Supplementary Materials

Molecular Biology of the Cell

Kaplan *et al*.

Figure S1: Three-dimensional resolution of STORM setup. Accumulated localization distributions of 24 representative single molecules from 3 different samples. Gaussian fits (red curves) gave standard deviations of 10 nm in-plane for the *XY* directions (left and middle plot) and 19 nm in-depth for the *Z* direction (right plot).

Figure S2: Representative examples x-y and x-z STORM image projections to visualize the different appearance of actin at CME sites compared to cortical actin. (A) and **(F)** X-y projections of super-resolved clathrin coats (red). Regions of interest (ROI) selected from STORM image in Figure 1A. **(B)** and **(G)** Corresponding super-resolved x-y projections of actin (cyan) to ROIs in (A) and (F). (**C)** and **(H)** Dual-color x-y projection from STORM images of clathrin (A, F, red) and actin (B, G, cyan). The yellow square inset shows a clathrin coated pit (CCP) with associated actin. The red squared inset shows actin at the cortex without a clathrin coated structure (CCS). **(D) - (J)** X-z STORM projections of red and yellow squared inset from C and H. The upper image shows clathrin (D, I, red) or no clathrin (E, J), the middle image shows actin (rainbow) and the lower image the merge of both images. In example images D and I actin is enriched and builds up at the CCP, therefore is distinguishable from the actin that extends away from the CCP. In examples E and J actin is not associated with a CCS and does not show a specific enrichment and accumulation as compared to the image in D and I. **(A) - (J)** All scale bars are 100 nm.

Figure S3: Representative examples of x-y STORM image projections of single and double clathrin structures. (A) Electron micrograph (image 1) shows a single clathrin coated pit in a SK-MEL-2 cell acquired by platinum replica EM. Image 2 shows a clathrin coated pit (red)

resolved by STORM of the same size as the pit in the electron micrograph. X-y STORM projections of diverse single clathrin coated structures and pits (image 2 to image 21) in SK-MEL-2 cells. **(B)** Electron micrograph (image 1) shows a double clathrin coated pit structure in a SK-MEL-2 cell acquired by platinum replica EM. Image 2 shows a double clathrin coated pit structure (red) resolved by STORM of the same size as the double pit in the electron micrograph. X-y STORM projections of diverse double clathrin coated structures and pits (image 2 to image 21) in SK-MEL-2 cells. The two clathrin coated structures that are forming a double structure are highlighted with a white dashed circle. **(A) - (B)** Scale bars all 100 nm.

Figure S4: Representative examples of ambiguous clathrin data, overview of 3D measurement methods for super-resolved clathrin and actin, and representative x-y projections.

(A) Representative examples of immunolabeled clathrin structures that cannot be identified as individual clathrin coated events. STORM images show x-y projection of clathrin structures. First two columns show structures that do not show a clear round or elliptical shape. Third image column shows clathrin structures that extend over the entire region of interest. Fourth image column shows very small punctate structures. **(B)** Illustration of method to obtain actin and clathrin height and extent of coverage from super-resolved CCP images. x-z projection of a merged STORM image of clathrin and actin with its corresponding normalized pixel intensity histogram projected onto the z-axis. The black line marks the 30^{th} percentile of the z height histogram, which we used to calculate the height of clathrin and actin. We found that this metric, the "full z width at 30^{th} percentile max," was more reliable than the full width at half max. These raw intensity profiles are noisy based on the image quality, which depends on the fluorescent

labeling quality of the target structure. Without filtering the profile, the measured height of clathrin and actin are underestimated. Numbers correspond to positions used to calculate height (in z) of clathrin (1. and 2.) and actin (4. and 5.) and their average positions (3. and 6.). Lower panel shows the same STORM image after a mean filter was applied to reduce noise in the histogram of z-position to improve the reliability of the clathrin and actin height readout. We measured the coverage of clathrin and actin profiles by subtracting the upper position of actin from the lower position of clathrin. **(C)** X-z projected STORM image of a clathrin coat with its corresponding normalized pixel intensity histogram projected onto the x-axis unfiltered. Due to the image noise the measurement of clathrin coat width misses most of the image. The right image shows the improved width measurement of the same clathrin coat after application of a median filter to smooth the histogram. All scale bars are 100 nm. **(D)** X-y STORM image projections corresponding to the x-z projections in Figure 1E. **(E)** Example of asymmetrically organized actin around a clathrin-coated pit. Normalized pixel intensity histograms of clathrin (red) and actin (blue) from x-z and y-z projections. Measurement is the distance of the peak actin signal from the center of the clathrin coat. Actin is asymmetrically organized since the distance from the x-axis to the center is large compared to the distance to the y-axis. **(F)** Violin plot of actin asymmetry values in isotonic condition for three endocytic stages. Differences are not statistically significant based on Mann-Whitney test. N = 289 (shallow), 405 (U-shape), and 602 (omega shape).

Figure S 5: Clathrin and actin scatter plots of geometric parameters obtained from xz and yz STORM image projections. (A) - (D) Data set of clathrin and actin parameters when cells were treated with isotonic media, n = 731. **(A)** Scatter plot of shape indices (blue dots) and

regression line (red) with an $r^2 = 0.92$. **(B)** Scatter plot of clathrin height (red) and regression line (black) with an $r^2 = 0.99$. **(C)** Scatter plot of clathrin width (red) and regression line (black) with an $r^2 = 0.93$. **(D)** Scatter plot of actin height (green) and regression line (red) with an $r^2 = 0.99$. **(E) - (H)** Data set of clathrin and actin parameters when cells were treated with hypotonic media. n = 473 **(E)** Scatter plot of shape indices (blue dots) and regression line (red) with an $r^2 = 0.94$. **(F)** Scatter plot of clathrin height (red) and regression line (black) with an $r^2 = 0.99$. **(G)** Scatter plot of clathrin width (red) and regression line (black) with an $r^2 = 0.95$. **(H)** Scatter plot of actin height (green) and regression line (red) with an $r^2 = 0.99$.

Figure S6: STORM images of N-WASP at clathrin-coated pits in SK-MEL-2 cells. Schematic of clathrin coat side profiles shows N-WASP localization. Merged STORM images show spatial localization of N-WASP-AF647 (rainbow) at clathrin coats (red) in x-z projections. **(A)** N-WASP localized at the base of clathrin coats. **(B)** N-WASP localized all over clathrin coats. Scale bars are 100 nm.

Figure S7: Hypotonic media treatment of SK-MEL-2 cells endogenously expressing CLTA-TagRFP-T^{EN} and DNM2-eGFP^{EN}. (A), (C) and **(E)** Normalized cumulative distribution data for CLTA-TagRFP-T^{EN} and DNM2-eGFP^{EN} fluorescence lifetimes when associated with or not associated with each other. Lifetimes were recorded under isotonic media conditions and then recorded after 2 min or 10 min of media exchange. Plot legend provides, respectively: mean lifetime ± SD, number of tracks, p-value determined in Kolmogorov-Smirnov test with significance compared to the control. **(B), (D)** and **(F)** show CME initiation rate, completion rate and percentage of persistent tracks plotted for sites in which DNM2-eGFP^{EN} and CLTA-

TagRFP-T^{EN} were associated with each other. Tables below show the corresponding statistics. Mann-Whitney two-tailed statistical test was used to compare CME initiation rates, completion rates, and persistent tracks. **(A)** and **(B)** Control conditions in which only isotonic media exchange was performed. n = 3-4 (cells) for each condition. Experiments were repeated 3 times. **(C)** and **(D)** Media were exchanged from isotonic to 225 mOsm hypotonic. For each of the following conditions: control, 2 min, or 10 min after media exchange, n = 6 (cells). **(E)** and **(F)** Media were exchanged from isotonic to 150 mOsm hypotonic. For each condition of the following conditions: control, 2 min and 10 min after media exchange, n = 6 (cells). **(G)** Unassociated tracks in 75 mOsm hypotonic media. **(C) – (G)** Experiments were repeated 5 – 6 times. **(H)** Statistics for CME initiation rates, completion rates and percentage of persistent tracks for CLTA-TagRFP-T^{EN} associated with DNM2-eGFP^{EN} corresponding to Figure 2, F-H.

