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Review

Exocytotic Vesicle Behaviour Assessed by Total Internal Reflection Fluorescence Microscopy

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The regulated trafficking or exocytosis of cargo-containing vesicles to the cell surface is fundamental to all cells. By coupling the technology of fluorescently tagged fusion proteins with total internal reflection fluorescence microscopy (TIRFM), it is possible to achieve the high spatio-temporal resolution required to study the dynamics of sub-plasma membrane vesicle trafficking and exocytosis. TIRFM has been used in a number of cell types to visualize and dissect the various steps of exocytosis revealing how molecules identified via genetic and/or biochemical approaches are involved in the regulation of this process. Here, we summarize the contribution of TIRFM to our understanding of the mechanism of exocytosis and discuss the novel methods of analysis that are required to exploit the large volumes of data that can be produced using this technique.

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Exocytosis functions to deliver cargo either into or across the plasma membrane of a cell. Often this delivery is constitutive; however, in many cases, the delivery of membrane proteins such as nutrient transporters, or soluble cargo such as hormones, is precisely regulated by environmental factors. The regulated exocytotic pathway involves membranous tubulo-vesicular or granule structures enriched in cargo being delivered to the plasma membrane. A variety of proteins and phospholipids required for exocytosis have been identified by numerous elegant *in vitro* biochemical and genetic studies. In contrast, the *in vivo* function of many of these molecules in exocytosis has been more elusive. More recently, continued development of fluorescent fusion protein constructs [green fluorescent protein (GFP) and its derivatives] has enabled the visualization of the exocytotic process in live cells. Thus, a more detailed understanding of the *in vivo* exocytotic trafficking process has emerged and defects in the 'behaviour' of exocytotic machinery have been assessed in relation to the identified proteins and phospholipids. One of the most powerful imaging techniques that has been employed in this regard is total internal reflection fluorescence microscopy (TIRFM), which delivers the necessary spatio-temporal resolution required to image such dynamic processes.

TIRFM or evanescent wave microscopy is an application of fluorescence microscopy that allows selective imaging of events on or close to the plasma membrane of a cell. By selectively illuminating only the basal surface of the sample, TIRFM overcomes the loss of resolution that occurs in standard epifluorescence microscopy because of the noise that results from the excitation of out of focus fluorescent molecules and unlike confocal techniques, there is no pinhole to reduce the amount of light getting to the camera. The physics behind TIRFM has been the subject of multiple excellent reviews [for a more detailed explanation of TIRFM physics and it its applications in cell biology, see reviews by Axelrod and others (1,2)]. Briefly, if a beam of light is travelling from a medium of higher to a medium of lower refractive index ($n1$, $n2$) and the angle of incidence (θ_i) is greater than the critical angle ($\theta_c = \arcsin(n2/n1)$), then the beam of light will be totally reflected [total internal reflection (TIR), Figure 1A]. This process creates a weak standing wave or evanescent field of electromagnetic radiation that decays exponentially from the interface between high and low refractive index materials (Figure 1A). Current TIRFM systems are capable of exciting fluorophores within this evanescent field or 'TIRF zone' with a penetration depth that is generally between 70 and 250 nm from the interface. In the context of cells grown on a glass coverslip expressing a GFP-fusion molecule, only GFP molecules on or close to the plasma membrane are imaged. This technique delivers greatly improved signal to noise relative to standard epifluorescence microscopy (Figure 1B), with obvious advantages for studying the later stages of exocytotic trafficking.

In this review, we attempt to take a generalized look at regulated exocytosis as a whole and how TIRFM has, and will, contribute to our understanding of this process. It is however, important to recognize the existence of multiple types of regulated exocytosis that are visibly

