Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes

Sang-Hee Shim1, Chenglong Xia1, Guisheng Zhong2, Hazen P. Babcock3, Joshua C. Vaughan4, Bo Huang5, Xun Wang6, Cheng Xu7, Guo-Qiang Bi6,5, and Xiaowei Zhuang3,4,6,7,8

1Department of Chemistry and Chemical Biology, 2Howard Hughes Medical Institute, 3Center for Brain Science, and 4Department of Physics, Harvard University, Cambridge, MA 02138; and 5Hefei National Laboratory for Physical Sciences at the Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, China

Edited by Stefan W. Hell, Max Planck Institute for Biophys. Chemistry, Goettingen, Germany, and accepted by the Editorial Board July 7, 2012 (received for review February 1, 2012)

Imaging membranes in live cells with nanometer-scale resolution promises to reveal ultrastructural dynamics of organelles that are essential for cellular functions. In this work, we identified photoswitchable membrane probes and obtained super-resolution fluorescence images of cellular membranes. We demonstrated the photoswitching capabilities of eight commonly used membrane probes, each specific to the plasma membrane, mitochondria, the endoplasmic recticulum (ER) or lysosomes. These small-molecule probes readily label live cells with high probe densities. Using these probes, we achieved dynamic imaging of specific membrane structures in living cells with 30–60 nm spatial resolution at temporal resolutions down to 1–2 s. Moreover, by using spectrally distinguishable probes, we obtained two-color super-resolution images of mitochondria and the ER. We observed previously obscured details of morphological dynamics of mitochondrial fusion/fission and ER remodeling, as well as heterogeneous membrane diffusivity on neuronal processes.

M
embrane structures, such as the plasma membrane, endosomes, lysosomes, mitochondria, the Golgi, and the endoplasmic recticulum (ER), perform a variety of functions in eukaryotic cells. To accommodate these functions, cellular membranes fold into various shapes, often with highly curved morphologies and nanometer-scale dimensions (1, 2). Examples include the filopodia protruding from the plasma membrane, the cristae of mitochondria, the cisternae of the Golgi, and the meshwork of the ER (1, 2). These intricate membrane structures often undergo rapid remodeling. Filopodia extend from and retract into the cell via cytoskeletal assembly and disassembly (1). ER tubules are pulled out of membrane sheets by molecular motors or polymerizing cytoskeletal filaments (2). Mitochondria constantly fuse and divide while moving along microtubules (3). Such dynamic processes play essential roles for maintaining the morphology and functions of these subcellular structures and organelles.

Ultrastructural characterization of membrane organelles has mainly relied on electron microscopy (EM), which provides both membrane contrast and nanometer-scale resolution. However, EM cannot be used to image live specimens and hence does not provide dynamic information directly. This difficulty may be overcome by the recently developed super-resolution fluorescence microscopy methods (4–7). Among these methods, stochastic optical reconstruction microscopy (STORM) (8) and (fluorescence) photoactivation localization microscopy [(F)PALM] (9, 10) take advantage of the use of photoswitchable probes and high-resolution localization of single molecules to surpass the diffraction limit. During the imaging process, only a sparse subset of the probes is switched on at any time such that their positions can be determined with high precision by finding the centroids of their images. After the coordinates of a sufficient number of probe molecules are accumulated, a super-resolution image can be constructed from these molecular coordinates. It has been further demonstrated that many conventional dyes can be used for super-resolution imaging based on photoswitching/bleaching and localization of single molecules (11–20). Using single-molecule-based super-resolution methods, 3D resolutions down to approximately 10 nm have been demonstrated for fixed samples (21–26), and live-cell imaging has also been achieved with spatial resolutions of 20–60 nm at time resolutions ranging from 0.5 s to 1 min (27–32).

To date, super-resolution imaging has been demonstrated mostly on protein-based fluorescent labels such as proteins tagged with dyes or genetically encoded fluorescent proteins. However, membrane proteins are often not distributed uniformly on the membrane, but instead form localized domains nonideal for general membrane imaging. Moreover, since probe density is a key determinant of the image resolution (27, 31), protein labels need to be expressed at high levels to achieve high resolutions, which can cause overexpression artifacts. An alternative to protein labels is small-molecule probes that directly bind to membrane structures, which have been widely used for specific labeling of membrane organelles (33). The cell permeability, small size, and high affinity of these probes for specific membrane organelles facilitate high-density labeling of live cells. Moreover, these small-molecule probes are commercially available and easy to use. However, it is unknown whether these probes are photoswitchable and suitable for super-resolution imaging.

In this work, we demonstrate super-resolution fluorescence imaging of membranes in live cells by using photoswitchable membrane probes. We identified the photoswitching capabilities of eight small-molecule probes, commonly used for labeling the plasma membrane, mitochondria, the ER, or lysosomes. Using these probes, we achieved 30–60 nm spatial resolution with temporal resolution down to 1–2 sec, and tens of independent snapshots when imaging live cells. The different spectral properties of these probes also allowed two-color super-resolution imaging of mitochondria and the ER. Utilizing these imaging capabilities, we captured ultrastructural dynamics of the plasma membrane, mitochondria, and the ER.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. S.W.H. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

1S.-H.S. and C. Xia contributed equally to this work.

2Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158.

