

Dynamic organization of lymphocyte plasma membrane: lessons from advanced imaging methods

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Summary

Lipids and lipid domains are suggested to play an essential role in the heterogeneous organization of the plasma membrane in eukaryotic cells, including cells of the immune system. We summarize the results of advanced imaging and physical studies of membrane organization with special focus on the plasma membrane of lymphocytes. We provide a comprehensive up-to-date view on the existence of membrane lipid and protein clusters such as lipid rafts and suggest research directions to better understand these highly dynamic entities on the surface of immune cells.

Keywords: lipid rafts; membrane domains; live cell imaging; lymphocytes; advanced imaging

The plasma membrane of eukaryotic cells, including the cells of the immune system, protects them from the uncontrolled entrance of materials to the cytoplasm and also acts as the communication interface with the environment. It is a complex structure formed of a large variety of proteins and lipids, both of which can be glycosylated. Proteins are the components predominantly involved in the recognition and processing of information required for a cell to survive and respond to environmental stimuli in the form of soluble or cell-associated ligands. Immune cells, in particular, have to respond to numerous different stimuli to fulfil their function, i.e. protection of the host organism against pathogens and cellular malformations.

Lipids are an important family of molecules constituting the plasma membrane. They form a selectively permeable bilayer and are responsible for the 'fluid' character of the membrane. The prevailing view since the 1970s is that different species of lipids and proteins are randomly distributed throughout the surface of cells, the hypothesis known as the fluid mosaic model.¹ Later, a more complex picture in which plasma membrane components can form domains with specific molecular compositions was proposed.^{2,3} Domain formation in cellular membranes was supported by the observation of lipid phase separation in model membranes studied with biophysical methods.⁴ The following decade was characterized by intense focus

on membrane organization, which crystallized into the 'lipid rafts' (LRs) hypothesis.⁵ In this model, Simons and Ikonen define LR as membrane domains where glycosphingolipids and phospholipids with highly saturated acyl chains laterally assemble into cholesterol-rich entities. Early research on LR was dominated by biochemical analysis of detergent-resistant domains, cholesterol depletion or sequestration studies and cholera toxin B-subunit patching techniques. The molecular basis of these methods, their limitations and the essential results achieved using immune cells have been reviewed recently.^{6,7} Despite the known limitations, biochemical experiments still provide the majority of information available in the field and one has to be extremely vigilant when interpreting such results.

In addition to the artificial character of early biochemical studies, the LR hypothesis was frequently criticized for failing to be adequately supported by data acquired in living cells.^{8,9} This was mainly the result of the limitations of available imaging techniques, a topic discussed in detail in the last section of this review. Recently, advanced imaging and biophysical techniques have been applied to study membrane organization and in general support the existence of heterogeneities in the plasma membrane of living cells.^{7,10,11} Based on these results, the current definition of LR now states that these are 'small (10–200 nm), heterogeneous, highly dynamic, sterol- and

sphingolipid-enriched domains that compartmentalise cellular processes'.¹² The following section briefly summarizes the results of advanced imaging and biophysical studies.

Small, dynamic lipid rafts of resting cells

The partitioning of specific probes to membrane domains was first used to determine the size and lifetime of LRs in the membranes of living cells. Segregation away from other (non-raft) molecules has been analysed to confirm these results. Many of these methods rely on obtaining information on the behaviour of different raft and non-raft molecules, such as their diffusion. Fluorescence correlation spectroscopy (FCS) recorded in spots of different size¹³ allowed advanced diffusion analysis of molecules with minimal perturbation of cells and the establishment of 'FCS diffusion laws'. Here, the diffusion of putative raft markers [e.g. fluorescein isothiocyanate-conjugated sphingomyelin, glycosylphosphatidylinositol-anchored green fluorescent protein (GFP-GPI), GFP-Thy1 or GFP-Akt-PH] was influenced by compartmentalization into small membrane domains ($< \phi 100$ nm).^{14–16} In contrast, largely cytoskeleton-dependent diffusion has been observed for a non-raft marker (TfR-GFP). No confinement was detected for phospholipids.^{14,15} Similar membrane domains affecting the diffusion of lipids and proteins were demonstrated in living cells using a combination of FCS and super-resolution nanoscopy (see below; ref. ¹⁷).

