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Modelling the movement of vesicles in cells

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Detection of the arrival of important proteins at the cell surface is a problem of intense biological interest. Here we create an ideal model of the arrival of proteins at the cell membrane, as seen by a total internal reflection fluorescence microscope.

Introduction

We explore the cell through the lens of a total internal reflection fluorescence (TIRF) microscope, in particular with a view to enable the automatic detection of events of interest occurring in sequences of images of live biological cells undergoing their regular metabolic processes. In order to benchmark and indeed enable the algorithms to be developed to do this image processing, ideal models of the events need to be derived, as even human experts cannot be certain or agree about the classification of regions of the TIRF imagery.

Biological Background

Cells control many aspects of their metabolism and other actions via the movement of proteins to and from their surfaces. Some of these are soluble, but many are embedded – they exist only within membranes – the structures that make up the cell surface (the plasma membrane) and also internal structures such as endosomes. Membranes are not solid, but rather fluid mosaics of molecules (lipids) in which embedded proteins and structures can move. Transport is then mediated by forming membrane parcels – often in the form of small, spherical vesicles of membrane, in which the proteins are embedded. These vesicles are formed by pinching off small "buds" of membrane from larger structures. These are then moved by diffusion or transported down filaments in the cell to the underside of the plasma membrane, where they are tethered by other proteins. The membrane vesicle is then fused with the main plasma membrane, whereupon its protein cargo is released onto the cell surface, whereupon it diffuses through the plasma membrane. Removal of these proteins happens via the reverse procedure – membrane vesicles are budded off from the underside of the plasma membrane, and move to fuse with internal membrane structures. In fact, this process is happening continuously, in a dynamic equilibrium, maintaining surface protein levels, until a stimulus or other event changes the rate of arrival, removal, loading or a combination of these to move the cell to a new dynamic equilibrium.

Due to their fundamental role in cell function, it is of great interest to study the behaviour of protein transport. This is often done by modifying the proteins of interest

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to add a fluorescent epitope, a small group of molecules which fluoresce upon light-stimulation, either upon binding with an antibody or natively. Fluorescent proteins such as enhanced green fluorescent protein (eGFP), are increasingly being used to enable the location of specific proteins to be defined in living cells. The discovery and development of the green fluorescent protein led to the award of the Nobel Prize for Chemistry for 2008 jointly to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien.

The movement of these fluorescent proteins in the cell are studied via a variety of means, however many of these rely on bulk measurements, which average the response of many thousands of cells. In order to understand the microscopic features of the vesicular transport of proteins, investigation of the subcellular features of the cells is required.

Traditional epifluorescence microscopy does not allow imaging to be restricted to events occurring at the cell membrane. TIRF microscopy however, allows the imaging of only the layer extending maximally a few hundred nanometres into the sample. This is achieved by utilising the evanescent wave that penetrates into the sample, despite the incident light being totally internally reflected.

Instead of varying sinusoidally with distance in all directions (like the incident wave), the electric field associated with an evanescent wave decays exponentially with distance from the interface, and thus there is a certain penetration depth beyond which the evanescent wave cannot excite the protein epitope to fluoresce (the TIRF zone). This is also a function of the intensity of the incident light, its wavelength and incident angle, and the particular epitope fluorescence properties, as well as the characteristics of the cell and microscope coverslip. For more details of evanescent waves and their use in TIRF microscopy, see for instance the review by Oheim [2].

As part of an ongoing effort to understand glucose transport and insulin response in cells, an automated image analysis system is being developed by the Garvan Institute of Medical Research, the University of New South Wales and the CSIRO [3]. Given the relatively high background of other events and structures visible in the TIRF images of the cells, even human experts could not always agree about whether a particular image sequence showed a fusion event or not. To build a classifier, or to use machine learning techniques, it is important to have positive controls. Thus we set out to build a model of an ideal approach, docking and fusion event as it would be seen in the TIRF image sequence.

