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REVIEW

Functional imaging of microdomains in cell membranes

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Abstract The presence of microdomains or rafts within cell membranes is a topic of intense study and debate. The role of these structures in cell physiology, however, is also not yet fully understood with many outstanding problems. This problem is partly based on the small size of raft structures that presents significant problems to their in vivo study, i.e., within live cell membranes. But the structure and dynamics as well as the factors that control the assembly and disassembly of rafts are also of major interest. In this review we outline some of the problems that the study of rafts in cell membranes present as well as describing some views of what are considered the generalised functions of membrane rafts. We point to the possibility that there may be several different 'types' of membrane raft in cell membranes and consider the factors that affect raft assembly and disassembly, particularly, as some researchers suggest that the lifetimes of rafts in cell membranes may be sub-second. We attempt to review some of the methods that offer the ability to interrogate rafts directly as well as describing factors that appear to affect their functionality. The former include both near-field and far-field optical approaches as well as scanning probe techniques. Some of the advantages and disadvantages of these techniques are outlined. Finally, we describe our own views of raft functionality and properties, particularly, concerning the membrane dipole potential, and describe briefly some of the imaging strategies we have developed for their study.

Keywords Rafts · Membrane microdomains · Imaging · Dipole potential · Signaling · Diffraction limit

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Introduction

What are membrane rafts?

Contemporary pictures of membranes (see e.g. Fig. 1 in O'Shea 2007) necessarily contain many different proteins, lipids and sugars all maintained in a highly regulated manner in intimate contact with both intracellular (cytoskeleton) and extracellular (matrix) structures. This organisation also includes spatial elements as cell membranes are now appreciated to be exceedingly well-organised structures containing localised 'microdomains' deployed laterally along the plane of the membrane that appear to confer added levels of biological control. An excellent review by Hancock (2006) represents a good overview of the complexities of these molecular and macro-molecular components and identifies some of the remaining challenges.

Lipid microdomains in both model and cellular membranes have been discussed for many years but it was a review by Simon and Ikonen (1997) that highlighted their potentially ubiquitous role in cell biology. Ironically, however, it was perhaps their coining of the term 'raft' to identify these microdomain structures in cells that had the most impact and in doing so provided a common forum

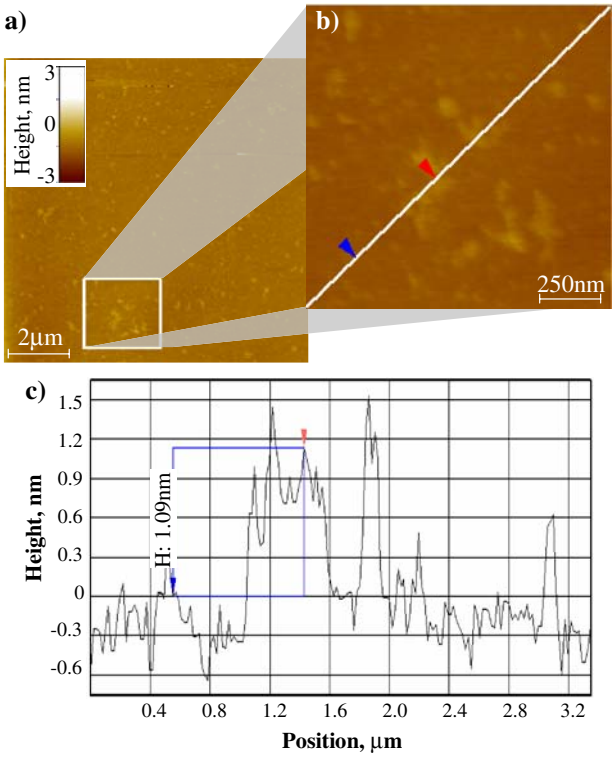

Fig. 1 Atomic force microscope image of membrane microdomains $PC_{80\%}$:$cholesterol_{20\%}$ bilayers. The heights of these raised regions above the bilayer were between 1.0–1.5 nm and therefore the thickness of the bilayer in these regions was ~5–5.5 nm. AFM image of $PC_{80\%}$:$cholesterol_{20\%}$ bilayer. The AFM image **a** is described with the *white box* denoting image **b**. The height differences between the *blue and red triangles* in image **b** are shown in *graph* **c**. All images were taken at room temperature and Tris 10 mM, pH 7.4). The $PC_{100\%}$ bilayers were prepared according to Richardson et al. (2007) and Rigby-Singleton et al. (2006) homogeneous with a bilayer thickness of ~4.0 nm. The microdomain found with $PC_{80\%}$:$cholesterol_{20\%}$ ranged from 55 to 267 nm in diameter and represented ~9.5% of the bilayer. The images were collected with Digital Instruments, (Veeco Metrology Group, Santa Barbara, CA, USA) Multimode and Dimension 3000 AFMs with a Nanoscope IIIa Controllers and silicon nitride cantilevers. The AFM images were height and size analysed using Scanning Probe Image Processor (SPIP) version 2.3232 (Image Metrology, ApS)

for a dialogue between cell biologists and membrane biophysicists. This led to the present consensus that rafts are ubiquitous membrane structures, typically 'detergent-insoluble', 'viscous patches', rich in cholesterol and sphingomyelin and about 40–150 nm in diameter in the plasma membranes of all eukaryotic cells. Rafts appear to be involved in very many biological processes and although they appear to be small in size they may constitute a relatively significant fraction of the plasma membrane. Although this represents a broad consensus it is not without many questions. Shaikh and Edidin (2006), for example, refer justifiably to some membrane structures as "non-raft microdomains" made up of phospholipids with polyunsaturated fatty acids (i.e. rather than cholesterol or sphingomyelin) and also state that a better nomenclature is required to clarify the possible heterogeneity of these membrane structures. For the sake of clarity, in the present article but with the comments of Shaikh and Edidin (2006) on nomenclature in mind, we will refer to membrane microdomains and rafts as essentially the same general structures and only if a particular property needs emphasising will we utilise one or other terms explicitly.

These latter comments illuminate the fact that cell membranes possess cocktails of lipids some of which do not undergo the lipid phase transitions at appropriate temperatures that are thought to predispose membranes to microdomain formation (Brown and London 1997). Coupled to earlier difficulties in purifying 'rafts' this led to trenchant questions on whether their biological existence was really genuine (although see Garner et al. 2007). While rafts are clearly present in artificial membranes, nevertheless there remains a small but intense debate as to whether they do indeed exist in cell membranes (Hancock 2006). This is compounded by the fact that rafts are not easily visualised (i.e. unequivocally) within cells, although they are clearly identifiable as 'microdomains' in model membrane systems (see also Brown 2001; Richardson et al. 2007) as illustrated in Fig. 1. Despite criticism there is a large and growing body of evidence and active research directed towards studies of membrane microdomains in cells (see Pike 2006 and for a comprehensive multi-specialist review see McIntosh 2008). In the present article, we adopt the standpoint that rafts are important and genuine components of cell membranes and will outline some of the problems of visualising them in both the artificial membranes and in living cells. This is done with a view to describe some strategies for making measurements of molecular interactions with and within rafts and providing further pointers to some of their roles in cell biology.