Using a different method to measure diffusion of membrane molecules, Kusumi and co-workers employed high-speed single particle tracking (SPT) to describe membrane organization.^{18,19} All tested membrane molecules were found to exhibit constrained diffusion in small compartments (30–300 nm). Such compartments are primarily defined by a sub-membranous actin cytoskeleton mesh that constrains the diffusion of molecules by acting as a picket-fence.²⁰ In contrast to FCS studies, 'non-raft' phospholipids (e.g. dioleoyl phosphatidylethanolamine) have been demonstrated to compartmentalize in a similar manner to their protein counterparts when analysed by high-speed SPT.¹⁸ Such discrepancies probably can be explained by the technical limitations of both methods. The FCS technique suffers from being a statistical multi-component method, whereas SPT can usually analyse only a limited number of molecules. High-speed SPT (25 microseconds/frame) also requires labelling of the analysed species with 20–40 nm gold particles, which can cause steric hindrance and influence the diffusion of small molecules such as phospholipids. Also, SPT observations do not exclude the existence of small domains within membrane skeleton-defined compartments.²¹

The hypothesis proposed that LRs in living cells mimic the liquid-ordered phase in model membranes.²² In simple terms, higher ordering of lipid acyl chains can provide

a specific environment for organization of membrane molecules into molecular assemblies such as receptor oligomers or signalling clusters.²³ The use of fluorescent probes that change their properties depending on the local environment has facilitated studies of the membrane's physical properties, e.g. membrane lipid order.²⁴ In this way, higher lipid ordering was reported for the areas of the membrane associated with active signalling and adhesive events.^{25–27} A specific pattern of increased lipid ordering was observed in the immunological synapse where macromolecular signalling assemblies and microclusters have been described to play an essential role in the process of T-cell activation.^{25,28,29} These studies demonstrate the heterogeneous distribution of lipid order in cellular membranes and indicate the importance of membrane ordering in cellular processes. The specific existence of nanoscopic ordered domains cannot be confirmed nor excluded using these methods because of the spatial resolution limits of conventional fluorescence microscopy.

Cholesterol as the guardian of fluidity in cellular membranes?

The continuous fluid character of lipid bilayers is essential for the integrity of the barrier formed between two aqueous solutions, such as the extracellular environment and cytoplasm. However, the interface between the fluid-phase and gel-phase regions that co-exist within a membrane would lead to leakage. Hence the lipid composition in cell membranes is likely to be controlled to prevent a fluid–gel transition. Cellular membranes are composed of a huge variety of lipids, many of which contain highly saturated acyl chains, and are enriched in the outer leaflet of the plasma membrane.³⁰ Together with the high proportion of sphingolipids in the outer leaflet, these lipids risk forming gel-like domains in the membrane because of their high transition temperatures. In other words, they have a high tendency to form gel-like structures even at physiological temperature of 37°, as confirmed by analysis of cells after cholesterol extraction.^{31–33} Coexistence of gel and liquid (fluid) lipid phases in membranes presents a danger for a cell because of the increased permeability of such a lipid bilayer to small molecules and ions (e.g. glucose or calcium ions).^{34,35} This is supported by the observation that cholesterol depletion leads to increased calcium mobilization in resting T cells, indicating increased permeability of treated cells.^{36,37} Further analysis of plasma membrane permeability in cholesterol-depleted cells and the existence of gel-like domains under physiological conditions is required for better understanding of cholesterol function in the membranes of living cells.

