Localized topological changes of the plasma membrane upon exocytosis visualized by polarized TIRFM

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The authors have requested the following corrections:

In the discussion of the polar coordinate system leading to Eq. (1), the azimuthal angle $\phi$ is ambiguously defined as “measured around the z axis.” A more conventional precise definition is “measured around the z axis starting from the x axis.” With this standard definition, the expression for $S$ in Eq. (1) should contain the factor $\sin^2 \phi$ instead of $\cos^2 \phi$. (The factor $\cos^2 \phi$ is correct only if $\phi$ is understood to be measured from the y axis.) All of the geometrical models considered in the paper are azimuthally isotropic, so the starting axis for measuring $\phi$ makes no difference whatsoever in the subsequent analysis.

Also, in the equation for orientational distribution $\eta(\text{plane})$, shown before Eq. (5), the delta function factor $\delta(\theta - \pi/2)$ is best written as $\delta(\cos \theta)$. This makes no difference for the particular case here, but a $\delta$-function distribution at any general polar angle $\theta_o$ must be written as $\delta(\cos \theta - \cos \theta_o)$.

We thank Dr. Nancy L. Thompson for suggesting these changes.
Localized topological changes of the plasma membrane upon exocytosis visualized by polarized TIRFM

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Total internal reflection fluorescence microscopy (TIRFM) images the plasma membrane–cytosol interface and has allowed insights into the behavior of individual secretory granules before and during exocytosis. Much less is known about the dynamics of the other partner in exocytosis, the plasma membrane. In this study, we report the implementation of a TIRFM-based polarization technique to detect rapid submicrometer changes in plasma membrane topology as a result of exocytosis. A theoretical analysis of the technique is presented together with image simulations of predicted topologies of the post-fusion granule membrane–plasma membrane complex. Experiments on dil-stained bovine adrenal chromaffin cells using polarized TIRFM demonstrate rapid and varied submicrometer changes in plasma membrane topology at sites of exocytosis that occur immediately upon fusion. We provide direct evidence for a persistent curvature in the exocytotic region that is altered by inhibition of dynamin guanosine triphosphatase activity and is temporally distinct from endocytosis measured by VMAT2-pHluorin.

Introduction

The plasma membrane–cytosol interface is a highly specialized domain in which unique and critical cellular functions occur. It is the site of biochemical and ionic signaling through receptors and channels that coordinates cellular processes within the extracellular environment. It is also the site of intercellular interactions, the locus of molecular components important for cell motility, and the site of numerous vesicular trafficking reactions including endocytosis, phagocytosis, viral budding, and exocytosis.

Total internal reflection (TIR) fluorescence (TIRF) microscopy (TIRFM) is superbly suited to visualize the plasma membrane–cytosol interface because the exponentially decaying evanescent field of TIR selectively illuminates the portion of the cell within a distance of 50–100 nm from the glass coverslip upon which the cells reside. TIRFM has been used to characterize the dynamics of fluorescently tagged secretory granules around the time of exocytosis and to visualize individual granule fusion events (Burke et al., 1997; Steyer et al., 1997; Oheim et al., 1998; Han et al., 1999; Tsuoi et al., 2000; Ohara-Imaiuzumi et al., 2002; Allersma et al., 2004, 2006; Shakiryanova et al., 2005; Silverman et al., 2005; Degtyar et al., 2007). However, the other partner to exocytosis, the plasma membrane, has been much less studied. In this study, we report the implementation of a TIRFM-based polarization technique to detect rapid submicrometer changes in plasma membrane topology as a result of exocytosis. The technique is based upon excitation of an oriented fluorescent membrane probe (the carbocyanine dye, diI) with a polarized evanescent field. DiI has been shown to embed in the membrane with its transition dipole moments nearly in the plane of the membrane (Axelrod, 1979). The technique in a nonimaging mode was pioneered by Thompson et al. (1984) to measure the orientational distribution of a probe (NPD-PE) incorporated within a model membrane. The technique was applied to time sequence imaging of dynamic orientational
changes in the plasma membrane itself on living cells labeled with diI (Sund et al., 1999). Recently, polarized TIRF (pTIRF) was used to demonstrate the spread of FM4-64 into the plasma membrane of PC12 cells (Taraska and Almers, 2004) and peptide-induced disorder in supported bilayers (Oreopoulos and Yip, 2009).

The technique involves taking successive images of a sample with orthogonal excitation polarizations in TIR mode: a p-polarization (p-pol; in the plane of incidence and perpendicular to the coverslip) and an s-polarization (s-pol; perpendicular to the plane of incidence and parallel to the coverslip) image. Regions, even submicroscopic ones, in which the membrane deviates from parallelism of the substrate are vividly highlighted by taking the ratio p-pol/s-pol (P/S) of the membrane-embedded diI fluorescence images excited by the two polarizations. This approach is generally applicable for visualizing dynamic topological alterations in the plasma membrane during important cellular processes.

In a theoretical section, we show that combination of p-pol and s-pol excited images reports the local fluorophore concentration independently of the orientation. We also examine the ability of pTIRF to highlight submicrometer morphological distortions in the plasma membrane in the presence of complicating factors including the finite evanescent field depth, near-field emission into the substrate, high aperture observation, membrane foreshortening, optical resolution limitation, and pixelation of the observation region.

In the experimental section, we describe that spatial and spatial resolution images of the granule membrane—plasma membrane complex in living bovine chromaffin cells taken as a repeated triplet sequence: p and s excitation of diI in the plasma membrane and also excitation (at a different wavelength) of the fluorescent protein—marked secretory granules. Guided by the theory section predictions, we demonstrate rapid and varied submicrometer changes in plasma membrane topology at sites of exocytosis that occur immediately upon fusion (within the time resolution of the imaging) and provide direct evidence for a persistent curvature in the exocytotic region after fusion that is subject to regulation. This occurs immediately upon fusion in at least 60% of events. The curvature decays with varying speeds from as fast as 450 ms to tens of seconds and is strongly affected by the dynamin GTPase inhibitor dynasore. Endocytosis was also imaged by optical techniques. We find that <10% of the fusion events stimulated with elevated K+ result in rapid endocytosis. Thus, most of these topological changes are kinetically distinct from endocytosis and reflect a varying and regulated time course for the flattening of a fused granule membrane into the plasma membrane.

**Results**

Theory of polarized excitation TIRF

To help interpret our experimental results on the plasma membrane dynamics of secretion from chromaffin cells, we calculate the p-pol and s-pol excited image intensities for a particular geometrical model of secretion: a spherical membrane (the secretory granule) fusing with a planar membrane (the plasma membrane) from within the cell interior, taking into account several possibly countervailing optical effects.

**Orientation versus concentration**

Idealized geometry. In general, TIRF images show a convolved mix of local fluorophore orientations with concentrations and distances from the substrate. What combination of p-pol and s-pol excited images reports purely orientational distributions, and what combination reports purely concentration and distances?