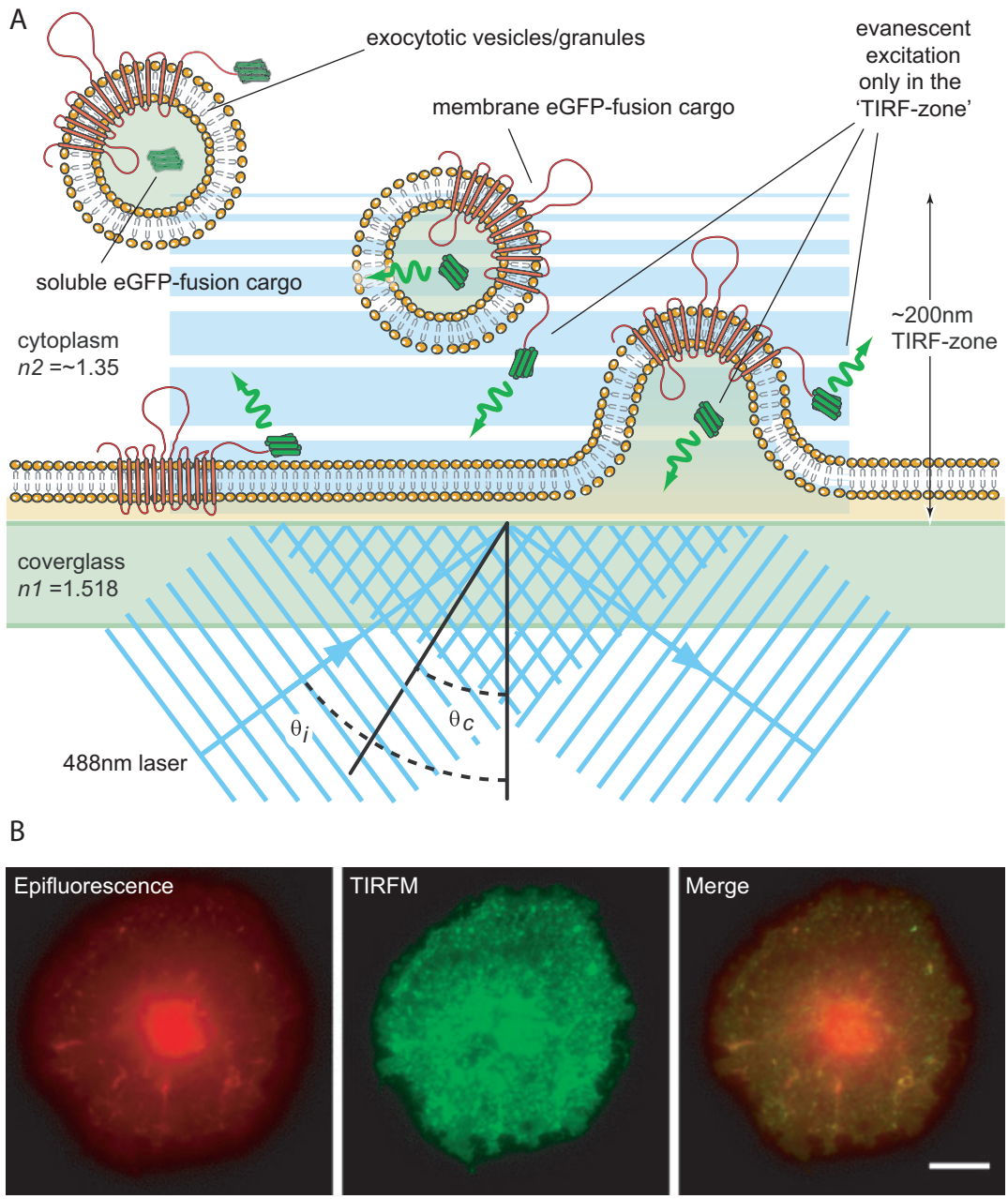

Figure 1: TIRFM. A) TIR of excitation energy introduced above the critical angle (θ_c) leads to the generation of an exponentially decreasing evanescent field capable of exciting fluorescent molecules. Prism or, as depicted here, objective-based TIRF microscope systems are capable of exciting fluorophores within the evanescent field or TIRF zone of between 70 and 250 nm from the interface between the higher (glass) and lower (cell) refractive indices (*n*). B) Epifluorescence versus TIRF microscopy. Images of a 3T3-L1 adipocyte expressing the glucose transporter GLUT4 fused at the carboxyl-terminus to eGFP (GLUT4-eGFP). Levels of GLUT4 at the plasma membrane are, at least in part, tightly controlled by regulated exocytotic processes. Reflected fluorescence was detected from GLUT4-eGFP by focussing on the basal surface (close to the coverslip) under either epifluorescent excitation (left, red) or TIR excitation (middle, green). A merged image is shown on the right highlighting the increase in visual information achieved by TIRF illumination. Scale bar represents 10 μm.

and biochemically distinct. The major exocytotic steps are true to all systems but the molecules involved and their regulation differ. The majority of cell types display one of the two distinct types of regulated exocytosis. Calcium-triggered exocytosis represents one of the most common and well-characterized form of regulated exocytosis. Particularly evident in neuronal and neuroendocrine cells, this form of exocytosis exhibits

precise regulation by calcium to initiate the exocytosis of soluble cargo into the extracellular environment. In models of calcium-triggered exocytosis, vesicles are thought to exist in a number of morphologically and biochemically distinct locations. A small percentage of vesicles exists in a pool that is morphologically attached to the plasma membrane, termed the predocked pool of vesicles (3). A subset of these morphologically attached vesicles, described as the readily releasable pool, are highly release competent and rapidly undergo release without any further modification (3,4). The remaining vesicles exist in the cytoplasm and have been defined as the reserve pool. These require a series of ATP, Ca^{2+} and time dependent processes to enable them to move to the membrane and gain release competence. In contrast, other forms of regulated exocytosis do not rely on calcium flux as an important trigger, such as in the exocytosis of the glucose transporter GLUT4 in muscle and fat cells. The distribution of exocytotic vesicles appears to be quite different in these cell types. Within the cell, GLUT4 is closely associated with several intracellular compartments including recycling endosomes, the trans-Golgi network and tubulo-vesicular elements (5,6). These tubulo-vesicular elements maintain a highly insulin-responsive GLUT4 compartment known as GLUT4 storage vesicles (GSVs). Insulin-stimulated GLUT4 exocytosis is achieved through the initiation of signalling cascades, particularly via PI3-kinase and protein kinase B (PKB) PKB/Akt (7,8) that regulate various steps of the trafficking of GSVs to the plasma membrane.

Vesicle Behaviours Observed by TIRFM

Studies using fluorescently tagged proteins coupled with TIRFM are contributing a wealth of new information on the *in vivo* exocytotic process [for a good example, see Ref. (9)]. However, in collating this review, it has become clear that there is considerable inconsistency in the terminology used to describe vesicle behaviour. For example, some behaviours are described by different terms whilst in other examples, differing behaviours are described by the same term. This has probably arisen through both the difficulty in correlating the inter-model, *in vitro* and *in vivo* observations, and the relative youthfulness of this field. It is useful to look at exocytotic vesicle behaviours in the context of three key steps which also correspond to key steps for regulation (Figure 2, see also Ref. (10)).