The original LRs hypothesis suggests that cholesterol together with lipids containing highly saturated acyl chains forms cholesterol-rich domains capable of preferentially

accommodating some proteins and excluding others.^{5,38} Cholesterol is also thought to promote the formation of such domains by making condensed complexes of a certain stoichiometry with other lipids.^{39–41} In contrast, Ivankin *et al.* recently observed that the stoichiometry of cholesterol–phospholipid complexes in binary mixtures depends exclusively on cholesterol concentration and that cholesterol was evenly distributed between liquid-ordered and liquid-disordered phases in the model membrane tested.⁴² The authors also suggest that the data acquired using binary mixtures of sphingolipids and cholesterol⁴⁰ can be interpreted in the same way and concluded that cholesterol cannot promote long-range ordering of lipids in membranes. Similar cholesterol distribution between heterogeneous populations of membrane fractions was also observed using biochemical techniques.⁴³ In contrast, slightly increased cholesterol content was reported in isolated detergent-resistant domains compared with general plasma membrane and in the immunisolates of activated receptor membranes in T cells compared with transferrin receptor domains.^{41,44} These controversies require further detailed analysis in complex mixtures of lipids (and proteins) such as plasma membrane-derived vesicles or viral capsules.^{45,46}

Rafts (domains) or transient macromolecular assemblies?

Lipid rafts are now believed to be rather small (~ 20 nm across), dynamic membrane domains in resting cells.^{12,17} In addition, immunoelectron microscopy of fast-frozen membrane sheets and live cell nanoscopy demonstrated the existence of protein islands (clusters) of similar size at the plasma membrane of resting cells.^{47–50} Often, a non-overlapping distribution of proteins to individual clusters was observed. Such distributions of membrane proteins were frequently changed after activation of the cells.^{47,48,51} Little or no co-localization of raft markers has been shown using immunoelectron microscopy on membrane sheets,^{52–54} which may reflect the heterogeneity of membrane domains reported previously.^{55,56} A recent comprehensive study of proteins in the yeast plasma membrane indicates a non-homogeneous distribution of all analysed proteins ($n > 70$). Two-colour imaging also demonstrated poor co-localization of most proteins in yeast membranes. Only proteins with identical or highly similar transmembrane domains showed a tendency to form similar distribution patterns (F. Spira, N. Mueller, R. Wedlich-Soldner, personal communication).

The small size of LR caused by the non-homogeneous distribution of proteins and lipids in plasma membranes and their enlargement after stimulation leaves room for speculation that membranes of resting cells contain highly dynamic complexes (or nanoclusters) composed of specific lipids and proteins capable of assembly into larger

microdomains after cell activation (Fig. 1). Such assemblies, or at least a fraction of them, are predicted to play an important role in cellular signalling events.⁵⁷ This hypothesis emanates from the observation that single molecules of some transmembrane receptors almost entirely cover the surface of lipid nanodiscs with a diameter of 10–13 nm.^{58,59} Similar large ‘space’ occupation can

