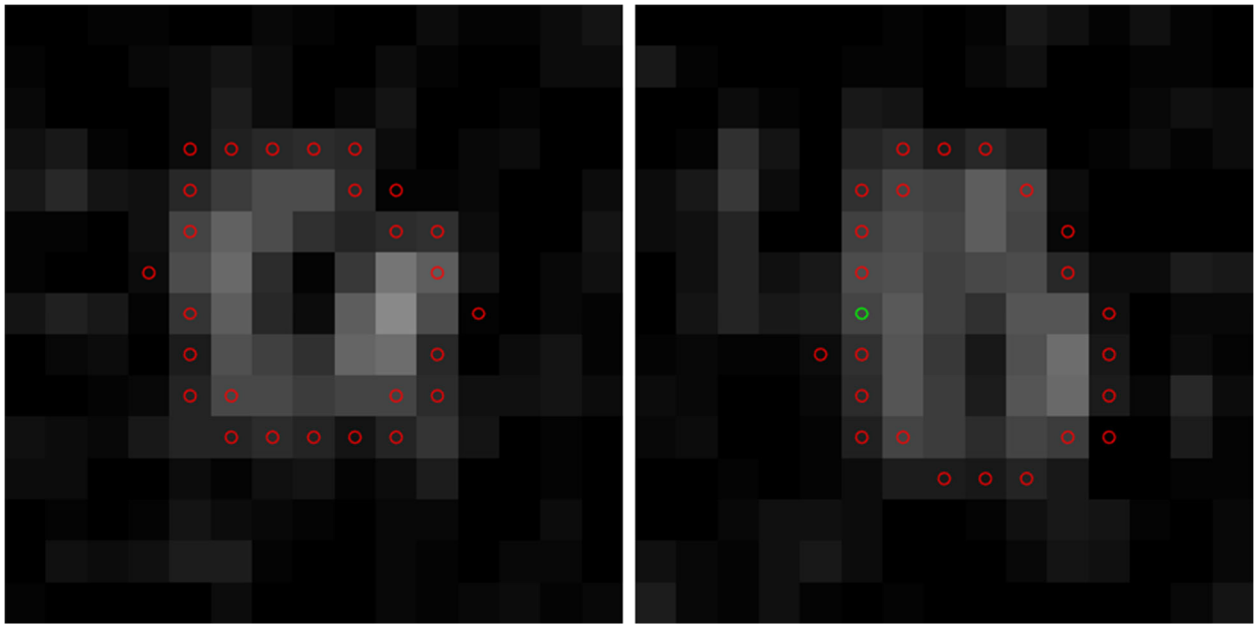
Automated analysis of the projected area during formation of clathrin pits in COS-7 cells. A. 175 traces detected from one COS-7 cell (imaged at 0.2 frames/second) are grouped and synchronized using the first detected ring pattern (black rectangle). Average images (top) and corresponding radial averages (bottom) are shown for each group (small, medium and large). Detected boundaries are shown by the black demarcations. The temporal evolution of the average area **(B)** and peak-to-peak separation of the Gaussian fits to the radial averages **(C)** are plotted for the three groups. Shaded areas represent the standard deviation. Scale bar, 100 nm.

Figure S5.