Figure S8: Effects of hypotonic media and concomitant CK666 treatment on membrane tether force and CME progression. (A) Membrane tether force measurements from atomic force microscopy in cells treated with DMSO (left) or 100 μM CK666 (right). n=19 cells in each condition. 4 biological replicates; minimum 3 tethers per cell. Tether force was measured 2-19 mins after addition of CK666. $p>0.5$ by t-test. Boxplot shows median with interquartile range. **(B)** Normalized fluorescence lifetime cumulative distribution data for CLTA-TagRFP-T^{EN} and associated DNM2-eGFP^{EN} for CK666 concentrations of 25 μM (CLTA: red line, DNM2: light green line), 50 μM (CLTA: orange line, DNM2: blue line), 100 μM (CLTA: purple line, DNM2: dark green line) and pre treatment (black line). Respective controls were 0.5% v/v (CLTA: red line, DNM2: light green line), 1% v/v (CLTA: orange line, DNM2: blue line), 2% v/v (CLTA:

purple line, DNM2: dark green line) DMSO and pre treatment (black line). Fluorescence lifetimes were acquired after 2 minutes of treatment. Experiments were repeated 2-3 times. Kolmogorov-Smirnov statistical test was used. Statistics are provided in Table S2. **(C)** Corresponding CME completion rates for CLTA-TagRFP-T^{EN} associated with DNM2-eGFP^{EN} in Figure 3, B-F. Barplots show mean ± SD **(D)** Percentage of persistent tracks for CLTA-TagRFP-T^{EN} associated with DNM2-eGFP^{EN} for corresponding imaging data in Figure 3, B-F. Barplots show mean ± SD **(E)** Corresponding CME initiation rates for CLTA-TagRFP-T^{EN} associated with DNM2-eGFP^{EN} in Figure 3, B-F. Barplots show mean ± SD **(F)** Statistics for clathrin coat height and width for data in Figure 3H when cells were treated with 2% v/v DMSO, 100 μM CK666 and 100 μM CK666 in 150 mOsm hypotonic media. **(G)** Plotted clathrin coat width in nm for data in Figure 3H. Barplots show mean ± SD. **(H)** Ratio of clathrin coat height to width for data in Figure 3H and S3G. **(C), (E)** and **(G)** Mann-Whitney statistical test was used.

Figure S9: Effect of hypotonic media treatment on actin organization. (A) STORM image of CF-680 immunolabeled clathrin-coated structures (red) on the ventral cell surface when 75 mOsm hypotonic media was applied for 5 min. **(B)** Same area of the cell as in (A) showing the STORM image of phalloidin-AF647 labeled actin cytoskeleton (rainbow). **(C)** Same area of the cell as in (A) showing the corresponding DNM2-eGFP^{EN} conventional microscopy image. **(D)** Merged clathrin and actin STORM images from (A) and (B). Color bar shows actin position in the z-dimension. **(A) – (D)** All scale bars are 5 μm. **(E) - (G)** STORM x-y projection images corresponding to images in Figure 4B. (E) corresponds to the shallow invaginations. (F) corresponds to the U-shaped invaginations. (G) corresponds to the omega-shaped invaginations. All scale bars are 100 nm. **(H)** Violin plot of actin asymmetry values in isotonic and 75 mOsm

hypotonic conditions. Differences are not statistically significant based on Mann-Whitney test. Data for isotonic conditions are also reported in Figure S4F. Hypotonic condition: N = 1090.

Figure S10: Representative examples x-y and y-z STORM image projections to visualize the different appearance of actin at CME sites compared to cortical actin in cells treated with hypotonic media. (A) and **(F)** Example regions of interest showing x-y projections of super-resolved clathrin coats (red). Regions of interest (ROI) selected from STORM image in Figure S9D. **(B)** and **(G)** Corresponding super-resolved x-y projections of actin (cyan) to ROIs in (A) and (F). (**C**) and **(H)** Dual-color x-y projection from STORM images of clathrin (A, F, red) and actin (B, G, cyan). The yellow square inset shows a clathrin coated pit (CCP) with associated actin and extends along the cell cortex. The red squared inset shows actin at the cortex without a clathrin coated structure (CCS). **(D) - (J)** Y-z STORM projections of red and yellow squared insets from C and H. The upper image shows clathrin (D, I, red) or no clathrin (E, J), the middle image shows actin (cyan) and the lower image the merge of both images. In example images D and I actin is enriched and builds up at the CCP, therefore is distinguishable from the actin that extends away from the CCP. In examples E and J actin is not associated with a CCS and does not show a specific enrichment and accumulation as compared to the image in D and I. **(A) - (C)** and **(F) - (H)** All scale bars are 500 nm. **(D), (E)**, **(I) - (J)** All scale bars are 100 nm. (**K**) Example ROI of super-resolved clathrin (magenta) that was used to generate an image mask (L) to select only actin Z coordinates associated with the cortex in the same ROI. (**L**) Generated binary image mask of the super-resolved clathrin ROI in K. (**M**) Image of actin Z coordinates of the same ROI as in K. The white outline highlights some example clathrin areas from the image mask in L. (**O**) Image of actin Z coordinates after the binary clathrin mask (L) was applied. White outlined

example areas are cleared of actin Z coordinates. (**P**) Histogram of standard deviations of binned actin Z coordinates excluding clathrin-associated actin Z coordinates. Actin z coordinates for this ROI are from a cell grown in isotonic media. (**Q**) Histogram of standard deviations of binned actin Z coordinates excluding clathrin-associated actin Z coordinates. Actin z coordinates for this ROI are from a cell grown in hypotonic media. (**R**) Median standard deviations of actin Z coordinates for three ROIs measured in a cell treated with isotonic, and a cell treated with hypotonic media, respectively. The experiments were conducted on 3 different days. Bar plot shows the median and lines the standard deviation.

Table S1: Lifetimes of endocytic tracks in isotonic and hypotonic conditions. P values determined by Kolmogorov-Smirnov test. Statistics in table of data shown in Figure 1, D and E.

Table S2: Lifetimes of endocytic tracks in cells treated with the small molecule inhibitor CK666. P values determined by Kolmogorov-Smirnov test. Statistics in table of data shown in Figure S8B.

Table S3: Lifetimes of endocytic tracks in cells in hypotonic conditions and treated with the small molecule inhibitor CK666. P values determined by Kolmogorov-Smirnov test. Statistics in table of data shown in Figure 3, B-F.

Supplementary movie 1: Movie of CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} (green) in SK-MEL-2 cells in isotonic media.

Supplementary movie 2: Movie of CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} (green) in SK-MEL-2 cells after 2 minutes of media exchange to 75 mOsm media.

Supplementary movie 3: Movie of CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} (green) in SK-MEL-2 cells after 10 minutes of media exchange to 75 mOsm media.

Supplementary movie 4: Movie of CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} (green) in SK-MEL-2 cells in isotonic media before treatment with CK666 and 150 mOsm hypotonic media.

Supplementary movie 5: Movie of CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} (green) in SK-MEL-2 cells in 150 mOsm hypotonic media with 100 µM CK666. Movie acquisition started 2 minutes after media exchange.

Data sheet 1: Data set of clathrin and actin values of height, width, shape index (aspect ratio) and maximum position in the Xz - and Yz - projections. Control data set. Cells were under isotonic media conditions.

Data sheet 2: Clathrin height and width under isotonic media conditions, CK 666 and 150 mOsm hypotonic media plus CK 666 treatment.

Data sheet 3: Data set of clathrin and actin values of height, width, shape index (aspect ratio) and maximum position in the Xz - and Yz - projections. Cells were under 75 mOsm hypotonic media conditions.

