One of the most intriguing issues in membrane biology is whether non-caveolar glycosphingolipid (GSL)-enriched domains actually exist in the plane of the plasma membrane. One reason for the high interest in the domains is that, if they exist, they would underscore the importance of lateral organization in the plasma membrane for complex activities such as signal transduction. It was postulated initially that lipid domains in Golgi membranes act as an apical sorting device in epithelial cells—based on the resistance of specialized lipid fractions and apically directed glycosylphosphatidylinositol (GPI)-anchored proteins to extraction with cold non-ionic detergent. Whether such domains actually exist in vivo has been debated vigorously since the inception of the idea. A number of different acronyms have been attached to the operationally defined membrane fractions, including DRM (detergent-resistant membranes), DIGs (detergent-insoluble glycolipid-enriched membrane domains), and several different forms of transport. However, such fractions can also be isolated from cells not exhibiting overt caveolae or expressing caveolin. This, together with the fact that the size of detergent-resistant membranes as established by electron microscopy is often much larger than individual caveolae suggests that caveolae are a subclass within the operationally defined detergent-resistant fractions.

It is certainly plausible that non-caveolar, GSL-enriched plasma membrane domains with a distinct lipid composition could form. Because of their special acyl chain compositions (long chains, very little unsaturation), GSLs, sphingomyelins and saturated phospholipids would preferentially associate laterally with themselves and with cholesterol. Indeed, in model membranes enriched in sphingomyelin and cholesterol, these components form a detergent-resistant liquid-ordered phase in which the acyl chains are ordered but the components remain in the plane of various membranes is simply tempting. In principle, microscopy should be able to determine whether the postulated rafts exist. This article focuses on recent microscopy experiments addressing this question. Several, but not all, results support the raft concept, but further definition of the structure, dynamics and function of lipid domains in various biological contexts is urgently required.

The notion that microdomains enriched in certain specialized lipids exist in membranes has been both attractive and controversial since it was first proposed that such domains, termed rafts, might act as apical sorting devices in epithelial cells. The observation that certain lipids are not extractable in cold non-ionic detergent supports the raft concept, but the nature of the in vivo correlate of such detergent-resistant membranes remains enigmatic. In principle, microscopy should be able to determine whether the postulated rafts exist. This article focuses on recent microscopy experiments addressing this question. Several, but not all, results support the raft concept, but further definition of the structure, dynamics and function of lipid domains in various biological contexts is urgently required.

Looking at lipid rafts?

Ken Jacobson and Christian Dietrich

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In principle, modern light-microscopic methods should provide key information on whether there is an *in vivo* correlate of the non-caveolar detergent-resistant membrane fraction. At this juncture, however, the answer is not completely clear because several recent papers come to opposite conclusions regarding the existence of rafts. In this article, we review some recent microscopy studies and their impact on the raft hypothesis.

**Recent microscopic evidence for rafts**

A number of recent light-microscopy papers that employ sophisticated strategies provide evidence supporting the raft concept. In an elegant study, Stauffer and Meyer\(^2\) produced green fluorescent protein (GFP)-tagged Src-homology 2 (SH2) domain constructs that transiently translocated from the cytoplasm to GM1-rich regions in the plasma membrane upon activation of rat haemopoetic leukaemia cells with antigen. Concomitant and transient redistribution of the Igf receptor to the GM1-enriched domains was also observed. This presumably reflects the recruitment of appropriate signalling kinases to such domains and would therefore provide an *in vivo* correlate of biochemical studies showing translocation of antigen-activated Igf receptor to detergent-resistant membranes enriched for the kinase Lyn\(^2\). In addition, Harder et al.\(^2\) have performed studies on BHK cells expressing, by transfection, a variety of GPI-anchored and transmembrane proteins. They showed that GPI-anchored proteins, such as placental alkaline phosphatase and Thy-1, colocalize with GM1-enriched domains. Transmembrane proteins, such as vta haemagglutinin, which are isolated in the detergent-resistant membranes, also colocalize in a patched pattern with GPI-anchored proteins, but others, for example the transferrin receptor, which are detergent-extractable, do not copatch. Copatching of components sequestered in rafts is thought to result from the affinity of rafts for one another. Patched raft components, such as Thy-1\(^2\), can ‘cap’ in fibroblasts, suggesting a linkage of these raft components, once patched, to the retrograde flow of the cortical cytoskeleton.