One priority related to all these foregoing comments is the necessity to understand the factors that control raft populations in living cells. The regulation of the formation and disassembly of plasma membrane rafts, however, is as yet, not well understood (we recently published the very first such formal model of this process: Richardson et al. 2007). Some of the problems related to this issue are described later. Similarly, we have explored further complexities in this process and have demonstrated recently that a number of factors appear to be involved, particularly some that involve membrane proteins and other extra-membrane factors (Davis et al. 2008).

What are membrane rafts for?

The importance of the raft paradigm is underlined by the fact that in recent years, it has been one of the most spectacularly productive areas of cell biology in general and

membrane research in particular (Hancock 2006, O'Shea 2005). Membrane microdomains (all types, rafts and 'non-rafts') are implicated in manifold biological functions, generically they are said to lead to a much more exquisite control of localised signaling events. In particular, rafts exhibit a number of features such as recruitment of GPI-linked proteins and are linked to cell signaling and vesicular trafficking including endocytosis/exocytosis and transcytosis as well as exhibiting less desirable characteristics such as acting as sites or targets for infection (e.g. as with HIV, see Cladera et al. 2001).

In terms of acting as localised sites for signaling activity, microdomains or rafts (perhaps in partnership with or structurally involved with the cytoskeleton, see e.g. Lenne et al. 2006) as separate nanoscopic (i.e. submicron) regions in the plasma membrane may help isolate reactants to prevent their interaction with others in the fluid-mosaic membrane or in contrast, they may promote interaction by constraining the reactants in close proximity within the raft domain. In each case proteinacious receptors and smaller ligands or protein–protein interactions underlie a biological response (or not). Thus, rafts may contain incomplete signaling pathways that are activated when a receptor or other required molecule is recruited into the raft. Rafts may also be important in limiting signaling, either by physical sequestration of signaling components (which also serves to limit non-specific interactions) or by suppressing the intrinsic activity of signaling proteins present within rafts. Each of these, however, are conceptually analogous. To put this in the context of the new biological subdiscipline known as *systems biology*, the raft concept offers another parameter (in effect a spatial tensor) with which to model biological control. Thus, a number of approaches to theoretical modelling incorporating this dynamical and structural organisation as a means of understanding signaling networks is an area of intense current study (e.g. Pike 2006; Silvius 2005; Bray et al. 1998).

Over the last few years we have developed a hypothesis that augments the hitherto prevailing view of what rafts do (i.e. sequestration of reactants/proteins, etc.) such that rafts also modulate membrane receptor activity by virtue of their elevated (or decreased) membrane dipole potential (described in O'Shea 2005 and discussed with new examples mentioned later). The membrane dipole potential is a relatively newly understood membrane property that appears to have a profound influence on the behaviour of membrane proteins located in regions of high potential such as that found in membrane rafts (Asawakarn et al. 2001; O'Shea 2003). The arrangements of this potential in a lipid bilayer is illustrated schematically in Fig. 2. The physical origin of the potential resides in the ester linkages of glycerophospholipids but the ceramide link as in sphingolipids and other separated partial charges as well as bound water

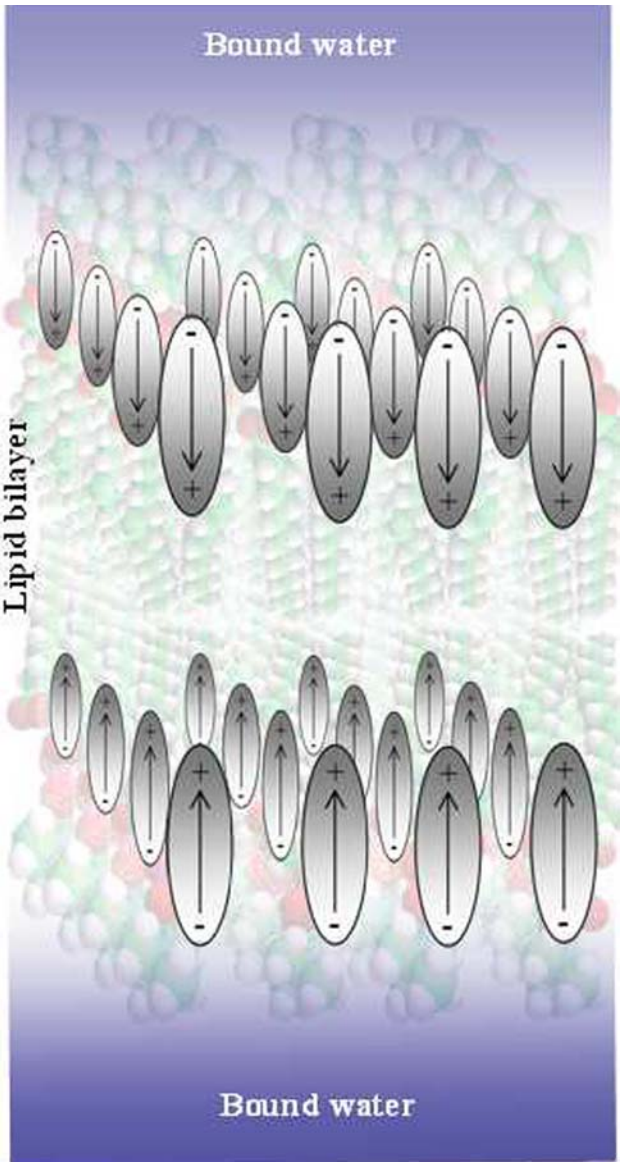

Fig. 2 Schematic arrangement of molecular dipoles in a membrane. The arrangement of the dipole represents a composite of all the charges and partial charges associated with membrane components. It has also been suggested that water molecules close to the membrane also contribute to the dipole potential and illustrated 'bound water'. This 'layer' is likely to be a single or just a few molecules in thickness. The average dipole vector is thought to be arranged at 45° to the membrane normal

appear also to be involved (for a comprehensive review of this membrane potential and its relationships to the others, see O'Shea 2004).

The view that localization of receptor and signaling systems within the raft environment modulates the behaviour of the systems is also taken by other authors (e.g. Bari et al. 2005, Filipp and Julius 2004), although these and most other authors have not implicated the membrane dipole potential as the molecular basis of the functional modulation. In all these cases, however, these suggestions offer an

interesting new concept by which the activities of receptor systems are controlled.

These foregoing research goals underline the many technological demands that cell biology makes; studies of membrane rafts requires spatial information usually from optical imaging and often with a very low photon economy. In other words, both their very small cross-sectional area (and perhaps also their transient dynamics, Hancock 2006) together with few signal photons (e.g. with raft-located fluorophores) may conspire to make studies of membrane raft properties rather difficult. Biological imaging in general, however, has expanded enormously over the last few years both in terms of different imaging modalities (i.e. technologies) as well as many different biological applications. Biological imaging presently and for the future will be required to incorporate the means for multi-parametric measurements at and beyond the diffraction limit through dense and heterogeneous assemblies of living cells. Visualising functionality of membrane rafts, even in single cells (as opposed to many interacting cells), however, presents a number of profound challenges that are not easily circumvented. We outline later some of the imaging-solutions that are available to studies of the functionality of membrane microdomains. The emphasis is placed on the biological experimental demands rather than the imaging technology per se.