Data sheet 4a: Completion rates of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-eGFP^{EN} tracks when cells are under isotonic and hypotonic media treatment. Data is used to plot Figure 2G and Figure S7B, D and F.

Data sheet 4b: Initiation rates of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-$eGFP^{EN}$ tracks when cells are under isotonic and hypotonic media treatment. Data is used to plot Figure 2F and Figure S7B, D and F.

Data sheet 4c: Percentage of persistent tracks of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-$eGFP^{EN}$ tracks when cells are under isotonic and hypotonic media treatment. Data is used to plot Figure 2H and Figure S7B, D and F.

Data sheet 5a: Completion rates of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-$eGFP^{EN}$ tracks when cells are treated with a combination of isotonic and hypotonic media treatment with the CK666 inhibitor. Data is used to plot Figure S8C.

Data sheet 5b: Initiation rates of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-$eGFP^{EN}$ tracks when cells are treated with a combination of isotonic and hypotonic media treatment with the CK666 inhibitor. Data is used to plot Figure S8E.

Data sheet 5c: Percentage of persistent tracks of associated CLTA-TagRFP-T^{EN} (magenta) and DNM2-$eGFP^{EN}$ tracks when cells are treated with a combination of isotonic and hypotonic media treatment with the CK666 inhibitor. Data is used to plot Figure S8D

Data sheet 6: Mean and median standard deviation of clathrin masked actin x,y, and z molecule localization list as a metric of actin cortex height, respectively. Data is plotted in Figure S10R.

Table S1: Lifetimes of endocytic tracks in isotonic and hypotonic conditions. P values determined by Kolmogorov-Smirnov test. Table refers to Figure 1, D and E.

CLT-associated	Mean (s)	SD (s)	N. tracks	P-value to control
Isotonic media	47	32	3760	
75 mOsm media, 2min	128	112	918	3.12E-10
75 mOsm media, 10 min	91	91	1153	9.16E-69

DNM2 - associated	Mean (s)	SD (s)	N. tracks	P-value to control
Isotonic media	39	32	3760	
75 mOsm media, 2min	125	112	918	5.02E-10
75 mOsm media, 10 min	82	83	1153	2.59E-64

CLT - only	Mean (s)	SD (s)	N. tracks	P-value to control
Isotonic media	17	12	1517	
75 mOsm media, 2min	20	15	742	0.0027
75 mOsm media, 10 min	19	14	954	0.0112

DNM2 - only	Mean (s)	SD (s)	N. tracks	P-value to control
Isotonic media	19	12	1182	
75 mOsm media, 2min	23	16	1312	3.56E-10
75 mOsm media, 10 min	23	17	2000	4.92E-08

Table S2: Lifetimes of endocytic tracks in cells treated with the small molecule inhibitor CK666. P values determined by Kolmogorov-Smirnov test. Statistics in table of data shown in Figure S8B.

CLT-associated	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
Pre CK666	68	49	1441	6	
CK666 25µM	66	45	744	6	0.7111
CK666 50µM	69	52	833	5	0.7925
CK666 100µM	79	66	835	8	0.0485

CLT-associated	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
Pre DMSO	65	50	2852	14	
DMSO 0.5% (v/v)	64	52	1469	5	0.2012
DMSO 1.0% (v/v)	666	57	1177	5	0.1405
DMSO 2.0%(v/v)	56	40	526	5	0.0011

DNM2-associated	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
Pre CK666	53	42	1441	6	
CK666 25µM	51	45	744	6	0.0064
CK666 50µM	55	48	833	5	0.5346
CK666 100µM	65	64	835	8	0.0034

DNM2-associated	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
Pre DMSO	51	46	2852	14	
DMSO 0.5% (v/v)	51	49	1469	5	0.5880
DMSO 1.0% (v/v)	54	52	1177	5	0.2858
DMSO 2.0% (v/v)	49	38	526	5	0.0583

Table S3: Lifetimes of endocytic tracks in cells in hypotonic conditions and treated with the small molecule inhibitor CK666. P values determined by Kolmogorov-Smirnov test. Statistics in table of data shown in Figure 3, B-F.

CLTA-tagRFP.t, associated **Pre-treatment to**	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
DMSO	59	47	2378	10	
DMSO + 150 mOsm	62	52	4335	12	0.093
150 mOsm	65	54	2173	9	3.88E-06
CK666 100µM	75	46	4219	10	1.18E-14
CK666 100µM + 150 mOsm	59	45	1405	9	0.902
CLTA-tagRFP.t, associated **Treatment**	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
DMSO	59	52	3028	10	
DMSO + 150 mOsm	80	87	2286	10	2.13E-13
150 mOsm	79	82	2762	8	7.15E-20
CK666 100µM	70	56	3124	10	7.46E-25
CK666 100µM + 150 mOsm	96	85	2783	10	1.12E-119
DMN2-eGFP, associated **Pre-treatment to**	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
DMSO	47	46	2378	10	
DMSO + 150 mOsm	50	52	4335	12	0.004
150 mOsm	51	51	2173	9	0.001
CK666 100µM	50	43	4219	10	2.57E-09
CK666 100µM + 150 mOsm	44	41	1405	9	0.094
DMN2-eGFP, associated **Treatment**	Mean (s)	SD (s)	N. tracks	N. cells	P-value to DMSO
DMSO	47	49	3028	10	
DMSO + 150 mOsm	74	86	2286	10	4.27E-26
150 mOsm	73	81	2762	8	3.51E-44

CK666 100μM	54	54	3124	10	8.39E-11
CK666 100μM + 150 mOsm	84	85	2783	10	1.94E-115