The TIRF zone is where the vesicles approach, dock and fuse to deliver their protein cargo to the surface, as shown in Figure 1. The events of interest here are as follows. The vesicle enters the TIRF zone and the epitopes start to fluoresce (Figure 1a). Fluorescence intensity increases as vesicle approaches membrane. The vesicle may then approach and attach to the underside of the plasma membrane – Docking (Figure 1b). Some vesicles then remain docked, or only approach the underside of the plasma membrane, and then recede again, characterised by a decrease in intensity. This approach and receding of the vesicle is termed “kiss-and-run”. Other docked vesicles go on to combine the vesicle membrane with that of the plasma membrane – Fusion (Figure 1c) – whereupon the embedded proteins in the vesicular membrane are released into the plasma membrane which can be thought of as a two-dimensional structure at this

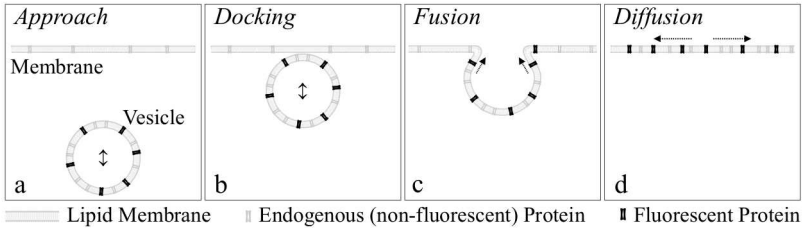

Figure 1: Side view of vesicle a) approaching, b) docking with and c) fusing with the plasma membrane, followed by d) diffusion of the embedded proteins, both endogenous and fluorescent out into the plasma membrane. The direction of diffusion of the proteins is shown with the dotted arrows.

scale. This fusion "spot" of high concentration of proteins then diffuses out to other areas of the plasma membrane (Figure 1d).

The Mathematical Model

Here we will consider the incorporation of a spherical vesicle embedded with eGFP into the plasma membrane. There are several questions of interest. What do the approach and docking events look like in a sequence of TIRF images? How long would it take for all the vesicle membrane to be incorporated in the plasma membrane during a fusion event? How big would the resulting eGFP spot on the plasma membrane be?

Vesicle Entry into the TIRF zone

The intensity at any point on the interface is due to any source perpendicularly below that point. The intensity of the source is attenuated by scattering and absorption of light by the cell, according to the Beer-Lambert law. The law states that there is a logarithmic dependence between the transmission (or transmissivity), T, of light through a substance and the product of the absorption coefficient of the substance, α, and the distance the light travels through the material (i.e. the path length), l. This may be written as

$$T = \frac{u}{u_0} = e^{-\alpha l},$$

where u_0 and u are the intensity (or power) of the incident light and that after the material, respectively.

Thus the intensity seen at the interface is due to the combination of the actual fluorescence of the point and also the distance from the point to the interface.

Let us consider a perfectly spherical vesicle radius r_v centred at perpendicular distance z_v from the interface, as shown in Figure 2. Rather than considering individual epitopes, let us assume that they are uniformly spread over the surface of the vesicle, such that any point on the surface of the sphere would have intensity u_0 if it were at the interface. The intensity due to a vesicle changes as it enters the TIRF zone. If the

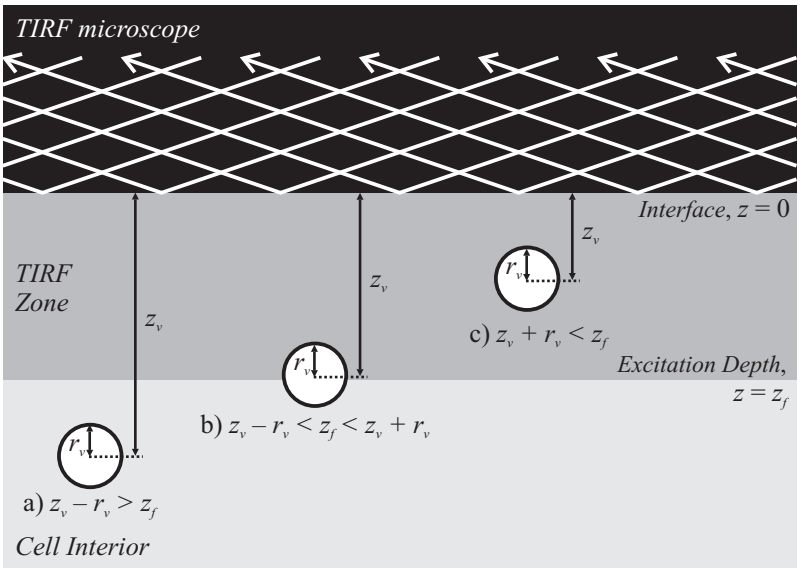

Figure 2: Cross-section of an ideal spherical vesicle in the cell interior, radius r_v, at depth z_v below the interface. In position a) the vesicle is fully below the TIRF zone, and so there is no contribution to the intensity at the interface. At b) the vesicle is partially in the TIRF zone, and so only the upper part contributes to the intensity at the interface. At c) the vesicle has fully entered the TIRF zone.

point is below the TIRF zone it is not activated and has zero intensity, and then the perceived intensity gradually increases as it approaches the interface, as less of its energy is absorbed and scattered by the intervening distance.