Imaging nanoscopic biological structures

To resolve spatially any detail (e.g. spectroscopic) of membrane rafts present major difficulties; the attendant questions of spatial resolution then becomes paramount when considering studies of rafts in vivo. These problems are not just constrained to such membrane-related problems as in general much of biology within cells operates beyond what is known as the Rayleigh–Abbe diffraction limit. This is a physical limit that for a uniformly illuminated objective is given by 0.61λ/NA (λ, wavelength and NA, numerical aperture). Thus, if two objects are illuminated under a laser beam the output is a sum of the point distributions. The Rayleigh criterion is illustrated in the simulations, shown in Fig. 3, indicates that the two points are deemed resolved when the first minimum of one distribution coincides with the maximum of the other, as shown in Fig. 3, and shows two points separated by half the Rayleigh criterion in which the object appears to be a single-extended object. Abbe's approach was slightly different but lends itself to image where the light and illumination are coherent. The Abbe criterion then states that the finest grating that can be imaged corresponds to the diffracted orders *just* passing through a grating. This yields Abbe's criterion of 0.5λ/NA, which is close to that of the Rayleigh criterion. These

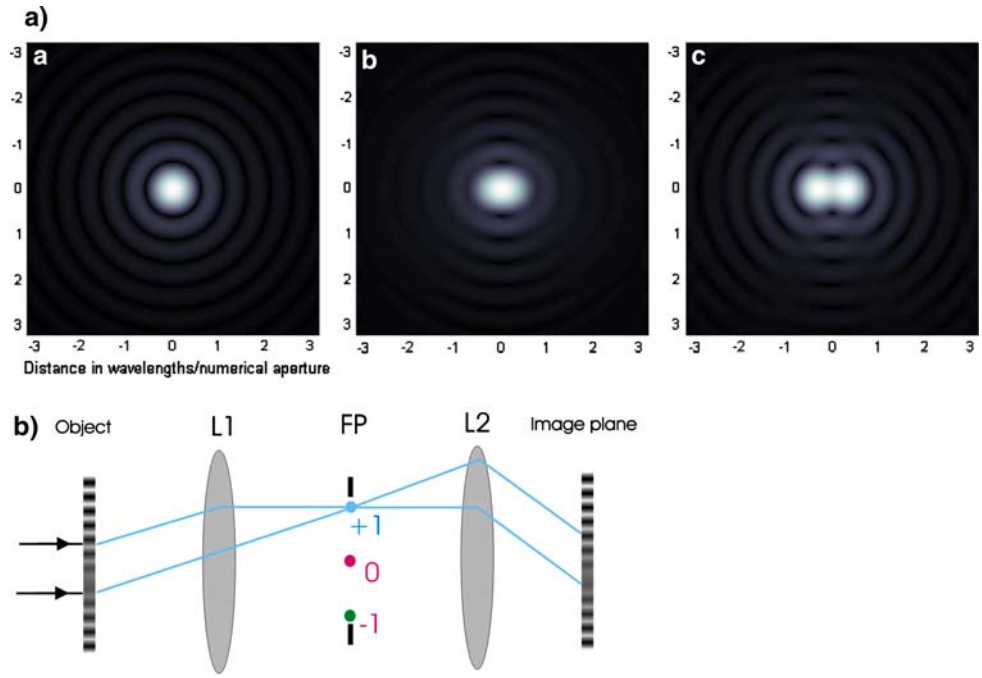

Fig. 3 **a** The Rayleigh resolution criterion. *a* Point spread function from single point object. *b* Two points separated by a distance equal to one half the Rayleigh resolution distance. *c* Two points separated by the Rayleigh distance. Note the contrast has been altered so that the sidebands are visible. **b** The Abbe resolution principle. The incident light (*black*) hits the grating and is diffracted into different diffracted orders. For clarity only the +1 diffracted order is shown in *blue*. These diffracted orders arrive at the so-called Fourier plane at the positions indicated. If the object grating is very coarse the diffracted orders arrive close to the zero order, on the other hand, for a very fine grating the +1 and −1 orders do not pass through the Fourier plane and are blocked. The diagram shows a situation where the diffracted orders just pass through the Fourier plane so they can be collected by L2 to form an image

criteria are both referred to as the 'diffraction-limit'. Their convenience is that they offer different approaches to techniques used to achieve resolution beyond the diffraction limit. Following discussions with Einstein in 1928, Synge, for example, is recorded to be the first to point out that if an aperture smaller than the wavelength was scanned close to the surface of a sample, the light passing through the aperture would not have sufficient distance to diffract before interacting with the sample and passing back through the aperture. This approach now known as NSOM (near-field scanning optical microscopy) achieves resolution of around 25 nm; the difficulties of controlling the probe very close to a sample, however, remain challenging. NSOM, however, is not really suitable for the deeper cellular imaging demands that cell biology also places on imaging technologies nevertheless it hold much promise for biological imaging.

Johnston (2007), for example, outlines the use of NSOM to study microdomains in simple model membranes. The spectacular resolution of NSOM lends itself to imaging membrane microdomains in cells and has been applied by Edidin and co-workers (Hwang et al. 1998) among others. Their analysis indicated membrane microdomain sizes ranging from ~70 to ~600 nm in diameter. With similar aims, we utilised AFM to visualise membrane microdomains (e.g. Richardson et al. 2007) as have several other groups (see e.g. Yuan et al. 2002). Some further discussion of this kind of application is outlined later but for a number of practical reasons (see e.g. O'Shea et al. 2008) microscopy in the far-field is a more desirable means to visualise membrane microdomains in living cells. NSOM is a scanning probe technique akin to atomic force microscopy (AFM) rather than optical microscopy (i.e. microscopy in the far field) but there are several techniques that have resolution comparable to near-field microscopy that have emerged in recent years. While, it is not possible to describe all of them some examples mentioned later are emphasised that could be valuable for studies with membrane microdomains.