Figure S1

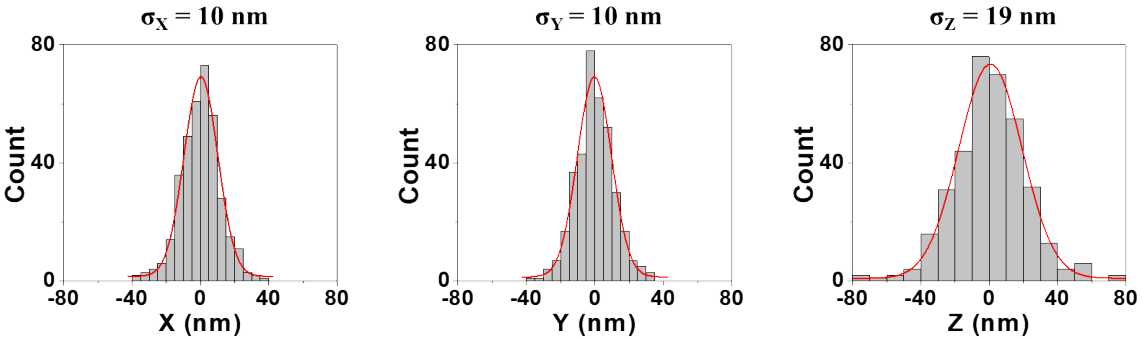

Figure S2

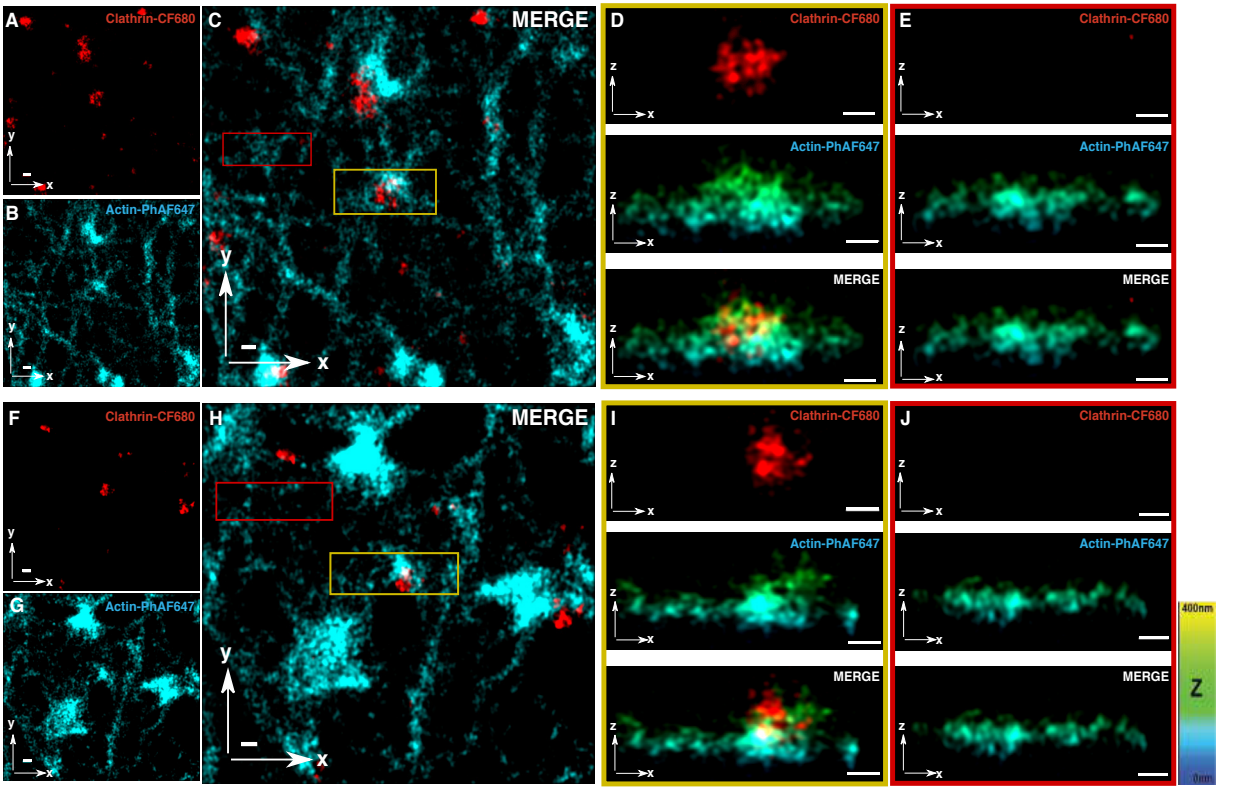
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Figure S3

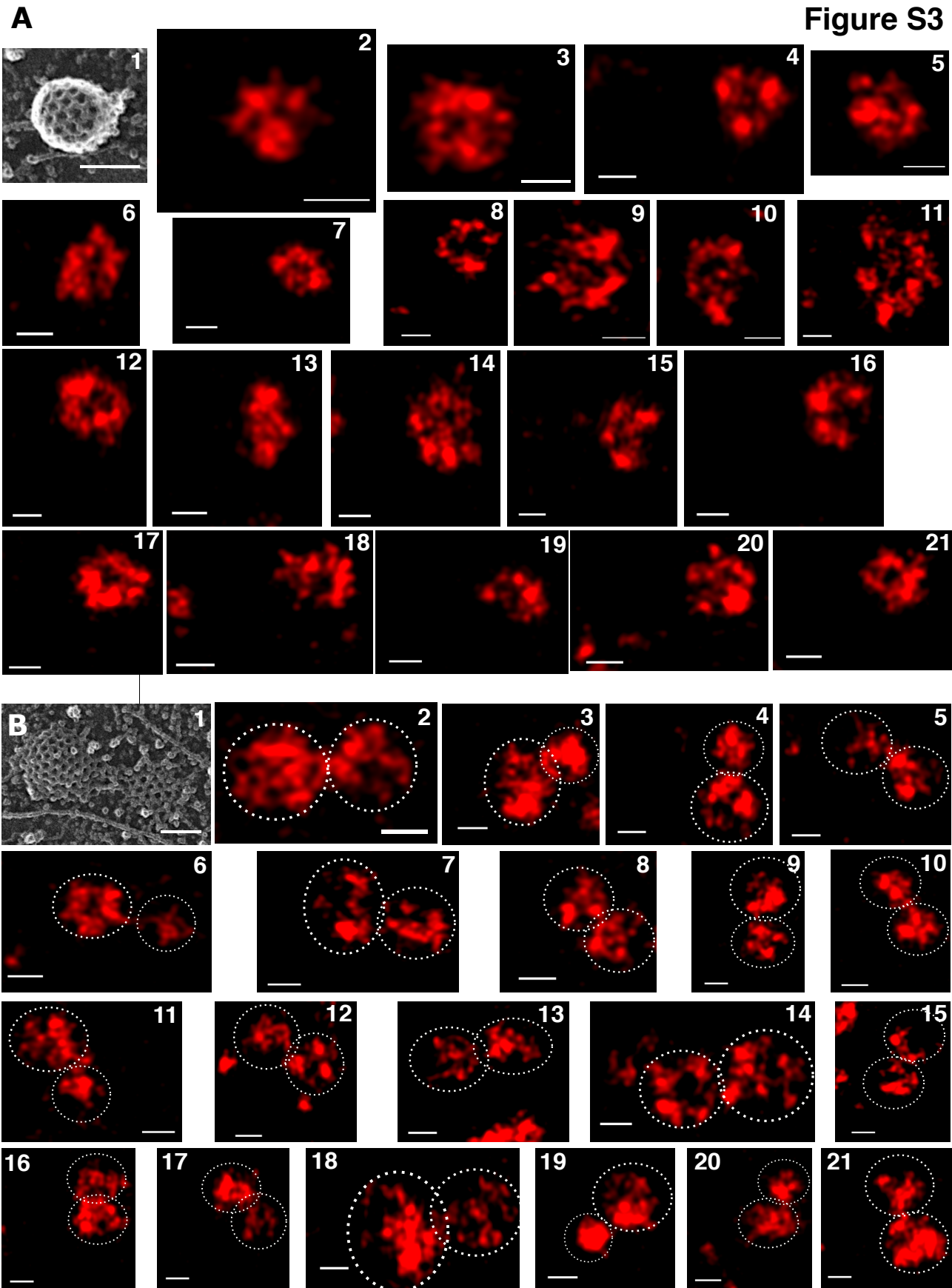
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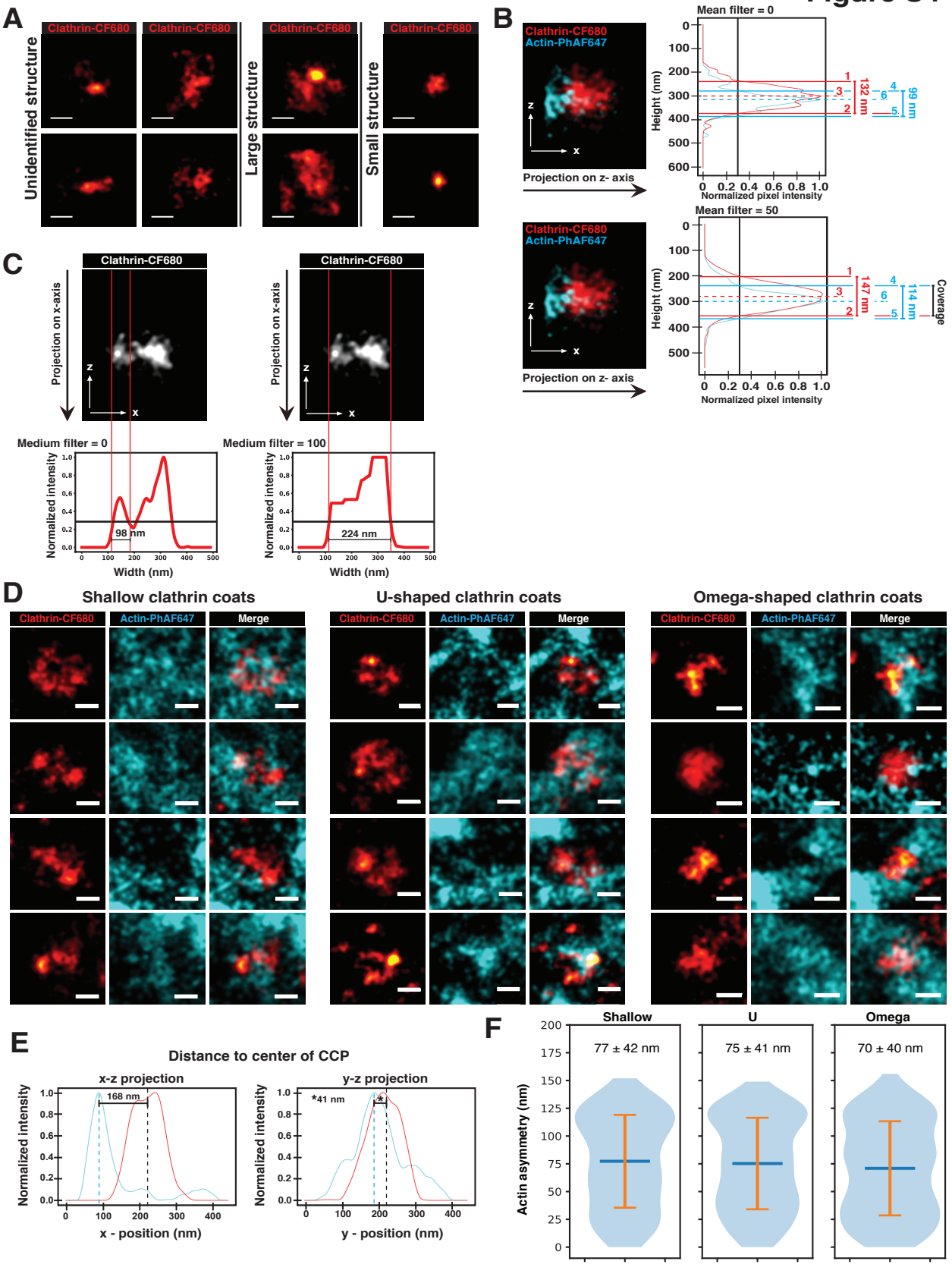
Figure S4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Small structure
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Projection on z- axis
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Normalized pixel intensity
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99 nm
Mean filter = 50
147 nm
114 nm
Coverage
C
Clathrin-CF680
Projection on x-axis
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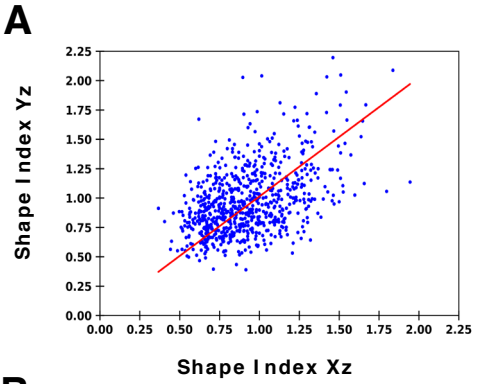
Medium filter = 100
Normalized intensity
98 nm
224 nm
Width (nm)
D
Shallow clathrin coats
U-shaped clathrin coats
Omega-shaped clathrin coats
Clathrin-CF680
Actin-PhAF647
Merge
E
Distance to center of CCP
x-z projection
y-z projection
168 nm
*41 nm
Normalized intensity
x - position (nm)
y - position (nm)
F
Shallow
U
Omega
77 ± 42 nm
75 ± 41 nm
70 ± 40 nm
Actin asymmetry (nm)