One recent paper utilizing energy transfer in a very novel way strongly supports the existence of rafts. When expressed in transfected CHO cells, the folate receptor (FR), a GPI-anchored protein, shows spectroscopic evidence of being clustered\(^2\). This conclusion is based on the principle that the emission from concentrated solutions of fluorophores is depolarized by homoenergy transfer\(^2\) (Fig. 1a); this effect can only be relieved by dilution, which separates the fluorophores. Varma and Mayor\(^2\) observed that the emission anisotropy (a measure of fluorescence depolarization) of a fluorescein-labelled folac acid analogue bound to the FR is the same for pixels with fluorescence intensities that vary by a factor of 200, and that the anisotropy could only be increased by either photobleaching the analogue or dilution with folac acid. These results can be used to argue that the FR is clustered into domains that are 70 nm in diameter (Fig. 1b). Moreover, coexpression of a second GPI-anchored protein, decay accelerating factor (DAF), leads to an increased anisotropy of the labelled FR, suggesting that FR and DAF can occupy the same raft (D. Mayor, pers. commun.). Consistent with the raft model, removal of cholesterol causes clusters to disperse; furthermore, a chimeric transmembrane form of the FR, lacking the GPI anchor, fails to exhibit clustering as judged by this assay.
Clustering of the FR is also supported by chemical crosslinking studies on similar FR-expressing cells\(^25\). These studies also concentrated on a chimeric GPI-anchored protein – growth hormone fused to the GPI anchor from decay accelerating factor – expressed in MDCK cells. Crosslinking indicated that clusters were composed of at least 15 molecules and that clustering was inhibited by cholesterol removal. By contrast, chemically crosslinking growth hormone fused to transmembrane anchors did not result in clustering in this assay.

Further support for the raft concept comes from a new fluorescence spectral technique exploiting the fact that GFP exhibits an excitation spectrum that is dependent on contact between adjacent GFPs. In HeLa cells, GPI-anchored GFP shows an excitation spectrum consistent with a loose clustering of these proteins\(^26\). The degree of clustering is significant compared with either soluble GFP or palmitoylated GFP but considerably less than that mediated by crosslinking with antibodies against GFP.

Single-particle tracking (SPT) studies, in which the lateral mobility of single or small groups of membrane molecules is followed by attaching a small gold or fluorescent particle to the component of interest, are also consistent with the raft concept\(^27\). These studies detect what are termed transient confinement zones (Fig. 1c) in which roughly one-third of both Thy-1, a GPI-anchored protein, and GM1, a GSL, are confined to zones 200–300 nm in diameter. The number and size of these zones diminish when GSL synthesis is metabolically inhibited, but the number of zones is unaffected by extraction of the cells with cold Triton X-100 (although their size is reduced somewhat), suggesting that the zones are detergent resistant. Furthermore, fluorescein phosphatidylethanolamine (Fl-PE), a fully saturated phospholipid analogue that should be accommodated to some extent by the ordered raft lipids, partitions into transient confinement zones but to a lesser degree than Thy-1 or GM1. The number, but not the size, of these zones markedly increases as温度 is lowered (K. Jacobson and C. Dietrich, unpublished), a condition that favours

![Possible lipid microdomains](image-url)

(a) Molecules are randomly distributed or clustered in such small microdomains, comprising just a few molecules (dashed boxes), that no appreciable fluorescence resonance energy transfer (FRET) occurs between labelled raft components. Such domains would be too small to mediate a biological function. (b) Upper panel: rafts of intermediate size (~100 nm) in which the lipids might be in a liquid-ordered phase that is enriched in cholesterol. One such domain could accommodate up to a maximum of ~600 proteins, assuming a molecular mass of 50 kDa. Lower panel: raft size could be modulated by antibody-induced crosslinking [here, for simplicity, depicted as mediated by a single layer of bivalent antibodies against the generic glycosylphosphatidylinositol (GPI)-anchored protein]. (c) Upper panel: ‘macrafts’ of the order of ~500 nm in dimension in which the lipids might be in a liquid-ordered phase that is enriched in cholesterol. The extent of raft development might be regulated by the membrane skeleton ‘fence’\(^29\). Lower panel: model scenario in which the membrane skeleton fence confines laterally mobile intermediate-sized rafts to small regions of the plasma membrane defined by the effective mesh size of the fence (of the order of 500 nm).
the formation of detergent-resistant membranes. This observation and the fact that both GPI-anchored and transmembrane isoforms of NCAM show similar-sized confinement zones suggest that the membrane-associated cytoskeleton, in some way, regulates raft size, possibly by confining diffusing small rafts (see Fig. 2c, lower panel).

Microscopic evidence that does not support the raft concept

Conventional fluorescence microscopy employing direct immunofluorescence or fluorescent ligand analogues to visualize GPI-anchored proteins gives fairly uniform staining of live cells, with little evidence of punctate patterns indicative of clusters packed with these proteins. If rafts do exist, there are several possible explanations for this negative finding. The rafts could be below the resolution limit of the light microscope (~250 nm) and not be sufficiently concentrated in the GPI-anchored protein to appear as fluorescent dots at the resolution limit. It could also be that rafts are large enough to be resolvable but are simply not enriched enough in the fluorescent component to appear as distinct entities. In addition, it is possible that the fluorescent antibody or ligand employed disrupts the putative raft localization.