The TIRF image is a picture of the intensity as a function of position at the interface. This means that the spherical vesicle will have a circular profile in the TIRF image, the intensity of which varies according to the relative depth of the different parts of the sphere. Additionally there is a contribution from both the upper and lower surfaces of the vesicle. The contribution from the lower surface will also be attenuated by the intervening vesicle – here we approximate all attenuation within the cell as having the same average attenuation constant, irrespective of the location.

Define the plane of the interface to be $z = 0$. Consider the surface of the vesicle as a sphere, radius r_v, centred at $(0, 0, z_v)$, described by

$$x^2 + y^2 + (z - z_v)^2 = r_v^2,$$

or, in cylindrical coordinates,

$$r^2 + (z - z_v)^2 = r_v^2.$$

Each point on the surface of the vesicle has intensity profile

$$u_0^* (r_v, \theta, z) = \begin{cases} u_0, & z \leq z_f \\ 0, & z > z_f \end{cases} = u_0 H (z_f - z),$$

where

$$H(t) = \begin{cases} 1, & t \geq 0 \\ 0, & t < 0 \end{cases},$$

is the Heaviside function, reflecting the entry of the point into the TIRF zone. The Heaviside step function, H, also called the unit step function, is a discontinuous function whose value is zero for negative argument and one for positive argument. It seldom matters what value is used for $H(0)$, since H is mostly used as a distribution. In this case however, we define the zero value to be one as this reflects the edge of the vesicle. Note, as the system is axisymmetric, the intensity profile is independent of θ.

Each point at the interface has an intensity made up of contributions due to the intensity from the upper part of the sphere, $z = z_v - \sqrt{r_v^2 - r^2}$, and the lower surface of the sphere, $z = z_v + \sqrt{r_v^2 - r^2}$.

The perceived (attenuated) intensity at the interface is thus

$$\begin{aligned} u\left(r,\theta,0\right) = & \\ & H\left(r_v - r\right) u_0^* \left(z_v + \sqrt{r_v^2 - r^2}\right) e^{-\alpha\left(z_v + \sqrt{r_v^2 - r^2}\right)} \\ & + H\left(r_v - r\right) u_0^* \left(z_v - \sqrt{r_v^2 - r^2}\right) e^{-\alpha\left(z_v - \sqrt{r_v^2 - r^2}\right)}, \end{aligned} \tag{0.1}$$

where α is the average absorption coefficient of the cell.

If the vesicle has fully entered the TIRF zone this reduces to

$$\begin{aligned} u\left(r,\theta,0\right) &= u_0 H(r_v - r) \left[e^{-\alpha\left(z_v + \sqrt{r_v^2 - r^2}\right)} + e^{-\alpha\left(z_v - \sqrt{r_v^2 - r^2}\right)}\right] \\ &= 2u_0 H(r_v - r) e^{-\alpha z_v} \cosh\left(\alpha\sqrt{r_v^2 - r^2}\right). \end{aligned}$$

Thus as the position of the vesicle changes, its perceived intensity in the TIRF image increases as it approaches or diminishes as it recedes. Of course these two processes do not need to occur over the same time scale. Docking is characterised by the vesicle centre, z_v, being approximately a single radius, r_v, below the interface, usually with some associated noise from thermally induced movements.

Fusion

When the vesicle is docked and the fusion process begins (Figure 1c), the vesicle is tethered in place by specialist proteins (SNARES). These form a circular pore, connecting the membrane of the vesicle to that of the plasma membrane.

The process of fusion of the vesicle membrane with the plasma membrane is limited by the rate at which the membrane can be transported across the boundary of the pore separating the vesicle from the plasma membrane. As an approximation, consider a spherical vesicle of radius r_v, tethered to the underside of the membrane, connected at the boundary, a circular pore of radius r_p, between the vesicle and plasma membrane (Figure 3).