Figure S5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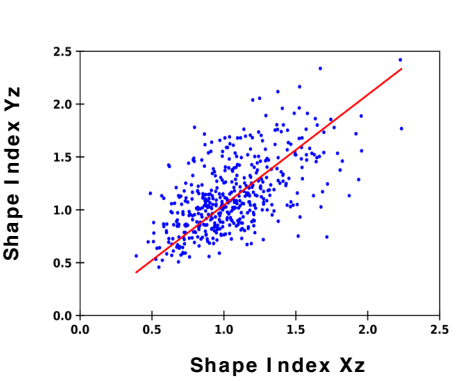
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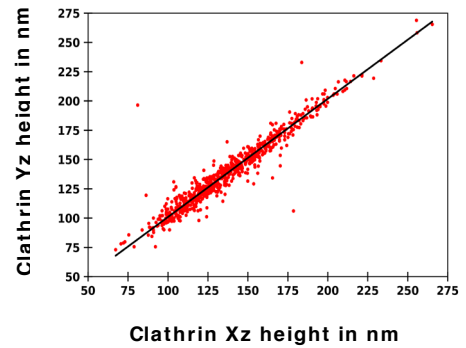
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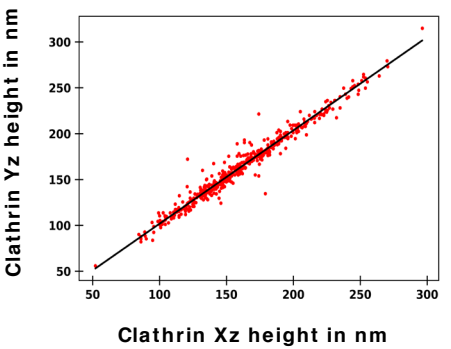
Clathrin Yz height in nm
Clathrin Xz height in nm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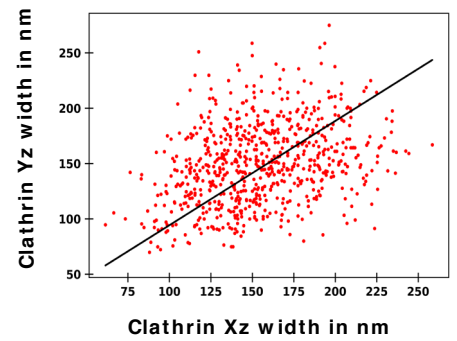
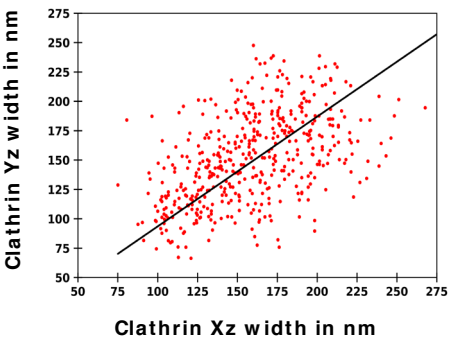
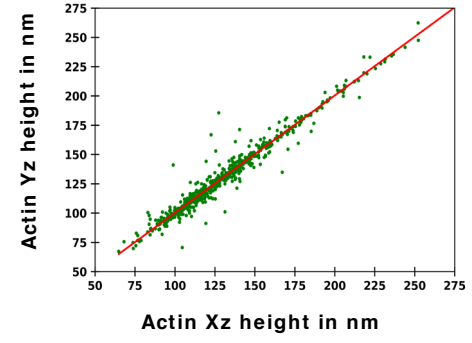
Clathrin Yz height in nm
Clathrin Xz height in nm