Optical techniques that are finding wide use for the study of rafts include FCS (Bacia et al. 2004), FRET (Silvius 2003) and single-molecule approaches (Mashanov and Molloy 2007) and each holds much promise in their different ways. A newer (but as yet with just a few examples) optical far-field method developed by Hell and coworkers (Sieber et al. 2007) has recently been applied to studies of localised behaviour in cell membranes. This technique is referred to as STED (an acronym from stimulated emission and depletion) microscopy and appears extraordinarily powerful. The underlying concept of this method breaches the Rayleigh resolution limit by narrowing the effective width of the imaging spot to below the diffraction limit. The principle is based on manipulating the fluorescence that is used to build up the image as it emerges from a small region from the centre of the diffraction limited spot. This is done by exciting fluorophores in a diffracted limited spot with one beam. A second beam, typically in the form of doughnut shape, is used to de-excite the fluorophores in the outer part of the diffraction limited spot. The process of stimulated emission is a similar process to that which occurs in a laser, where one beam of light causes de-excitation of an excited molecule. In the case of the laser, the purpose of this process is to amplify the light output. In STED microscopy, the purpose is to depopulate the excited states (i.e. STED) but only from the outer parts of the light distribution in the illumination spot. The result of the depletion process is that the only excited molecules remaining are in a small region at the centre of the optical distribution. The resulting fluorescence that arises from spontaneous emission thus comes from a small area, so that the effective spot size is considerably smaller than the diffraction limited spot. This technique can be used to achieve lateral resolution close to the values quoted for the NSOM earlier. The system is compatible with most scanning confocal microscopes and a STED module option is commercially available offering lateral resolution of ca. 50–70 nm. An implementation of STED microscopy to study cell membranes (Sieber et al. 2007) indicates that syntaxins appear to be organised into supra-molecular complexes of the order of 60–70 nm. These early applications indicate that the dynamics of raft formation and architecture are accessible using STED microscopy and it will be of much interest when applied to studies of localised microdomains in other systems.

Finally, an optical method that has recently emerged known as stochastic reconstruction microscopy (STORM), however, offers excellent resolution (Huang et al. 2008; Bates et al. 2007). This technique was first conceived as photo-activated localization microscopy and known by the acronym as PALM by Eric Betzig, based at the Janelia Farm Research Campus in Virginia, USA (see O'Shea et al. 2008). The technique takes advantage of the ability to locate a singular object with far greater precision than one can image two objects (i.e. which is the basis of single-molecule tracking e.g. Mashanov and Molloy 2007). The approach renders fluorophores 'dark' or switches them off with a one beam (usually from a red laser) and switched on with a second beam (green). The imaging process goes through a series of cycles; firstly, the fluorophores are switched off. The second laser is used to switch on some of the fluorophores, the novelty of this implementation is to illuminate for a sufficiently short time so that only a small proportion of the fluorophores are excited i.e. switched on in the field of view. This means that the active molecules are separated by a distance greater than the diffraction limited resolution. The active molecules can then be located with great accuracy by illuminating with the red beam until they are switched off. The result from a single cycle is a

sparse image with a few pixels positioned with very fine precision. By repeating this several times, the random (stochastic) nature of the process of switching the molecules on and off again repeatedly, means that multiple sparse picture of different random points are recovered. Whole image reconstruction simply involves adding all these separate images together, so a more detailed (i.e. heavily populated) fluorescence image can be assembled (Huang et al. 2008). This technique can be used to obtain resolution of the order of 20 nm. Technical problems with this approach, however, reside in the large number of image acquisition cycles necessary which means that the imaging is slow and that the sample is subject to a very high photon dosage.

Imaging and functional analysis of membrane microdomains in:

- (1) artificial membranes
- (2) cells

(1) *Artificial membranes*: Fig. 1 stated earlier illustrates an AFM image of membranes made up from PC and cholesterol (5 mol%); microdomain structures are clearly evident as indicated by the topographical plot in the lower portion of the figure. It is relatively simple, therefore, to prepare membranes made up of various cocktails of lipids to identify the characteristics of the constituent lipids that predispose or oppose the formation of the microdomains. Similar images are possible using fluorescence microscopy as shown in Fig. 4.

These images illustrate membrane properties that are closely related to the longstanding and large body of work that has accumulated documenting the phase transition behaviour of membranes made up of different lipids. The membrane phase transition, however, is an ensemble property detected as the singular 'melting temperature' (e.g. Silvius 1982) or change in membrane microviscosity (Hwang and Li 1999). Whereas, the membrane microdomain population is essentially a local (spatially) property (i.e. as shown in the Fig. 1, 4). Clearly any formal model requires both thermodynamic and kinetic (i.e. diffusional) parameters to accommodate experimental data that includes spatial co-ordinates. This was the approach we took to establish the effect of the (e.g.) cholesterol/phospholipid (PC) ratio on the number and size of the microdomains. It was of interest also to identify whether microdomains of discrete sizes would exist i.e. as separately defined populations. In fact our model was able to predict that no singular or discrete (i.e. spatially defined) populations based on size would exist, rather than a continuum of microdomain sizes exists or at a given cholesterol/PC ratio. We also considered whether a steady-state model based on thermodynamic

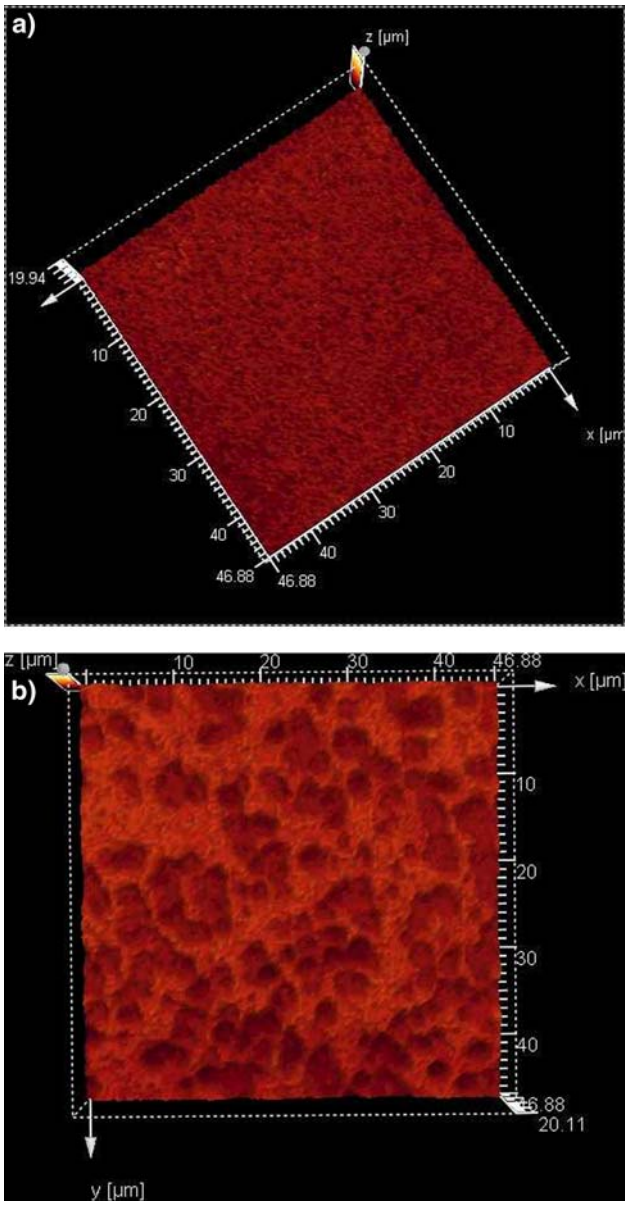

Fig. 4 Fluorescence images of phospholipid bilayers exhibiting **a** no microdomains and **b** microdomains. Fluorescent micrograph images of model membrane bilayers labelled with FPE (excitation 488 nm, emission collected at 520 nm). Two images are illustrated, the upper image **a** is obtained with a pure PC membrane labelled with FPE (at 0.2 mol%). The lower image **b** is obtained from membranes containing $PC_{80\%}$:$cholesterol_{20\%}$ also labelled with FPE (at 0.2 mol%). The fluorescence contrast in image **b** arises from the partial exclusion of the fluorescent phospholipid FPE from the microdomains