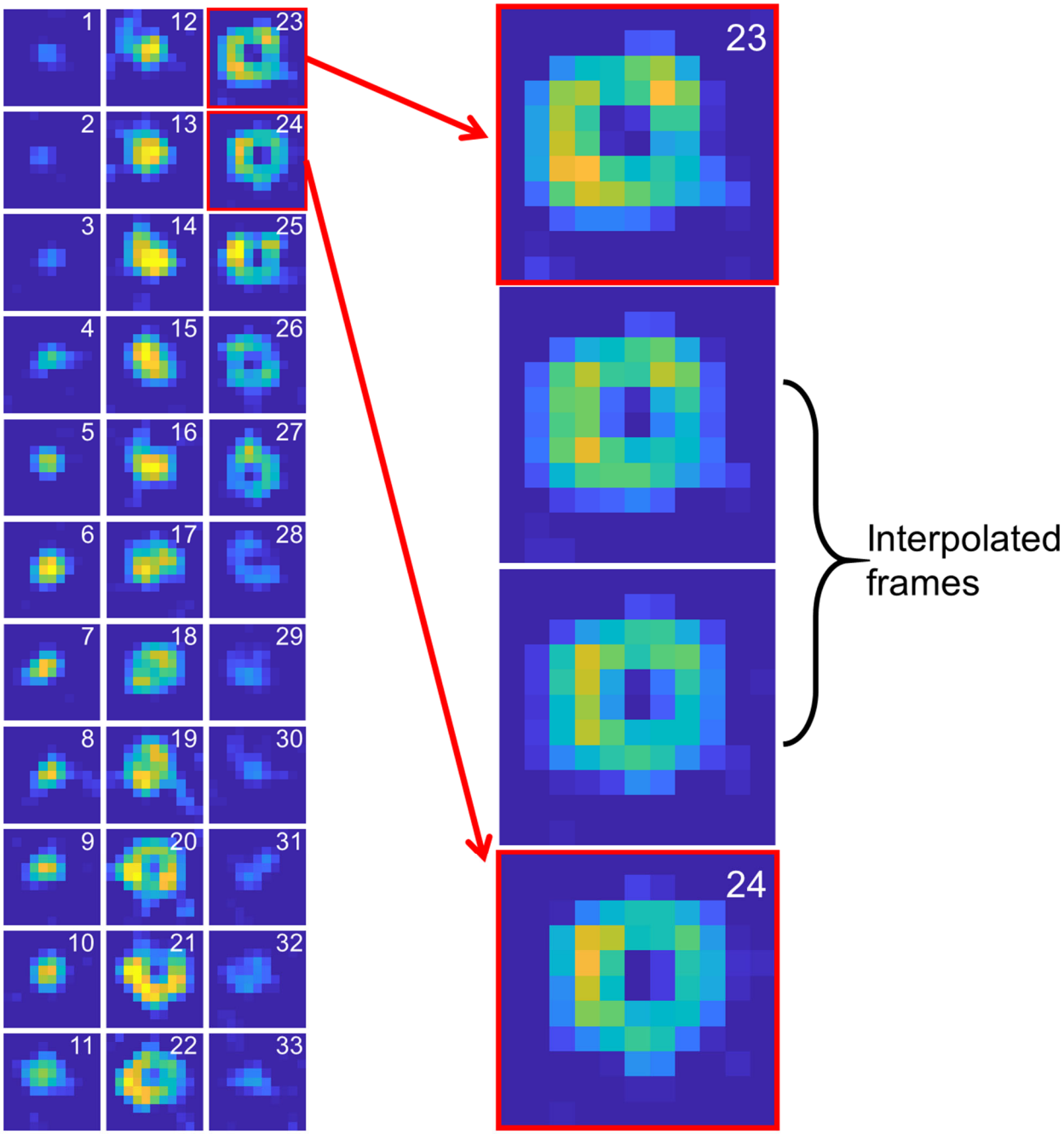
Fluorescent spots that appear as individual structures under diffraction-limited imaging may correspond to multiple independent clathrin-coated pits. A. Three examples of diffraction-limited fluorescent spots comprising multiple clathrin-coated structures. Top rows show TIRF snapshots of individual diffraction-limited spots (marked by white arrowheads) at different time points. Bottom rows correspond to high-NA TIRF-SIM images of the same region, where green arrowheads mark multiple structures in close proximity that cannot be resolved in the TIRF channel (top row). **B.** Plots show the fluorescence intensity traces of the diffraction-limited spots in A.

Figure S6.



