Correlative super-resolution fluorescence and metal-replica transmission electron microscopy

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We combine super-resolution localization fluorescence microscopy with transmission electron microscopy of metal replicas to locate proteins on the landscape of the cellular plasma membrane at the nanoscale. We validate robust correlation on the scale of 20 nm by imaging endogenous clathrin (in two and three dimensions) and apply the method to find the previously unknown three-dimensional position of the endocytic protein epsin on clathrin-coated structures at the plasma membrane.

Super-resolution localization microscopy (for example: photo-activated localization microscopy, PALM1, or direct stochastic optical reconstruction microscopy, dSTORM2) can locate fluorescently labeled biomolecules at a resolution of 10–20 nm using multiple differently colored3,4 or genetically encoded probes3,5,6. Interferometric PALM (iPALM)6 adds an axial resolution of ~10 nm, offering the best resolution among three-dimensional (3D) implementations. However, structure determination with localization microscopy is limited by labeling densities that do not satisfy the Nyquist sampling criterion, which requires a labeling frequency that is at least twice the frequency of the structure being resolved. Furthermore, molecules that are not fluorescently labeled are invisible to fluorescence microscopy, which results in images that lack their underlying cellular context. In contrast, platinum replicas viewed by transmission electron microscopy (TEM) provide 2-nm-resolution images of the structural features of cells7.8. Identification of proteins in these images is usually done by ~15-nm-diameter immunogold labeling. However, gold is bulky, which can cause sparse labeling and obscure the structural details beneath it, and gold is sometimes indistinguishable from dark electron microscopy (EM) areas. Thus, super-resolution localization microscopy and metal-replica TEM are complementary methods that can be combined to determine protein positions within a rich cellular context at the resolution of several nanometers in two or three dimensions.

Correlative super-resolution microscopy with EM is becoming an important control for verifying super-resolution data9. In these cases, empirical coarse overlays of images are sufficient. However, there is a lack of techniques that reproducibly correlate at the nanometer scale across large areas and are robust enough to allow statistical analysis of protein locations with previously unknown positions. Prior attempts have encountered three major difficulties: fluorescent probes that are incompatible with EM sample preparation, structural deformation between imaging modes and a lack of alignment markers that are stationary at the nanoscale5,10. We have found that thin, coverslip-bound samples, such as cell membranes, avoid these problems when grown on custom-designed gold nanoparticle-embedded coverslips; this allows super-resolution 2D and 3D PALM imaging to be combined with metal-replica TEM techniques7,11. With this combined technique we report reproducible nanoscale correlation across 20-µm images and statistical analysis of protein localizations on single clathrin-coated structures across the membrane.

Cells to be imaged by fluorescence super-resolution microscopy-metal-replica TEM (Fig. 1) were grown on coverslips containing gold nanorods embedded under an ~20-nm layer of silica. The silica-covered nanorods have strong photoluminescence owing to surface plasmon resonance and are visible in iPALM. They extended above the flat surface of the coverslip (Supplementary Fig. 1) and transferred with the platinum replica, which allowed them to be used as nanometer-scale iPALM-TEM correlation markers in the xy plane. Proteins were labeled with Alexa Fluor 647 antibody (AF647; Invitrogen), and the membrane was labeled with myristoylated pcSFP2, which allowed for iPALM-to-TEM alignment in the z dimension.

First we examined 2D correlation across entire cell membranes (Fig. 2a,b). We mapped the xy PALM positions of AF647 antibody-labeled clathrin heavy chain (clathrin-AF647) onto the TEM images (Fig. 2b and Supplementary Fig. 2) with a first-degree polynomial spatial warp transformation that registers positions of gold nanoparticles in PALM and TEM data. For eight cells, 6–22 nanoparticles were used for each transformation (mean = 16 nanoparticles), and the mean registration error for nanoparticles in each cell ranged from 3.6 nm to 10.1 nm. xy localizations from clathrin-AF647 in the PALM image matched the 2D shapes of the clathrin-coated structures (CCSs) in TEM (Fig. 2c,d). AF647 is linked to clathrin through a 20- to 25-nm antibody complex; therefore, the fluorescence of a structure is generally larger than that of the TEM image. To assess how the fluorescence mapped to the geometric clathrin lattice observable in TEM, we created average 2D fluorescence density maps of clathrin-AF647 xy PALM localizations with respect to 2D TEM structures.
Figure 1 | Brief Communications. (a) Cells are grown and transfected with a membrane label (myristoylated psCFP2) on a coverslip containing gold nanorods embedded under an ~20-nm layer of silica. (b, c) Cells are fixed and sonicated to disrupt the upper cell membrane and expose the inner surface of the plasma membrane (‘unroofing’). (d, e) The protein of interest is immunolabeled, and the sample is imaged with iPALM. The imaged area is marked with a diamond scribe, and a low-magnification differential interference contrast image is acquired. (f, g) The sample is dehydrated and then coated with a thin layer of platinum and carbon to create a replica of the membrane surface. (h) The replica is separated from the coverslip, rinsed and transferred to a TEM grid. Glass-embedded gold nanorods visible in iPALM adhere to and are transferred with the platinum replica. (i) The same cells imaged in iPALM are imaged with 2D tiling at multiple angles (to create 3D tomograms) in TEM.