Studies on fixed MDCK cells using fluorescence resonance energy transfer (FRET), which detects molecular proximity by donor-to-acceptor energy transfer when molecules are within 10 nm or less (Fig. 1b), showed little evidence of clustering of a transfected GPI-anchored protein – S' nucleotidase – expressed in a steady-state distribution at the apical surface. In this study, solutions containing antibodies conjugated to either Cy3 (donor) or Cy5 (acceptor) in varying ratios were used to label the cells, providing variable acceptor-to-donor ratios. FRET was measured as a function of surface density of the enzyme and at increasing ratios of acceptor to donor. Predictions of the results were constructed from previously published theoretical models of energy transfer in a two-dimensional membrane for clustered, mixed clustered and random and totally random distributions of S' nucleotidase. The results were consistent with a random distribution of this GPI-anchored protein and suggest that rafts are either very small (containing only one or two enzymes; Fig. 2a) or that the raft comprises the entire apical membrane. This is an especially striking result because it is based on the same principle, namely energy transfer between proximate fluorophores, but leads to a conclusion diametrically opposed to that concerning the clustering in rafts of other GPI-anchored proteins.

Another fluorescence microscopy study also appears to be inconsistent with the existence of rafts in the plasma membrane. Pagano and coworkers used changes in the emission spectrum of a bodipy sphingomyelin analogue (C5 DMBSM) to study where this lipid probe concentrates in membranes. As the probe becomes more concentrated, its emission shifts from green to red. Thus, the existence of rafts would have been expected to lead to small regions of more red fluorescence superimposed on the green emission. At low concentrations of C5 DMBSM, only green emission is seen in the plasma membrane, indicating the absence of domains containing high concentrations of the probe. Under the same conditions, however, a relative concentration of the probe was indicated by red fluorescence from very early endosomes near the plasma membrane. It is quite possible, however, that the presence of the bodipy moiety at the end of a shortened acyl chain of this sphingomyelin analogue creates a more bulky probe that does not pack well into the ordered environment postulated for rafts.

Conclusions and outlook

Although each of the recent light-microscopy studies appears to be technically sound, specific caveats, arising from the particular methodologies, can be raised against each. Many are performed by transfecting the GPI-anchored protein into the cells – thus expression levels will not be the same as in the wild-type cells. The FRET study finding no evidence for rafts used the photobleaching energy transfer method, which is more conveniently done with fixed cells; fixation could disrupt fragile lipid rafts. For both the fluorescence microscopy and SPT studies, labelled antibodies or other ligands are generally required, which have the potential to perturb the system. In particular, the SPT studies require a gold particle coated with antibody (or cholera toxin for GM1) that is 30 or 40 nm in diameter, giving several membrane-binding sites per bead, which could cause some aggregation of very small rafts.

Keeping these limitations in mind, the various experimental results support a number of models for raft structure. Fig. 2 gives a range of possible raft models. ‘Vanishingly small’ rafts are shown in Fig. 2a and are so small that they would have no significant biological function. Intermediate, ~100 nm scale, rafts are depicted in Fig. 2b (upper panel) that might, under crosslinking conditions, cluster together (Fig. 2b, lower panel) because of inter-raft affinity. Indeed, it is possible that the membrane constitutively contains many small domains and is poised to facilitate different functions by oligomerization-induced clustering. Finally, larger-scale macromdomains (of the order of 500 nm or larger) are depicted schematically in Fig. 2c (upper panel). The occurrence of larger domains could arise from large areas of the membrane being composed of such components or from cold detergent extraction causing coalescence of numerous smaller rafts. The possible involvement of the cytoskeleton underlying the membrane in the regulation of intermediate-sized raft mobility is depicted in Fig. 2c (lower panel).

We feel that the accumulating evidence favours the existence of some kinds of raft, but the important details regarding size and composition in different biological contexts remain to be elucidated. Indeed, the negative results and the arguments about the size of these domains indicate that the formation, stability and partitioning of molecules into rafts depends on subtle details. There is reason to
hope that the discordant microscopic results will be reconciled and a much clearer picture of lipid rafts in the plane of the membrane will emerge soon.

Furthermore, the application of additional tools, including laser tweezers and image-correlation spectroscopy, should enhance the definition of these potentially most interesting plasma membrane structures. Among the most pressing issues are:

- the dynamics and size of rafts and the molecular determinants of these characteristics;
- further definition of the affinities of various membrane components for rafts, how such affinities might be regulated, and the related issue of the duration that particular components reside in a given raft;
- the relationship of putative rafts to the actin-based cytoskeleton;
- how the outer monolayer, containing GPI-anchored proteins, GSLs, sphingomyelin and cholesterol, is coupled to the inner monolayer into which acylated signal-transduction components insert.

References