C
Clathrin Yz width in nm
Clathrin Xz width in nm


G
Clathrin Yz width in nm
Clathrin Xz width in nm


D
Actin Yz height in nm
Actin Xz height in nm

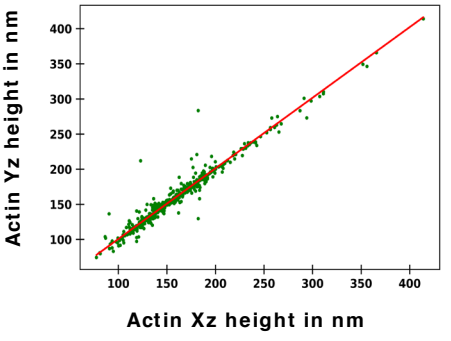

H
Actin Yz height in nm
Actin Xz height in nm

Figure S6

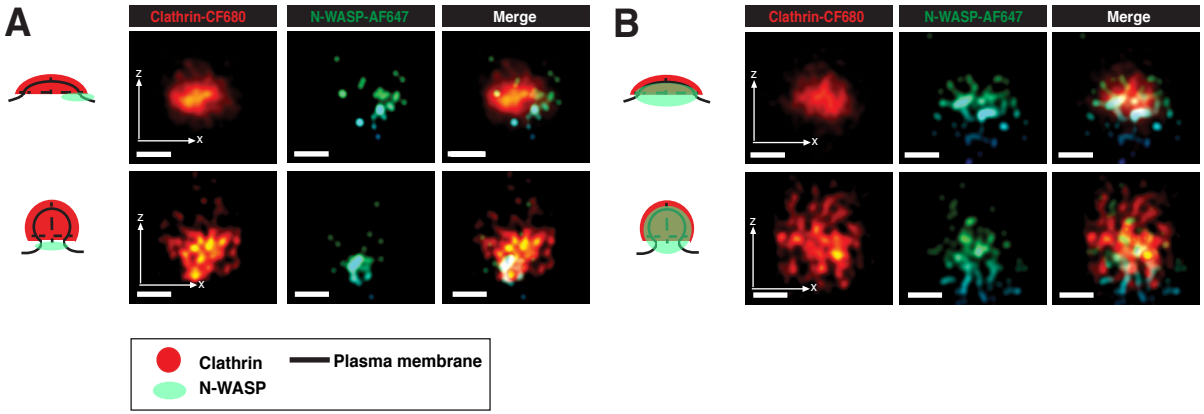
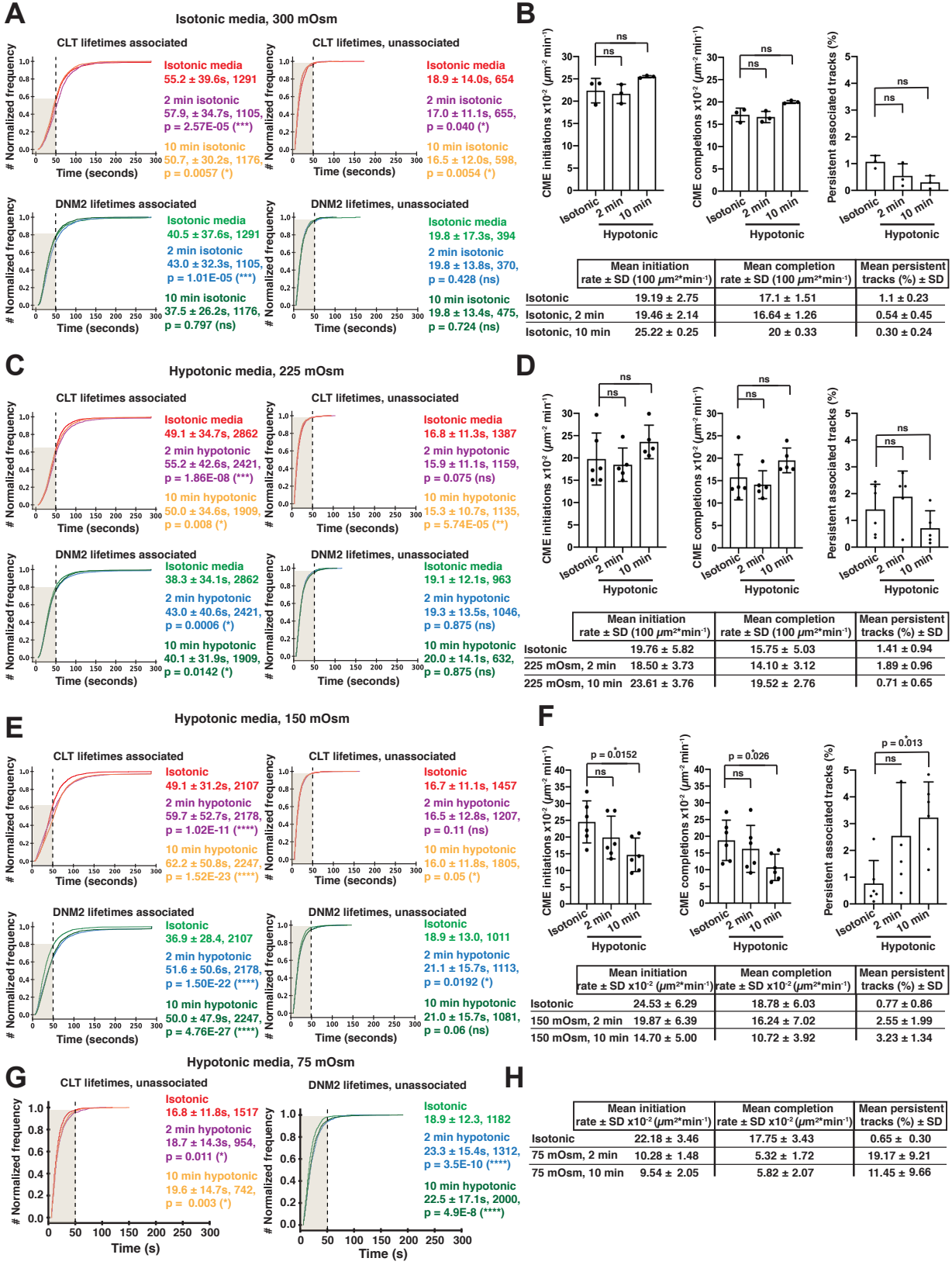

A
Clathrin-CF680
N-WASP-AF647
Merge
B
Clathrin-CF680
N-WASP-AF647
Merge
Clathrin
N-WASP
Plasma membrane

Figure S7



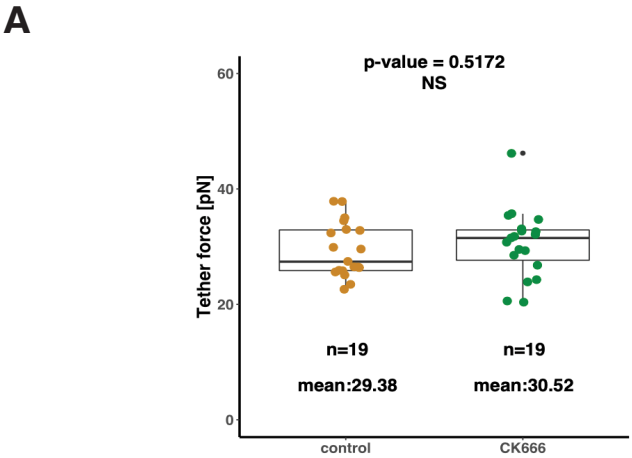
	Mean initiation rate ± SD (100 μm^2*min^{-1})	Mean completion rate ± SD (100 μm^2*min^{-1})	Mean persistent tracks (%) ± SD
Isotonic	19.19 ± 2.75	17.1 ± 1.51	1.1 ± 0.23
Isotonic, 2 min	19.46 ± 2.14	16.64 ± 1.26	0.54 ± 0.45
Isotonic, 10 min	25.22 ± 0.25	20 ± 0.33	0.30 ± 0.24