(Gaussian convolution introduces a statistical sampling distribution to the model that incorporates errors such as those due to PALM-to-TEM registration, PALM localization and visually defining the perimeters of CCSs in TEM. Even with these errors, the average fluorescence density maps demonstrate the close 2D correlation obtainable by this method. We further correlated the fluorescence to 3D tomograms by matching features between the tomogram and the 2D TEM image. We attained correlation in the axial dimension (z) by aligning the iPALM z position of a fluorescent membrane marker (myristoylated psCFP2) to the TEM membrane plane (Fig. 2g). Gold nanoparticles could not be used for the z-dimension alignment because they shift in z with respect to the rest of the replica during lifting. 3D correlations of CCSs (Fig. 2h and Supplementary Videos 1 and 2) show that the shapes of the pits in the z dimension in iPALM images match the shapes of the corresponding pits in the TEM tomograms. Occasionally, there were regions in which the fluorescence membrane marker did not align with the TEM membrane (<10% membrane area). These regions tended to be where there was a wide (~1-µm) non-adherent domed membrane patch visible in iPALM that likely collapsed during critical-point drying or replica transfer to the

Figure 2 | Correlation of clathrin fluorescence and TEM structures. (a) 2D TEM tiling of an unroofed cell replica. (b) iPALM image of AF647-labeled clathrin mapped onto the TEM image. (c, d) Higher-magnification image of green box (c) and orange box (d) in b. (e) Average 2D fluorescence density maps of 154 flat or slightly domed clathrin structures (green) and 116 highly domed clathrin structures (orange). Radial scans (bottom right) are shown with standard error of pixel sampling. (f) Geometric models of a flat disk (purple; top) or hemispherical shell (blue; bottom). Radial scans of the images generated by these geometric models are shown at the right compared with the data from e. (g) Myristoylated psCFP2 (blue) and clathrin-AF647 (magenta) fluorescence are shown mapped onto the z projection of a tomogram. A magnified z slice along the orange dashed line is shown. (h) Individual clathrin structures are shown in xy (z projection) and yz dimensions (tomogram slice). (i) 10 histograms of fluorescence positions of myristoylated psCFP2 (blue) and clathrin-AF647 (magenta) in the z dimension were produced from 96 separate CCSs (3 tomograms) and shown ordered by height. The local membrane plane and the top of the corresponding CCSs as observed by TEM are marked in orange, and each histogram is aligned to bring this local TEM membrane plane to 0. The membrane and clathrin data are separated below for clarity. Scale bars, 5 µm (a, b) and 200 nm (c, d, g, h).
Figure 3 | Location of epsin 1 in clathrin-coated structures. (a) Epsin-AF647 fluorescence (magenta) correlated to TEM images (grayscale) in the xy dimension. The seven selected images all contain a clathrin structure in the center as evidenced by the geodesic mesh pattern. (b,c) Average fluorescence density maps of epsin for short (b; green; n = 159) and tall (c; orange; n = 84) clathrin structures, as in Figure 2 for clathrin. (d) Radial scans of the images in b (green opaque) and c (orange opaque) compared to their respective clathrin radial scans (Fig. 2; lighter hues). The widths of the profiles depict the standard error of pixel sampling from each separate structure. (e) 97 1D histograms of epsin-AF647 (magenta) and psCFP2 (cyan) fluorescence localizations along the z dimension placed in order of height. The plane of the plasma membrane and the top of the CCS as observed in TEM are shown in orange. All histograms are aligned to the position of the plasma membrane. (f) Three CCSs with epsin-AF647 shown as z projections through the tomogram. (g,h) Isosurface models (produced by IMOD freeware) of the tomograms viewed down the z axis (g) and y axis (h). Scale bars, 100 nm.