considerations can be formulated to describe our experimental results. It became obvious, however, that attempts to formulate a steady-state model to describe the experiments, since all experimental measurements made were at steady state. We found that the predictions of such thermodynamics based models could not explain the experimentally observed distributions of microdomain sizes. Thus, a

simple thermodynamic approach based on minimization of the Gibbs free energy for the system of cholesterol clusters was not appropriate for our most simple of systems, essentially because it does not take into account the different mobilities of the clusters of molecules, which exhibit hugely varying sizes in such systems. We also noted that there are other circumstances in which it is inappropriate to apply equilibrium thermodynamics to determine equilibrium constants (i.e. diffusion-limited reactions). AFM approaches, however, are essentially only able to resolve long-lived microdomain structures, whereas there is evidence that some microdomains are short-lived (i.e. less than a second) labile structures with their own attendant properties that have biological relevance (reviewed by Hancock 2006).

The length scale explored by the AFM imaging technique is vastly superior to those available to fluorescence microscopy which are of course limited by the optical diffraction-limit described above. In terms of data collection, however, AFM has not been wholly successful due to more practical limitations rather than any theoretical restrictions. In particular, in our hands AFM did not lend itself easily for interrogating large areas of membrane due to slow scan rates. Predicted scan rates of more-advanced instruments however would seem to offer far better possibilities for such studies (M. Miles, Bristol, UK; personal communication). This is not a problem with fluorescence-based methods of imaging artificial membrane microdomains, with many novel approaches developed including some in our own laboratories (Goh et al. 2005). One virtue of fluorescence approaches over AFM, therefore, is that often the field of image view in the latter is large and even if data averaging for image acquisition is necessary, a large area of membrane may not be interrogated (see Richardson et al. 2007). This is not a problem with far-field optical (i.e. fluorescence) imaging for which large fields of view are typical, although there are other problems that tend to dominate the practical aspects of cellular (membrane) imaging. When visualizing membrane microdomains using fluorescence, for instance, the signal to noise ratio (SNR) and particularly the origins of image contrast are very important considerations. With cellular membranes, this may not be a major problem as fluorescent antibodies to raft-resident proteins may be employed (e.g. see later). In other words, if the fluorescent antibodies target epitopes solely located within rafts very selectively then there will be little background fluorescence and detection can be optimized with the limits based solely on the detection of the emitted photons. With artificial membranes, however, discriminating factors are less easy to identify. It would be necessary, for example, that a fluorophore sequestrates either selectively into the raft domain or into the fluid-mosaic membrane in order that contrast in the image can be observed. Fortunately, there are some remedies to this problem, Ishitsuka et al. (2005), for example, offer a nice approach using fluorescent lipids. Fluorescent-cholesterol analogues appear also to suffice (e.g. dehydroergosterol, DHE see e.g. Parker et al. 2004) to illuminate cholesterol-rich domains as shown in Fig. 5. This essentially 'lights-up' the microdomain but some DHE also occupies the fluid-mosaic membrane as well which would then be considered 'noise'. Nevertheless, the SNR is still very acceptable and microdomain imaging is feasible. If measurements of the number density, size, etc., are sought (see Richardson et al. 2007) then that would be an appropriate method of improving image contrast, although the diffraction limit remains in force. By time-resolving measurements of local fluorescence anisotropy, utilizing acceptor–donor pairings for FRET (e.g. DHE and DPH by Parker et al. 2004) and assembling images, it is also possible to determine the lipid fluidity of the microdomains and lipid exchange dynamics. This would be a function of local cholesterol concentration as well as the degree of acyl chain unsaturation and so is quite revealing. If a more detailed identification of microdomain functionality is sought, however, then DHE does not yield such information. Knowledge of other membrane properties deemed desirable would include some information of surface charge/potential and/or membrane dipole potential. With this in mind, we developed the use of fluorophores such as fluoresceinphosaphatidylethanolamine (FPE) and di-8-anepps to label membranes at indicator concentrations to measure the membrane surface and dipole potentials, respectively, particularly with a view to determine molecular interactions with membranes (O'Shea 2003; Cladera and O'Shea 2001). FPE may be preferentially located within rafts or in the fluid-mosaic membrane, for example, as it is essentially a user-prepared fluorescent phospholipid (see Wall et al. 1995a). By manufacturing this molecule with either saturated or unsaturated acyl chains of defined lengths, it is possible to locate preferentially within rafts or not depending on the experimental goals as shown in Fig. 4a and b and in a similar manner to that described by Ishitsuka et al. (2005). The added virtue however is that the spectroscopic yield from this essentially non-invasive probe offers information on the local dielectric constant adjacent to the membrane surface and the membrane surface electrostatic potential (see Wall et al. 1995a). This probe was designed, however, as a tool to measure intermolecular interactions with both the artificial (see Wall et al. 1995a) and cellular membranes (see Wall et al. 1995b) and so in addition facilitates measurement of such quantities with rafts (e.g. Asawakarn et al. 2001). This is regularly utilized with living cell membranes (Wall et al. 1995b) and also when implemented within fluorescence imaging systems for studies of molecular interactions with rafts/microdomain within cells (Cladera et al. 2001).

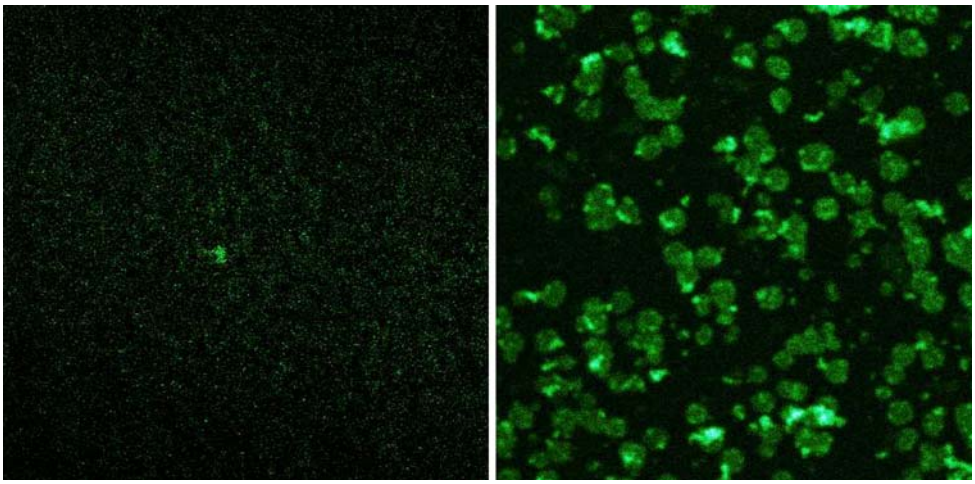

Fig. 5 Fluorescence images of cholesterol in phospholipid bilayers exhibiting microdomains. Fluorescent micrograph images of model membrane bilayers labelled with DHE (excitation was set at 325 nm and emission at 375 nm) see eg. Parker et al. (2004). Two images are illustrated, the LHS image was obtained with membranes containing $PC_{67\%}$:$cholesterol_{33\%}$ (at 0.5 mol%). The RHS image represents a zoomed region of the LHS image to show more clearly the 'cholesterol-rich' microdomains

Related to these approaches and in order to improve image quality for functional studies of rafts, we have also a development programme for imaging technologies that has included multi-photon surface-wave microscopy (e.g. Goh et al. 2005) as well as TIR and TIRF techniques (Byrne et al. 2008).