To calculate the answer, we set up the following polar coordinate system (Fig. 1): the sample plane (at which TIR occurs) is the x-y plane, and the optical axis of the microscope is the z axis. The TIR evanescent field propagates in the x-y plane along x, s-pol excitation is polarized along y, and p-pol excitation is polarized along z (actually, a small amount of p-pol intensity is polarized in the x direction, dependent on the incidence angle of the TIR beam, but we will ignore it for simplicity). In this coordinate system, θ and φ are the polar angle (measured from the z axis) and the azimuthal angle (measured around the z axis), respectively.

For a particular fluorophore whose dipole is oriented in the θ and ϕ, the probability of excitation caused by p-pol or s-pol light is proportional to the square of the component of the dipole along the z or x axis, respectively. These square components are proportional to cos²θ and sin²θ cos²φ, respectively. Given that the fluorophore becomes excited, the emission can be resolved into two components, one along the z axis (i.e., perpendicular to the substrate) and the other in the x-y plane (i.e., parallel to the substrate). The square of each of these components, proportional to cos²θ and sin²θ, each contribute to the emitted light that is collected by the objective with efficiencies Qₜ and Qₛ, respectively.

An actual sample, of course, contains numerous fluorophores with their dipoles possibly pointing in different directions. Assume that the normalized orientational distribution of dipoles is η(θ, φ). The emission light intensities P and S gathered by an objective (and imaged at a single pixel) from p-pol and s-pol excitation, respectively, are

\[
P = \int C \eta(\theta, \phi) \cos^2 \theta (Q_\perp \cos^2 \theta + Q_\parallel \sin^2 \theta) \sin \theta d\theta d\phi
\]

\[
S = \int C \eta(\theta, \phi) \sin^2 \theta \cos^2 \phi (Q_\perp \cos^2 \theta + Q_\parallel \sin^2 \theta) \sin \theta d\theta d\phi.
\]

(1)

where C is a number proportional to the local effective total amount of fluorophore at the pixel under view. C implicitly includes decaying effect of the evanescent field in the z direction such that fluorophores at larger distances from the surface simply contribute less toward C. C also implicitly includes the foreshortening effect of tangential viewing, all the multiplicative constants and efficiencies of absorption and emission that convert concentration into units of light intensity. The assumptions in Eq. (1) are that no polarizer is used in the emission system, the dichroic mirror introduces no polarization bias, the absorption and emission dipoles are parallel to each other, and
The plane of incidence (which contains both the incident and reflected beams) is the \(x\)-\(z\) plane. The incident propagating beam (traveling up along \(z\)) is transversely polarized in either the \(x\) or \(y\) directions, which correspond to \(p\)-pol and \(s\)-pol, respectively. The evanescent field is polarized either primarily in the \(z\) direction (for \(p\)-pol) or entirely in the \(y\) direction (for \(s\)-pol).

The orientational distributions are not a function of \(z\) distance to the TIR substrate. Clearly, ratio \(P/S\) reports only upon the orientational distribution \(\eta(\theta, \phi)\) because the \(C\) factors cancel upon taking the ratio. The goal of the following derivation is to find the opposite: a combination of \(P\) and \(S\) that only reports on \(C\) and does not depend on \(\eta(\theta, \phi)\).

All of the possible biases of the objective toward gathering light from one excited dipole orientation or another are contained in the factors \(Q_p\) and \(Q_s\). For a very high aperture (NA \(\geq 1.40\)), these factors are within 20% of each other (see Materials and methods; Fig. 2B), so we will set them both equal to unity in Eq. (1). This leads to collapse of the integrand factor involving \(Q_p\) and \(Q_s\) to unity. Note that this step is valid only for 1.40, 1.45, and 1.49 NA objectives, and even then, it is just an approximation. The normalization requirement is that

\[
\int \eta(\theta, \phi) \sin \theta d\theta d\phi = 1. 
\]

We now assume azimuthally symmetry in fluorophore orientation so that \(\eta(\theta, \phi) = \eta(\theta)\); i.e., there is no particular orientation preference for \(\phi\) in the \(x\)-\(y\) plane. This is expected to be the case in many biological organelles (plasma membrane, secretory granules, etc.) if we gather light from a large enough area. The normalization (Eq. 2) then becomes

\[
\int \eta(\theta) \sin \theta d\theta = \frac{1}{2\pi}. 
\]

Then, Eq. (3) gives

\[
P = \int C\eta(\theta) \cos^2 \theta \sin \theta d\theta = 2\pi \int C\eta(\theta) \cos^2 \theta d\theta = 2\pi \frac{C}{2}. 
\]

\[
S = \int C\eta(\theta) \sin^2 \theta \cos^2 \theta \sin \theta d\theta 
\]

\[
= \frac{\pi}{2} C \left[ \eta(\theta) \sin \theta d\theta - \left( \frac{P}{2} \right) \right] = \frac{C - P}{2}.
\]

Therefore,

\[
P + 2S = C.
\]

This expression is true regardless of the exact form of orientational distribution \(\eta(\theta)\) (as long as it is azimuthally symmetric). The sum \(P + 2S\) is proportional only to the effective concentration. As long as we view with a very high aperture objective and bin together a large enough image area to ensure that the orientational distribution within the area is azimuthally symmetric, \(P + 2S\) is approximately proportional only to the local concentration convolved with the evanescent exponential decay, and invariant with respect to the details of azimuthally symmetric orientation. Because \(Q_p\) and \(Q_s\) undulates as a function of \(z\) (Fig. 2), there is no algebraic combination of \(P\) and \(S\) that is exactly invariant to orientation for all geometrical shapes of the membrane.

We can plug in a particular azimuthally symmetric \(\eta(\theta)\) function of interest here: the idealized cases of the fluorophore diI in a planar plasma membrane parallel to the substrate and on a spherical granule much smaller than the size over which \(P\) and \(S\) are evaluated. We assume for now that diI intercalates into lipid bilayer membranes with its dipole moments parallel to the local membrane surface (Axelrod, 1979). They are (in properly normalized form)

\[
\eta(\text{plane}) = \frac{1}{2\pi} \delta(\theta - \pi/2) 
\]

\[
\eta(\text{sphere}) = \frac{1}{4\pi} 
\]

where \(\delta\) is the standard spike \(\delta\) function. Using Eqs. 3 and 4 (with \(C = 1\) for both plane and sphere, thereby implicitly assuming the same surface area for both) gives

\[
P(\text{plane}) = 0, 
\]

\[
S(\text{plane}) = \frac{1}{2}, 
\]

\[
P(\text{sphere}) = \frac{1}{3}, 
\]

\[
S(\text{sphere}) = \frac{1}{3}. 
\]

The ratio \(P/S\) changes from 0 to 1 in going from a pure plane to a pure sphere. This distinction provides a very sensitive test of local orientation. The sum \(P + 2S\) remains constant at 1 and provides an orientationally independent means to measure relative local concentrations.