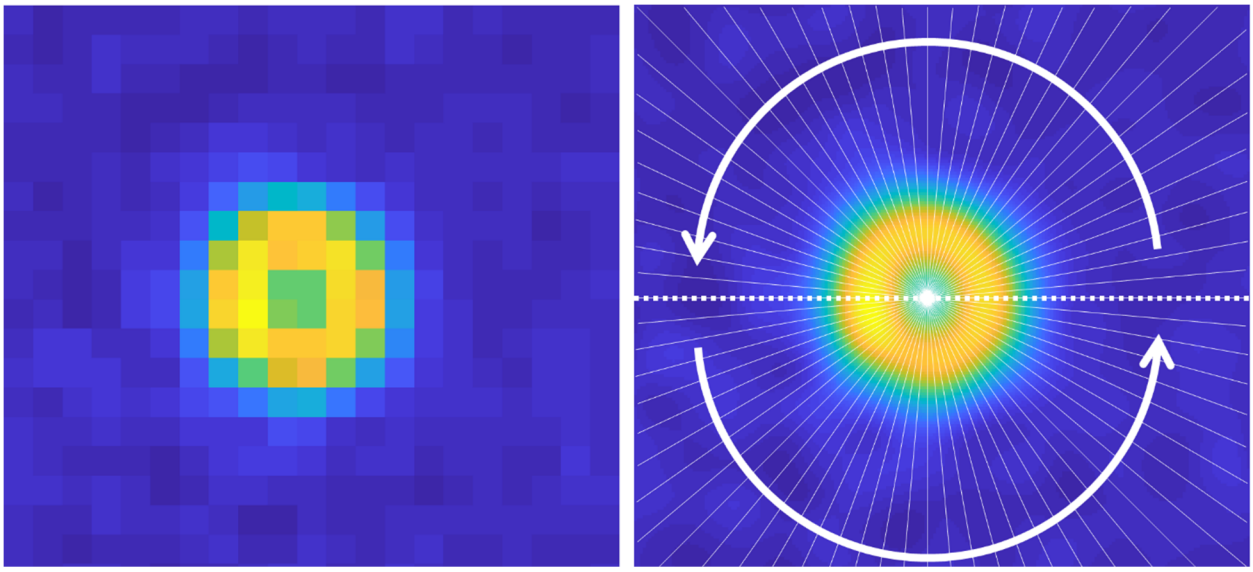
Red circles in each image correspond to the edges detected by the Canny edge-detection algorithm. The pixels within this boundary are used to define the designated area of the structure (left). When the Canny algorithm fails to close the boundary (right), the user has the option to manually assign the missing edge pixels (green circle).

Figure S7.



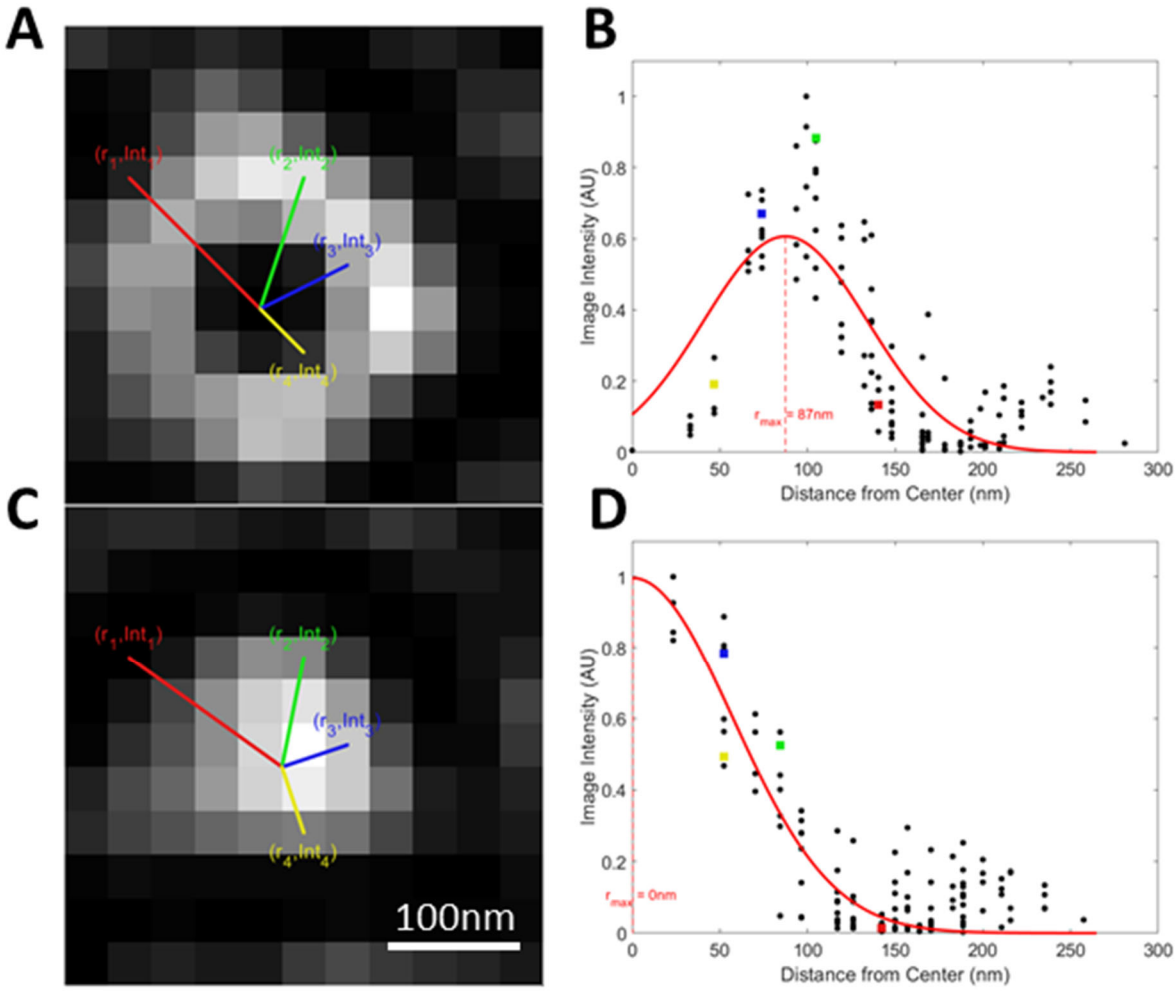
33 consecutive TIRF-SIM images corresponding to formation and dissolution of a clathrin-coated pit in a COS-7 cell expressing clathrin-mEmerald (left). Blowup image shows the linear interpolation linking frames number 23 and 24 (right).