TEM grid. In these cases, there was clear membrane misalignment (Supplementary Fig. 3 and Supplementary Video 3) and CCSs were not analyzed. Myristoylated psCFP2 was rarely found inside of clathrin structures but was often found in nonclathrin vesicles (Supplementary Video 3), a result suggesting that myristoylated proteins may be excluded.

We assessed the clathrin-AF647 fluorescence along the z dimension with 1D fluorescence histograms of 96 CCSs (Fig. 2). Histograms were normalized and ordered by height (as measured from TEM). For the range of heights, clathrin fluorescence extends from 20–30 nm above the plasma membrane to ~25 nm above the top of the clathrin structure observed by TEM. These distances are consistent with fluorophores attached to a 25-nm antibody complex that coats the top of a dome. The small differences between fluorescence and TEM data in zx results from errors discussed above as well as possible drying effects from the TEM preparation. We attribute the low registration error to the thin fixed sample adhered to the coverslip for both iPALM imaging and TEM preparation.

After validating our method with clathrin, we applied it to the endocytic protein epsin 1. Epsin binds clathrin, the lipid phosphatidylinositol-4,5-bisphosphate, ubiquitinated cargo, eps15 homology domains and the adaptor complex AP2, and it has been proposed to generate membrane curvature, facilitate vesicle scission, traffic ubiquitinated cargo and regulate actin6,12–16. With such diverse functions under discussion, it is unclear how epsin functions in endocytosis. Epsin is highly colocalized with clathrin in live cells when examined by diffraction-limited fluorescence microscopy (Supplementary Fig. 4). Understanding the physical location of epsin within clathrin structures at the nanometer scale would help to unravel epsin’s role in endocytosis.

In 2D correlative images for the CCSs (n = 327) observed in three cells, AF647-labeled epsin 1 (epsin-AF647) appeared at the outer perimeter of most structures (81%), including both flat structures and highly curved pits (Fig. 3a and Supplementary Figs. 5 and 6). There were, however, cases (18%) in which epsin was distinctly found at the center. Less than 1% of CCSs had no detectable epsin-AF647. Because epsin-AF647 was often visible as less than five puncta per structure, it was necessary to average the signals in order to assess the 2D probability density for 159 slightly domed (or flat; Fig. 3b) and 84 highly domed (Fig. 3c) structures, as we previously did for clathrin. We compared the average probability density map for epsin to that of clathrin for short and tall structures (Fig. 3d). In both cases, the fluorescence from epsin-AF647 dipped lower in the center and reached its maximum further from the center than did the fluorescence from clathrin-AF647. This confirms the visual analysis that epsin is statistically concentrated at the perimeter of CCSs in the xy plane. Control experiments using two-color PALM (or dSTORM) imaging of clathrin–Atto 488 and epsin-AF647 in intact versus ‘unroofed’ cells (the latter having the upper cell membrane removed for visualization of the inner plasma membrane) demonstrated that our protein locations were not changed during our unroofing preparation or correlative imaging pipeline (Supplementary Fig. 7).

Epsin could also be labeled with a photoconvertible fluorescent protein such as mEos3 (Supplementary Fig. 8), but observing the native protein and the higher resolution afforded by the brighter AF647 make it superior for statistical analysis.

We constructed tomograms (Supplementary Videos 4 and 5) and analyzed the position of epsin-AF647 in the z dimension (Fig. 3e). Strikingly, the position of epsin-AF647 with respect to CCSs in the z dimension looks similar to that of clathrin-AF647. This indicates that epsin is not located exclusively at the base of CCSs but instead samples the entire height of the CCSs during all stages of endocytosis. To illustrate this, we created 3D isosurface models of TEM tomograms and epsin-AF647 iPALM renderings (Fig. 3f–h and Supplementary Video 6). These models show that although the fluorescence is at the outer perimeter of CCSs in the xy plane, it appears anywhere along the height of clathrin pits.

Our data provide a map of proteins at single endocytic structures and help form models of these proteins’ functions. For example, epsin has been suggested to induce curvature of
CCSs$^{12,15}$. We show that epsin 1 is enriched and depleted in regions of equivalent curvature (the edge and center of flat CCSs, or the side and top of clathrin pits). Our data also contradict previous suggestions that epsin is part of the complex located at the bottom rim of CCSs$^{13,16}$. A complete map of all the molecules associated with these structures is possible with this method and will provide a comprehensive structural view of endocytosis in cells.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

K.A.S., G.S. and J.W.T. designed the experiments. K.A.S. and G.S. performed the experiments. G.S. and K.A.S. processed data. K.A.S. analyzed the results. K.A.S. and J.W.T. wrote the manuscript. S.B.v.E. designed plasmids. J.W.T. and H.F.H. oversaw the project. All authors discussed the results and commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture and transfection. PC12-GR5 cells were maintained at 37 °C and 5% CO₂ in T75 flasks (Sarstedt 83-1813-002) in poly(l-lysine) before the cells were plated. (Denton Vacuum). These coverslips were cleaned and coated with poly(l-lysine) before the cells were plated.