This idealized calculation must be altered to include numerous real effects: finite evanescent field depth, exocytotic structures containing both truncated spherical and planar regions, granule size on the edge of optical resolvability, and pixelation. These effects, and a general approach to handling them, are considered in detail in Materials and methods. Based
Prediction was tested with HEK293 cells stained with diI. According to the idealized theory (Eq. 5), the decrease in $P/S$ ratio reaches a greater maximum for larger granules in the course of fusion with the plasma membrane. The range of $P/S$ predictions derived from this realistic image simulation (with parameters specified in Fig. 4 A) agree with the range of experimentally observed $P/S$ values on bovine chromaffin cells (see Test of theory on…). The agreement does not mean that the actual indentations are truncated spheres, but it does suggest that the actual indentations are quite significant. The solid and dashed lines in the $P/S$ graphs of Fig. 4 A correspond to different granule radii, 150 and 250 nm. For a constant-sized, pixelated region of integration, the $P/S$ ratio reaches a greater maximum for larger granules in the course of fusion with the plasma membrane.

Fig. 4 B shows that $P+2S$ is rather insensitive to the depth of indentation until the indentation is beyond a hemisphere. Therefore, any large changes in $P+2S$ indicate either (a) a deep indentation, (b) an actual increase in fluorophore concentration, or (c) a different geometry than assumed by the sphere/planar model. Large granules (radius $R = 250$ nm) do show a decrease in $P+2S$ in the middle of the indentation depth range, as the simulated image develops a larger dark hole at its center.

Test of theory on model systems

According to the idealized theory (Eq. 5), the $P/S$ ratio should equal 0 if the diI transition dipoles are parallel to the membrane and the membrane is parallel to the coverslip substrate. This prediction was tested with HEK293 cells stained with diI. The plasma membrane was imaged with p- and s-polarized 514-nm excitation. The $P/S$ ratio of the plasma membrane apposed to the glass coverslip was not uniform. The minimum $P/S$ ratio was $0.304 \pm 0.017$ ($n = 6$ cells) and was assumed to represent areas parallel to the coverslip. This value could be greater than zero for several reasons. (a) The absorption and/or emission dipoles of diI are not parallel to the bilayer but are at a somewhat oblique angle. A $P/S$ ratio of 0.3 is predicted for if the transition dipoles are oriented an angle $\beta$ of 69° rather than 90° from the normal to the bilayer. This value for $\beta$ was used to generate the curves shown in Fig. 4. (b) The p-pol evanescent field is not purely perpendicular to the glass interface but in reality is elliptically polarized in the x-z plane with a weak x component (Axelrod, 2001) of ~6.8% of the total p-intensity at the incidence angle used in this study. This x component would cause a deviation of $P/S$ from zero but not nearly accounting for a $P/S$ of 0.3 and was not considered in the calculations. (c) The evanescent field obtained with through the lens TIRFM is contaminated by impurely polarized propagated light because of internal reflections in the objective (Mattheyses and Axelrod, 2006). The impurity accounts for ~10% of the total intensity at the substrate surface and increasing amounts further into the aqueous phase but was not considered in the calculations. (d) The diI absorption and emission dipole moments are not totally parallel to each other, as discussed in Axelrod (1979). However, because the 1.49 NA objective is relatively insensitive to the orientation of the emission dipole, this nonparallelism probably has little effect.
Eq. 5 also predicts a \( P/S \) equal to 1 for an isotropic distribution of fluorophore dipoles. This distribution was approximated by diI-labeled beads. The beads gave a \( P/S \) ratio of 0.890 ± 0.042 (\( n = 21 \)), much larger than for the planar membrane on HEK cells and only slightly less than the theoretical value of 1.0. The quantitative discrepancy from theory can be explained. The bead sample does not really qualify as an isotropic distribution of diI; the beads are on the same order of size as the evanescent field depth and are thereby illuminated nonuniformly, with the part closest to the substrate (the part presumably with a small \( P/S \) ratio) contributing the most to the fluorescence intensity.

In summary, emissions obtained with p- and s-polarized excitations with diI in defined geometries are similar to the predictions of the theory. Most importantly, the \( P/S \) ratio is much greater (threefold) for diI on a spherical bead compared with diI in a bilayer parallel to the coverslip.

**pTIRFM detects topological changes of the plasma membrane at sites of exocytosis**

Chromaffin cells were transfected with neuropeptide Y (NPY)–cerulean (Cer) to label secretory granules and subsequently stained with diI immediately before imaging. As discussed in Materials and methods, a sequence of three images (NPY-Cer, s-polarized diI, and p-polarized diI) was acquired. The footprint of a chromaffin cell is predominantly parallel to the glass coverslip as indicated by the emission pattern of size as the evanescent field depth and are thereby illuminated nonuniformly, with the part closest to the substrate (the part presumably with a small \( P/S \) ratio) contributing the most to the fluorescence intensity.

In summary, emissions obtained with p- and s-polarized excitations with diI in defined geometries are similar to the predictions of the theory. Most importantly, the \( P/S \) ratio is much greater (threefold) for diI on a spherical bead compared with diI in a bilayer parallel to the coverslip.

**Fig. 3. Simulated Images.** Based on Eq. 6 and a custom IDL program, the expected intensity patterns \( P \) and \( S' \) for p-pol and s-pol excitation, respectively, are shown for a diI-labeled spherical granule fusing with (and truncated by) a diI-labeled planar plasma membrane. In this particular simulation, most of the sphere is still intact; only the lower one fourth of its radius is truncated off. The schematic line drawing (white) shows a side view of this configuration at the same scale as the simulated images. The effects of finite evanescent depth, optical resolution limit, and nonparallelism of the diI dipole with the membrane are all included in generating these \( P \) and \( S' \) patterns, thereby simulating what appears at the CCD array image plane. The pixelation of the CCD array is superimposed along with an outline showing which pixels are actually used to integrate the total intensities \( P \) and \( S \). The ratio \( P/S \) and the sum \( P'+2S' \) are also shown. The corresponding pixel by pixel ratios and sums on experimental data (as pixelated by the camera) are used to determine lateral positions of diI/membrane morphology features at the time of exocytosis. However, extended temporal tracking of the p-pol and s-pol ratios and sums uses the spatially integrated values \( P \) and \( S \) (without the primes) before forming the \( P/S \) and \( P+2S \) combinations. The predictions of the simulations are sensitive to the assumed parameters, which are set close to the actual or expected experimental values: granule radius = 150 nm; Airy disk half-width (out to first minimum) = 211 nm; evanescent field depth = 110 nm; side length of CCD array pixel (as projected onto the image) = 73 nm; angle \( \beta \) between membrane normal and diI dipole = 69°. The \( P \) and \( S' \) images are shown with the same grayscale; the \( P/S' \) and \( P'+2S' \) each have their own gray scales.