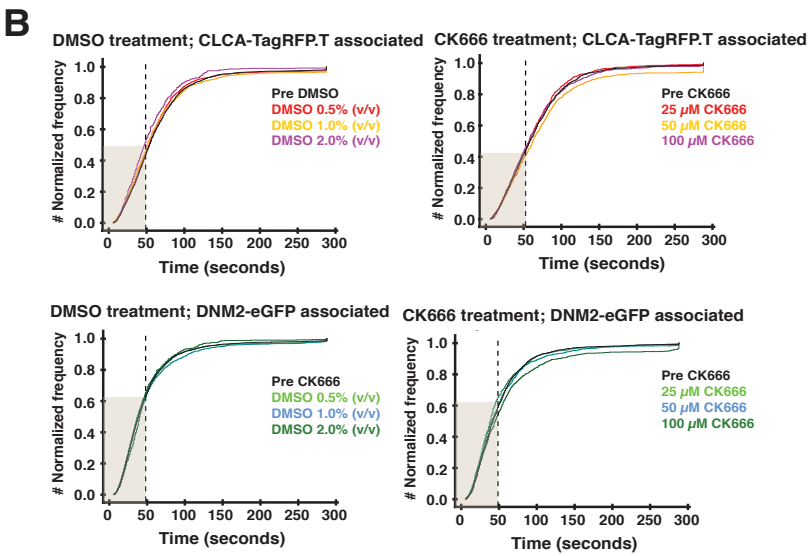
	Mean initiation rate ± SD (100 μm^2*min^{-1})	Mean completion rate ± SD (100 μm^2*min^{-1})	Mean persistent tracks (%) ± SD
Isotonic	19.76 ± 5.82	15.75 ± 5.03	1.41 ± 0.94
225 mOsm, 2 min	18.50 ± 3.73	14.10 ± 3.12	1.89 ± 0.96
225 mOsm, 10 min	23.61 ± 3.76	19.52 ± 2.76	0.71 ± 0.65

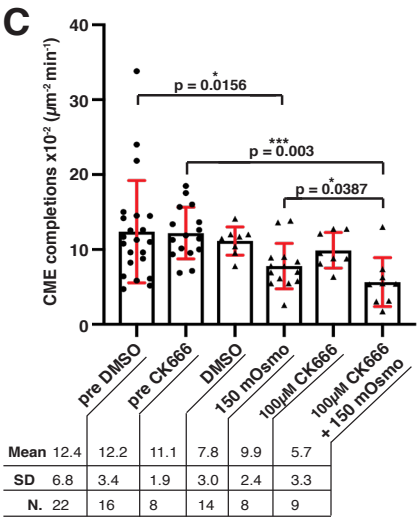
	Mean initiation rate ± SD x10^{-2} (μm^2*min^{-1})	Mean completion rate ± SD x10^{-2} (μm^2*min^{-1})	Mean persistent tracks (%) ± SD
Isotonic	24.53 ± 6.29	18.78 ± 6.03	0.77 ± 0.86
150 mOsm, 2 min	19.87 ± 6.39	16.24 ± 7.02	2.55 ± 1.99
150 mOsm, 10 min	14.70 ± 5.00	10.72 ± 3.92	3.23 ± 1.34

	Mean initiation rate ± SD x10^{-2} (μm^2*min^{-1})	Mean completion rate ± SD x10^{-2} (μm^2*min^{-1})	Mean persistent tracks (%) ± SD
Isotonic	22.18 ± 3.46	17.75 ± 3.43	0.65 ± 0.30
75 mOsm, 2 min	10.28 ± 1.48	5.32 ± 1.72	19.17 ± 9.21
75 mOsm, 10 min	9.54 ± 2.05	5.82 ± 2.07	11.45 ± 9.66

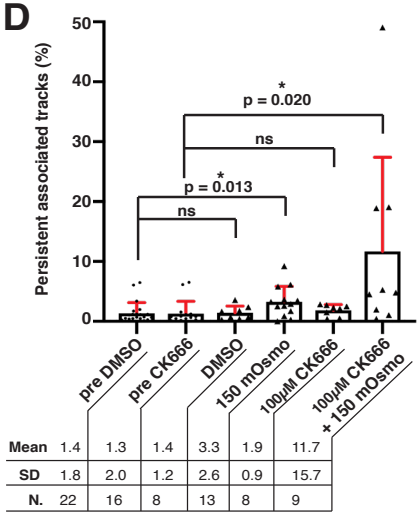
Figure S8







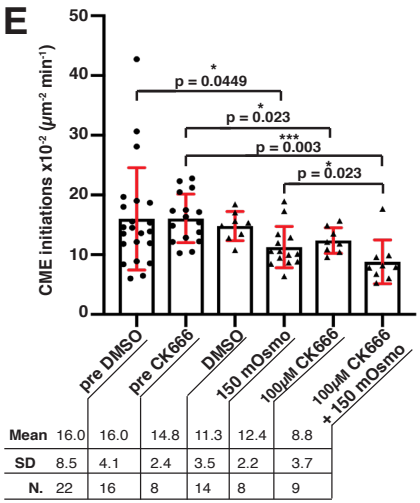
	pre DMSO	pre CK666	DMSO	150 mOsmo	100μM CK666	100μM CK666 + 150 mOsmo
Mean	12.4	12.2	11.1	7.8	9.9	5.7
SD	6.8	3.4	1.9	3.0	2.4	3.3
N.	22	16	8	14	8	9



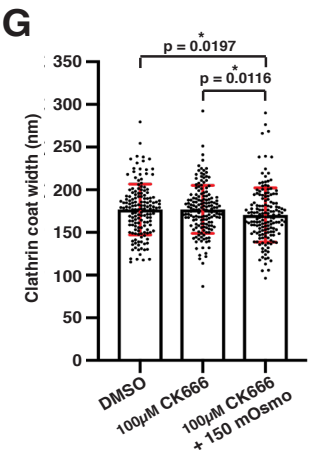
	pre DMSO	pre CK666	DMSO	150 mOsmo	100μM CK666	100μM CK666 + 150 mOsmo
Mean	1.4	1.3	1.4	3.3	1.9	11.7
SD	1.8	2.0	1.2	2.6	0.9	15.7
N.	22	16	8	13	8	9

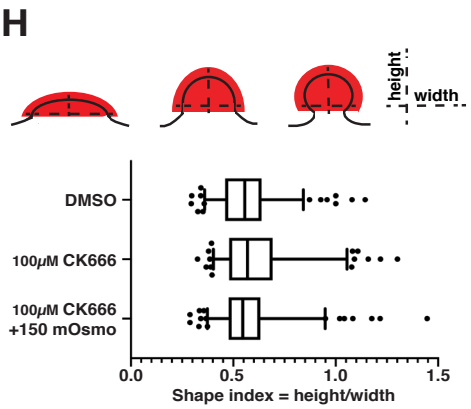
F

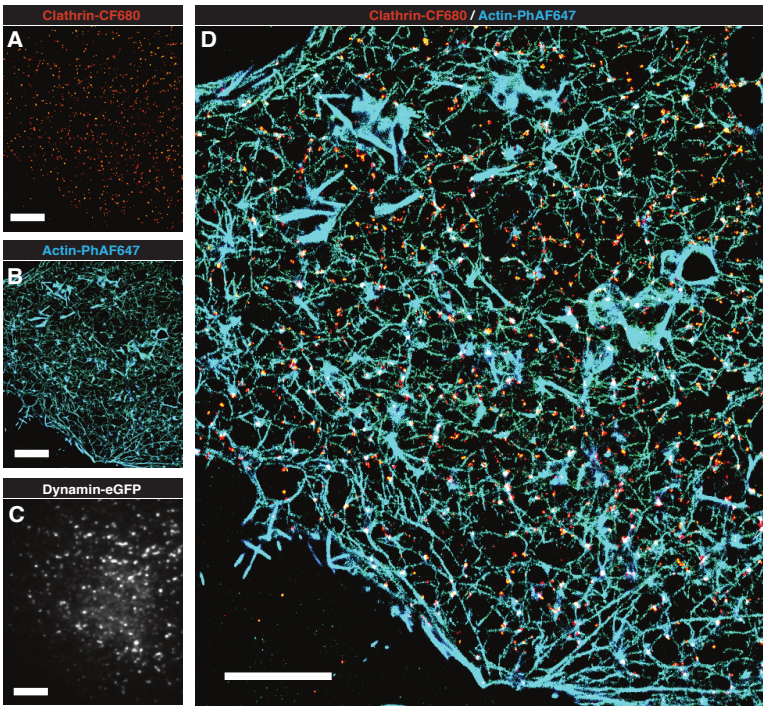
	Mean height (nm)	SD (nm)	N. of values	Mean width (nm)	SD (nm)	N. of values
DMSO	98	21	154	177	30	154
100μM CK666	106	27	158	177	28	158
100μM CK666 + 150mOsmo	96	24	159	171	32	159

