New and Notable

A Deeper Look into Single-Secretory Vesicle Dynamics

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Synaptic nerve terminals and neuroendocrine cells rely on continuous vesicle cycling to support sustained exocytosis and transmitter release. Whereas incomplete fusion and local refilling are widely accepted as a recycling mechanism for synaptic vesicles, the way by which large, dense core, peptide and hormone-containing vesicles release their cargo from neuroendocrine cells has been the subject of intense debate during the past few years. But even within a given cell, not all vesicles are equal. Recent work focusing on the mobility (Ng et al., 2003), spatial organization (Rizzoli and Betz, 2004), and temporal sequence (Duncan et al., 2003) of vesicle recruitment during ongoing exocytosis has uncovered a surprising heterogeneity in the mobilization of even close-by vesicles. It remains a major challenge to integrate the different steps leading from vesicle formation, filling, and transport to vesicle docking at the plasma membrane into a coherent sequence of events that explains where and when secretory vesicles arrive at their to-be fusion sites. Such studies would largely benefit from the ability to follow, in a live cell, single secretory vesicles throughout their entire life cycle.

Imaging individual secretory vesicles is not an easy task. With the exception of mast cell granules, most secretory vesicles are submicrometer (μm) objects and their image is spread out by the microscope optics in 3-D to the size of the point-spread function. Images of close-by vesicles will overlap and blur into a fuzzy haze. As vesicles rarely come alone, but flock together in large numbers in the perinuclear region and in proximity of the plasma membrane, single-vesicle tracking is more like spotting the one in the crowd. This can be achieved either by a restriction of fluorescence labeling to a few vesicles (e.g., using FM-dyes or an inducible construct of GFP-tagged neuropeptides, Ng et al., 2003; Levitan, 2004), or by the restriction of the excitation or observation volume (or a combination of these). Tracking of the spatial and temporal dynamics of single-vesicle fusion and re-uptake has become possible with a ''shortsighted'' microscope (Lang et al., 1997; Steyer et al., 1997; Oheim et al., 1998). Total internal reflection of a laser results in an evanescent field skimming the glass-water interface. Total internal fluorescence microscopy (TIRFM) permits the selective illumination of a ~200 nm thin optical section at the bottom of the cell adhering to a planar glass substrate. Through its confinement to a monolayer of near-membrane vesicles without interference from cytoplasmic regions, this technique permits near-membrane vesicle motion to be tracked over time. Although evanescent-field excited single-vesicle fluorescence can be related to some extent to axial vesicle motion (see however, Rohrbach, 2000; Loerke et al., 2002; Schapper et al., 2003) most recent studies restricted their analysis of vesicle motion to lateral (x-y) movement (Johns et al., 2001; Desnos et al., 2003; Duncan et al., 2003; Ng et al., 2003; Tarasaka et al., 2003; Yang et al., 2003; Fix et al., 2004; Ñeco et al., 2004; Ohara-Imaizumi et al., 2004; Tarasaka and Almers, 2004; Tran et al., 2004). A similar 2-D low-background high-contrast view of the near-membrane space is obtained when the entire upper-half of the cell is ripped off by a brief ultrasound pulse to leave an ''unroofed'' cell in which secretory vesicles are still capable of release (Avery et al., 2000). Both techniques are inherently limited to the lower plasma membrane of cultured cells and thus give little information on what is going on deeper inside the cell.