3To whom correspondence may be addressed. Email: gobi@ustc.edu.cn or zhuang@chemistry.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1201882109/-/DCSupplemental.
Results

Imaging the Plasma Membrane with Lipophilic Cyanine Dyes. Carboxyanine dyes with long alkyl chains such as DiI, DiD, and DiR have been widely used as labels for the plasma membrane (see Fig. S1A for chemical structures). Due to their lipophilic nature, incubating live cells with dye solutions for a few minutes is sufficient to label the plasma membrane with high probe density (33). Interestingly, we found that DiI, DiD, and DiR exhibited photoswitching behaviors without an exogenous switching agent. Under 561-nm (for DiI), 657-nm (for DiD), or 752-nm (for DiR) illumination, these probes fluoresced and rapidly switched off to a dark state; the dark-state molecules could then be reactivated to the fluorescent state by 405-nm illumination (Fig. 1A). Similar laser excitation schemes have been used for switching other dyes (13, 17). This reversible photoswitching behavior were suitable for STORM imaging of the plasma membrane (Fig. 2A and Fig. S2 A and B).

We performed STORM imaging of the plasma membrane on live hippocampal neurons using DiI (Fig. 2B). DiI-labeled neurons were imaged with 561-nm illumination at a 500-Hz camera frame rate. A roughly constant number of activated DiI molecules per camera frame were maintained with ramping 405-nm illumination. The fast switching rates of DiI allowed us to collect a super-resolution image in 15 sec or less. Compared to the raw and deconvoluted conventional images, substantial resolution improvement was observed in the STORM image (Fig. 2B and Fig. S3A). The enhanced resolution allowed us to measure the widths of the dendritic spine necks in live neurons (Fig. 2C).

During STORM imaging, individual DiI molecules were switched off in approximately two camera frames, emitting 720 photons per frame on average. By measuring the spread of the localization distributions of individual fixable DiI molecules in fixed cells, we determined the localization precision to be 17 nm, measured in SD, corresponding to an image resolution of 40 nm measured in full width at half maximum (Table S1). Another key factor affecting the final image resolution is the label or localization density within the structure. Because DiI molecules diffuse on the plasma membrane in live cells, a single probe molecule is able to sample different locations on the membrane and contribute multiple independent localizations for mapping out the underlying structure. Based on the Nyquist sampling criterion, which equates the resolution limit to twice the average distance between neighboring localizations, a Nyquist resolution limit can be defined as $2/(\text{localization density})^{1/2}$ for 2D images (27). For the live neuron images, we found the Nyquist resolution limit to be approximately 40 nm for the 15-sec STORM snapshots (eight independent snapshots) and approximately 70 nm for 5-sec snapshots (24 independent snapshots).

Time-resolved STORM images of DiI captured extension and retraction of filopodia or dendritic spines (Fig. 3A). In addition to the morphological dynamics of the plasma membrane, molecular motion within the membrane can also be monitored by tracking individual probe molecules. The use of photo-switchable probes allows a high density of molecular trajectories to be accumulated over time (34). In Fig. 3B, we present a subset of the DiI trajectories that lasted for 15 frames or more, from which we calculated the local diffusion coefficients (See Fig. S4 for the full distribution of the trace length). Diffusion of DiI was slower in thin dendritic structures such as filopodia or spines, in comparison to the mobility measured in the shaft (Fig. 3C), consistent with previous results (35). Moreover, the high density of molecular traces allowed us to determine the local distribution of diffusivity within filopodia or spines (Fig. 3D). The mobility of DiI decreased as the molecules approached the tips of filopodia or spines (Fig. 3E),
showed substantially improved resolution (Fig. 2). For up to 30 independent snapshots per movie. Compared to the conventional images, the STORM images had similar widths [Fig. 4B, 102 ± 16 nm (SD, N = 24) prior to fission; 108 ± 12 nm (SD, N = 11) after fusion]. These thin tubular structures could persist for more than 10 sec. While

Imaging Mitochondria with Cationic Rosamine and Carbocyanine Fluorophores. Mitochondria consist of two layers of membranes: the outer membrane forms a smooth tubular outline of the organelle; the inner membrane forms deeply invaginated tubules and lamellae called cristae. Many mitochondrial probes are cell-permeant cations that accumulate on the inner membrane of mitochondria via electrostatic interactions (33). We identified three photoswitchable mitochondrial probes that can be classified into two groups: (i) MitoTracker Orange and MitoTracker Red are cationic rosamine dyes; (ii) MitoTracker Deep Red is a cationic carbocyanine (Fig. S1B). We obtained STORM images of mitochondria in live BS-C-1 cells using MitoTracker Red with a procedure similar to that used in DiI imaging at a 500-Hz frame rate (Fig. 2B). Per imaging frame, each dye molecule emitted on average 790 photons and was localized with 13-nm localization precision, corresponding to 30-nm resolution (Table S1). Images acquired in 10 sec exhibited localization densities that correspond to a Nyquist resolution limit of approximately 30 nm with <15% variation among different cells and experiments. Such resolutions could be achieved for up to 30 independent snapshots per movie. Compared to the raw and deconvolved conventional images, the STORM images showed substantially improved resolution (Fig. 2B and Fig. S2C and D).

Because mitochondria tend to move fast in cells, we further improved the time resolution by increasing the camera frame rate to 900 Hz and by activating a higher density of probes per frame such that their images partially overlap. We analyzed the overlapping images of individual molecules by a multiemitter fitting algorithm (37). Under these imaging conditions, we obtained a Nyquist-based resolution of approximately 40 nm in 2 sec. The improved temporal resolution allowed us to capture mitochondrial fission and fusion intermediates (Fig. 4).

Time-lapse STORM images revealed thin, extended tubular intermediates connecting neighboring mitochondria both prior to fission and after fusion (Fig. 4A and Movie S1). The average width of these tubular structures was 104 ± 15 nm (SD, N = 35) (Fig. 4B). Notably, such tubules tend to have uniform widths over an extended length of several hundred nanometers. Multiple fission and fusion events (Fig. 4C) were captured in the movie.
fission and fusion events could also be observed in the conventional fluorescence movies, the tubular intermediates were not clearly resolved in the diffraction-limit images (Fig. 4C, ii).

