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A novel fluorescence microscopic approach to quantitatively analyse protein-induced membrane remodelling

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Membrane remodelling or the bending and rupture of the lipid bilayer occurs during diverse cellular processes such as cell division, synaptic transmission, vesicular transport, organelle biogenesis and sporulation. These activities are brought about by the localized change in membrane curvature, which in turn causes lipid-packing stress, of a planar lipid bilayer by proteins. For instance, vesicular transport processes are typically characterized by the cooperative recruitment of proteins that induce budding of a planar membrane and catalyse fission of the necks of membrane buds to release vesicles. The analysis of such membrane remodelling reactions has traditionally been restricted to electron microscopy–based approaches or force spectroscopic analysis of membrane tethers pulled from liposome-based model membrane systems. Our recent work has demonstrated the facile creation of tubular model membrane systems of supported membrane tubes (SMrTs), which mimic late-stage intermediates of typical vesicular transport reactions. This review addresses the nature of such an assay system and a fluorescence-intensity-based analysis of changes in tube dimensions that is indicative of the membrane remodelling capacity of proteins.

Keywords.   Fluorescence microscopy; membrane remodelling; membrane tubes

1.   Introduction

The hydrophobic effect confers lipids with the ability to self-assemble into a stable bilayer. The lipid bilayer was chosen over the course of evolution as the material to contain life for its remarkable ability to resist rupture (Antonny *et al.* 2015). Nevertheless, a well-orchestrated and a controlled process of membrane bending and rupture manifests in diverse cellular processes such as cell division, synaptic transmission, vesicular transport, organelle biogenesis and sporulation. In the case of vesicular transport, such a controlled process of bending and rupture or 'membrane fission' typically involves the application of localized curvature stresses to first deform the planar membrane into a bud and later to constrict the neck of the bud to cause scission and release of a vesicle (Antonny 2006). Since these shape changes require the lipid bilayer to deviate from its preferred planar configuration, membrane budding and fission are energetically unfavourable processes, and cells have evolved multiple proteins and mechanisms to catalyse such processes. The identification, understanding and recreation of such mechanisms represent an extensively researched topic in contemporary cell biology. The complex environment of the cell, however, presents a hurdle in discovering and appreciating the design principles by which a singular protein or combinations of proteins manage membrane remodelling. Thus, methods that allow reconstitution of membrane remodelling using purified proteins on model membrane systems of defined compositional and topological complexity as templates have emerged as an attractive approach to address these outstanding questions.

2.   Traditional templates to analyse membrane remodelling

The use of liposomes, the preferred model system for most membrane-based reconstitution studies, has provided a wealth of information on the ability of proteins to cooperatively bind and remodel lipid bilayers (Takei *et al.* 1998; Kinuta *et al.* 2002; Boucrot *et al.* 2012; Inaba *et al.* 2016). Nevertheless, visualizing liposome remodelling requires tedious and often artefact-prone use of electron microscopic methods. The use of alternative model membrane systems has considerably helped overcome many of the challenges inherent in liposome-based assays. Supported lipid bilayers with excess reservoir (SUPER) is an assay system that presents investigators with the option of analysing membrane budding and fission using simultaneous microscopic and biochemical approaches (Pucadyil and Schmid 2008, 2010; Neumann *et al.* 2013).

In contrast to liposomes and SUPER templates, membrane tethers pulled from a giant unilamellar vesicle or SUPER templates using micromanipulators or optical traps have led to a significant advancement in specifically understanding the pathway to membrane rupture and fission (Frolov *et al.* 2003; Bashkirov *et al.* 2008; Roux *et al.* 2010; Morlot *et al.* 2012; Shnyrova *et al.* 2013; Bassereau *et al.* 2014). Membrane tethers provide a convenient option to assess forces exerted on the membrane using force spectroscopy. Using the knowledge of tension in the vesicle and the bending rigidity of the membrane, such methods allow precise estimation of forces necessary to deform the membrane tube (Koster *et al.* 2003). The use of such model membrane systems has significantly contributed to our understanding of the physics of membrane curvature generation (Baumgart *et al.* 2011), mechanism by which proteins sense membrane curvature (Sorre *et al.* 2012) and the pathway to membrane fission (Morlot *et al.* 2012; Shnyrova *et al.* 2013; Renard *et al.* 2015; Simunovic *et al.* 2017).

3. Supported membrane tubes

The traditional methods to form membrane tethers demand a high degree of technical expertise and are experimentally challenging, since they allow monitoring a single membrane tether at a time. In contrast, a simpler method to produce membrane tethers is by subjecting hydrated lipids spotted on a passivated glass coverslip to the flow of buffer (figure 1). The flow-induced extrusion of the lipid reservoir results in the formation of long membrane tubes, which eventually settle down and get pinned on to defects on the glass surface. Our recent efforts have led to the systematic characterization of such templates, referred to as supported membrane tubes (SMrT) (Dar *et al.* 2015; Holkar *et al.* 2015; Pucadyil and Holkar 2016; Dar *et al.* 2017). SMrT templates can be formed from any lipid that can self-assemble into a bilayer, and the initial stages in the formation of these templates appear similar to how vesicles respond to hydrodynamic flow (Rossier *et al.* 2003). In many ways, SMrT templates are architecturally similar to the previously described motor protein-pulled membrane tethers (Roux *et al.* 2002; Koster *et al.* 2003; Leduc *et al.* 2004) but can be set up with far less biochemical complexity, technical skill and expertise.