(2) *Imaging membrane microdomains in cells*: Many of the same techniques utilised to visualise microdomains in artificial membranes may also be brought to bear on living cells (see e.g. Ishitsuka et al. 2005). This is very satisfying as it allows direct comparison between model and real systems. Unfortunately, there are still a number of problems, not least as sometimes the results from these respective experimental systems appear to be contradictory. The difference between cell membranes and model artificial membranes reside in the presence of proteins in cell membranes, some authors suggest, therefore, that this underlies the different properties of the respective experimental systems (see e.g. Nichols 2005). Imaging studies tend, therefore, to be undertaken in one or the other of these systems, although a number of laboratories such as Schwille and co-workers (Bacia et al. 2004) like ourselves (e.g. Richardson et al. 2007, O'Shea 2003) strive to utilise both systems to make comparisons.

A number of techniques have evolved for cellular raft/microdomain study, AFM and NSOM techniques (Hwang et al. 1998) as mentioned earlier, Schwille and co-workers utilise fluorescence correlation spectroscopy (FCS) and of course multi-photon techniques lend themselves to their identification (Gaus et al. 2003). Pralle et al. (2000) suggest, for example, that rafts in cell membranes exist at around 26 ± 13 nm in diameter. This is clearly not quite the same picture that is obtained with model systems (e.g. Fig. 1) in which the microdomain size varies from a few molecules (i.e. a few nm) to much larger assemblies approaching sub-micron dimensions. A more recent study concurs in part with both these assertions and points to additional complexity in the cellular system. Thus, Hess et al. (2007) using fluorescence photo-activation localization microscopy (PALM see later), were able to image distributions of tens of thousands of hemagglutinin (HA) molecules at sub-diffraction resolution (approx. 40 nm) in live and fixed fibroblasts. The HA molecules were found to form irregular clusters on length scales from approx 40 nm (i.e. in agreement with Pralle et al. 2000) up to many micrometers implying these clusters represent rafts of similar dimensions.

The approach our research group has taken to visualise membrane rafts in cells has been to take advantage of their elevated membrane dipole potential above that of the surrounding membrane lipid. Thus, a fluorescent probe that responds to the membrane dipole potential such as di-8-anepps will exhibit contrast between raft domains and fluid-phase membrane on that basis (O'Shea 2003, 2005). We considered this particularly useful as we suggested that this relatively recently understood membrane concept would modulate protein conformation within membranes (see e.g. Cladera and O'Shea 1998). As this membrane potential is much larger in the microdomain than in the fluid phase membrane, we suggested that migration of membrane proteins to and from rafts could well be an important principle in cellular control (O'Shea 2005). An example of an image accrued in this manner is shown in Fig. 6. These images are relatively stable over long periods of time and so indicate that the microdomains are relatively stable over time. The added advantage of utilising an indicator of the elevated

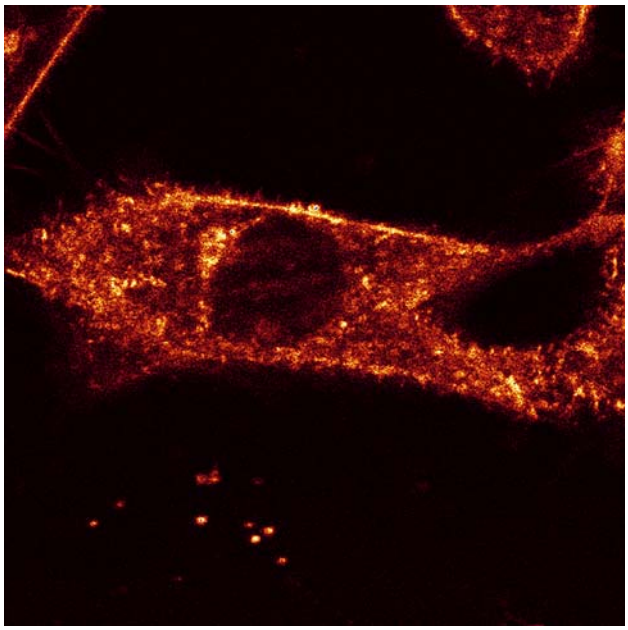

Fig. 6 Functional imaging of microdomains in cells. A fluorescent image of a single fibroblast labelled with di8-anepps and acquired using laser scanning confocal microscopy (excitation 520 nm emission collected at 580 nm). The contrast in the image arises from effects of membrane dipole potential on fluorescence. Regions of greatest intensity are most likely associated with regions of elevated membrane dipole potential found in membrane microdomains (Asawakarn et al. 2001; O'Shea 2003). Thus this image indicates that such structures are also prevalent in the intracellular membranes

levels of dipole potential in membrane microdomains, however, is that when molecules interact with the microdomains they cause small changes in the dipole potential and so their interaction can be observed (Asawakarn et al. 2001). The technique our laboratory pioneered by utilising small changes of the membrane electrostatic potential as an indicator of molecular interactions is also available for similar such studies (Cladera et al. 2001). We have exploited both these technologies for a number of years (e.g. O'Shea 2003, 2005) but the power of it is that we can compare regional differences in the same cell by imaging the molecular interactions.

Is the assembly and disassembly of cell membrane microdomains, lipid (sterol) or protein mediated?

The origin of membrane microdomain structures is considered by most researchers to be most logically a consequence of or to lie somewhere within the phase behaviour of membrane lipids. Thus, in addition to conventional liquid crystalline (l_c) phases (Ayuyan and Cohen 2008), membranes also exhibit cholesterol-spingomyelin rich liquid-ordered (l_o) phases. The l_o phase is characterized by a high degree of acyl chain order and high-T_m (T_m = transition i.e. melting temperature) lipids with saturated acyl chains. These properties lead to these phases being to some extent resistant to extraction using non-ionic detergents such as Triton X-100 or Brij98 at lower temperatures. Detergent-resistant membranes (DRMs) were found to be enriched in sphingolipid–cholesterol l_o microdomains. Analysis of DRMs from cellular systems are useful indicators of proteins associating with rafts but the experimental system have received much critical examination (see McIntosh 2008 for reviews). The correlation between detergent resistance and domain formation in vivo, however, remains a topic of trenchant debate. The simplicity of artificial membranes may not be wholly appropriate as models for cell membranes that are rich in protein and have two asymmetric layers each with many different types of lipid. Other suggestions include the possibility of raft-forming lipids such as cholesterol being delivered to the membrane via cholesterol transport systems (e.g. Orlowski et al. 2007). We take the view, however, that all such possibilities may well feature in cell membranes, perhaps with different mechanisms being responsible for microdomains possessing different properties. For as outlined later, it seems that it is difficult to generalise the properties of rafts/microdomains.