Given the extended length of the tubular intermediate with a relatively constant width, such membrane tube is likely constricted by a protein tubular structure. A potential candidate protein is Drp1, a dynamin-family protein required for mitochondrial division in mammalian cells (3, 38). Indeed, in the in vitro assemblies of liposome and Dnm1, the Drp1-homolog in yeast, Dnm1 was found to assemble outside membrane tubes and constrict them to a luminal diameter of approximately 90 nm (39), similar to the widths of the fusion and fission intermediates measured here.

**Imaging the ER and Lysosomes with BODIPY Dye-Conjugates.** BODIPY dyes are neutral and nonpolar. Therefore, BODIPY conjugates tend to be more cell permeant than charged or polar dyes, and are often used to label intracellular structures (33). We tested two BODIPY-conjugated probes: (i) ER-Tracker Red, a BODIPY TR conjugate of glibenclamide which binds to potassium channels enriched in the ER; (ii) LysoTracker Red, a BODIPY 564/570 linked to a weak base that is highly selective for the acidic membrane of lysosomes (Fig. S1 C and D) (33). Again, labeling procedures for these probes are similar to that of DiI. We found both BODIPY TR and BODIPY 564/570 to photoswitch in live cells; they could be imaged and switched off by 561-nm illumination and reactivated by 405-nm light (Fig. 1 C and D).

STORM imaging of the ER and lysosomes were conducted using a procedure similar to that used for DiI or MitoTracker Red (Fig. 2 C and D). At a 500-Hz frame rate, ER-Tracker Red and LysoTracker Red emitted an average of 820 photons per frame, providing a localization precision of 13–15 nm, corresponding to a resolution of 30–35 nm (Table S1). We obtained STORM images within 10 sec for the ER and 1 sec for lysosomes with localization densities that correspond to a Nyquist resolution limit of 30–40 nm for 30–80 snapshots. STORM images of the ER showed an intricate meshwork of tubules and sheets (Fig. 2C). While these structures were largely resolvable in conventional images, the width of the ER tubules were often below the diffraction limit, but could be determined from STORM images (Fig. 2C). Likewise, lysosomes were often resolvable from each other in conventional images, but their sizes and shapes were better determined in STORM images (Fig. 2D).

Dynamics of ER-remodeling were observed using ER-Tracker Red (Fig. 5). In a time-series of 10-sec snapshots (Fig. 5A and Movie S2), we observed extending ER tubules (Fig. 5A). When multiple snapshots were collapsed into a single image with each localization colored by the time of appearance (Fig. 5B), extending tubules could be easily identified by color. The newly extended tubules appeared thinner than older tubules (Fig. 5C); the average widths were 84 ± 15 nm (SD, N = 22) for the newly extended tubules and 127 ± 32 nm (SD, N = 34) for tubules that existed for at least 2 min. When we increased the dye activation rate as in the case of mitochondrial imaging, we were able to increase the imaging speed to 2 sec per image without compromising the spatial resolution (Fig. 5 D and E and Movie S3).

**Two-Color Imaging of Mitochondria and the ER.** The different spectral properties of the probes described above allow for multicolor imaging. For instance, the emission maxima of MitoTracker Red and ER-Tracker Red differ by 16 nm. They can be distinguished using a ratiometric method by splitting their emission into a short- and a long-wavelength channel; the probe identity can then be determined from the intensity ratio of these two channels (30, 32, 40).

Using this approach, we obtained two-color STORM images of mitochondria and the ER in live cells (Fig. 6 and Movie S4). Although we achieved a comparable Nyquist resolution (25–35 nm) to that of the single-color images, the image quality was somewhat degraded from single-color images due to the color cross-talk (16% from ER to mitochondria, 28% from mitochondria to ER with the color assignment defined in Fig. S5).

Correlative dynamics of the two structures were observed from these images. ER tubules were often present at the sites of mitochondrial contraction and fission (Fig. 6), suggesting that ER tubules are involved in constriction of mitochondria. This observation is consistent with the previous hypothesis that ER-induced mitochondrial constriction facilitates the recruitment of Drp1 at the mitochondrial fission site (41).

**Discussion**

Cell membranes often exhibit nanometer-scale morphologies and undergo dynamic remodeling. Super-resolution fluorescence imaging can reveal previously unknown ultrastructural dynamics of these structures. Here, we identified several photoswitchable, small-molecule membrane probes and demonstrated their utility in super-resolution STORM imaging of the plasma membrane, mitochondria, the ER, and lysosomes in living cells. The images revealed nanometer-scale morphological dynamics of neuronal processes, mitochondria, and the ER, as well as nonuniform membrane diffusivity in neurons.