In this issue of the *Biophysical Journal*, Li and co-workers describe an alternative procedure that permits a deeper look into the cell while maintaining single-vesicle resolution. They sequentially acquire z-image planes (NA 1.65, designed for TIRFM) of neuropeptide-Y-DsRed or Acridine-orange labeled PC12-cell granules, remove low spatial-frequency out-of-focus blur by high-pass filtering, and use iterative deconvolution to clear up images. A stack of sixteen z-image planes (at 5-ms exposure time) spaced 150 nm apart is used to calculate a set of 3-D granule-coordinates $\mathbf{x}_i = (x_i, y_i, z_i)$ that are obtained every 5 s by fitting a 3-D centroid to the deconvolved single-vesicle image stack. They analyze those granules located closer than 1 μm from the plasma membrane and a distant population and calculate the 3-D diffusion coefficient ($D^{(3)}$) from a plot of the mean-squared displacement versus time $n \cdot \Delta t$, where Δt is the time between two points x_i. Imaging single secretory granules in an equatorial section, far from the bottom of the cell usually probed with TIRFM, they find a reduced 3-D mobility for near-membrane granules with a median short-range diffusion coefficient $D^{(3)}$ in the order of 3×10^{-4} $\mu m^2\ s^{-1}$, similar to previous estimates of $D^{(2)}$ from TIRF measurements (2×10^{-4} $\mu m^2\ s^{-1}$). This is good news, because TIRFM has often been criticized for looking at the ''wrong'' site of the cell that is facing the glass coverslip. Also, these near-membrane granules displayed a caged diffusion with cage radii confirming previous TIRF estimates.

Barriers to the diffusion of proteins and lipids play an important role in generating functionally specialized domains of the plasma membrane. Such

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barriers have been reported at the light and electron microscopic level, leading in the 1980s to the formulation of the cortical actin barrier hypothesis, in which filamentous (F-) actin acts as a fusion clamp that is released during stimulation. Furthermore, actin plays an important role in endocytosis (for review, see Brodin, 2000). Although actin has been shown to be an important constituent of the near-membrane space, its impact on the regulation of vesicle mobility and the time course of secretion has remained controversial (see Eitzen, 2003; Sakaba and Neher, 2003 for details). Whereas micromolar [Ca^{2+}] clearly induce a change in the F-actin distribution, F-actin disassembly is not required for secretion and recovery from pool depletion (Sullivan et al., 2000; Sakaba and Neher, 2003). In neuroendocrine cells, despite the presence of abundant F-actin locally condensed into stress fibers in the cellular footprint region, TIRF imaging of vesicle single dynamics did not confirm an exclusion of mobile granules from the subplasmalemmal volume (Lang et al., 2000; Oheim and Stühmer, 2000). However, granule mobility was almost blocked when F-actin was stabilized, and was diminished upon F-actin disassembly, suggesting the requirement of both intact actin rails and dynamic changes in cortical actin to propel secretory granules to their fusion sites. In particular, the fastest-moving granules require actin, probably by providing tracks for the movement of myosins (Desnos et al., 2003). Furthermore, studying granule motion in z-direction (normal to the substrate and plasma membrane), Axelrod and co-workers (Johns et al., 2001) found in bovine chromaffin cells that the granule mobility decreased by two orders of magnitude over a distance of less than a granule diameter (Johns et al., 2001). They concluded that ''a system of tethers or a heterogeneous matrix severely limits granule motion in the immediate vicinity of the plasma membrane.''