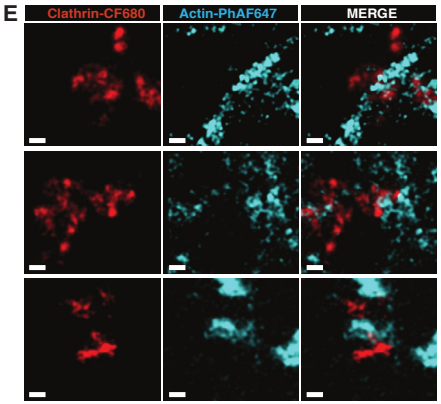


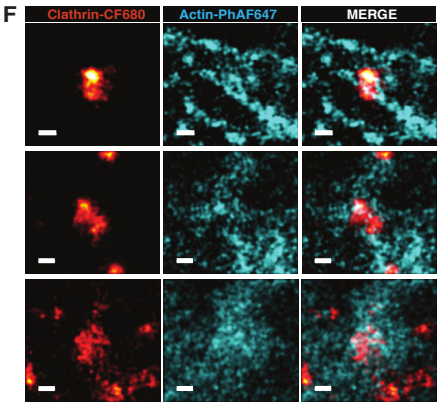
	pre DMSO	pre CK666	DMSO	150 mOsmo	100μM CK666	100μM CK666 + 150 mOsmo
Mean	16.0	16.0	14.8	11.3	12.4	8.8
SD	8.5	4.1	2.4	3.5	2.2	3.7
N.	22	16	8	14	8	9

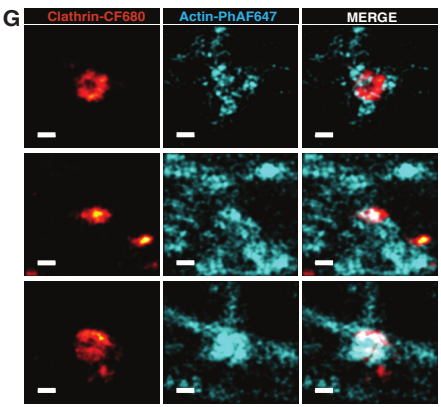





Clathrin-CF680
A
Actin-PhAF647
B
Dynamin-eGFP
C
Clathrin-CF680 / Actin-PhAF647
D


E
Clathrin-CF680
Actin-PhAF647
MERGE


F
Clathrin-CF680
Actin-PhAF647
MERGE


G
Clathrin-CF680
Actin-PhAF647
MERGE

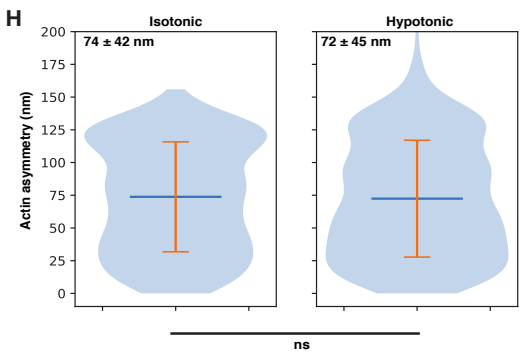
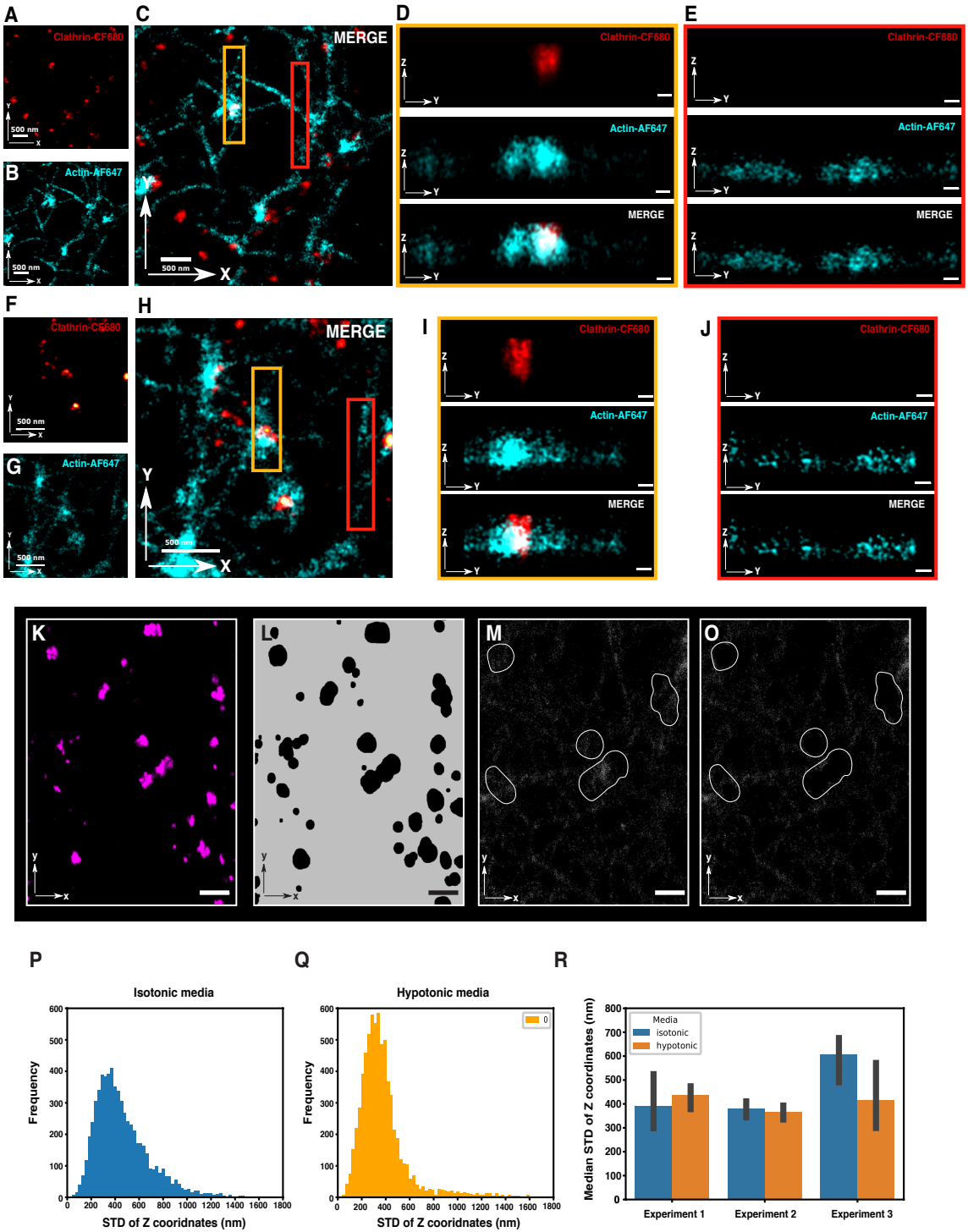

H
Isotonic
Hypotonic
74 ± 42 nm
72 ± 45 nm
Actin asymmetry (nm)
200
175
150
125
100
75
50
25
0
ns

Figure S10


A
Clathrin-CF680
500 nm
B
Actin-AF647
500 nm
C
MERGE
500 nm
D
Clathrin-CF680
Actin-AF647
MERGE
E
Clathrin-CF680
Actin-AF647
MERGE
F
Clathrin-CF680
500 nm
G
Actin-AF647
500 nm
H
MERGE
500 nm
I
Clathrin-CF680
Actin-AF647
MERGE
J
Clathrin-CF680
Actin-AF647
MERGE
K
L
M
O
P
Isotonic media
Frequency
STD of Z cooridnates (nm)
Q
Hypotonic media
Frequency
STD of Z cooridnates (nm)
R
Median STD of Z coordinates (nm)
Media
isotonic
hypotonic
Experiment 1
Experiment 2
Experiment 3