STORM imaging of these probes was performed in buffered medium supplemented with an oxygen scavenger system, but no exogenous chemical additives such as thiol reagents was added. Without the oxygen scavenger, the probes photoswitched similarly but bleached substantially faster, and therefore the overall
observation time was reduced by 10–20 fold. Although these membrane probes all photoswitched under the same buffer conditions, their switching mechanisms could potentially be different. The carbocyanine dyes (DiI, DiD, DiR, and MitoTracker Deep Red) were possibly switched to a dark state by forming a thiol adduct with endogenous thiols (e.g. Glutathione exists in animal cells at approximately 5 mM), as shown for Cy5 (42). The rosamine dyes (MitoTracker Orange/Red) may be switched with a nearly identical width (28 nm, SD, N = 246). Mitochondria are more dynamic, moving with speeds of approximately 20 nm/s during fusion and approximately 50 nm/s at other times (45). For a spatial resolution of 30–60 nm and a time resolution of 2 sec, movement speeds up to 15–30 nm/s are tolerable and allowed us to capture mitochondrial fission/fusion dynamics without substantial motion blur. Indeed, STORM images of mitochondria revealed fusion/fission intermediate structures previously unobserved in cells. The widths of the tubular fusion/fission intermediates measured in live cells (104 ± 15 nm, SD, N = 35) is similar to the width of the tubular structure of mitochondrial dynamin assembled in vitro (39), suggesting a molecular underpinning of the fission/fusion intermediates. We also observed intermitochondria tubes in fixed cells with a nearly identical width (102 ± 28 nm, SD, N = 44), suggesting that the tubular structures were not substantially blurred by motion. On the other hand, the cristae structure was partially blurred. Only the cristae with relatively large spacing appeared to be resolved in the live-cell STORM images (Fig. 4), as in previous live-cell images by structured illumination microscopy (46). The more tightly packed cristae with smaller spacing, observed in EM and fixed-cell STORM images (Fig. S7), were not well resolved in the live-cell images.

Finally, since biological structures are typically three-dimensional, extending the high image resolution into the third dimension could help resolve these structures better. Indeed, the cristae of mitochondria in fixed cells were better resolved in the 3D STORM images than in the corresponding 2D projection images (Fig. S7). The z coordinates of the molecules can be obtained by a variety of means for 3D super-resolution imaging (21–26), and indeed 3D STORM has been demonstrated in live cells (31). However, the use of diffusing membrane probes presents an additional challenge, especially when the shapes of the single-molecule images are used to determine their z-positions. Further increasing the camera frame rate or strobing the excita-
tion laser should help overcome this problem and allow 3D super-resolution imaging of membrane organelles in living cells.

**Materials and Methods**

**Sample Preparation.** Immediately before imaging, BS-C-1 cells and hippocampal neurons were labeled by incubating live cells with culture medium containing one of the membrane probes for 0.5–3 min. For two-color imaging, cells were labeled sequentially with MitoTracker Red and then ER-Tracker Red.

**Image Acquisition and Analysis.** Labeled cells were imaged with continuous illumination at 561, 657, or 752 nm for imaging and 405 nm for probe activation in an oblique-incidence geometry on an Olympus IX-71 inverted microscope. The single-molecule images were recorded at 500–900 Hz frame rates on an EMCCD camera (Ixon 860; Andor). For ratiometric two-color imaging, the fluorescence was split by a 624-nm longpass dichroic mirror into two channels and imaged on two halves of the same camera. The movies were analyzed by custom-written STORM analysis software as we previously described (31), or using the DAOSTORM (37) or a similar multimitter fitting software (47). See SI Materials and Methods for details.

**ACKNOWLEDGMENTS.** This work is supported in part by the US National Institutes of Health and the Collaborative Innovation Award of Howard Hughes Medical Institute (X.Z.), and National Natural Science Foundation of China and National Basic Research Program of China (G-Q.B.). X.Z. is a Howard Hughes Medical Institute investigator. S.-H.S is in part supported by the Mary Fieser fellowship. J.C.V. is supported in part by a Burroughs-Wellcome Career Award at the Scientific Interface.

**REFERENCES**

Supporting Information

Shim et al. 10.1073/pnas.1201882109

SI Text

SI Materials and Methods. Cell culture. BS-C-1 cells [CCL-26; American Type Culture Collection (ATCC)], an African green monkey kidney epithelial cell line, were maintained in a 5% CO₂ atmosphere at 37 °C in Eagle Modified Minimum Essential Medium (ATCC), supplemented with 10% fetal bovine serum (Invitrogen). Cells were passaged every 2–3 d and maintained for up to 20 passages. For imaging, cells were plated into eight-well chambered coverglasses (LabTek-II; Nalgene Nunc), previously cleaned with 1 M aqueous potassium hydroxide, at a density of 16,000–30,000 cells per well.

Hippocampal neuron culture was prepared largely as described previously (1). Briefly, hippocampi from embryonic 18 CFW mice (Charles River) were isolated and digested with trypsin, and neurons were plated onto poly-L-lysine/laminin-coated 12-mm coverslips in a plating medium. Three hours after plating, the coverslips with neurons were reversely placed into 24-well culture chambers with prepared glia. Five μM cytosine-β-D-arabinofuranoside was added to the culture to inhibit the growth of glial cells three days later. The neurons were fed twice a week with Neurobasal medium (Invitrogen) containing B-27 Supplement (Invitrogen) until use.

Live-cell labeling and imaging buffer. Live BS-C-1 cells and neurons were stained immediately before imaging. Approximately 36–48 h after plating, BS-C-1 cells were rinsed with Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (4.5 g/mL) and no phenol red (Invitrogen). Then, the cells were incubated in staining solution containing 10 μM DiI/DiD/DiR (Invitrogen), 0.2–1 μM MitoTracker Orange/Red/Deep Red (Invitrogen), 10–20 μM ER-Tracker Red (Invitrogen), or 1 μM LysoTracker Red (Invitrogen) in DMEM for 0.5–3 min. The stock solutions of all dyes were prepared by dissolving the dye powder in anhydrous DMSO at 25 mM for DiI/DiD/DiR and at 1 mM for other probes. For double-staining of mitochondria and the ER, cells were incubated in 0.2–0.5 μM MitoTracker Red in DMEM for 30 sec, washed two to three times with DMEM, and then incubated in 20 μM ER-Tracker Red in DMEM for 3 min. After staining, the cells were washed two to three times with DMEM and immediately used for imaging. Imaging medium was prepared with DMEM supplemented with 2% glucose, 6.7% FCS, and 1 M HEPES (pH 7.4), and an oxygen scavenging system (0.5 mg/mL glucose oxidase and 40 μg/mL catalase). BS-C-1 cells were imaged at room temperature.