4.   Quantitative analysis of protein-induced remodelling of tubular membrane templates

Based on the premise that the brightness of a diffraction-limited membrane tube labelled with a fluorescent lipid is directly proportional to the net membrane area within the field of illumination (Kunding *et al.* 2008) (figure 2), wide-field fluorescence microscopy can be used to conveniently and quantitatively measure the extent of change in the radial dimension of the tube in response to membrane remodelling.
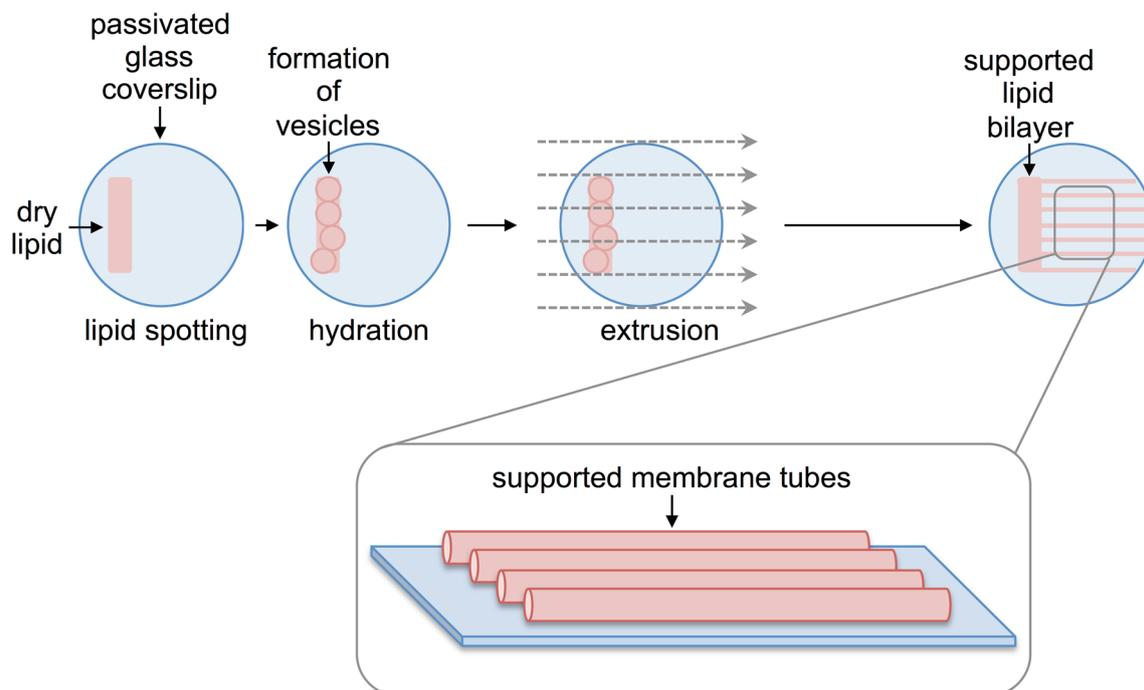
Figure 1.   Schematic of generation of SMrT templates. A lipid mix spotted on a passivated glass coverslip when hydrated inside the flow cell forms large vesicles that are extruded by flow of buffer to generate an array of membrane tubes.
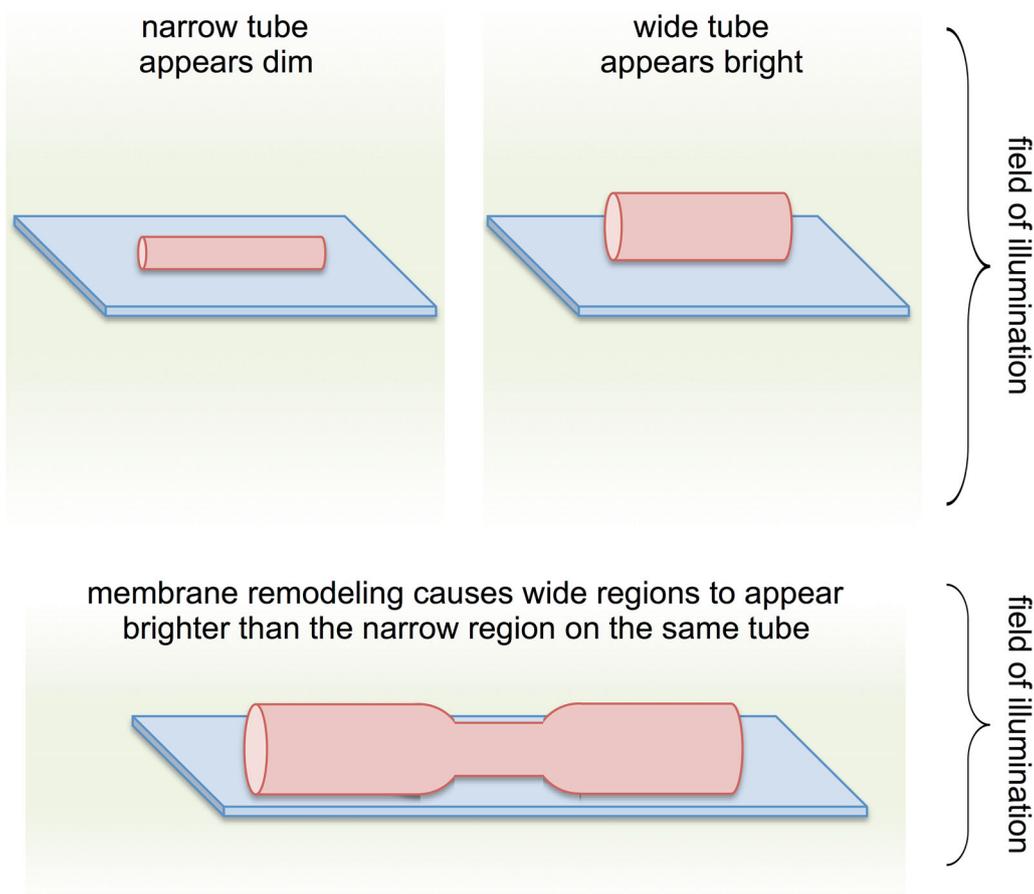
Figure 2. Schematic showing the basis for a fluorescence-intensity-based analysis of membrane remodelling. Fluorescence from a diffraction-limited membrane tube labelled with a fluorescent lipid is proportional to the net membrane area within the field of illumination.