**Fig. 4. Exocytosis visualized by polarized TIRFM.** Anantharam et al.
The dotted vertical line at 6% elevation reflects rapid endocytosis. However, the following experiments indicate that rapid endocytosis is a relatively rare event during elevated K+ stimulation and can only account for a small fraction of topological events after fusion.

Figure 4. Simulated P/S and P+2S as a function of spherical indentation depth $z$. The geometry of the indentation structure is schematically shown at the bottom. The individual P and S intensities are also shown. The exact Q1 versus z and Q(1) versus z collection efficiencies, as calculated from Hellen and Axelrod (1987) for NA = 1.49, were used to generate these curves. Parameters assumed in the simulation are $R = 150$ nm (solid) or $R = 250$ nm (dashed) radius spherical indentation, dil labeling in both the indentation and planar areas, convolution with a point-spread function of half-width 211 nm to simulate the optical resolution limit, an evanescent field depth of 110 nm, an angle $\beta$ between dil dipoles and the normal to the plane of the membrane of 69° (see Test of theory…), and a distance between the TIR substrate and the planar parts of the structure of 50 nm. Images generated with these parameters are then pixelated in simulation with a pixel size of 73 nm. Only those pixels whose centers fall with a radius of 4-pixel widths (292 nm) from the center of the image are counted toward total intensities P and S. The values for P and S that would be expected from a completely flat membrane are seen as the ordinate intercepts at $z/R = 0$. The curves are changed only slightly if the collection efficiencies Q1 and Q(1) are equal at all z positions.

The cartoon (Fig. 5 C) depicts an interpretation of these measurements. Immediately upon fusion, the granule membrane retains its curvature, and dil rapidly diffuses into the fused granule membrane. Indeed, it has been shown in PC12 cells that within 30 ms of fusion, lipid diffusion is not significantly restricted between the granule and plasma membrane (Tarasko and Almers, 2004). The P/S ratio increases because of the curvature; the P+2S emission decreases because the granule membrane is recessed from the surrounding plasma membrane. Subsequently, the fused granule–plasma membrane complex flattens over time. The theoretical analysis indicates that because of the limit of resolution, the ability to detect a decrease in emission because of a fused and recessed granule membrane is dependent on the size of the fusing granule. A granule of 150-nm radius, just under the median granule radius 175 nm (Coupland, 1968), would show no decrease in P+2S (Fig. 4) during fusion. However, a granule of >250-nm radius could result in a measurable decrease in P+2S during fusion. Indeed, granules of this size or larger account for 15% of the epinephrine-containing granules in bovine adrenal chromaffin cells (Coupland, 1968).

There is variety in both the magnitude and duration of the local P/S and P+2S changes detected upon exocytosis (Fig. S3). This is likely to reflect differences in both the initial topology of the fused granule–plasma membrane complex upon exocytosis and its subsequent fate (e.g., flattening of the complex into the plasma membrane or endocytosis). The responses of the membrane to fusion are summarized in a cumulative frequency histogram (Fig. 6 A, control). The dotted vertical line at 6% P/S change indicates the level above which the ratio is significant (see Materials and methods). The P/S ratio increased significantly in 60% of the events ($n = 50$) within 0.45 s of fusion. Fusions without evident changes in P/S may reflect transient topological changes that occurred faster than the temporal resolution of the technique. Indeed, preliminary data with a faster image acquisition rate of 10 Hz suggests that ~80% of fusion events are associated with an increase in P/S. When the P/S does increase with fusion, there is a tendency for it to subsequently decline (Fig. 6 B). For example, after 31.5 s, 70% of the events had declined to baseline (Fig. 6 B).

P+2S measurements (proportional to the total dil covalently linked to the membrane position in the evanescent field) were aligned to the frame immediately before fusion and averaged for fusion events that had significant changes in P/S (Fig. 6 C, control). Superimposed on a gradual decline of P+2S that reflects photobleaching was an immediate but transient dip (Fig. 6 C, arrow) immediately after fusion. This dip probably reflects the occasional occurrence of a dil-labeled granule membrane that remains recessed after fusion (Fig. 5).
The relationship between the fusion-induced topological changes and endocytosis was investigated with vesicular monoamine transporter 2–pHluorin (VMAT2-pHL). VMAT2 is normally present on bovine chromaffin granules and mediates the transport of catecholamine into the granule. Transfected VMAT2 is punctate with 80% colocalization with endogenous dopamine β-hydroxylase, a marker for the granule (Wick et al., 1997). The chromophore in VMAT-pHL is luminal and normally quenched by the low luminal, pH 5.5 (Holz et al., 1983). We found that upon fusion, the VMAT2-pHL gives a bright punctate spot (Fig. 7 A) that often lasts for tens of seconds with some diffusion away from the fusion site. To detect endocytosis, the pH of the extracellular solution was repeatedly changed every 10 s between 7.4 and 5.5 (MES buffer substituted for Hepes; Ferguson et al., 2007). Baflomycin was present (unless otherwise indicated) to inhibit reacidification of granules that underwent endocytosis (Fernández-Alfonso and Ryan, 2004).

The low extracellular pH quenches surface pHluorin but not pHluorin that had undergone endocytosis as shown in Fig. 7 A. Single endocytotic events were readily detected (two events; Fig. 7 A, circles and arrows). Less than 10% of fusion events were associated with rapid endocytosis in control cells (Fig. 7 B). It is unlikely that there were additional events that the method failed to detect as a result of insufficient VMAT2-pHL because granules with the most VMAT2-pHL were not preferentially detected (Fig. 7 C). Because at least 60% of the fusion events showed distinct topological changes upon fusion, most of them do not correspond to rapid endocytosis. In fact, endocytosis would be predicted to result in an increase in P+2S (as well as in P/S; Fig. 4 B, right), an event that has almost never been observed in control cells (Fig. 6 C and Fig. S4). Thus, the topological changes occurring immediately after exocytosis most likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane.

Endocytosis could not be detected in the absence of baflomycin (Fig. 7 B), indicating that acidification normally occurs within seconds. This finding indicates that endocytic vesicles that form at the sites of exocytosis of dense core granules in chromaffin cells are likely to be able to take up catecholamine soon after formation. Acidified vesicles may sequester catecholamine that enters the cell through the plasma membrane catecholamine transporter after release.

**Topological changes upon exocytosis are regulated by dynamin**

Dynamin not only plays an important role in clathrin-dependent and -independent endocytosis but has also been implicated in the regulation of protein release from secretory granules (Tsukoi et al., 2004). Dynamin also tubulates lipid membranes in vitro (Sweitzer and Hinshaw, 1998), raising the possibility that its membrane-shaping function regulates the topology of the granule–plasma membrane complex after fusion.

The role of dynamin in the local topological changes after fusion was investigated by inhibiting the GTPase activities of
both of which are expressed in chromaffin cells. A brief membrane-permeant inhibitor of dynamin 1 and dynamin 2, dynasore (Macia et al., 2006). Dynasore is a Student’s unpaired datasets are significantly different at every point after time 0 (P < 0.05 by t test). Numbers are presented ± SEM.