Figure S8.



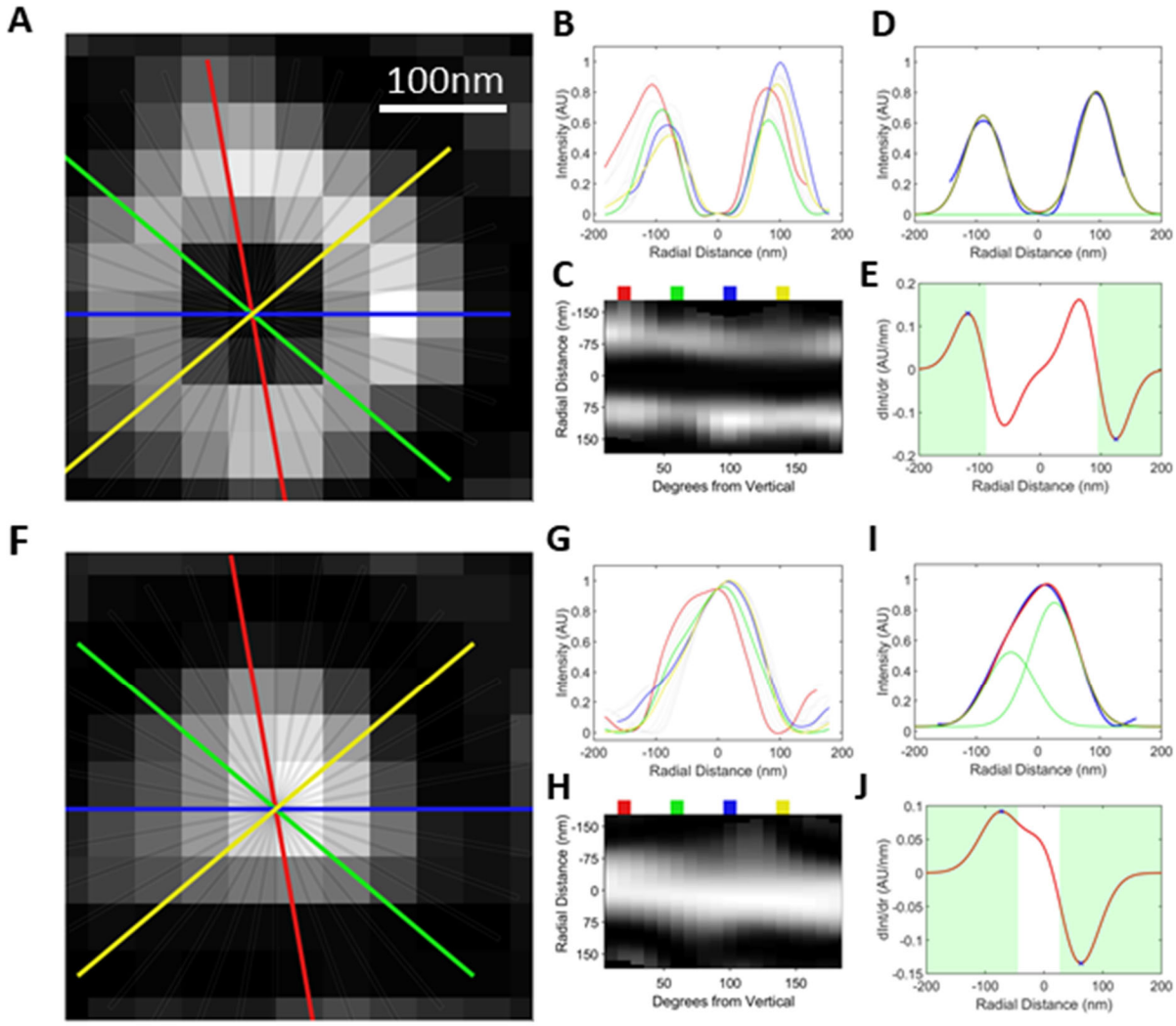
An average clathrin-coated pit image (left) and its upscaled version (right). Kymographs are calculated along the radial white lines separated by 5º. Radial averages are determined for different conditions by averaging the radial kymographs.