In their study, Li et al. corroborate and detail some of these observations, but they equally compare the 3-D mobility near-membrane and deeper cytoplasmic granules. They find an increased short-range diffusion coefficient, radius of caged diffusion and traveled distance for cytoplasmic granules when compared to near-membrane granules. Latrunculin-B treatment decreased the mobility of near-membrane granules, but left that of cytoplasmic granules unaltered. Interestingly, the stimulation-dependent augmentation in the directed movement of near-membrane granules observed under control conditions is absent after disruption of the actin cortex. Li's observations complement earlier data from PC12 cells, in which Ba^{2+} increased granule mobility without detectably modifying F-actin (Ng et al., 2002). In contrast, recent work in the Calyx nerve terminal (Sakaba and Neher, 2003) indicates that recruitment of synaptic vesicles after pool depletion requires actin polymerization but a dynamic actin reorganization is not necessary. One possibility is that the transport of large-dense core granules to their fusion sites demands a more elaborate transport system involving actin reorganization, whereas small clear synaptic-like vesicles rather rely on an intact actin rails. Thus, although probably not affecting the initial rate of release in both neuronal and neuroendocrine secretion, the physical mobilization of vesicles secretory granules occurs to facilitate sustained release. Given the timing relative to their physiological trigger, vesicle-actin interactions are more likely to be rate-limiting during asynchronous release of neuropeptide- and hormone-containing granules than during fast synaptic signaling. Given the heterogeneity of vesicle-cytoskeleton interactions and the striking differences between differentiated and nondifferentiated cells (Ng et al., 2002), future work must directly visualize actin-vesicle interactions. It will now be important to study the pathways underlying stimulation-dependent actin-vesicle interactions in cells expressing GFP-tagged actin (Ackermann and Matus, 2003) and to investigate to what degree actin filaments, voltage-gated Ca^{2+} channels and rab and SNARE proteins cluster together to define sites of vesicle docking and fusion (DePina and Langford, 1999; Desnos et al., 2003; Ñeco et al., 2004). Li's work should provide the methodological basis to carry such studies beyond the immediate proximity of the membrane.

Finally, it is noteworthy that the merit in using the NA 1.65 objective primarily lies in its compatibility with TIRF imaging. In as much as—for wide-field imaging far from the coverslip—both the resolution and the solid angle of detection are limited by the critical angle for total internal reflection, $\theta_c = \arcsin(n_1/n_2)$, rather than the objective's NA, the limiting parameter is the sample's refractive index, $n_1 \approx 1.35$. Over a conventional NA 1.4 oil immersion lens, the NA-1.65 objective only has a slightly larger collection efficiency, which stems from the use of a higher refractive-index coverslip, $n_2 = 1.78$, instead of 1.52. Thus, the far-field collected fraction, $\Omega_{NA} = (4\pi r^2)^{-1} \int_0^{2\pi} d\phi \int_0^{\theta_{NA}} r^2 d\theta dr = \frac{1}{2}(1 - \cos\theta_{NA})$, of light isotropically emitted from a dipole located at a distance $z \gg \lambda$ increases from 0.305 to 0.312. Here, θ_{NA}, z, λ, and r are $\arcsin(NA/n_2)$, the fluorophore distance, the emission wavelength, and an arbitrary radius, respectively. One additional concern is that the use of the NA 1.65 objective comes at the price of a higher spherical aberration. Focusing micrometric distances into the cytoplasm will engender spreading of light along the optical axis and thus degrade the signal/noise ratio (Inoué and Spring, 1997). In contrast, although providing similar resolution than a spinning-disk confocal microscope (Hiraoka et al., 1990, Kreitzer et al., 2003), wide-field detection with image deconvolution offers the advantage of detecting a higher number of fluorescence photons per excitation photon. This is primarily because out-of-focus fluorescence is not rejected but is assigned back to the plane of origin and constitutes useful signal, resulting in an increase in signal/noise

ratio (Swedlow et al., 1993). An attractive perspective is to combine deconvolution-assisted epifluorescence and evanescent field imaging on the same setup by chopping between filling the objective's back pupil and spot (or ring) illumination of the periphery, which should enable a direct comparison between 3-D and 2-D molecule dynamics in cytoplasmic and near-membrane compartments. A last note of caution comes from biological optics (Pawley, 2002): quantitative 3-D microscopy relies on the assumption of the cell as a homogenous refractive index. Clearly this is a simplifying, and probably oversimplifying, approximation as the nucleus, granules, and other organelles are prominent on phase contrast and dark-field images, the contrast of which depend on refractive-index changes. One should be aware of that the presence of these subcellular organelles will engender wave-front and optical-section distortions, thus limiting the attainable precision of single-particle tracking studies.