Some studies have indicated lipid rafts (or some populations of lipid rafts) become rapidly redistributed and assemble locally in response to extracellular signals and how components of some raft-based signaling domains undergo rapid and regulated rearrangements. These types of behaviour appear to influence signal quality, duration and strength, particularly in neuronal systems (e.g. Golub et al. 2004). Such interpretations highlight the potential role of the dynamical properties of signaling domains resident within lipid rafts, and suggest that processes of raft trafficking and assembly take central roles in mediating spatial and temporal control of signaling. These views, however, are slightly inconsistent with some imaging studies in which raft populations appear much more long-lived. This points, therefore, to the possibility that not all cellular rafts are alike (see also later) and that we still do not understand fully what rafts really are!

It must be conceded that there remains several major questions regarding the mechanisms employed in cell biology that facilitate the assembly and disassembly of membrane rafts with much of the existing data making generalisations rather difficult. Thus, for example, the increased import of cholesterol and/or sphingomyelin would in all likelihood lead to a greater surface area of the plasma membrane exhibiting raft-like structure (e.g. Orlowski et al. 2007; Baumgart et al. 2007). This would not, however, offer spatial control over the locality of where the rafts would appear and suggests that in addition we

should look for more sophisticated mechanisms. It might seem that it is not simply a matter of changing the overall (i.e. total) level of cholesterol and/or sphingomyelin in the plasma membrane that is the predominant factor in their biological control (O'Shea 2005). On the other hand, recent work reported by Baumgart et al. (2007) indicates large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles indicates that such mechanisms will occur if appropriate conditions are set up. Collins and Keller (2008) show that mechanisms of microdomain induction between the outer and inner leaflets of membranes do not necessarily require the participation of membrane proteins. This is interesting as it addresses the question of trans-bilayer communication as well as the need (or not) of membrane proteins in the manufacture of membrane rafts. Clearly, there are many important questions in need of resolution if we are to grasp fully the nature of membrane microdomain functionality in cellular membranes. Recent studies in our laboratory directed towards these interesting questions, particularly regarding the identity of molecular or physical factors that control the assembly and disassembly of cell membrane microdomains also raises a number of potential answers to some of these outstanding questions.

These latter comments are based on some work that is in the process of being published (Davis et al. 2008) in which we investigate the effects of glycosaminoglycans on the membrane organisation. The basis of this is relevant to the case of microdomain (i.e. possibly not raft-oriented) signaling through T-cells (Davis et al. 2004). At the start of an immune response, T-cells are activated when antigen molecules bind to the receptors on their surface. Stimulation of T-cells usually occurs when stable contacts referred to as 'immunosynapses' that form between the T-cell and so-called antigen-presenting cells. There is a striking degree of spatial organisation within the synapse; for example, molecules involved in the adhesion of the interacting cells, and activators and inhibitors of the signaling cascade, segregate and take on highly specific patterns. Moreover, because several of the proteins recruited to the T-cell synapse are found in detergent-resistant membrane fractions, various functions have been ascribed to lipid rafts in organizing these proteins during T-cell-receptor signaling (Drbal et al. 2007). This instance of a signaling machine in which the appropriate spatial distribution of its components is likely to be central to its function is a promising place to look for direct evidence of physiological mechanisms of lipid microdomain function. This adds to a growing body of knowledge that implies that there are many types of lipid micodomain with many different functions; some may be lipid mediated and some may be protein mediated. Whether we call each of these rafts or microdomains possessing different 'flavours', however, is in need of rationalisation such that a coherent nomenclature is agreed and established and a constructive dialogue promoted and not hindered. Thus, there may well be a variety of different types of membrane microdomain present within biological membranes and this may well be the origin of the present uncertainty within the academic community.

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References

Asawakarn T, Cladera J, O'Shea P (2001) Effects of the membrane dipole potential on the interaction of saquinavir with phospholipid membranes and plasma membrane receptors of caco-2 cells. J Biol Chem 276:38457–38463. doi:10.1074/jbc.M103269200

Ayuyan AG, Cohen FS (2008) Raft composition at physiological temperature and pH in the absence of detergents. Biophys J 94:2654–2666. doi:10.1529/biophysj.107.118596

Bacia K, Scherfeld D, Kahya N, Schwille P (2004) Fluorescence correlation spectroscopy relates rafts in model and native membranes. Biophys J 87:1034–1043. doi:10.1529/biophysj.104.040519

Bari M, Battista N, Fezza F, Finazzi-Agrò A, Maccarrone M (2005) Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells implications for anandamide-induced apoptosis. J Biol Chem 280:12212–12220. doi:10.1074/jbc.M411642200

Bates M, Huang B, Dempsey GT, Zhuang X (2007) Multicolor super-resolution imaging with photo-switchable fluorescent probes. Science 317:1749–1753

Baumgart T, Hammond AT, Sengupta P, Hess ST, Holowka DA, Baird BA et al (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. Proc Natl Acad Sci USA 104:3165–3170. doi:10.1073/pnas.0611357104

Bray D, Levin MD, Morton-Firth CJ (1998) Receptor clustering as a cellular mechanism to control sensitivity. Nature 393:85–88. doi:10.1038/30018

Brown DA (2001) Seeing is believing: visualization of rafts in model membranes. Proc Natl Acad Sci USA 98:10517–10518. doi:10.1073/pnas.191386898

Brown DA, London E (1997) Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem Biophys Res Commun 240:1–7. doi:10.1006/bbrc.1997.7575

Byrne GD, Pitter MC, Falcone FC, Stolink S, Somekh MG (2008) Total internal reflection microscopy for live imaging of cellular uptake of sub-micron non-fluorescent particles. J Microsc 231:168–176

Cladera J, O'Shea P (1998) Intramembrane molecular dipoles affect the membrane insertion and folding of a model amphiphilic peptide. Biophys J 74:2434–2442

Cladera J, O'Shea P (2001) Generic techniques for fluorescence measurements of protein–ligand interaction; real-time kinetics and spatial imaging. Protein–ligand interactions. Oxford University Press

Cladera J, Martin I, O'Shea P (2001) The fusion domain of HIV gp41 interacts specifically with heparan sulfate on the T-lymphocyte cell surface. EMBO J 20:19–26. doi:10.1093/emboj/20.1.19

Collins MD, Keller SL (2008) Tuning lipid mixtures to induce or suppress domain formation across leaflets of. Proc Natl Acad Sci USA 105:124–128. doi:10.1073/pnas.0702970105