A distinct advantage with SMrT templates is the availability of an *in situ* calibration standard in the form of a planar supported bilayer at the source of every SMrT template preparation using one which can equate fluorescence intensities to membrane area and therefore to tube radius (Pucadyil and Holkar 2016). For this purpose, SMrT templates are labelled with trace amounts of a specific fluorescent lipid probe *p*-Texas Red-DHPE, which displays partitioning properties that are insensitive to membrane curvature and fluorescence properties that are insensitive to protein binding (Jung *et al.* 2009; Hsieh *et al.* 2012).

5. Applications

SMrT templates are housed inside a flow cell, thus making it convenient to flow in proteins and accurately monitor reaction kinetics. Since the membrane tube is supported on a coverslip and not free-standing, out-of-focus movements are reduced and the assay system is amenable to quantitative light microscopic analysis. Contrary to traditional techniques that generate a single membrane tube at a given time, SMrT templates represent an array of membrane tubes and overcome the experimental challenge in recording statistically significant numbers of membrane remodelling events. Due to the ill-defined nature of the lipid reservoir at the source, extrusion by buffer flow results in the formation of tubes with highly variable dimensions. In conjunction with the planar supported bilayer present at source, SMrT templates represent an assay system that displays membrane surfaces with variable degree of curvatures, thus allowing the systematic characterization of how the membrane curvature influences the binding and scaffolding of proteins (Dar *et al.* 2015; Holkar *et al.* 2015; Pucadyil and Holkar 2016). Furthermore, any localized change in tube dimension causes a change in tube fluorescence and allows for the convenient monitoring of membrane remodelling (Dar *et al.* 2015). The arrayed architecture of SMrT templates allows for a high-throughput analysis of membrane fission, which manifests in the tube undergoing scission at multiple points along its length. Unlike free-standing membrane tubes where the first scission event results in tension-induced retraction of the tube, the presence of multiple pinning sites restricts such effects and allows scoring for multiple scission events in a single microscope field (Dar *et al.* 2015).

6. Future perspectives

The vastly simplified lipid compositions used for the design of the assay systems described earlier fail to mimic the compositional heterogeneity inherent in biological membranes. Quite understandably, an important question then is whether such assays misrepresent real biological phenomena. Remarkably, the bulk of literature on reconstitution of protein-induced membrane remodelling, membrane fusion and fission suggests otherwise. In fact, while simplified model membrane systems may fail to recapitulate certain processes, rarely do they misrepresent protein function. This is because model membranes and biological membranes are formed by the same principles of self-assembly and that the two display very similar material properties. So if a cellular process under investigation recognizes or responds to a change in the bulk physical properties such as charge, shape and tension of the membrane, then the assay system described earlier is a good starting point, and it offers the definite possibility of a systematic identification and evaluation of the diversity of proteins that exhibit the capacity to remodel membranes. Naturally, having identified these, the real challenge and excitement is to decipher how such activities manifest in and regulate functions of complex living systems.

Acknowledgements

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