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REFERENCES

Ackermann, M., and A. Matus. 2003. Activity-induced targeting of profilin and stabilization of dendritic spine morphology. *Nat. Neurosci.* 6:1194–1200.

Avery, J., D. J. Ellis, T. Lang, P. Holroyd, D. Riedel, R. M. Henderson, J. M. Edwardson, and R. Jahn. 2000. A cell-free system for regulated exocytosis in PC12 cells. *J. Cell Biol.* 148:317–324.

Brodin, L., P. Low, and O. Shupliakov. 2000. Sequential steps in clathrin-mediated synaptic vesicle endo cytosis. 2000. *Curr. Opin. Neurobiol.* 10:312–320.

DePina, A. S., and G. M. Langford. 1999. Vesicle transport: the role of actin filaments and myosin motors. *Microsc. Res. Tech.* 47:93–106.

Desnos, C., J. S. Schonn, S. Huet, V. S. Tran, A. El-Amraoui, G. Raposo, I. Fanget, C. Chapuis, G. Menasche, G. de Saint Basile, C. Petit, S. Cribier, J. P. Henry, and F. Darchen. 2003. Rab27A and its effector MyRIP link secretory granules to F-actin and control their motion towards release sites. *J. Cell Biol.* 163:559–570.

Duncan, R. R., J. Greaves, U. K. Wiegand, I. Matskevich, G. Bodammer, D. K. Apps, M. J. Shipsont, and R. H. Chow. 2003. Functional and spatial segregation of secretory vesicle pools according to vesicle age. *Nature.* 422:176–180.

Eitzen, G. 2003. Actin remodeling to facilitate membrane fusion. *Biochim. Biophys. Acta.* 1641:175–181.

Fix, M., T. J. Melia, J. K. Jaiswal, J. Z. Rappoport, D. You, T. H. Sollner, J. E. Rothman, and S. M. Simon. 2004. Imaging single membrane fusion events mediated by SNARE proteins. *Proc. Natl. Acad. Sci. USA.* 101:7311–7316.

Hiraoka, Y., J. W. Sedat, and D. A. Agard. 1990. Determination of three-dimensional imaging properties of a light microscope system. Partial confocal behavior in epifluorescence microscopy. *Biophys. J.* 57:325–333.

Inoué, S., and K. R. Spring. 1997. Video Microscopy, 2nd ed. Plenum, New York.

Johns, L. M., E. S. Levitan, E. A. Shelden, R. W. Holz, and D. Axelrod. 2001. Restriction of secretory granule motion near the plasma membrane of chromaffin cells. *J. Cell Biol.* 153:177–190.

Kreitzer, G., J. Schmoranzer, S. H. Low, X. Li, Y. Gan, T. Weimbs, S. M. Simon, and E. Rodriguez-Boulan. 2003. Three-dimensional analysis of post-Golgi carrier exocytosis in epithelial cells. *Nat. Cell Biol.* 5:126–136.

Lang, T., I. Wacker, J. Steyer, C. Kaether, I. Wunderlich, T. Soldati, H. H. Gerdes, and W. Almers. 1997. Ca^{2+}-triggered peptide secretion in single cells imaged with green fluorescent protein and evanescent-wave microscopy. *Neuron.* 18:857–866.

Lang, T., I. Wacker, I. Wunderlich, A. Rohrbach, G. Giese, T. Soldati, and W. Almers. 2000. Role of actin cortex in the subplasmalemmal transport of secretory granules in PC-12 cells. *Biophys. J.* 78:2863–2877.

Levitan, E. S. 2004. Using GFP to image peptide hormone and neuropeptide release in vitro and in vivo. *Methods.* 33:281–286.

Loerke, D., W. Stühmer, and M. Oheim. 2002. Quantifying axial secretory-granule motion with variable-angle evanescent-field excitation. *J. Neurosci. Meth.* 119:65–73.