Davis DM, Dustin ML (2004) What is the importance of the immunological synapse? Trends Immunol 25:323–327. doi:10.1016/j.it.2004.03.007

Davis B, Duggan J, Horn E, Vere K Richens J, O'Shea P (2008) Modulation of leucocyte plasma membrane microdomains by heparan sulfate. Cell (submitted)

Drbal K, Moertelmaier M, Holzhauser C, Muhammad A, Fuertbauer E, Howorka S et al (2007) Single-molecule microscopy reveals heterogeneous dynamics of lipid raft components upon TCR engagement. Int Immunol 19:675–684. doi:10.1093/intimm/dxm032

Filipp D, Julius M (2004) Lipid rafts: resolution of the "fyn problem"? Mol Immunol 41:645–656. doi:10.1016/j.molimm. 2004.04.011

Gaus K, Gratton E, Kable EP, Jones AS, Gelissen I, Kritharides L, Jessup W (2003) Visualizing lipid structure and raft domains in living cells with two-photon microscopy. Proc Natl Acad Sci USA 100:15554–15559

Goh JYL, Somekh MG, See CW, Pitter M, Vere K, O'Shea P (2005) Two-photon fluorescence surface wave microscopy. J Microsc 220:168–175. doi:10.1111/j.1365-2818.2005.01525.x

Golub T, Wacha S, Caroni P (2004) Spatial and temporal control of signaling through lipid rafts. Curr Opin Neurobiol 14:542–550. doi:10.1016/j.conb.2004.08.003

Hancock JF (2006) Lipid rafts: contentious only from simplistic standpoints. Nat Rev Mol Cell Biol 7:456–462. doi:10.1038/nrm1925

Hess ST, Gould SJ, Gudheti MV, Maas SA, Mills KD, Zimmerberg J (2007) Dynamic clustered distribution of hemagglutinin resolved at 40 nm in living cell membranes discriminates between raft theories. Proc Natl Acad Sci USA 104:17370–17375

Huang C-H, Li S (1999) Calorimetric and molecular mechanics studies of the thermotropic phase behavior of membrane phospholipids. Biochim Biophys Acta 1422:273–307

Huang B, Wenqin Wang, Mark Bates, Xiaowei Zhuang (2008) Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. Science 319:810 –813

Hwang J, Levi A, Gheber LA, Margolis L, Edidin M (1998) Domains in cell plasma membranes investigated by near-field scanning optical microscopy. Biophys J 74:2184–2190

Ishitsuka R, Sato SB, Kobayashi T (2005) Imaging lipid rafts. J Biochem 137:249–254. doi:10.1093/jb/mvi041

Johnston LJ (2007) Nanoscale imaging of domains in supported lipid membranes. Langmuir 23:5886–5895. doi:10.1021/la070108t

Lenne P-F, Wawrezinieck L, Conchonaud F, Wurtz F, Boned A, Guo X-J et al (2006) Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. EMBO J 25:3245–3256. doi:10.1038/sj.emboj.7601214

Mashanov GI, Molloy JE (2007) Automatic detection of single fluorophores in live cells. Biophys J 92:2199–2211. doi:10.1529/biophysj.106.081117

McIntosh TJ (ed) (2008) Lipid rafts, Vol. 398: methods in molecular biology™ series. Humana Press, Totowa

Nichols B (2005) Cell biology: without a raft. Nature 436:638–639. doi:10.1038/436638a

O'Shea P (2003) Intermolecular interactions with/within cell membranes and the trinity of membrane potentials: kinetics and imaging. Biochem Soc Trans 31:990–996

O'Shea P (2004) Membrane potentials; measurement, occurrence and roles in cellular function. In: Walz D et al (eds) Bioelectrochemistry of membranes. Birkhauser Verlag, Switzerland, pp 23–59

O'Shea P (2005) Physical landscapes in biological membranes: physico-chemical terrains for spatio-temporal control of biomolecular interactions and behaviour. Philos Trans R Soc A 363:575–588. doi:10.1098/rsta.2004.1509

O'Shea P (2007) Membrane potentials and membrane probes. In: Larijani et al (eds) Chemical biology. Wiley, pp 67–84

O'Shea P, Somekh MG, Barnes WL (2008) Shedding light on life: visualising nature's complexity. Phys World 21:29–34

Orlowski S, Coméra C, Tercé F, Collet X (2007) Lipid rafts: dream or reality for cholesterol transporters? Eur Biophys J 36:869–885. doi:10.1007/s00249-007-0193-8

Parker A, Miles K, Cheng KH, Huang J (2004) Lateral distribution of cholesterol in dioleoylphosphatidylcholine lipid bilayers: cholesterol–phospholipid interactions at high cholesterol limit. Biophys J 86:1532–1544

Pike LJ (2006) Rafts defined. A report on the keystone symposium on lipid rafts and cell function. J Lipid Res 47:1597–1598. doi:10.1194/jlr.E600002-JLR200

Pralle AP, Keller E-L, Florin K, Simons, Hörber JKH (2000) Sphingolipid–cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol 148:997–1008. doi:10.1083/jcb.148.5.997

Richardson G, Cummings LJ, Harris H, O'Shea P (2007) Towards a mathematical model of the assembly and disassembly of membrane microdomains: comparison with experimental models. Biophys J 92:4145–4156. doi:10.1529/biophysj.106.090233

Rigby-Singleton S, Davies MC, Harris H, O'Shea P, Allen S (2006) Visualizing the solubilization of supported lipid bilayers by an amphiphilic peptide. Langmuir 22:6273–6279

Shaikh SR, Edidin MA (2006) Membranes are not just rafts. Chem Phys Lipids 144(1):1–3

Sieber JJ, Willig KI, Kutzner C, Gerding-Reimers C, Harke B, Donnert G, Rammner B, Eggeling C, Hell SW, Grubmüller H, Lang T (2007) Anatomy and dynamics of a supramolecular membrane protein cluster. Science 317:1072–1076. doi:10.1126/science.1141727

Silvius JR (1982) Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins, in lipid-protein interactions. Wiley, New York

Silvius JR (2003) Fluorescence energy transfer reveals microdomain formation at physiological temperatures in lipid mixtures modeling the outer leaflet of the plasma membrane. Biophys J 85:1034–1045

Silvius JR (2005) Lipid microdomains in model and biological membranes: how strong are the connections? Q Rev Biophys 38:373–383. doi:10.1017/S003358350600415X

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572. doi:10.1038/42408

Wall J, Golding CA, Van Veen M, O'Shea P (1995a) The use of fluoresceinphosphatidylethanolamine (FPE) as a real-time probe for peptide-membrane interactions. Mol Membr Biol 12:183–192. doi:10.3109/09687689509027506

Wall J, Ayoub F, O'Shea P (1995b) Interactions of macromolecules with the mammalian cell surface. J Cell Sci 108:2673–2682

Yuan C, Furlong J, Burgos P, Johnston LJ (2002) The size of lipid rafts: an atomic force microscopy study of ganglioside GM1 domains in sphingomyelin/DOPC/cholesterol membranes. Biophys J 82:2526–2535