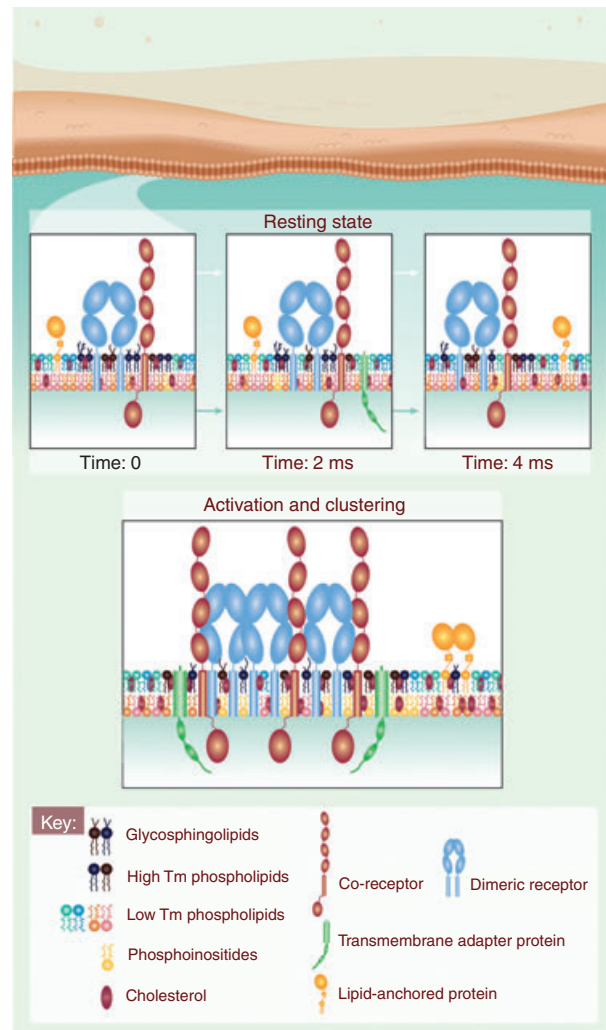


Figure 1. Lipid–protein clusters in the plasma membrane. A cross-section through a putative plasma membrane cluster composed of few proteins and lipids in resting cells. The lipid composition of the cluster rapidly changes but the general enrichment in high melting temperature (T_m) lipids is maintained. Effector proteins transiently associate with the clusters with a short dwell time (1–10 milliseconds; ms). Protein–protein interactions stabilize and cause the expansion of the cluster during activation (recognition of ligand) but have little impact on small dynamic clusters in resting cells. Activated supramolecular clusters increase the dwell time of associated effector proteins (e.g. enzymes or adapters; > 100 ms) in these assemblies. High T_m lipids and cholesterol tend to accumulate in the clusters probably as the result of putative weak interactions with transmembrane domains of proteins.

be predicted for multi-subunit immunoreceptors [such as the T-cell receptor (TCR)] as illustrated in scale in Fig. 2(a). Dimers or oligomers of such voluminous receptors will occupy even larger areas of the membrane (Fig. 2b). For immunoreceptors, dimerization and higher order membrane organization in the absence of stimulus or in the presence of weak ligands has been proposed.^{51,60–62} Hence, the presence of only a few receptors and tens of lipids is sufficient to form an assembly with a diameter of ~ 10 nm, a size notably similar to that of putative LRs in resting cells.

Only a few examples of specific integral membrane protein–lipid assemblies have previously been reported. These include caveolin-1 binding to cholesterol and sphingolipids⁶³ or synaptophysin to cholesterol.⁶⁴ Other examples of the involvement of lipids in integral membrane protein function are being investigated (G. van Meer, personal communication).

There are now numerous studies showing the existence of discrete protein nanoclusters in resting cells.^{47,49,50,65} Such pre-existing protein clusters tend to co-assemble in close proximity after stimulation but do not actually fuse.⁵¹ In T cells, the existence of nanoscale assemblies of individual proteins before and their association during the early phase of activation indicates the involvement of forces capable of segregating individual signalling molecules.⁶⁶ Protein–protein interactions were suggested to be the sole force responsible for the clustering of membrane proteins.⁶⁷ Such a proposition is in conflict with previous observations of the clustering and activation of peripheral

inner membrane leaflet proteins (such as kinases Fyn and Lck) after extracellular domain cross-linking of GPI-anchored proteins lacking transmembrane and cytoplasmic domains.^{68–70}

Specific membrane protein–lipid interactions may also facilitate molecular clustering. No live cell experimental evidence is available for lipid involvement in membrane protein clustering. Therefore, the understanding of the protein–lipid relations in higher order organization of membranes will require detailed analysis of such events in living cells. In addition to chemical and *in vitro* analysis tools, such as photoactivatable lipids⁶³ and quantitative lipid analysis by mass spectrometry,⁴¹ high-speed, super-resolution imaging techniques will be required. How we can combine the advantages of individual optical techniques with modern biochemistry to better understand lipid and protein organization of plasma membranes is discussed in the following sections.