Two to three weeks after plating, hippocampal neurons were rinsed with Neurobasal medium, incubated in DiI solution (approximately 10 μM in Neurobasal medium) at 37 °C for 1–2 min, and washed two to three times with Neurobasal medium. The neurons were allowed to recover in an incubator maintaining a 5% CO₂ atmosphere and 37 °C for at least 15 min before imaging. Labeled neurons were imaged in Neurobasal medium supplemented with 4% of 1 M HEPES (pH 7.4) and an oxygen scavenging system. While imaging, the neuron samples were placed in a stage incubator (Chamlide TC; LCI) set at 37 °C.

Fluorescence microscope. Most STORM experiments were performed on an Olympus IX-71 inverted optical microscope, except for DiR imaging. Here, we used three continuous wave lasers at wavelengths of 405 nm (CUBE 405-50C; Coherent), 561 nm (Sapphire 561-200; Coherent), and 657 nm (RLC-300-656; Crystalaser) controlled individually by mechanical shutters (Uniblitz LS6TS; Vincent Associates). The laser intensities were automatically controlled by an acousto-optic tunable filter (AOTF PCAOM NI VIS; Crystal Technology) for the 561- and 657-nm lines and by direct digital modulation of the laser power supply for the 405-nm line. All laser lines were combined and coupled into an optical fiber (Oz Optics), and the fiber output was collimated and focused at the back focal plane of a high numerical aperture (N.A.) oil immersion objective (100× UPlanSApo, NA1.4; Olympus). The dye molecules were imaged with excitation light of 561 nm for DiI, MitoTracker Orange/Red, and all 640D1Y probes, or 657 nm for DiD and MitoTracker Deep Red. The excitation beams were reflected by longpass dichroic mirrors: ZT561RDC (Chroma) for 561 nm and T660LPXR (Chroma) for 657 nm. At the sample, the maximum laser intensity was 10 kW/cm² for the 561-nm line and 6 kW/cm² for the 657-nm line. Upon 561-nm or 657-nm illumination at high intensities, majorities of the probes were turned off to a dark state. Then, a low intensity of 405-nm illumination (typically 0–3 W/cm² at the sample) was used to activate the probes back to the fluorescence state. The 405-nm intensity was adjusted to maintain an approximately constant number of activated probes per frame and to compensate for the loss of fluorophores due to photobleaching. Except for Movie S6, the sample was continuously illuminated with the imaging and activation lasers. We typically imaged flat cell peripheries with approximately 1 μm thicknesses using a highly-oblique-incidence illumination geometry to reduce background. The fluorescence was collected by the same objective and imaged onto a back-illuminated EMCCD camera (iXon DU-8860; Andor) running at 503 Hz with a 128 × 128 pixel region of interest (ROI) or at 900 Hz with a 64 × 128 pixel ROI. Fluorescence emission was filtered with the following filters: BLP01-561R (Semrock) for DiI and MitoTracker Orange; FF01-630/92 (Semrock) for MitoTracker Red, ER-Tracker Red and LysoTracker Red; ET705/72m (Chroma) for DiD and MitoTracker Deep Red.

For two-color imaging, both MitoTracker Red and ER-Tracker Red were excited using 561-nm light reflected by a longpass dichroic mirror (ZT561RDC; Chroma). The fluorescence emission was filtered by a longpass filter (BLP01-561R; Semrock), then split by a dichroic longpass (FF624-Di01; Semrock) mounted in a commercial image-splitting device (Dual-View; Photometrics). The two wavelength channels were further filtered by bandpass filters: ET595/50 (Chroma) in the short wavelength channel and FF01-630/92 (Semrock) in the long wavelength channel. Both channels were recorded by the EMCCD camera running at 503 Hz.

To stabilize the focus during data acquisition, an IR beam from an 830-nm fiber-coupled diode laser (LPS-830-FC; Thorlabs) was reflected from the coverglass-water interface and directed to a quadrant photodiode. The position readout of the quadrant photodiode provided a feedback to a piezo objective positioner (Nano-F100; MadCity Labs). DiR was imaged on a nearly identical Olympus IX-71 microscope equipped with a 752-nm krypton laser (Innova 302C; Coherent), as described previously (2). The 752-nm line (approximately 1 kW/cm² at the sample) was reflected by a Q770DCXR dichroic (Chroma), and the fluorescence was filtered with an HQ800/60m emission filter (Chroma), and collected at a 33 Hz frame rate. The focus was locked by using a 975-nm IR laser (PL980P330J; Thorlabs), detecting the reflected beam from the coverglass-water interface with a quadrant photodiode and then compensating for the z-drift using a piezo stage (NZ100C; Prior Scientific).
**STORM image analysis.** Two-dimensional live-cell STORM movies were analyzed using DAOSTORM software as previously described (3). DAOSTORM determines multiple molecular coordinates from overlapping images of individual molecules by iterating the following routine: (i) From the original image, candidate molecules were identified and fit with a Gaussian function of a fixed width; (ii) The residual image was inspected to identify extra molecules; (iii) Multiple overlapping Gaussian functions were then used to fit the original image with the updated list of candidate molecules including the extra molecules from the residual image. Three-dimensional STORM movies were analyzed using a custom-written software in C++ that fits individual molecular images to an elliptical Gaussian function with variable widths. The centroid positions of the Gaussian were used to determine the lateral coordinates of the molecules and the Gaussian widths were used to determine the axial positions of the molecules as described previously (4). Sample drift was negligible in live-cell images because of the relatively short imaging time. For fixed-cell images, both lateral and axial drifts were measured and corrected by image correlation as described previously (4). Finally, STORM images were rendered with each localization plotted as a 2D Gaussian whose width is weighted by the inverse square root of the number of detected photons. Background signals coming from cellular autofluorescence and non-specifically bound probes appear as scattered localizations at low local densities, and were removed by a local density filter as previously described (5).