Figure S9.



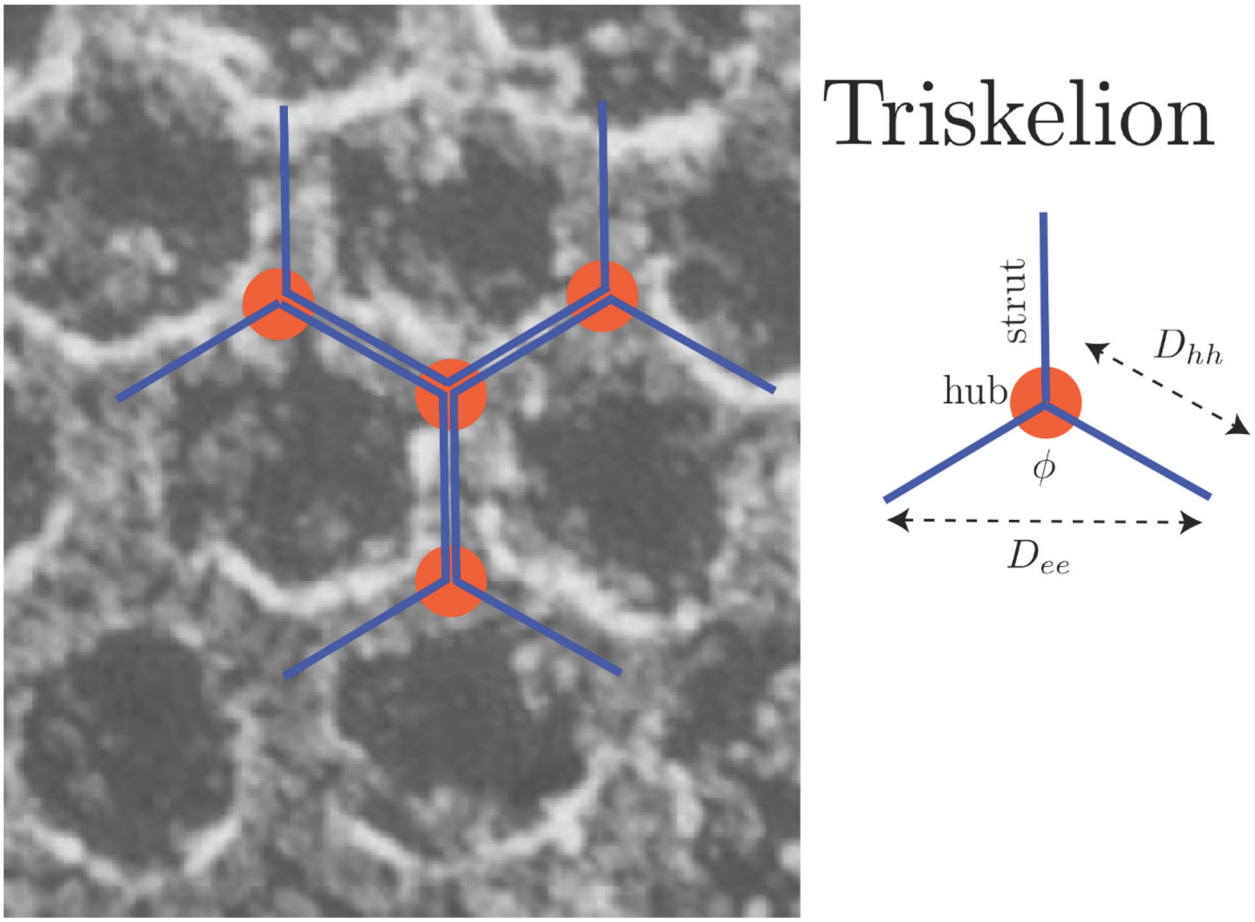
A & C. For each time point in the trace, the analysis is performed within the 11x11 pixel region around the center of the structure. **B & D.** A coordinate pair (r_i , Int_i) is defined for each pixel in the cropped image, where r_i is the distance from the center of the ith pixel to the center of the structure and Int_i is the intensity of that pixel. Plotting these coordinates gives a radial profile of the structure. (Colored squares in B, D are the same colored pixels indicated in A, C respectively). To this profile, a Gaussian distribution is fit using least squares. The mean value of this Gaussian is the radial distance of maximum intensity (r_{max}). When this r_{max} value is above a threshold (empirically chosen to be 60 nm), we can be confident that the structure displays obvious curvature (**B**). Below this threshold we find that curvature cannot be resolved (**D**).