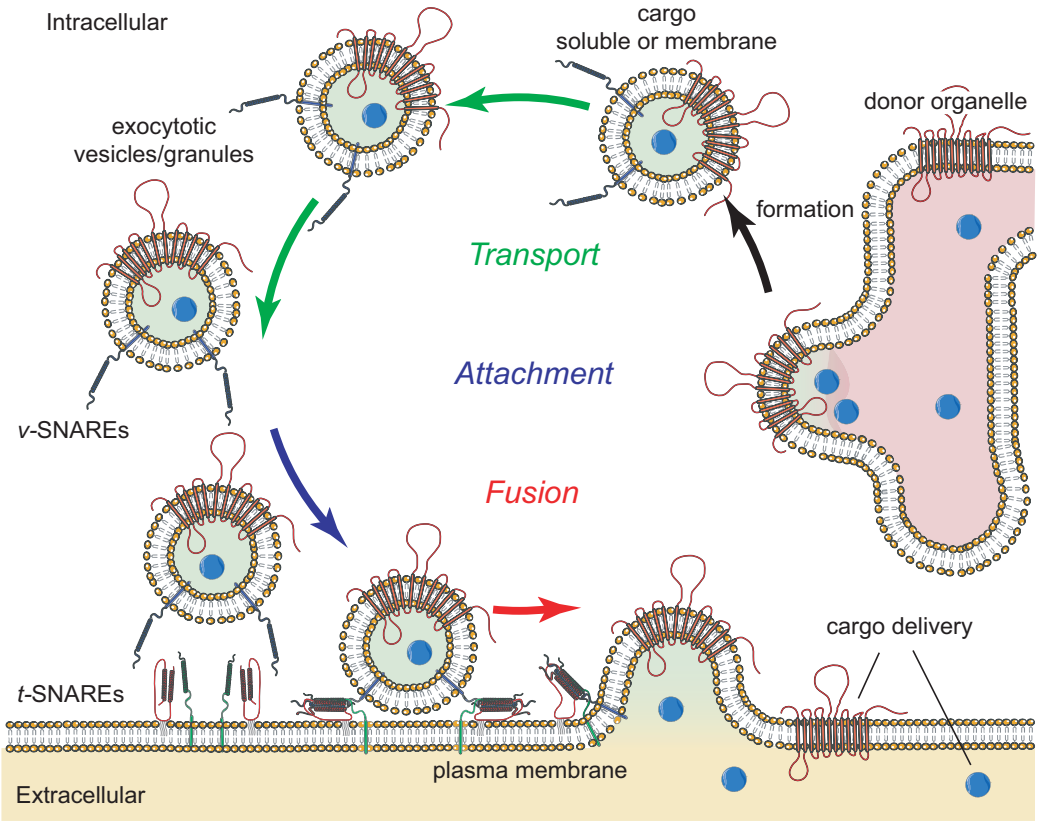

Figure 2: Model of exocytosis. Exocytotic vesicles or granules enriched in soluble or membrane-bound cargo form from a donor organelle. These then undergo transport, which encompasses the movement from the donor organelle to the target membrane where the vesicle undergoes attachment, followed by fusion that allows delivery of cargo to the plasma membrane or external environment.

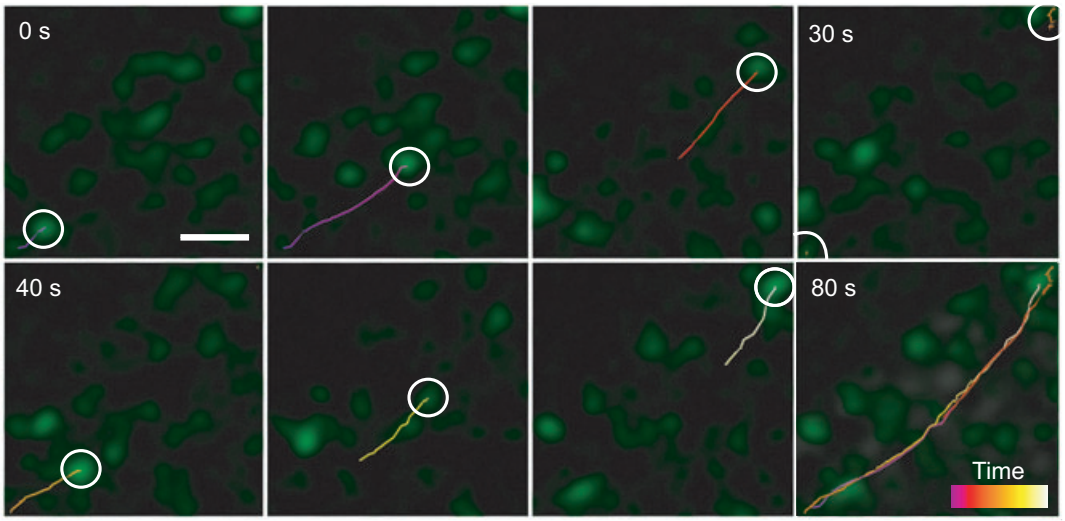

Figure 3: Vesicle behaviours in the TIRF zone – transport. TIRFM images of a region of an L6 myoblast expressing GLUT4-eGFP. A vesicle structure moves, in a largely linear manner, from bottom left to top right of the image over ~30 seconds at an average of 0.420 mm/second (maximum 1.2 mm/second). Shortly after, a second vesicle structure moves along the same 'track' at an average of 0.390 mm/second (maximum of 0.9 mm/second). Scale bar represents 1.5 μm.

Exocytosis begins with the generation of cargo-enriched structures from donor organelle membranes (Figure 2). These exocytotic vesicles must then undergo *transport* to a site where exocytosis will be completed. Transport is the first exocytotic behaviour readily identified by TIRFM. Visually, transport corresponds to unrestricted behaviours or linear movements whereby vesicles or granules can be trafficked through the cytosol often along cytoskeletal elements towards their target membrane. Examples of such transport behaviours can be seen in Figure 3. Close to the target membrane, often well within the TIRF zone, granules or vesicles may undergo a*ttachment*. Attachment is the next exocytotic vesicle behaviour that is readily identified. Visually, attachment corresponds to a restriction in movement and is likely to encompass the biochemically identified processes described as tethering and docking. Examples of attachment behaviours can be seen in Figure 4. Attachment facilitates the correct targeting of vesicles and engages the two membrane interfaces through direct protein–protein interactions preparing the vesicles for the final delivery of cargo in a process known as *fusion*. Fusion defines the process by which the lipid bilayers merge either completely or partially and can be readily identified by TIRFM. Examples of such fusion behaviours can be seen in Figure 5.

Thus, in the context of transport, attachment and fusion, a number of key *in vivo* similarities between models of exocytosis are observed. A variety of vesicle behaviours that can be attributed to transport have been described using TIRFM in most cell types. It is important to note, however, that the nature of TIRFM limits the observations to those that occur within ~250 nm of the coverslip and thus not all transport behaviours are observable. Transport behaviours include 'long-range directed movements' (11) (see examples in Figure 3), 'short range directed movements', as well as 'appearances' and 'disappearances' or more 'random movements' (12–15).