**Localization precision and Nyquist resolution.** To quantify the spatial resolution achieved with each of the membrane probes, we experimentally determined both the localization precisions and the Nyquist resolution limits based on the localization densities. For measuring localization precisions, BS-C-1 cells were labeled with fixable membrane probes (CM-DiI, MitoTracker Red, ER-Tracker Red and LysoTracker Red; Invitrogen) at a labeling density low enough to distinguish individual probes in the STORM images. The labeled cells were fixed with 3% PFA and 0.1% glutaraldehyde for 10–15 mins at 37 °C to immobilize the probes. The fixed cells were imaged with the same imaging conditions used in live-cell experiments. The fixed probes typically emit 10–20% fewer photons than the corresponding probes in living cells and hence slightly underestimate the localization precision. The distributions of multiple localizations obtained from individual fixed probes were used to determine the localization precisions.

To determine the Nyquist resolution limits based on localization densities, the STORM images were thresholded by Otsu’s method (6) to measure the area of the structure of interest and to determine the number of localizations within the area of interest. Then, the Nyquist resolution was computed as $2\sqrt{\text{area}}$ (7). The widths of candidate molecules including the extra molecules from the residual image were measured from STORM images by fitting Gaussian functions. The widths of candidate molecules were paired and assumed to belong to the same molecule. The peaks in the two channels within a radius of 0.5 pixels (78 nm) were paired and assumed to belong to the same molecule. The photon numbers detected in the two channels ($n_{\text{long}}, n_{\text{short}}$) were determined for individual molecules and used to determine whether the molecule was the ER dye or the mitochondrial dye. For calibration purposes, we first determined the distributions of ($n_{\text{long}}, n_{\text{short}}$) for samples singly stained with either the ER dye or the mitochondrial dye (Fig. S5). Two types of single-color samples were imaged: (i) The ER dye or the mitochondrial dye adsorbed on coverslips; (ii) live cells labeled with either the ER dye or the mitochondrial dye. For probes adsorbed on coverslips, the detected photons from a single probe formed a distribution along a characteristic ratio $n_{\text{long}}/n_{\text{short}}$ with expected spread due to the finite emission bandwidths of the two dyes and the shot noise (Fig. S5A). In live cells, the $n_{\text{long}}/n_{\text{short}}$ ratio of the mitochondrial dyes was largely unchanged, but the ER dyes split into two populations with blue- and red-shifted spectra (Fig. S5B). Because the ER dye binds to potassium channels enriched in ER, the two spectrally distinct populations may indicate two different states of the dye-protein complex.

**Width measurements of various tubule structures.** The widths of spine necks, intermitochondrial tubules, and ER tubules in Figs. 2A, 4B and 5C were measured from STORM images by fitting transverse profiles of localization with Gaussians. To obtain the transverse profiles, regions of interests were chosen with boxes of various widths (all >200 nm) that enclose roughly straight tubes with constant widths. The localizations within the box were counted and binned with 20-nm intervals. The histograms were fit with Gaussians and full-width-at-half-maximum (FWHM) values were reported. The same method was used throughout the paper except for Fig. S3 (see the figure caption for the specific method used in this figure).

**Color assignment for two-color images.** Because the spectral separation of the ER dye (ER-Tracker Red) and the mitochondrial dye (MitoTracker Red) is only 16 nm, we used a ratiometric method to assign the colors of images of these dyes (10–13). Each probe was detected in two channels: a short wavelength channel and a long wavelength channel separated by a 624-nm longpass dichroic mirror. After identifying peaks corresponding to individual molecules in each channel, the molecular coordinates in the long wavelength channel were overlaid on the short wavelength channel by using the transformation map obtained from calibration images of 100-nm fluorescent beads (Tetraspeck; Invitrogen). Peaks in the two channels within a radius of 0.5 pixels (78 nm) were paired and assumed to belong to the same molecule. The photon numbers detected in the two channels ($n_{\text{long}}, n_{\text{short}}$) were determined for individual molecules and used to determine whether the molecule was the ER dye or the mitochondrial dye. For calibration purposes, we first determined the distributions of ($n_{\text{long}}, n_{\text{short}}$) for samples singly stained with either the ER dye or the mitochondrial dye (Fig. S5). Two types of single-color samples were imaged: (i) The ER dye or the mitochondrial dye adsorbed on coverslips; (ii) live cells labeled with either the ER dye or the mitochondrial dye. For probes adsorbed on coverslips, the detected photons from a single probe formed a distribution along a characteristic ratio $n_{\text{long}}/n_{\text{short}}$ with expected spread due to the finite emission bandwidths of the two dyes and the shot noise (Fig. S5A). In live cells, the $n_{\text{long}}/n_{\text{short}}$ ratio of the mitochondrial dyes was largely unchanged, but the ER dyes split into two populations with blue- and red-shifted spectra (Fig. S5B). Because the ER dye binds to potassium channels enriched in ER, the two spectrally distinct populations may indicate two different states of the dye-protein complex.
We assigned individual localizations in the two-color images to the ER and mitochondrial color channels using a statistical approach as previously reported (11). Briefly, we estimated maximum likelihood of probe identities based on the \((n_{\text{long}}, n_{\text{short}})\) values of individual localizations using calibration data sets derived from live cells singly labeled with the ER or mitochondrial probes. From the two calibration data sets, 2D frequency histograms of \((n_{\text{long}}, n_{\text{short}})\) \(p_{\text{ER}}(n_{\text{long}}, n_{\text{short}})\) and \(p_{\text{MT}}(n_{\text{long}}, n_{\text{short}})\) (MT: mitochondria)] were obtained with bins of 50 photons. The probe identity of any given \((n_{\text{long}}, n_{\text{short}})\) was determined with a maximum-likelihood estimator: the estimated color, \(T(n_{\text{long}}, n_{\text{short}})\) was simply chosen as the probe with larger probability; i.e.,