Figure S10.



For boundary detection, we calculate the profiles of 36 lines running through the center of the clathrin-coated structure (only 18 lines are shown in **A** and **F**) in each time point. The profiles can be viewed as line plots (**B, G**) or as images showing the profile intensity on one axis and the angle of the profile line (from vertical) on the other (**C, H**). The line plots (**B, G**) and the angles marked with a colored boxes (**C, H**) are the representations of the corresponding colored profiles (**A, F**). To calculate the area of the clathrin coat, we take the average of these profiles and fit it to the sum of two Gaussian distributions (which are required to have the same standard deviation) using least squares (**D, I**; blue is the average profile; red is the fitted Gaussians; green are the fitted Gaussian separated). The average extent of the clathrin coat is chosen to be the maximum and minimum slopes of the fit. We take the derivative of the fit (**E, J**; red line). In order avoid finding a slope which is on the interior of the profile, we consider slopes which exterior to the Gaussian peaks (**E, J**; shaded green regions). The maximum slope in the left region and the minimum slope in the right region define the average diameter of the clathrin coat (**E, J**; blue crosses).

Figure S11.



Schematic representation of a clathrin triskelion superimposed to an electron micrograph.

Movie S1. Assembly of a T=7 polyhedron according to the constant curvature model, where the position of each new triskelion is determined using the self assembly model. The coat growth is shown in the *x-z* and *x-y* planes in **A** and **B**, respectively. In **A**, the color bar represents normalized evanescent TIR field intensity. Fully assembled pentagons are highlighted with pink. **C.** Simulated high-NA TIRF-SIM images corresponding to the clathrin coats shown in A and B. The red line shows the detected boundary. **D.** Projected area (i.e., area within the detected boundary) is plotted with respect to the number of triskelions integrated into the coat. Scale bars, 20 nm.

Movie S2. Assembly of a T=9 polyhedron according to the constant curvature model, where the position of each new triskelion is determined using the self assembly model. The coat growth is shown in the *x-z* and *x-y* planes in **A** and **B**, respectively. In **A**, the color bar represents normalized evanescent TIR field intensity. Fully assembled pentagons are highlighted with pink. **C.** Simulated high-NA TIRF-SIM images corresponding to the clathrin coats shown in A and B. The red line shows the detected boundary. **D.** Projected area (i.e., area within the detected boundary) is plotted with respect to the number of triskelions integrated into the coat. Scale bars, 20 nm.

Movie S3. TIRF-SIM movie of clathrin coat dynamics acquired at the amnioserosa tissue of a late Drosophila embryo expressing clathrin-mEmerald. Further zoom-in (blue box) shows growth and dissolution of an individual clathrin-coated pit (arrowhead).

Movie S4. Average clathrin-coated pit trace obtained from COS-7 cells expressing clathrin-mEmerald (left; $N_{traces} = 34$), where the black line is the detected boundary. The maximum projected area (area within the boundary) is reached at 75% of completion. For each image the radial average (red circles) is plotted on the right panel. Gaussian fits (green and blue) and corresponding peak-to-peak distances are shown between 40-96% completion.

Movie S5. Average clathrin-coated pit trace obtained from BSC-1 cells expressing clathrin-EGFP (left; $N_{traces} = 81$), where the black line is the detected boundary. The maximum projected area (area within the boundary) is reached at 69% of completion. For each image the radial average (red circles) is plotted on the right panel. Gaussian fits (green and blue) and corresponding peak-to-peak distances are shown between 40-96% completion.

Movie S6. TIRF-SIM (left) and TIRF (right) images of multiple clathrin-coated structures that appear as a single spot under diffraction-limited imaging. The movie corresponds to the second example in Figure S5.