Attachment behaviours have been described in a number of the earliest TIRFM studies of exocytosis in chromaffin cells and are generally more restricted than transport behaviours. Populations of granules close to the plasma membrane were seen to have 'restricted' or 'caged' movements also described as 'docked' or 'less mobile' – the vesicles move but within a limited volume. In a number of studies, these vesicles were released upon the application of a secretory stimulus suggesting that these vesicles constitute the readily releasable pool (12–15) confirming that these attachment behaviours may follow transport and precede final fusion. Such attachment behaviours have now been described in most models of regulated exocytosis (16–18). More recently, studies in chromaffin cells have endeavoured to better resolve these granule attachment behaviours by measuring individual vesicle movements using more sophisticated analysis that took into account the limit of resolution, noise and relative *z* position. Using VAMP2-GFP (vesicle associated membrane protein 2, synaptobrevin) and pro-atrial natriuretic peptide (ANP)-GFP, Allersma et al. measured the 'jittering' motion of granules describing tethered or caged granules as those close to the plasma membrane with restricted motion (19). Interestingly, removal of ATP (to inhibit priming) reduced the granule movement, whereas Ca^{2+} influx stimulated an increase in movement prior to fusion. Small increases in the lateral movement of granules have also been observed

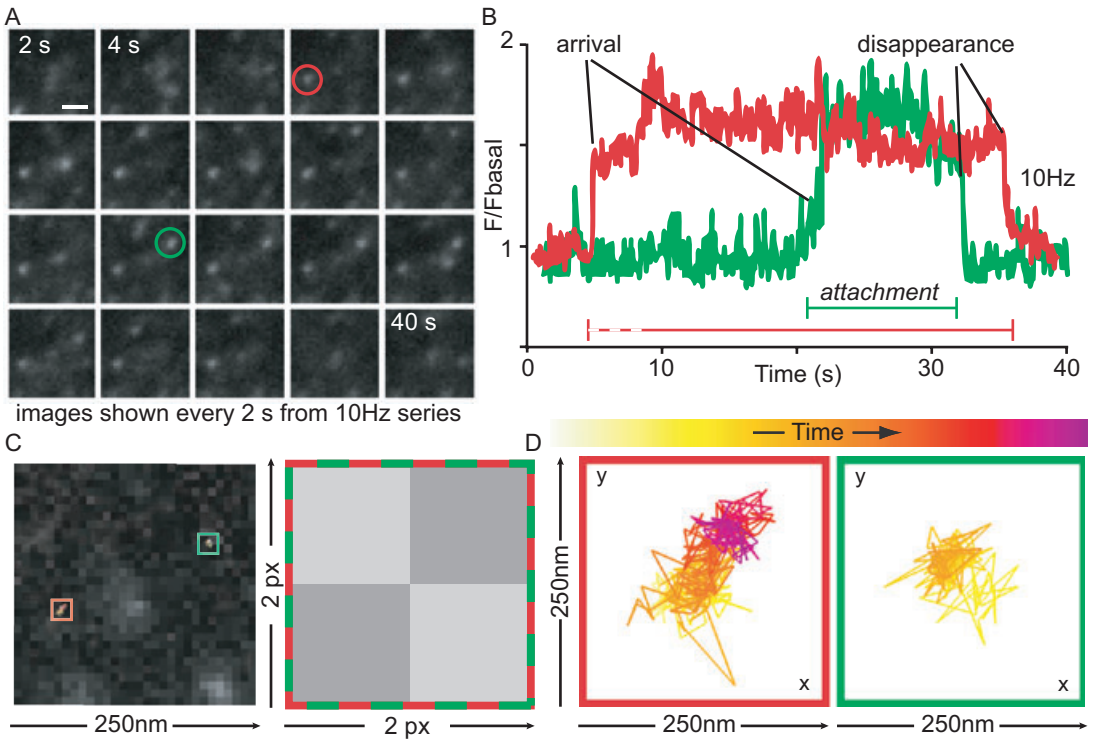

Figure 4: Vesicle behaviours in the TIRF zone – attachment. A) Representative TIRFM images (every 2 seconds) of a region of a 3T3-L1 adipocyte (imaged at 10 Hz) highlighting two GLUT4-eGFP labelled vesicles in the TIRF zone, denoted by red and green circular regions of interest (ROI) in the first frame they appear. B) The normalized fluorescence within these red and green circular ROIs highlights the arrival and disappearance of these vesicles and the time–course of their attachment. C) The tracks of each vesicle are shown in the denoted by the 2 × 2 pixel square red and green ROIs. D) The regions enlarged are shown. Whilst attached, the movement of these vesicles is highly restricted as shown by the tracks. The tracks are colour coded based on time (C). Scale bar, 2 μm.

in pancreatic β-cells immediately prior to fusion. This has led to the suggestion that movement to hotspots of fusion or specific Ca^{2+} concentration may be an important regulated step (20). These data also suggest that a lack of movement may not be the best indicator of vesicles that are fusion competent and fully engaged with the fusion machinery. Moreover, the restricted movement described by some as docking has been demonstrated to be insensitive to SNARE cleavage (see below), again suggesting that these vesicles have not engaged with the fusion machinery (21). Thus, while these granules display a stationary behaviour that has been assumed to be consistent with membrane attachment, it appears that stimulated movement of these granules is still required prior to engagement of the final fusion apparatus. This highlights the importance of using care when attempting to apply ideas that have emerged from biochemical and morphological electron microscopy (EM) studies to behaviours observed in live cells.