The SNARE proteins maintain this pore at a constant radius, but allow the incorporation of the vesicle membrane into the plasma membrane, with a flux of the membrane

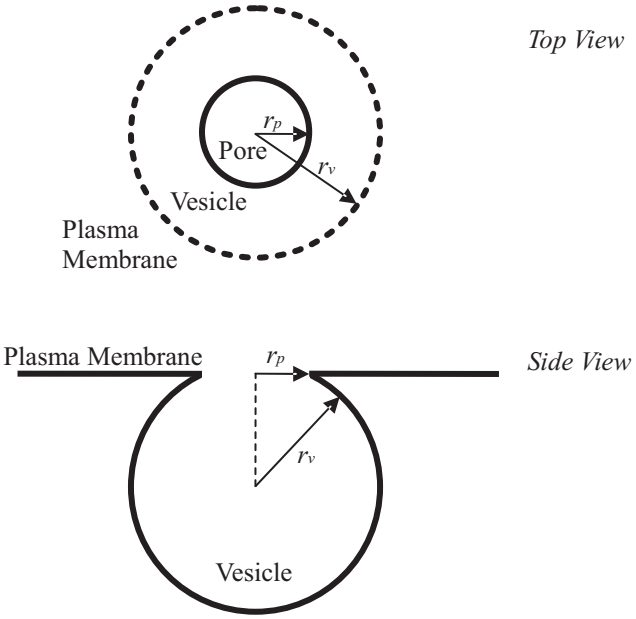

Figure 3: The initial condition of the vesicle fusing with the plasma membrane. The pore, which is the boundary between the vesicle and the plasma membrane is a circle of radius r_p in the plane of the plasma membrane. The vesicle is a sphere of radius r_v, truncated at the pore.

across the pore boundary at rate ϕ (ms^{-1}) (i.e. ϕ square metres of membrane passes per metre of boundary per second). Due to symmetry, the incorporated membrane then forms a ring around the pore in the plane of the plasma membrane.

We consider the initial state to be where the amount of vesicle membrane incorporated into the plasma membrane is the area of the pore itself. When the remaining vesicle area decreases to that of the pore itself, the pore itself is covered and we consider the vesicle to be fully incorporated into the membrane, as in Figure 4.

We are trying to determine the outer radius of the ring as a function of time as the vesicle fuses. From this we will also be able to determine how long it will take for the entire vesicle membrane to be incorporated.

Let the outer radius of the ring of incorporated membrane be r. We can determine the initial value of r by considering that the area of the ring is initially equal to the area of the pore (this being the amount of vesicle membrane incorporated initially).

So

$$\pi r^2 - \pi r_p^2 = \pi r_p^2 r = \sqrt{2} r_p.$$

The increase in the area of the ring, A, is due to the flux of vesicle membrane across the circumference of the pore (and is equal to the decrease in the surface area of the vesicle),

$$\frac{dA}{dt} = \phi 2\pi r_p.$$

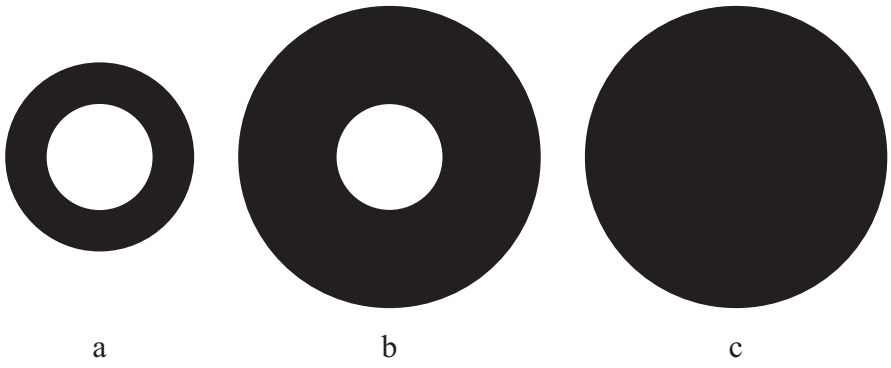

Figure 4: Top view of the vesicle membrane being incorporated into the plasma membrane. The pore, and vesicle below are shown in white, and the incorporated membrane is shown in black. a) The initial view, where the ring of incorporated membrane has an area equal to that of the pore, b) the ring just before the completion of fusion, where all of the vesicle membrane except an amount equivalent to the pore area has been incorporated (note the optical illusion – the white circle representing the pore is the same size in both a) and b)!); c) shows the completed fusion, where the entire surface area of the vesicle has now become a circular area in the planar plasma membrane.