The increase in P/S strongly suggests that there is more dil-labeled membrane at the site of fusion, which is consistent with superimposed curvature from the fused granule and flat plasma membrane. A likely structure is a fused granule membrane that retains curvature (R > 1.0; Fig. 4; and Fig. 8 C, b). P+2S tended to undergo photobleaching in control but much less so for events in the presence of dynasore. This is also consistent for the structure proposed in Fig. 8 B (b) because a significant amount of the dil is subject to lower intensity excitation because of the decaying evanescent field in the unflattened granule membrane compared with the plasma membrane. Dil molecules in the fused but recessed granule membrane would be exposed on average to one third the excitation intensity compared with dil in the plasma membrane (Schmoranzer et al., 2000).

Electron microscopy indicates that the inhibition of dynamin GTPase activity by dynasore arrests clathrin-mediated endocytosis with clathrin-coated structures that have connections to the plasma membrane with narrow or broad necks (Macia et al., 2006; Newton et al., 2006). The imaging of the topological changes after fusion suggests that dynasore causes the formation of similar structures within 0.5 s of fusion.

Lateral displacement of localized topological change in plasma membrane and the last granule position before fusion

Postfusion increases in P/S occurred close to the last position of the granule before fusion, but they were often not overlapping. A striking example of this lateral displacement is shown in Fig. 9 A and Fig. 5 A. There was a 254-nm displacement from the center of the solid white circle (last position of granule before fusion) to the center of the dashed white circle (position of increased P/S). We determined the lateral displacements for fusion events in which there was a clear increase in P/S using a center of mass calculation as described in Materials and methods (Fig. 9 B). The mean displacement of the P/S change from the last position of the granule was 122 ± 15 nm. Such displacements are consistent with the finding that granules move tens to hundreds of nanometers within 100 ms of fusion (Allersma et al., 2006; Degtyar et al., 2007).

dynasore with dynasore (Macia et al., 2006). Dynasore is a membrane-permeant inhibitor of dynamin 1 and dynamin 2, both of which are expressed in chromaffin cells. A brief preincubation with dynasore caused significant changes in the topological responses that follow exocytosis. An example of a typical response is shown in Fig. 8. P/S immediately increased after fusion then decreased somewhat but remained elevated for tens of seconds. Most remarkably, P+2S increased, a response that is rarely observed in control cells (Fig. S4).

The topological changes in the presence of dynasore and controls are summarized and compared in Fig. 6. P/S changes in the presence of dynasore tended to be larger (Fig. 6 A), with a greater fraction of fusion events in the presence of dynasore associated with an immediate increase in P/S (86% dynasore and 60% control; Fig. 6 A). Moreover, P/S increases decayed more slowly to baseline in the presence of dynasore (Fig. 6 B). For example, by 31.5 s, 70% of the P/S changes in the control but only 35% in the presence of dynasore had returned to baseline. Most dramatically, P+2S tended to increase after fusion and stay elevated for tens of seconds (Fig. 6 C). Significant increases in P+2S occurred in 14 of 25 events (56%) in which P/S was elevated in the presence of dynasore but in only 1 of 29 events (3%) in control (Fig. S4).

Figure 6. The magnitude and duration of P/S and P+2S changes at the sites of fusion differ in control and dynasore-treated cells. (A) A cumulative frequency histogram was generated to compare the frequency and magnitude of P/S changes observed with fusion in control (30 granules) and dynasore (27 granules)-treated cells. A greater fraction of fusion events show an associated increase in P/S with dynasore. The percent increase was calculated by taking the difference between the P/S at a particular frame (P/S), and the mean P/S of 10 prefusion frames (Ave pref) divided by the mean; ((P/Si − Ave pref)/Ave pref). The two histograms are significantly different (P < 0.05 by Mann-Whitney test). (B) The decay of the initial increase in P/S after granule fusion was followed. Only those events that showed an increase of at least 6% in P/S (compared with the prefusion baseline) in the first 0.45 s after fusion and that could be followed for 31.5 s after fusion were considered (control, n = 29; dynasore, n = 25). The fraction of events with a P/S increase that subsequently declines to the baseline within 4.5, 9, 13.5, 18, 22.5, 27, and 31.5 s are plotted for the two conditions. (C) The P+2S of fusion events with a significant increase in P/S were aligned to their prefusion frames and averaged (control, n = 29; dynasore, n = 25). The data were also normalized to the mean of 10 prefusion frames. The arrow indicates a transient dip in P+2S in control cells. In dynasore-treated cells, an increase in P+2S is usually observed. The two datasets are significantly different at every point after time 0 (P < 0.05 by Student’s unpaired t test). Numbers are presented ± SEM.
with the substrate, many of the considered factors work in opposite directions so the amplitude of the increase is not easily anticipated. In the case of $P+2S$, the theoretical results are also complicated: some geometries and optical setups can lead to an increase and others to a decrease. Nonetheless, an appropriate model can provide the basis for a reasonable and reliable interpretation of the experimental results.

Direct visualization of fusion pore expansion

Recent work supports the idea that events associated with the fusion pore can take distinct pathways. This has important implications for the extent to which plasma membrane morphology is reordered upon fusion. The classical pathway or model holds that subsequent to fusion pore formation, granules merge completely with and flatten into the plasma membrane. Some granule membrane constituents can diffuse away. The granule lipid and some of its proteins are internalized, and the granule membrane rebuilt through poorly understood intracellular sorting mechanisms (Winkler, 1977). Soluble protein content is likely added during interaction with the Golgi. This pathway may be preferred after intense stimulation in chromaffin cells (Fulop et al., 2005). In the second pathway of fusion/recycling, a fusion pore is also formed through which some or all of the granule cargo is released. However, the pore is not fully expanded as in the classical model but rather is closed after a variable amount of time. Thus, the granule does not collapse fully into the plasma membrane, and the empty or nearly empty granule cavity is recaptured largely intact (Tarska et al., 2003; Tsuboi and Rutter, 2003; Perrais et al., 2004; Tsuboi et al., 2004; Fulop et al., 2005). This model has been termed granule cavity recapture or “cavicapture” (Henkel and Almers, 1996).

Discussion

In previous studies, we and others have investigated granule motions that immediately precede fusion but without concomitant high spatial and temporal resolution of localized plasma membrane topology. In this study, using a combination of dual labeling and pTIRFM, we have succeeded in visualizing fusion by imaging the release of granule contents and the accompanying changes in the local plasma membrane topology. We have extended the theory of pTIRF for measurement of membrane topology, taking into account the emissions captured by a high NA objective and have predicted the emissions from topological changes anticipated in exocytosis. Experimentally, we have demonstrated with high spatial and temporal resolution imaging of plasma membrane diI with p-TIRFM that the technique is able to capture rapid and varied submicrometer changes in topology at sites of exocytosis in chromaffin cells.