The behaviours observed above ultimately enable a number of vesicles/granules to undergo fusion with the plasma membrane. Fusion of fluorescently labelled vesicles with the plasma membrane observed by TIRFM has been described as a 'sudden brightening and a rapid spreading' of the signal (22) or as a 'sudden diffuse cloud' (23) as seen in Figure 5. These behaviours appear to be characteristic of the majority of fusion events described in all cell types (24). The sudden increase in signal seen in many studies has been speculated to arise from evanescent excitation being more efficient closer to the interface (see Figure 1) or from the pH sensitivity of the GFP that is predicted to increase in fluorescence 1.7-fold during the pH change from 5.5 within the acidified granule to 7.4 in the extracellular environment (22). Either way, it is a characteristic common to most forms of exocytosis and one described by many authors (24–26). The potential change in pH between the lumen of the exocytotic vesicle/granule and the extracellular environment has actually been exploited to identify fusion events using the pH-sensitive GFP derivative pHluorin (27) better. With a lumenal pHluorin-fusion construct, the fusion event from insulin granules (23) or GSVs (25,26) appears as a rapid increase in the vesicles' fluorescence intensity followed by spreading of the signal, as the fluorophore diffuses laterally into the cell membrane (Figure 5). This characteristic fusion signature has been useful in the development of automated approaches to analyse these events (discussed below).

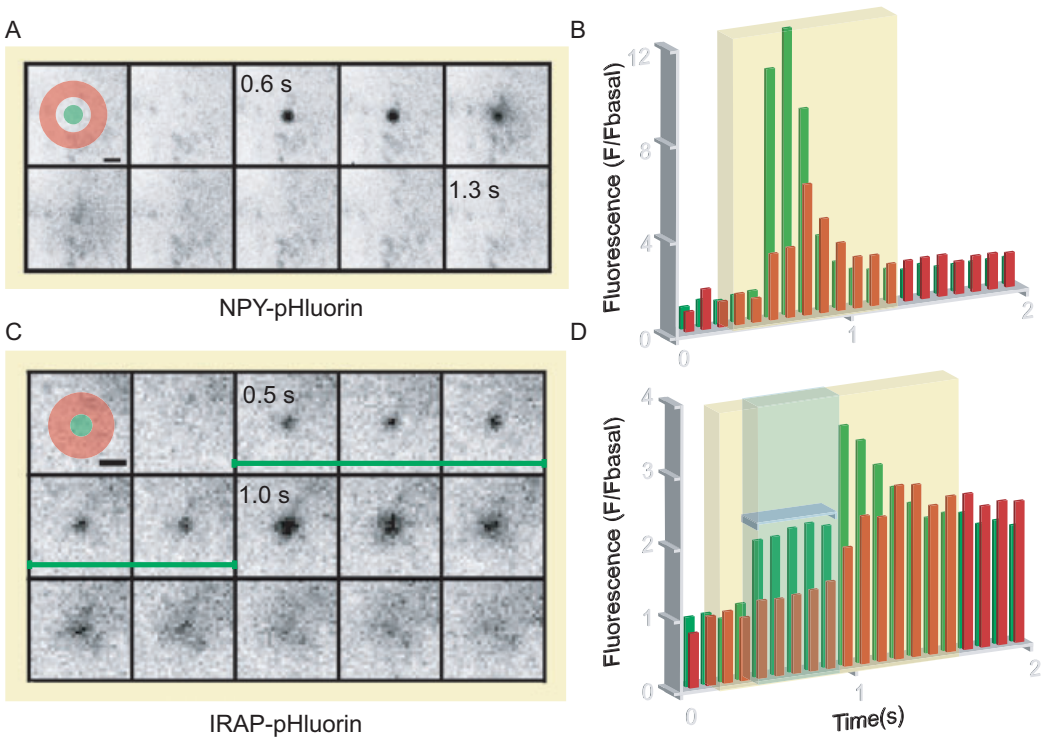

Figure 5: Vesicle behaviours in the TIRF zone – fusion. A and B) Representative fusion events from calcium-triggered and non-calcium triggered models of exocytosis are shown. Pancreatic β-cell, MIN6, expressing soluble cargo NPY-pHluorin, was imaged at 10 Hz. C and D) Apipocyte, 3T3-L1, expressing membrane attached cargo IRAP-pHluorin imaged at 10 Hz. The normalized fluorescence profiles of two regions of interest (red and green) within these events are shown in (B) and (D) respectively, with the highlighted region (yellow), corresponding to the images shown (A or C). Comparing the fluorescence for the centre of the events (green) and annular ring outside the centre (red) highlights the lateral diffusion away from the site of exocytosis. In (A) the marker is soluble and rapidly diffuses away from the site of exocytosis with fluorescence rapidly returning to basal levels. In (B) the vesicle exhibits attachment behaviour prior to fusion at $t = 1$ second. In (D) the fluorescence remains above basal as the marker is tethered to the membrane. Scale bar, 1 μm.

In pancreatic β-cells, Tsuboi, Rutter and colleagues have demonstrated that there is heterogeneity in the way that cargo is released. This appears to be related to both the nature of the cargo and the size of the fusion pore. They describe three classes of pancreatic β-cell fusion: kiss and run – a small fusion pore releasing only some lumenal content, mixed kiss and run – where some lumenal and some membrane content may be released, and full kiss and run – where all soluble cargos can be released (23,28,29). This arose from the observation that different granule membrane markers display different behaviours upon fusion, even though they reside in the same pool of granules. Vesicle membrane markers such as VAMP2-GFP diffuse rapidly and radially from a fusion site, whereas GFP-phogrin, another membrane marker, generally does not. Unexpectedly, large lumenal cargo such as tissue plasminogen activator (tPA)-mRFP or neuropeptide Y (NPY)-venus could remain within a vesicle lumen after the fusion event (23,28,29). Others suggest that the majority (87%) of fusing insulin granules labelled lumenally with syncollin-GFP undergo full fusion. The remaining events remained visible for extended periods – described as kiss and linger (30). Another type of fusion common to some cell types is compound fusion. In cells that display this type of exocytosis, the vesicles fuse with each other as well as with the plasma membrane either before (multivesicular fusion, e.g. mast cells) or after (sequential fusion, e.g. pancreatic acinar cells) the opening of the primary fusion pore [for review see Ref. (31)].