Unroofing. One day after transfection, cells were rinsed with imaging buffer (130 mM NaCl, 2.8 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4), briefly incubated in one part stabilization buffer (70 mM KCl, 30 mM HEPES at pH 7.4, 5 mM MgCl₂ and 1 mM DTT) and three parts 0.01% (w/v) poly(l-lysine) for 10–15 s, and then incubated for 10–15 s three times in a fresh solution made of one part stabilization buffer and three parts water. The coverslips were then placed in stabilization buffer containing fixative (2% paraformaldehyde, Electron Microscopy Sciences) immediately before sonication. Sonication was done with a Branson Sonifier 450 with a 1/8” tapered microtip. The tip was positioned 5 mm above the coverslip, and a single 400-ms pulse at the lowest output setting resulted in approximately 1 cm² of unroofed cells on the coverslip. The cells were then placed into fresh stabilization buffer and fixed for 20 min.

Immunolabeling. Fixed unroofed cells were rinsed with PBS and placed into blocking buffer (PBS with 3% (w/v) bovine serum albumin, BSA) for 1 h. The unroofed cells were then incubated for 1 h in blocking buffer containing 2 μg/mL primary antibody (R-20, Santa Cruz Biotechnology, or X22, Thermo Scientific). The sample was rinsed with blocking buffer before secondary antibody labeling for 30 min (2 μg/mL Alexa Fluor 647 donkey anti-goat, Invitrogen A11035; Alexa Fluor donkey anti-mouse, Invitrogen A21447; or Atto 488 donkey anti-mouse) in blocking buffer. Atto 488 donkey anti-mouse was created with Atto 488 NHS ester (Sigma, 4-molar excess) and unlabelled donkey anti-mouse IgG (Abcam ab6707) and purified through a Superdex 75 10/300 GL size-exclusion column (GE Healthcare). Finally, the sample was washed with blocking buffer and PBS and was post-fixed for 20 min. For immunolabeling intact cells, 0.2% Triton X-100 was added to the blocking buffer, and cells were incubated for 2 min in permeabilization buffer (PBS with 3% (w/v) BSA, 0.5% Triton X-100) before blocking.

iPALM. After immunolabeling, cells were rinsed, placed in blinking buffer (50 mM Tris, 10 mM NaCl, 0.1 g/mL glucose, 0.8 mg/mL glucose oxidase, 40 μg/mL catalase, 71 mM 2-mercaptoethanol) and covered with an 18-mm #1.5 coverslip. The two coverslips were sealed with epoxy (Elmer’s) and Vaseline (Unilever). iPALM was performed as previously described with the z-axis measurement range extended to 750 nm (ref. 3). Typical iPALM data acquisition consisted of 40,000–80,000 frames acquired with iXon DU-897E EM-CCD cameras (Andor). Acquisition was performed in frame transfer mode, and the laser excitation was constantly active during acquisition. AF647 was imaged with a 637-nm MRL-III-640 laser (OptoEngine) at ~3,000 W/cm² with 20- to 30-ms exposure using LP02-647RU and FF01-720/SP filters. psCFP2 was imaged with 488-nm Cyan 488 laser excitation (Newport, Spectra Physics) at ~400 W/cm² with 50-ms exposure using LP02-488RS and FF01-520/35 filters. An additional quad notch filter NF01-405/488/561/635 was used for all imaging. Filters were from Semrock. 

Electron microscopy. Samples were critical-point dried and were coated with platinum and carbon as previously described. The coated samples were imaged with 10× phase contrast light microscopy to locate the regions of interest (ROIs). A pioloform negative for mycoplasma contamination. Transfections were performed with Lipofectamine 2000 (Invitrogen) and 2 μg of plasmid after 1 d of growth on coverslips. The myristoylated pmCherryCl-Epsin1 was obtained from Addgene (#22228). pmEos3-Epsin1 were both made by replacing EGFp from pEGFFC1-Epsin1 with mCherry (Clontech #632524) or mEos3 (ref. 19) using AgeI and BsrG1. For live imaging, cells were transfected with 1 μg each of pEGFP-LCa (clathrin light chain A) and pmCherryCl-Epsin1.