The approach used in the theoretical development of pTIRF and the computer simulations should be generally useful in a wide range of studies of plasma membrane morphological dynamics. We show how, under a set of common assumptions and optical conditions, the ratio $P/S$ reports purely upon local membrane orientation, and the linear combination $P+2S$ reports purely on local concentrations (convolved with distance to the glass coverslip). For a model of the membrane geometry of exocytosis (a spherical shell truncated by and fusing with a plane), we have derived the expected $P/S$ and $P+2S$ measures, taking into account many practical factors: granule radius, evanescent field depth, optical resolution limitation, size of integration area, and detector pixelation. Although it is intuitively expected that $P/S$ should increase whenever the membrane is nonparallel...
within hundreds of milliseconds to seconds after fusion, is regulated by dynamin 1, and is independent of clathrin (Artalejo et al., 2002; Fulop et al., 2008). The electrophysiological approaches that have studied this process were not designed to localize individual exocytotic and endocytotic sites in real time. In this study, individual exocytotic and (rapid) endocytosis events were imaged with VMA T2-pHL. Only \( \leq 10\% \) of the fusion events were associated with endocytosis within 30 s of fusion. Because at least 60% of the fusion events showed distinct topological changes upon fusion, the changes do not correspond to rapid endocytosis. They likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane. The fused granule membrane sometimes flattens into the plasma membrane in less than a second but often retains curvature for tens of seconds.

We also routinely detected less than a one–granule diameter shift between the last observed position of the granule and the topological change. This process occurs within hundreds of milliseconds to seconds after fusion, is regulated by dynamin 1, and is independent of clathrin (Artalejo et al., 2002; Fulop et al., 2008). The electrophysiological approaches that have studied this process were not designed to localize individual exocytotic and endocytotic sites in real time. In this study, individual exocytotic and (rapid) endocytosis events were imaged with VMAT2-pHL. Only \( \sim 10\% \) of the fusion events were associated with endocytosis within 30 s of fusion. Because at least 60% of the fusion events showed distinct topological changes upon fusion, the changes do not correspond to rapid endocytosis. They likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane. The fused granule membrane sometimes flattens into the plasma membrane in less than a second but often retains curvature for tens of seconds.

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Another possibility is that the granule fuses with the plasma membrane and only partially flattens into the plasma membrane, retaining curvature without immediate endocytosis. This type of behavior has been observed after fusion of cortical granules in sea urchin eggs (Terasaki, 1995). The fate of the granule membrane immediately upon exocytosis in mammalian cells has been indirectly surmised but has been difficult to directly image because of the small size of the granules and the rapidity of the events.

In this study, pTIRFM was adapted to monitor the transient changes in the plasma membrane upon exocytosis in chromaffin cells. Upon exocytosis (sudden release of NPY-Cer), there was an immediate and significant localized increase in the \( P/S \) ratio in \( >60\% \) of the events. Recent, higher frequency imaging indicates that \( \sim 80\% \) of the fusion events have significant increases in \( P/S \).

The increase in \( P/S \) likely reflects the union of the granule and plasma membranes with this new entity retaining some of the granule curvature. The topological changes decay with a varying time course from less than a second to many tens of seconds, probably representing different outcomes after the initial fusion event.

One possible outcome for the granule membrane after fusion is recycling by rapid endocytosis. This process occurs...
The experiments support the notion that the granule membrane and plasma membrane become a unique entity upon fusion whose dynamics and function are specifically regulated.

Materials and methods

Theoretical details: NA effects

The emission pattern of a dipole near a surface is rather complex, with both the intensity and polarization as a function of both the observation angle and the fluorophore orientation. A microscope objective, which gathers light over a wide range of observation angles, will thereby see an intensity and polarization that is a strong function of its NA. Aspects of the relevant classical physical optics theory have been worked out by several groups of researchers, notably by Burghardt and Thompson (1984), who examined how much fluorescence is captured by a nearby microscope objective from a fluorophore in a dielectric material sandwiched between two other (possibly different) dielectric materials. Using the same general approach but with some modifications, Hellen and Axelrod (1987) calculated the emission intensity patterns from a fluorophore located in, e.g., water at or near a glass substrate interface possibly coated with an intermediate material. The graphical results and discussion in this study are developed from digital integration of the relevant equations in Hellen and Axelrod (1987).

The emission intensity patterns can be graphically shown on a polar plot (Fig. 2 A). The emission intensity in any particular direction is denoted by the radial distance from the center, at which a fluorophore is located (Fig. 2 A, small circle). Two patterns are shown: one produced by an excited fluorophore with its dipole oriented parallel to the surface (Fig. 2 A, thicker line) averaged over all azimuthal angles; the other with its dipole oriented perpendicular to the surface. Note that the perpendicular dipole emits very little light end-on along the z axis toward the objective (out of view below). Both orientations produce sharp intensity peaks at an angle corresponding to the critical angle. Both orientations also emit light into the glass at angles greater than the critical angle, but the intensity of these supercritical angles drops to zero at an observation angle skimming along the interface. The supercritical intensity is due entirely to the interaction of the dipole’s near field (a nonpropagating component of the dipole field, strongest within a wavelength of the fluorophore) with the surface. For objectives with a small NA, fluorescence from parallel dipole clearly dominates. For larger NA, fluorescence from a perpendicularly dipole becomes increasingly important.

Fig. 2 A assumes that the dipole resides right upon the surface (z = 0). For dipoles a little farther out into the solution, the intensity pattern changes as the dipole’s near field interacts less with the surface. The pattern also changes somewhat because reflection of emitted light off the surface gives rise to interference effects. The collection efficiency (proportional to the amount of light collected by an objective) for each dipole thereby depends on z. The collection efficiency is denoted as Q_∥ and Q_⊥, respectively.

Fig. 2 B shows the ratio of Q_∥/Q_⊥ as a function of the distance from the dipole to the surface (in nanometers) for a selection of different NAs. Q_∥/Q_⊥ increases with increasing NA as expected. For 1.49 NA objectives, Q_∥/Q_⊥ is fairly close to 1 (actually 1.0 ± 0.2, depending on the fluorophore’s distance from the surface). Therefore, 1.49 NA objectives see both parallel and perpendicular excited fluorophore dipoles (roughly) equally well; they don’t care what the orientation is of an excited dipole. What matters is only the total number of excited fluorophores in a region corresponding to a detector pixel regardless of their orientation. Another consequence of Q_∥/Q_⊥ being close to 1 is that rotational diffusion of the fluorophore after excitation has little effect on the total amount of gathered light.