\[
T(n_{\text{long}}, n_{\text{short}}) = \begin{cases} 
\text{ER} & \text{if } p_{\text{ER}} > p_{\text{MT}} \\
\text{MT} & \text{if } p_{\text{ER}} < p_{\text{MT}} \end{cases} \tag{S1}
\]

Then, the confidence level \(C\) was calculated as follows:

\[
C = \begin{cases} 
\frac{p_{\text{ER}}}{p_{\text{ER}} + p_{\text{MT}}} & \text{if } p_{\text{ER}} > p_{\text{MT}} \\
\frac{p_{\text{MT}}}{p_{\text{ER}} + p_{\text{MT}}} & \text{if } p_{\text{ER}} < p_{\text{MT}} \end{cases} \tag{S2}
\]

A threshold confidence level of 0.70 was used to define regions of specific color assignment shown in Fig. S5C. We also excluded regions where the calibration data contained less than four events within the bin. This method discarded 20–40% of total localizations as uncertain points. The color cross-talk from the ER channel to the mitochondria channel was determined as 16%, while the cross-talk from the mitochondria channel to the ER channel was 28%.

Fig. S1. Chemical structures of the eight photoswitchable membrane probes, each specific for the plasma membrane (A), mitochondria (B), the ER (C) or lysosomes (D). For each probe, wavelengths at its excitation and emission maximum are displayed as “Ex” and “Em”, respectively.
Fig. S2. STORM images of live BS-C-1 cells labeled with DiD, DiR, MitoTracker Orange, or MitoTracker Deep Red. (A and B) Conventional (Left) and STORM (Right) images of the plasma membrane stained with DiD (A) and DiR (B). (C and D) Conventional (Left) and STORM (Right) images of mitochondria labeled with MitoTracker Orange (C) and MitoTracker Deep Red (D). The conventional fluorescence images were taken prior to STORM imaging with a low excitation intensity that did not switch off the probes appreciably. Scale bars, 1 μm.
Fig. S3. Comparison of conventional (i), deconvolved conventional (ii), and STORM (iii) images of the plasma membrane of the hippocampal neuron (A) and mitochondria in the BS-C-1 cell (B) in Fig. 2 A and B in the main text. The profiles (iv) along the red dash line show enhanced resolution of the STORM images (red lines) as compared to that of the conventional (blue lines) and deconvolved conventional (green lines) images; in particular, the approximately 90-nm-thick neck of a dendritic protrusion in A and the approximately 70-nm-wide gap between adjacent mitochondria in B were blurred in both of the conventional images, but clearly resolved in the STORM images. STORM images were collected immediately after acquiring the conventional fluorescence images. Due to the time lapse between the conventional and STORM images, the structures could have moved slightly. The deconvolved images were obtained from the conventional image using a plug-in to ImageJ (http://www.optinav.com/Iterative-Deconvolution.htm) and the point-spread-function obtained from single-molecule images of DiI or MitoTracker Red. The intensity profiles from the conventional and deconvolved images present intensity values from individual pixels where the red dash line is placed. The STORM profiles were generated by integrating the intensity of the STORM images within a 70-nm-wide box. These profiles, binned with 40-nm intervals, were then fitted with Gaussians to determine the width in FWHM, as indicated in the plots. Scale bars, 1 μm.

Fig. S4. Length distribution of all trajectories of DiI molecules detected in the live neuron in Fig. 3. Note that the subsequent analysis used only long trajectories with more than 15 steps to obtain diffusion coefficients with reasonable accuracy.
DiI (green) and fixed fluorophores (red) were generated this way and fitted to 2D Gaussians to determine the molecular localizations, as plotted in each panel here. Noise (also set based on experimental values) was then added to the image. Molecular images of 2,000 probes were generated with photon numbers above 5000 were excluded. Photon numbers below 150 were undetected due to an intensity threshold used for peak identification. The measured widths of the tube structures with diffusing probes converges to approximately 30 nm, which is equal to the realistic width of a 100-nm tube probed with fixed fluorophores.