Sample preparation for TEM was performed on a JEOL 1400 running SerialEM free-ware and equipped with an XR-111 CCD camera (Advanced
Microscopy Techniques) Montages of entire unroofed cells were produced at 15,000× with 10% overlap. Single-axis tilt series (−60° to 60°, 1° increments) were collected at 8,000×. The montages were stitched together, and the tilt series were reconstructed into tomograms using IMOD software17,18.

**Correlation.** The 25 × 45–nm gold nanorods were identified in TEM micrographs. The procedure used for two-color PALM alignment23 was also used to register two-color PALM images to the TEM images. For image correlations we used a POLYWARP1 image transformation defined as

\[
X' = K_{x00} + K_{x01} \cdot X + K_{x10} \cdot Y + K_{x11} \cdot X \cdot Y
\]

\[
Y' = K_{y00} + K_{y01} \cdot X + K_{y10} \cdot Y + K_{y11} \cdot X \cdot Y
\]

where X and Y are the PALM gold coordinates being transformed to the TEM gold coordinates, X′ and Y′. K coefficients were calculated with a least-squared solution.

After xy PALM data were aligned to the 2D TEM data, the 10-nm gold nanospheres were used as markers to align the 2D TEM/iPALM correlation to the 3D tomogram (in the xy plane) using linear regression or POLYWARP1. z alignment was performed by manually aligning the membrane iPALM marker with the membrane in TEM tomograms across the entire tomogram.

**Fluorescence probability density maps.** 2D EM micrographs were analyzed in ImageJ23, and elliptical regions of \(r_x\) and \(r_y\) (radii along the x and y axes) were drawn to best fit the shape of visible clathrin lattices. Clathrin structures were split into two categories: (i) flat or slightly curved domes and (ii) highly invaginated pits. Clathrin structures were omitted from this analysis only if they were at the edge of the cell, did not fit well to an ellipse, were within 0.5 μm of a gold nanorod or were right next to another clathrin structure. In Matlab (MathWorks), the coordinates of PALM localizations were mapped onto the coordinates of the ellipses and assigned a fractional position from −2 \(r_x\) to 2 \(r_x\) in the x dimension and −2 \(r_y\) to 2 \(r_y\) in the y dimension. The fractional coordinates from each ellipse were binned into a 40 × 40 2D histogram. The resulting image was normalized before the images were resampled to have 10-nm pixels (to simulate the fluorescence density maps produced by our data).

**z-axis histograms.** The height of each structure was determined by selecting the local plasma membrane plane and the top of the clathrin structure in TEM tomograms. A 1D histogram of the z position of all iPALM localizations associated with each structure was mapped with respect to the TEM coordinates. Finally, histograms were aligned by their bottom TEM coordinate (the plasma membrane) and arranged in order of height (Figs. 2i and 3e).

**Two-color localization microscopy (for Supplementary Fig. 7).** Unroofed cells were prepared as for correlative microscopy. Intact cells were rinsed with imaging buffer and fixed with 2% paraformaldehyde. Immunolabeling clathrin heavy chain with Atto 488 and epsin 1 with AF647 is described above. Fixed and immunolabeled cells were placed in blinking buffer and imaged with a Nikon N-STORM super-resolution microscope system (N-1245) equipped with a CFI SR Apochromat TIRF 100× oil objective, an 80-mW 488-nm laser, a 125-mW 647-nm laser and an Andor Ixon Ultra DU-897 camera. Two-color images were acquired in TIRF at full laser power with 20-ms frames. Final super-resolution images were localizations from 20,000–30,000 frames (each color).

**AFM.** Tapping-mode AFM images of clean coverslips were obtained in air with a multimode atomic force microscope driven by a NanoScope V controller and E scanner (Veeco/Bruker) using AC160TS cantilevers (Olympus) with a typical resonance frequency of 300 kHz and a nominal spring constant of 42 N/m. Images were analyzed using NanoScope software (version 7).

**Live TIRF.** Cells were grown on poly(t-lysine)-coated 25-mm round coverslips of #1.5 thickness (Warner Instruments) and transfected with pEGFP-LCa (clathrin light chain A)20 and pmCherryC1-Epsin1. Cells were imaged by total-internal-reflection fluorescence (TIRF) in imaging buffer as described previously22. Each image shown is an average of five subsequent 100-ms frames each 500 ms apart.

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