Ñeco, P., D. Giner, S. Viniegra, R. Borges, A. Villaroel, and L. M. Gutierrez. 2004. New roles of myosin II during the vesicle transport and fusion in chromaffin cells. *J. Biol. Chem.* 279:27450–27457.

Ng, Y.-K., X. Lu, A. Gulacsi, W. Han, M. J. Saxton, and E. S. Levitan. 2003. Unexpected mobility variation among individual secretory vesicles produces an apparent refractory neuropeptide pool. *Biophys. J.* 84:4127–4134.

Ng, Y. K., X. Lu, and E. S. Levitan. 2002. Physical mobilization of secretory vesicles facilitates neuropeptide release by nerve growth factor-differentiated PC12 cells. *J. Physiol.* 542:395–402.

Ohara-Imaizumi, M., C. Nishiwaki, T. Kikuta, S. Nagai, Y. Nakamichi, and S. Nagamatsu. 2004. TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic beta-cells: different behaviour of granule motion between normal and GK diabetic rat beta-cells. *Biochem. J.* 381(Pt. 1):13–18.

Oheim, M., D. Loerke, W. Stühmer, and R. H. Chow. 1998. The last few milliseconds in the life of a secretory granule. Docking, dynamics and fusion visualized by total internal reflection fluorescence microscopy (TIRFM). *Eur. Biophys. J.* 27:83–98.

Oheim, M., and W. Stühmer. 2000. Tracking individual granules through the actin cortex. *Eur. Biophys. J.* 29:67–89.

Pawley, J. B. 2002. Limitations on optical sectioning in live-cell confocal microscopy. *Scanning.* 24:241–246.

Rizzoli, S. O., and W. J. Betz. 2004. The structural organization of the readily releasable pool of synaptic vesicles. *Science.* 303:2037–2039.

Rohrbach, A. 2000. Observing secretory granules with a multiangle evanescent-wave microscope. *Biophys. J.* 78:2641–2654.

Sakaba, T., and E. Neher. 2003. Involvement of actin polymerization in vesicle recruitment at the Calyx of Held synapse. *J. Neurosci.* 23:837–846.

Schapper, F., J. T. Gonçalves, and M. Oheim. 2003. Fluorescence imaging with two-photon evanescent-wave excitation. *Eur. Biophys. J.* 32:635–645.

Steyer, J. A., H. Horstmann, and W. Almers. 1997. Transport, docking and exocytosis of single secretory granules in live chromaffin cells. *Nature.* 388:474–478.

Sullivan, R., M. Burnham, K. Torok, and A. Koffer. 2000. Calmodulin regulates the disassembly of cortical F-actin in mast cells but is not required for secretion. *Cell Calcium.* 28:33–46.

Swedlow, J. R., J. W. Sedat, and D. A. Agard. 1993. Multiple chromosomal populations of topisomerase II detected in vivo by time-lapse, three-dimensional wide-field microscopy. *Cell.* 73:97–108.

Taraska, J. W., and W. Almers. 2004. Bilayers merge even when exocytosis is transient. *Proc. Natl. Acad. Sci. USA.* 101:8780–8785.

Taraska, J. W., D. Perrais, M. Ohara-Imaizumi, S. Nagamatsu, and W. Almers. 2003. Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc. Natl. Acad. Sci. USA.* 100:2070–2075.

Tran, V. S., A. M. Marion-Audibert, E. Karatekin, S. Huet, S. Cribier, K. Guillaumie, C. Chapuis, C. Desnos, F. Darchen, and J. P. Henry. 2004. Serotonin secretion by human carcinoid BON cells. *Ann. N. Y. Acad. Sci.* 1014:179–188.

Yang, D.-M., C.-C. Huang, H. Y. Linm, D. P. Tsqi, L. S. Kao, C. W. Chi, and C. C. Lin. 2003. Tracking of secretory vesicles of PC12 cells by total internal reflection fluorescence microscopy. *J. Microsc.* 209:223–227.