Thus, it is clear that some form of transport, attachment and fusion behaviour occurs in all these cell types to permit exocytosis of cargo. From the calcium-triggered exocytosis of large lumenally laden granules to the insulin-stimulated exocytosis of small membranous glucose transporter vesicles, numerous molecules regulate these steps and TIRFM is being used to identify the key regulatory molecules

Molecules Controlling Vesicle Behaviour

Cytoskeletal elements

Cytoskeletal structures have been shown to play a role in exocytosis in multiple cell types and in all stages of the process including transport, attachment and fusion.

Lizunov et al. identified and described GSV transport behaviours associated with microtubular elements (11), and these are behaviours seen, although to a lesser extent, also by others (32). Consistent with microtubules being important for transport to the plasma membrane, disruption of microtubules with nocodazole restricted long-range movements of GSVs in adipocytes (33). Latrunculin B treatment, which disrupts actin remodelling, also impairs insulin-stimulated GSV exocytosis in 3T3L1 adipocytes, but did not prevent the transport of GLUT4-GFP into the TIRF zone demonstrating that insulin regulates the transport of GSVs to the cell surface. However, the final fusion event was inhibited, as indicated by the lack of characteristic fusion events and an accumulation of GSVs in the TIRF zone (34). These observations suggest that actin remodelling is a step required after transport or entry into the TIRF zone but before the fusion event (34).

The disruption of actin has been shown to inhibit the transport and attachment of granules in both PC12 cells (35) and adrenal chromaffin cells (36). These observations suggest that transport along actin filaments is necessary for movement to the site of fusion. Consistent with this idea, reduced expression of the motor protein myosin Va in pancreatic β-cells reduced the number of insulin granules in the TIRF zone (37). Similarly, transport and attachment behaviours were seen to be disrupted in cells containing defective myosin Va (38). A potentially similar role for other myosins including myosin Vc is also emerging from TIRFM studies (39). Actin has also been implicated in regulating the fusion event itself. Modification of cortical actin through overexpression of actin or actin-related protein 3 or via treatment with cytochalasin D or Latrunculin B was seen to alter cargo release during fusion, suggesting that actin is involved in regulating fusion pore stability (40). Interestingly, actin depolymerization was shown some time ago (through EM studies) to increase granule fusion in pancreatic β-cells leading to the hypothesis that cortical actin acts as a physical barrier preventing access of granules to the fusion machinery (41). Taken together, these data suggest that both an intact actin cytoskeleton and its dynamic rearrangement are crucial for granule exocytosis. The cytoskeleton therefore plays a crucial role in the regulation of exocytosis. The data suggest a generalized model in which the cytoskeleton is required for long-range movements delivering vesicles to the cortical actin network, that is then involved in attachment and fusion at multiple levels, possibly in a cell-type dependent manner.

SNARE proteins

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are a family of proteins thought to be crucial for membrane fusion (42,43). These proteins are present in both the donor (v-SNARE) and target (t-SNARE) membranes. These two groups interact in a zipper like manner to overcome repulsive forces and bring the two membranes into tight apposition. The formation of a SNARE complex is generally considered to define docking events as opposed to other modes of attachment (although a clear behaviour for docking remains elusive). The use of TIRFM to study the role of SNARE proteins in exocytosis has contributed a lot to further our understanding of how these proteins are involved in exocytosis.

In pancreatic β-cells, treatments that interfere with SNARE protein interactions reduced the number of visible fusion events in both first (1–120 seconds) and second phase (>120 seconds) secretion. This was attributed to defects in attachment (docking) as granule entry into the TIRF zone was reduced in these treated cells (44). Syntaxin1–SNAP25 clusters were shown to colocalize with insulin granules in the TIRF zone and thus these SNARE proteins have been described as defining docking and thus fusion sites. In cells from syntaxin1A knockout animals, the number of docked granules was reduced, as was first phase secretion (identified by counting visible fusion events). The Goto-Kakizaki diabetic rat (a non-obese strain developing diabetes early in life) has reduced granule numbers in the TIRF zone (described as reduced docking) correlating with a reduction in syntaxin1 and SNAP25 clusters (45,46). Interestingly, second phase secretion did not appear to be affected by the absence of syntaxin1 (47), suggesting that various SNARE isoforms may be involved in granule fusion in a temporally regulated manner. Similar observations have been made in adipocytes where it was found that there is a redundancy in the requirement for particular v-SNAREs, with either VAMP2, VAMP3 or VAMP8 being sufficient for normal GLUT4 exocytosis in the absence of the other two (48). Toonen et al. demonstrated that strong docking and fusion required the t-SNARE syntaxin1 but not the v-SNARE VAMP (49). Use of Tetanus Toxin - Long Chain (TeNt-LC) toxin (cleaving VAMP) led to increases in lateral movement suggesting that this step may be dependent on VAMP (50). TIRFM was used to identify plasma membrane SNARE clusters consisting of GFP-SNAP25 and syntaxin 1. These were seen to be somewhat mobile and a SNAP-25 deletion construct (equivalent to that produced by botulinum neurotoxin A) decreased granule fusion and increased lateral mobility again suggesting the importance of attachment and potential attachment-fusion hotspots (26).

Exocyst

The exocyst or Sec6/Sec8 complex (51) has also been implicated in the process of attachment. Overexpression of truncated, dominant negative, exocyst components reduced the number of granules identified in the TIRF zone relative to those seen in control cells (52). 'Tight' attachment of granules has been shown to be dependent on the Sec1p homologue Munc18-1 (49). Munc13-1 conversely has been linked to weaker attachment states (50).