Theoretical details: specific geometry of exocytotic fusion model and optical resolution limitations

The goal is to calculate a theoretical P and S fluorescence from a realistic model of the fusion event as seen through a microscopic and recorded by a charge-coupled device (CCD) array rather than the idealized plane and sphere model. The exocytotic fusion structure we use to describe the stages of secretory granule fusion with the plasma membrane is modeled in this study as spherical surface of radius R truncated by a flat plane, as schematically shown near the bottom of Fig. 3 (the theoretical method to be described is also applicable to other geometries). This fusion structure is both complicated and on the same order of size as the microscope resolution.
and the evanescent field depth, so the idealized model discussed in the text (submicronscopic pure sphere or pure plane) is not adequate for several reasons. Although the distribution of dill [1,1'-dioctadecyl-3,3',3'-tetra-methylinocarbocyanine perchlorate] may be azimuthally symmetric as integrated over the whole structure (as required in the previous section), it may not be so symmetric in resolvable subregions. The TIRF illumination intensity, which decreases exponentially with distance from the substrate, may vary significantly over the indentation produced by the fusion. The local azimuthal asymmetry, tangential foreshortening, and uneven illumination may be partially resolvable, but these features are significantly blurred by structure by optical resolution limitations. The dill dipole may not lie exactly in the plane of the membrane. Lastly, the actual image on a digital camera is noticeably pixelated over the size of a fusion site (several hundred nanometers).

To take these effects into account, we do not use the orientational distribution $\eta(\theta, \phi)$ approach as in the idealized model, but instead we consider the vectorial components ($M_s$, $M_p$, and $M_t$) of the dipole moment, $M$, of dill at each location on the fusion structure. The dill dipoles are assumed to be distributed with azimuthal symmetry at some fixed angle, $\beta$, relative to the normal to the membrane at each location (e.g., for dill dipoles exactly parallel to the local membrane plane, $\beta$ would be 90°). Any location on the spherical part of the structure can be identified by its polar coordinates ($\theta$, $\phi$). The azimuthal angle of any particular dipole around the local membrane normal is denoted by $\psi$. Use of rotation matrices allows calculation of the vectorial components for both the spherical and planar parts of the fusion structure. For the spherical part,

$$M_s(\text{sphere}) = \cos \theta \cos \psi \sin \beta \cos \phi - \sin \theta \sin \psi \sin \beta,$$

$$M_p(\text{sphere}) = \cos \theta \sin \psi \sin \beta + \sin \theta \cos \psi \cos \beta,$$

$$M_t(\text{sphere}) = \sin \theta \sin \beta \cos \phi + \cos \theta \sin \beta.$$

For the planar part,

$$M_s(\text{plane}) = \sin \beta \cos \psi,$$

$$M_p(\text{plane}) = \sin \beta \sin \psi,$$

$$M_t(\text{plane}) = \cos \beta.$$

Because the fluorescence is to be viewed with a very high aperture objective through which the emissions from all excited fluorophores are collected with equal efficiency regardless of orientation, the only important issue is how many dipoles are excited by a particular excitation polarization at each point in the fusion structure. That number is proportional to the square of the component of the absorption dipole in the polarization direction of the excitation, averaged over the azimuthal angles $\theta$ around the local membrane normal. The number of s-pol and p-pol excited fluorophores at any location is thereby proportional to $\langle M_s^2 \rangle$ and $\langle M_p^2 \rangle$, respectively, where the brackets denote averaging over $\theta$. The squaring step for the spherical part leads to numerous cross terms, which all become zero upon averaging over $\theta$, leaving only the square terms. Tangential viewing along the edge of the sphere must also be taken into account, whereby more membrane is in the view of a particular image pixel by a multiplicative factor of $\cos \theta$. Under these conditions, the raw emission intensities $S'$ and $P'$ (meaning before evanescent field depth and optical resolution are taken into account) for s-pol and p-pol, respectively, are (with all constants suppressed)

$$S'(\text{sphere}) = \frac{1}{2 \cos \theta} \left( \cos \theta \sin \phi \sin \beta + 2 \sin \theta \sin \phi \cos \beta + \cos \theta \sin \beta \right).$$

$$P'(\text{sphere}) = \frac{1}{2 \cos \theta} \left( \sin \theta \sin \phi \sin \beta + 2 \cos \theta \cos \phi \cos \beta \right).$$

$$S'(\text{plane}) = \frac{1}{2} \sin \beta.$$

$$P'(\text{plane}) = \frac{1}{2} \cos \beta.$$

Based on these formulas, we created computer simulations of p-pol and s-pol excited images using a program custom written in interactive data language (IDL). The depth of the spherical indentation is measured by the distance $z_2$ from the planar membrane to the top of the sphere, varying from 0 (no indentation) to 2R [a complete sphere with the fusion pore almost closed]. For $z_2 > R$, the local contribution to the fluorescence intensity comes from three separate locations: the planar membrane tucked underneath the truncated sphere (assumed to be flat against the substrate), the lower hemisphere of the sphere, and the upper hemisphere. In general, the fluorescence contributions (given for each point on the fusion structure by Eq. 6) are weighted by the exponentially decaying evanescent intensity. The two simulated fluorescence images (one for p-pol and one for s-pol) generated over a square area with a side of 2R are convolved with an optical point-spread function [an Airy disk] with a width expected from the objective's NA of 1.49. The images are binned into theoretical pixels of a size (relative to the spherical radius) corresponding to the actual experimental pixel size as would be seen by a CCD camera in our actual optical system. Fig. 3 shows examples of these simulated point-spread function–convolved images along with a grid indicating the pixelation for $z_2 = 1.75R$. The total integrated intensity of all the pixels within a defined radius in each simulated image (the same radius as actually used on experimental data) is denoted as $P$ and $S$, respectively, and the combinations $P/S$ and $P+2S$ are plotted in Fig. 4 as a function of indentation depth $z_2$. For this simulation, the orientation of the dill dipole was estimated to be 69° from the normal to the bilayer to match experimental measurements in HEK cells in regions where the plasma membrane is presumed to be flat (see Results).

**Chromaffin cell preparation and transfection**

Chromaffin cell preparation from bovine adrenal medulla and transient transfection were performed as described previously (Wick et al., 1993; Holz et al., 1994). Cells were plated onto 25-mm coverslips [refractive index 1.51] that had been coated with poly-D-lysine and calf skin collagen to promote cell adhesion. Cells were transfected with plasmid encoding NPY-Cer by Ca++ phosphate precipitation (Wilson et al., 1995). The super-elliptic Pfluorin was inserted into the luminal-facing domain (between transmembrane domains 1 and 2) of the rat VMAT2 sequence (Liu et al., 1992). The parent NPY plasmid was provided by W. Almers (Vollum Institute, Oregon Health and Science University, Portland, OR). NPY-Cer is a soluble luminal marker of chromaffin granules that is released upon exocytosis. Experiments were performed 3–7 d after transfection.