**Fig. 55.** Calibration measurements of MitoTracker Red (cyan) and ER-Tracker Red (red) for color assignment in two-color imaging. (A) Photon number distribution in the two channels (long and short wavelengths) measured for MitoTracker Red (cyan) and ER-Tracker Red (red) adsorbed on coverslips. All events with photon numbers above 5000 were excluded. Photon numbers below 150 were undetected due to an intensity threshold used for peak identification. (B) The measured widths derived from simulated STORM images for tube structures with varying actual widths. The x-axis of the plot represents the measured width of the tubes probed with fixed fluorophores and the y-axis represents the corresponding values derived from diffusing fluorophores. The diffusion coefficient, $D$, of the diffusing probes was set to be equal to those of MitoTracker Red (Middle) and DiI (Bottom). For each camera frame at 503 Hz, a diffusion trace with 500 steps ($\Delta t$ each step) was generated within the boundary of the tube structure based on these diffusion coefficients. Then, a point-spread-function (PSF) with realistic photon numbers based on experimental values was translated along the trajectory to generate a molecular image. Noise (also set based on experimental values) was then added to the image. Molecular images of 2,000 probes were generated this way and fitted to 2D Gaussians to determine the molecular localizations, as plotted in each panel here. (B) The measured widths derived from simulated STORM images for tube structures with varying actual widths. The x-axis of the plot represents the measured width of the tubes probed with fixed fluorophores and the y-axis represents the corresponding values derived from diffusing fluorophores. The diffusion coefficient, $D$, of the diffusing probes was set to be equal to those of MitoTracker Red ($D = 0.3 \mu m^2/s$). The simulated camera frame rates were 909 Hz (blue), 503 Hz (green), or 101 Hz (red). Specifically, for any given frame rate and actual tube width, STORM images were generated for $D = 0$ and $D = 0.3 \mu m^2/s$ as in A. Then, from the transverse profiles of localization, the widths of the tube images (in FWHM) were measured by fitting the localization profiles with Gaussians. (C) Same as in B except that the diffusion coefficient of the diffusing probes was set to be equal to that of Dil ($D = 1.1 \mu m^2/s$). The simulated camera frame rates were 503 Hz (blue), 251 Hz (green), or 126 Hz (red). Note that the measured widths of the tube structures with diffusing probes converges to approximately 30 nm, which is equal to the localization accuracy of the probes. (D and E) Simulated STORM images of grid structures with grid sizes ranging from 10–40 nm for diffusing fluorophores with diffusion coefficient $D = 0.3 \mu m^2/s$ (D) and $D = 1.1 \mu m^2/s$ (E). The images were simulated considering the realistic photon numbers detected from the probes and the realistic noise at camera frame rate of 503 Hz in a way similar to that described in A, except that the confining structure is changed from tubes to grids. Scale bars, 100 nm in (A) and 50 nm in (D and E).
**Fig. S7.** 3D STORM images of mitochondria in a fixed BS-C-1 cell. The cell was labeled with MitoTracker Red while alive and subsequently fixed. (A and B) A 2D projection image of mitochondria (A) and its corresponding z cross-section image with z = −50–0 nm (B). The focal plane is at z = 0. (C) Longitudinal profiles of localizations of the red-boxed region in A. (D) Localization profiles of the red boxed region in B. To generate the localization profiles, the molecular coordinates within the boxed region were projected to an axis perpendicular to most of the cristae in the region, indicated as “x” in A and B. Scale bars, 200 nm.

**Movie S1.** STORM movie of mitochondria labeled with MitoTracker Red in a live BS-C-1 cell. The same field of view as shown in Fig. 4C. Two-sec snapshots are displayed at 5× real time. Scale bar, 500 nm.

**Movie S2.** STORM movie of the ER labeled with ER-Tracker Red in a live BS-C-1 cell. The same field of view as shown in Fig. 5A. The movie runs in real time with 10-sec snapshots.

Movie S1 (MOV)
Movie S3. STORM movie of the ER network labeled with ER-Tracker Red in a live BS-C-1 cell. The same field of view as shown in Fig. 5D. The movie runs in real time with 2-sec snapshots.
Movie S3 (MOV)

Movie S4. Two-color STORM movie of mitochondria (green) and the ER (magenta) in a live BS-C-1 cell. The field of view here is the same as that shown in Fig. 6. The movie runs at 5× real time with 10-sec snapshots. Scale bar, 500 nm.
Movie S4 (MOV)

Movie S5. Five-min-long STORM movie of mitochondria labeled with MitoTracker Red in a live BS-C-1 cell imaged with continuous illumination. The mitochondrial morphology largely remained unperturbed within the first minute. Then, the elongated mitochondria slowly turned into spherical or disk-like shapes, indicating stress on mitochondria. The video runs at 10× real time with 5-s snapshots.
Movie S5 (MOV)
Movie S6. Twelve-min-long STORM movie of mitochondria labeled with MitoTracker Red in a live BS-C-1 cell imaged with strobed illumination. The 405 and 561-nm illumination was turned on for two frames followed by six frames of dark period with no light. The excitation sequence effectively reduces the amount of light exposure to the cell by 4 fold. The mitochondrial morphologies remained largely unchanged throughout the movie. The video runs at 10× real time with 20-sec snapshots.

Table S1. Photoswitching properties of the four representative membrane probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Photon number per frame*</th>
<th>Localization accuracy in SD (FWHM)*</th>
<th>Number of switching cycles</th>
<th>On-off duty cycle†</th>
<th>Contrast ratio‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiI</td>
<td>720</td>
<td>17 (40) nm</td>
<td>27</td>
<td>0.0006</td>
<td>≥1000</td>
</tr>
<tr>
<td>MitoTracker Red</td>
<td>790</td>
<td>13 (30) nm</td>
<td>74</td>
<td>0.0017</td>
<td>≥1000</td>
</tr>
<tr>
<td>ER-Tracker Red</td>
<td>820</td>
<td>15 (35) nm</td>
<td>28</td>
<td>0.0003</td>
<td>≥1000</td>
</tr>
<tr>
<td>LysoTracker Red</td>
<td>820</td>
<td>13 (30) nm</td>
<td>26</td>
<td>0.0003</td>
<td>≥1000</td>
</tr>
</tbody>
</table>

*We report these numbers per frame even though single switching events typically last a couple frames on average. Because these probes can diffuse on the membrane, combining photons from multiple consecutive frames could effectively reduce the localization precision due to motion blurring.

†The on-off duty cycle is defined as the fraction of time that the molecules spend in their on-state before photobleaching.

‡The contrast ratio is defined as the ratio of fluorescence intensities between the fluorescent state and the dark state.