Synaptotagmins

Synaptotagmins are a family of proteins thought to be important for facilitating fusion. Martens et al.

elegantly demonstrated, that upon calcium binding, synaptotagmin 1 induces positive membrane curvature in liposomes that promotes membrane fusion (53). Lynch et al. then demonstrated that knockdown of endogenous synaptotagmins in PC12 cells completely inhibited vesicle fusion (54). This was rescued by overexpression of wildtype (WT) and mutant forms of synaptotagmin 1 demonstrating that it is important for both calcium-triggered membrane fusion and regulation of fusion pore diameter. Similar observations were made by Tsuboi and Fukada, supporting the premise that synaptotagmin regulates both fusion and the size of the fusion pore. (24).

Signalling and phospholipids

Some of the earlier studies of GLUT4 exocytosis using TIRFM actually investigated the timing and importance of insulin-stimulated PI3-kinase signalling using GLUT4-YFP and a CFP-$PH^{PKB/Akt}$, a tagged pleckstrin homology domain with some specificity towards the class I PI3-kinase product, PtdIns(3,4,5)P_3. Upon stimulation with insulin, the PH domain construct was rapidly recruited to the plasma membrane (a ~70 nm TIRF zone) which preceded, and was essential for, the subsequent 'translocation' of GLUT4-YFP to the same zone (55). The role of class II PI3-kinase product PtdIns3P was also investigated by assessing GLUT4-eGFP movement into the TIRF zone of L6-myoblasts expressing reduced levels of the class II enzyme PI3KC2α (56). These were crude studies not differentiating between transport, attachment or fusion steps. Subsequently, TIRFM has mostly been used to investigate later steps of GSV exocytosis, particularly, to understand which of the steps may be the elusive insulin-regulated step. A combination of fixed and live cell TIRFM approaches was used with inhibitors of PKB/Akt and PI3-K signalling to identify the key regulated steps. Insulin-stimulated PI3-kinase and PKB/Akt activities were required to promote GSV 'recruitment' into the ~250 nm TIRF zone (57). This recruited signal was also observed to become more diffuse and less punctate consistent with GLUT4 located on the plasma membrane rather than on GSVs, a process that, whilst requiring PI3-K activity, appears independent of PKB/Akt (57). A series of studies from Xu and colleagues have described similar observations whilst also further defining the individual behaviours and key regulated steps. By tracking hundreds of individual GLUT4-eGFP labelled GSVs in variously treated 3T3-L1 adipocytes it was seen that attachment described as 'docking' or 'becoming immobile within the TIRF zone' was dependent on insulin-stimulated PI3K signalling and on PKB/Akt phosphorylation of the Rab-GAP AS160 (TBC1D4). By measuring the 'dwell time' (time in the TIRF zone) of attached vesicles, another insulin-dependent step was identified as insulin reduced the dwell time prefusion (16). Using TDimer-IRAP-pHluorin, a red and green fluorescent construct to mark GSVs and characterize fusion events better, it was confirmed that the role of AS160 is not to regulate the fusion event but it is required for efficient docking (25) (Figure 4). This insulin-dependent docking and reduction in tethering/docking time prefusion was also seen in a study of hundreds of GLUT4-eGFP labelled GSVs into a 100-nm TIRF zone (58). These authors also saw an accumulation of static GSVs in the plasma membrane that colocalized with clathrin (58).

Rabs

Rab proteins are a member of the Ras superfamily of monomeric G proteins and are thought to play regulatory roles in almost all stages of vesicle trafficking, with different Rabs regulating different steps.

Tsuboi and Fukuda characterized the fusion event further using rat pheochromocytoma cells (PC12) containing granules labelled with fluorescently tagged NPY. Small GTPases like Rab3A, Rab27A and Rab33A GTPases were identified on dense core vesicles in PC12 cells and their role in exocytosis was tested using siRNA-mediated silencing. Rab3A and Rab27A, but not Rab33A, were proposed to participate in attachment as the number of 'static vesicles in the TIRF zone' was significantly reduced (59). The Rab27a effector, granuphilin, is suggested to be a negative regulator of attachment, as granuphillin knockout β-cells were seen to have fewer total granules within the TIRF zone as a result of increased rates of fusion (60).

Summary

The data derived from TIRFM support an overall model of exocytosis whereby vesicles are transported along the cytoskeleton to the cortical actin network. Here, they are moved by molecular motors such as myosins to sites of fusion where the relevant SNARE proteins engage to bring the membranes into tight apposition. This step may be facilitated by the exocyst complex, although more work is required to understand how this complex is involved. In calcium-triggered secretion, calcium binding to synaptotagmins induces the necessary membrane curvature that provides the energy to drive fusion. The fusion that occurs then may be regulated by a number of proteins including actin. Future work will undoubtedly dissect these processes further.

Automated Approaches to Image Analysis (Fusion as Identified by TIRFM)

Accurately identifying and characterizing transport, attachment and fusion behaviours requires imaging at high spatial and temporal resolution to generate vast amounts of data. High speed is required as, for example, some attachment behaviours may last for less than half a second and fusion events can be complete in less than 300 milliseconds. Large fields of view of high magnification are required to record representative numbers of events from structures close to or beyond diffraction limits. While manual annotation of TIRFM data is possible and has been used in small studies, with the improvement in imaging capabilities it is now possible capture tens of thousands of frames from a single cell. The accurate annotation

of such data is an arduous task and indeed is now the major rate-limiting factor in the TIRFM workflow. TIRFM data are not readily analysed by the available image analysis tools; consequently, a number of groups have developed their own tools for mining these large data sets. Semi-, or indeed, full-automation of TIRFM data analysis promises to deliver more objectivity and reproducibility.

Different automated approaches have been used to study vesicle fusion in particular. Fusion is a vesicle behaviour that is generally readily identified in most cellular models of exocytosis and has a number of characteristic features (see Figure 5). At a crude level, fusion has been monitored by measuring the accumulation of a specific vesicular protein on the plasma membrane. As fusion increases, so does membrane fluorescence too (see Figure 6, red line). This technique has proved particularly useful in adipocytes (56,61) where the small size of vesicles (~80 nm) can hinder the visualization of individual fusion events. In some early TIRFM studies, fusion events were inferred by the rapid disappearance of a labelled structure in the TIRF zone with occasional observation of dye release (12) or by a disappearance preceded by a transient increase in fluorescence (29).