Solving this, noting that the pore radius is constant and the initial area of the ring is πr_p^2,

$$A = 2\pi r_p \int_0^t \phi d\tau + \pi r_p^2.$$

(We have allowed for the possibility here that the flux may not be constant in time).

We can then manipulate this to determine the outer radius of the ring as a function of time:

$A = 2\pi r_p \int_0^t \phi d\tau + \pi r_p^2 = \pi r^2 - \pi r_p^2 r = \sqrt{2r_p \left(\int_0^t \phi d\tau + r_p \right)}$.

So, at what time is the vesicle fully incorporated, t_{fusion}, and what is the outer radius of the ring, r_{max}, at this point? The condition is that the remaining surface area of the vesicle is equal to that of the pore, or conversely that the area of the ring is equal to that of the original surface area of the vesicle, less the area of the pore, that is,

$$A = \pi r_{max}^2 - \pi r_p^2 = 4\pi r_v^2 - \pi r_p^2.$$

So

$$r_{max} = 2R_0$$

and the timing is given by

$$2\pi r_p \int_0^{t_{fusion}} \phi d\tau + \pi r_p^2 = 4\pi r_v^2 - \pi r_p^2$$

$$\int_0^{t_{fusion}} \phi d\tau = \frac{2r_v^2 - r_p^2}{r_p}.$$

If we consider the flux to be constant in time, then the time it takes to fully fuse the vesicle is

$$t_{fusion} = \frac{2r_v^2 - r_p^2}{\phi r_p}.$$

Discussion

In this study we have used an ideal mathematical model to determine the apparent intensity in TIRF imagery of the approach, docking or possible recession of protein embedded vesicles.

Here we have only considered the direct, vertical motion of the vesicles as they approach the underside of the plasma membrane, as this is the main determinant of the perceived surface intensity, however the model is not limited to this, and could as easily be deployed for transverse motions. It is also thought that the protein embedded vesicles may not be undergoing completely free motion within the cell, but may be transported using molecular motors, so this ideal model may have utility in the quantification of these processes using TIRF image sequences.

In reality, we would probably use the model for fusion backwards – measuring the fusion times of vesicles viewed under the microscope, estimating also the size of the vesicle from the radius of the spot formed when fully fused. This gives us insights into the mechanical properties of the membranes themselves – the flux rates relative to the pore sizes formed by the SNARE proteins.

Once the vesicle has fused with the plasma membrane, the embedded proteins then diffuse out of the initial spot onto the rest of the membrane. This can also be modelled, but that's another story!

Thus we can build a "TIRF view" of the vesicle, as it approaches, docks, and recedes from the membrane using equation (0.1). The dynamics of the fusion of the vesicle with the plasma membrane and diffusion of the proteins in the membrane (beyond the scope of this article) can then be combined with this to produce simulated fusion events as shown in Figure 5. As can be seen in the figure, the results are promising, and this ideal system is in use in the development of the automated fusion detector.

Acknowledgements

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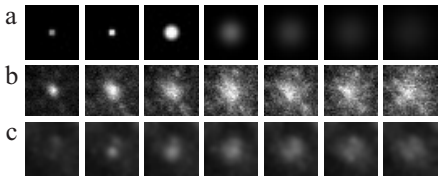

Figure 5: Sequential images of a single vesicle as it docks at and then fuses with the PM — a TIRF view. Sequence a) shows the ideal model, with time and scaling matching the experimental TIRF sequences. These images were additionally smoothed using a Gaussian filter and pixelated to replicate the additional effect of the TIRF microscopy lens and camera system. Sequences b) and c) are TIRF images taken of L6 myoblast cells labelled with GLUT4-eGFP, scale 126.6666 nm/pixel; time course 113 ms between images (images courtesy of the Microscope Imaging Unit at the Garvan Institute of Medical Research, Sydney, Australia).

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