**Perfusion**

Experiments were performed in a physiological salt solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 15 mM Hepes, pH 7.4, at −28°C. Solution was delivered to individual cells through a pipet (100-μm inner diameter) using positive pressure from a computer-controlled perfusion system (DAD-6VM, ALA Scientific Instruments). Generally, cells were perfused with PSS for 10 s and stimulated to secrete with elevated K$^+$-containing solution (95 mM NaCl, 56 mM KCl, 5 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 15 mM Hepes, pH 7.4). Where used, dynasore was added directly to PSS to a final concentration of 80 mM (Macia et al., 2006; Newton et al., 2006). Bafilomycin A1 (GE Healthcare) was added to PSS to a final concentration of 1 mM. Dil-C18 (Avanti Polar Lipids, Ala-Chir) was dissolved in ethanol to make a 1 mM stock solution. Dil was added directly to cells bathed in PSS at a 1:50 dilution. The cells were quickly washed several times in PSS and used immediately.

**dil-labeled beads**

To label beads with dil, 200-nm diameter nanospheres (Duke Scientific) were incubated in ethanolic dil and washed in PSS. TIRFM

Prismless [through the objective] TIRFM was obtained by directing an Argon ion (514 nm) or solid-state (442 nm) laser (CVI Melles Griot) through a custom side port to a side-facing dichroic mirror z442/514rpc and z442/514em filter (Chroma Technology Corp.) on an inverted microscope (IX70; Olympus) with the 1.5x internal magnifying lens in the emission path. The beam was focused on the periphery of the back focal plane of 60×1.49 NA oil immersion objective (Olympus), and its polar coordinates were recorded from the normal to confirm that the laser beam was incident on the coverslip at ~70° from the normal, giving a decay constant for the evanescent field of ~110 nm. The band widths of the emissions were 450–490 nm (Cer) and 530–600 nm (Dil). Digital images were captured on a cooled EM CCD camera (Andor iXon; Andor Technology).

**Excitation polarization optics**

Fig. 5 shows the optical setup used to create the p-pol and s-pol 514-nm beams, superimpose their paths, and further superimpose the 442-nm beam on that path. Each of the three beam types is mechanically shuttered independently. The system is programmed to step through a sequence of three openings [one at a time], repeating the cycle without additional delay. The camera takes an exposure synchronous with each shutter opening. Images were acquired at ~6.4 Hz with 50x exposures and 100 gain (EM setting). At 6.4 Hz, the full cycle of three exposures had a period of 454 ms.


Coupland, R.E. 1968. Determining sizes and distribution of sizes of spherical bodies such as chromaffin granules in tissue sections. Nature. 217:384–388. doi:10.1038/217384a0


Figure S1. **Optical system.** The 514-nm beam, vertically polarized as it emerges from a 100-mW air-cooled argon laser (CVI Melles Griot), is split into two orthogonal linear polarizations by passing through a quarter wave plate (QWP), which makes the polarization circular, and then into a polarizing cube (PC), which separates the circular polarization into linear vertical (v) and horizontal (h) components. These two paths are shuttered separately and recombined into one path by a second polarizing cube. The vertical and the horizontal paths eventually become the s-pol and p-pol excitation beams, respectively, at the TIR surface. The 442-nm beam, also separately shuttered, joins the same path via a dichroic mirror (DM1), which reflects 442 nm and transmits 514 nm. The lenses L1 and L2 are weak (long focal lengths), chosen and positioned to ensure that both colors (514 and 442 nm) will ultimately both focus at the microscope’s back focal plane (BFP) as required by objective-based TIR, despite the chromatic dispersion of subsequent lenses. Lens L3 converges and diverges the beam to illuminate a wide area on a dual galvanometer mirror system (GM). Lens L4 refocuses the beam after reflection at the microscope’s dichroic mirror (DM2) to an off-axis position at the back focal plane, and the objective recollimates it at a high incidence angle to create TIR at the sample plane (SP). The GM is positioned at a field diaphragm plane created by L4 (i.e., a plane complimentary to the sample plane, so rotations of the GM can move the off-axis position of illumination at the back focal plane and thus the angle of incidence at the sample) while still maintaining the TIR illumination at the center of the field of view. DM2 is housed in the standard filter cube, but it has dual-band specifications: efficient reflection at 442 and 514 nm and transmission in the 460–490- and 530–600-nm range.
Figure S2. **pTIRFM imaging of dil in the plasma membrane of chromaffin cells.** (A) Chromaffin cells expressing NPY-Cer and stained with dil were exposed sequentially to 442 s, 514 s, and 514 p-polarized excitation. The corresponding emission images are displayed left to right. Because the footprint of the chromaffin cell is predominantly parallel to the glass coverslip, we expected the fluorescence from the s-pol to be brighter than that from the p-pol over most of the cell surface. (middle and right) Indeed, this is evident despite disproportionate brightening of the dil-p image to better visualize details. Importantly, the p-pol image revealed details not evident in the s-pol image. (right) This is particularly true for cell edges (white arrows). We interpret these brighter regions to reflect a plasma membrane orientation oblique or perpendicular to the coverslip interface. A white circle is drawn around an NPY-Cer granule soon to undergo exocytosis (Fig. 5). Bar, 1.5 µm. (B) Pixel to pixel P/S and P+2S images were calculated and aligned to the preceding NPY-Cer image. The P/S image is sensitive to the orientational distribution of dil. It is insensitive to local fluorophore concentration and illumination intensity. The P/S highlights areas of the membrane with higher p with respect to s. The edge of the cell is evident as well as line through the middle of the cell (white arrows). The P+2S image is sensitive to local dil concentration convolved with the TIRF illumination intensity and is not sensitive to the dil orientation. The P+2S shows a more uniform fluorescence. Together, the P/S and P+2S images give us complementary pieces of information that can be used to discern topological attributes of the cell membrane.
Figure S3. **Plasma membrane topological changes are variable.** The topological responses of the membrane to fusion of a granule and release of NPY-Cer were varied. (A) In some cases, there was a short-lived increase in P/S at the site of fusion that rapidly fell close to baseline. In other cases, the change in P/S was more long lived. (B and C) The long-lived change either declined slowly over time (B) or leveled off above the baseline and did not decline (C). (A and C) An increase in P/S is sometimes accompanied by a brief change (usually decline) in P+2S.
Figure S4. Membrane P+2S in control and dynasore-treated cells 1 s after fusion. With dynasore, an increase in P+2S is observed within 1 s after fusion in 56% of events (14 out of 25 events). In control cells, this increase is rarely observed (1 out of 29 events). The percent increase was calculated by taking the difference between the P+2S at a particular frame (P+2Sf) and the mean P+2S of 10 prefusion frames (Ave.pre) divided by the mean [(P+2Sf - Ave.pre)/Ave.pre]. The sets of data are significantly different (P < 0.005 by Mann-Whitney test). The P+2S for only those events with a significant increase in P/S with fusion are included in the analysis. Changes in P+2S had a standard deviation of 3% (calculated as described in Materials and methods for P/S). The dotted lines at −6 and 6% are twice the standard deviation. Changes above or below these lines have at least a 95% probability of being significantly different from before stimulation. These data suggest that there is more diI-labeled membrane at the site of fusion with dynasore, which is consistent with superimposed curvature from a fused granule joined to a flat plasma membrane (Fig. S4 C, b).