More recently, the characteristic fusion signature (Figure 5) has been proposed to permit automated identification (62) and a number of groups have developed tracking-based approaches to achieve this (16,58). The approach is based on the premise that the end of vesicle track will represent either the movement of the vesicle out of the evanescent field, or a fusion event. Thus, the end of a vesicle track can be probed for a fusion signature. Bai et al. define fusion by a rapid increase in fluorescence accompanied by an increase in fluorescence in an annular ring representing radial diffusion (16) (see Figure 5) while Vallotton et al. use tracking in combination with matched filtering, an approach that classifies events based on their relation to one or more template or characteristic events (63). We have recently developed a novel computer vision system based on the rapid, automated screening of time-lapse sequences for areas that show rapid changes in intensity. Images are assembled in a 3D stack and the absolute difference between consecutive frames is calculated. Regions that are more variable have higher values and consist mainly of fusion events and moving vesicles. Connected regions representing candidate events are then extracted from the highly variable parts of the 3D volume and are then described by a set of novel domain-specific descriptors. Similarity scores are calculated between genuine fusion events and fusion candidates using principal component analysis to identify true fusion events (34,64). This approach has made automated analysis of large image sequences possible; in the example shown (Figure 6), ~1600 fusion events were identified during the 40 min of imaging (24 000 frames; 10 Hz) before and after addition of the secretory stimulus. Such accurate automated identification of all fusion events will allow a better understanding of how upstream regulatory inputs affect exocytosis. However, the detailed course of individual vesicle fusion events, a progressive spreading of the local intensity (Figure 5), also holds considerable information on the fusion mechanism itself (22). Thus, the combination of automated fusion detection with analysis of individual event kinetics will greatly enhance the ability to extract relevant information not only upstream but also downstream of fusion.

Conclusions

TIRFM reveals a lot of information about the behaviour of exocytotic vesicles. However, it is important that care is taken when interpreting and comparing TIRFM data.

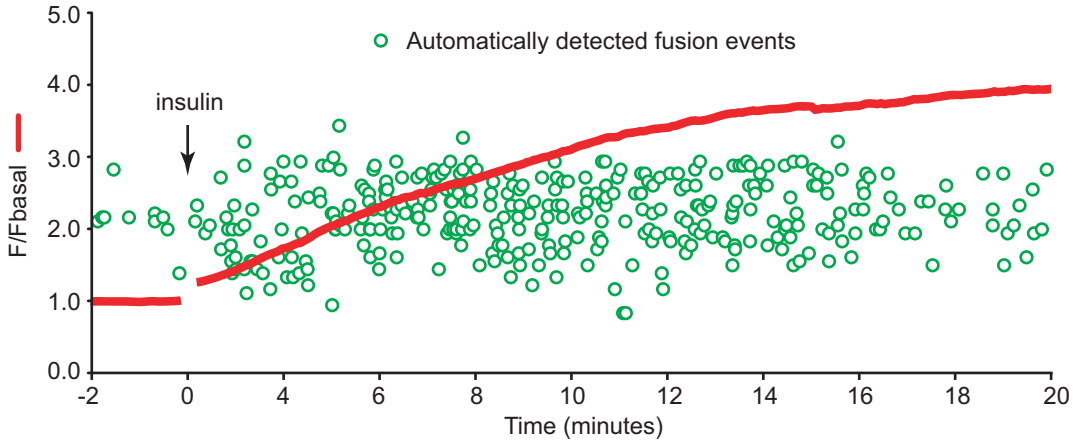

Figure 6: Automated detection of fusion events. A 3T3-L1 adipocyte expressing a VAMP2-pHluorin$^{(lumenal)}$ construct was imaged at 10 Hz before and after addition of 100 nM insulin. The change in normalized fluorescence detected in the TIRF zone for the whole cell is shown (red). This represents changes due exocytosis but also includes processes such as endocytosis and/or any photobleaching. Individual exocytotic fusion events that have been automatically identified are shown (green) as they occurred in time.

For example, Bai et al. demonstrated a critical effect of temperature on attachment kinetics. GSVs were seen to dock for ~12 seconds at 20°C, whereas this time reduced to ~3 seconds at 35°C (16). Many TIRFM studies are not performed at 37°C. Furthermore, Tsuboi and colleagues, as described earlier, demonstrated how different reporter constructs can reveal different aspects of exocytosis (23,29). Thus the choice of reporter can greatly influence experimental outcome. This is emphasized in a more recent study of four cargo-fusion constructs behaving differently in pancreatic β-cells (65). In addition, labelling stoichiometry, particularly of differing pools of exocytotic vesicle, will affect the results. Indeed secretion from chromaffin and pancreatic β-cells is thought to predominantly occur from freshly formed vesicles (66,67), the ones perhaps less likely to be carrying a fully matured GFP marker molecule. It is becoming clearer that the timing of observations is also critical. Clearly, first and second phase insulin secretions in pancreatic β-cells are mechanistically and behaviourally quite different (47) just as the attachment and fusion of various cargo-carrying structures could be quite different before, during and after an exocytotic stimulus in all cell types. Finally, as highlighted above, the varied terminology used to describe similar vesicle behaviours also adds a level of complexity to a comprehensive understanding these steps of exocytosis (e.g. although fusion is often 'blocked' when a molecule involved in transport is inhibited, the fusion process itself may not be the critical affected step).

In conclusion, it is clear that TIRFM will continue to reveal much about the behaviour of exocytotic vesicles particularly with the advances in fusion-construct design, imaging speed and spatial resolution, like that of live cell TIRF-photo-activated localization microscopy (PALM) microscopy (68). Advances in image analysis will also aid better understanding of the varied regulated steps. This is particularly true for fusion where automated analysis of thousands of events could reveal different classes of behaviours and kinetics. Accurate identification of all fusion events will allow the attachment behaviours immediately prior to fusion to be firmly characterized perhaps revealing the true *in vivo* nature of tethering and docking.

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