New imaging methods

While the techniques discussed in the previous sections have been tremendously useful in gaining insight into membrane organization, they all suffer from a variety of drawbacks, which limit the scope of information that can be extracted. For example, electron microscopy, while having the highest available spatial resolution, is not applicable to live-cell imaging. Fluorescence microscopy suffers from limited spatial and temporal resolution. Common quantitative techniques such as fluorescence

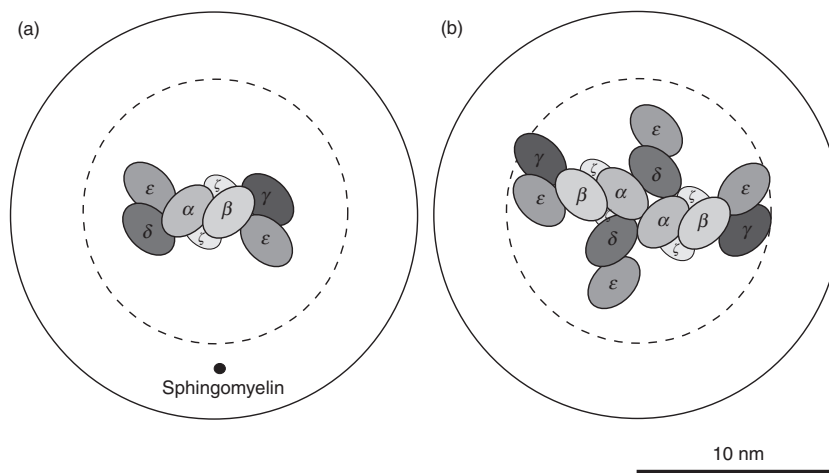


Figure 2. Extracellular domains of multi-subunit immunoreceptors cover a large area of the plasma membrane. Lipid rafts (LRs) are now believed to be rather small (~ 20 nm across) membrane domains. For comparison, we present a scheme with a circular area of 20-nm diameter surrounding a 13-nm lipid nanodisc indicated with a dashed line. Shaded, ellipsoid objects represent subunits of the T-cell receptor (TCR) in scale according to the crystal structures of N-terminal immunoglobulin-like domains of this receptor.^{88,89} A single TCR (a) covers approximately one-quarter of a nanodisc, whereas its predicted dimer⁶² (b) extends to cover one-fifth of the 20-nm domain. The spingomyelin headgroup is illustrated for comparison. Glycosphingolipids occupy even larger areas of the membrane because of additional sugar residues attached to ceramide. Based on these size comparisons, we propose the existence of small (~ 10 nm across), highly dynamic clusters containing specific lipids and protein(s) in resting cells rather than stable domains.

recovery after photobleaching or conventional FCS rely on ensemble measurements, which mask the subtle behaviour of individual molecules and sub-populations. In this section we review the newest cutting-edge fluorescence microscopy methods applicable to these problems; these are divided into super-resolution imaging methods and advanced quantitative measurements of molecular dynamics.

Nanoscopy

In 2008, super-resolution fluorescence microscopy (nanoscopy) was named 'Method of the Year' by *Nature Methods* because of its ability to resolve structures on the scales of tens of nanometres while retaining high molecular specificity and live-cell compatibility. These microscopes are becoming increasingly common and are now commercially available. The most widely publicized approaches to nanoscopy are stimulated emission depletion (STED) microscopy,⁷¹ and the two related techniques of photoactivation localization microscopy (PALM)⁷² and stochastic optical reconstruction microscopy (STORM).⁷³ A schematic depiction of these methods has been published recently.⁵⁷

In STED, fluorophores are excited in a similar way to a conventional confocal microscope, i.e. by focusing an excitation laser into a small spot. A second 'depletion' laser beam is formed into a 'doughnut' shape and overlapped with the excitation beam. This depletion beam causes fluorophores at the edge of the excitation spot to be turned off, which reduces the excitation volume.^{57,74} As an example, STED microscopy revealed that the acetylcholine receptor exist in 55-nm clusters, which were not resolved by conventional microscopy.⁷⁵ Clustering was determined quantitatively and serves as an excellent example of the generation of previously un-acquirable quantitative data. The clusters were found to be sensitive to cholesterol depletion by methyl β -cyclodextrin; however, because of the global effects of cholesterol depletion, its specific function in these clusters will require further investigation.

Despite its promise of delivering super-resolution images, one of the most interesting benefits of nanoscopy is its ability to shrink the scale at which quantitative information can be collected. For instance, STED allows FCS to be conducted on sub-resolution membrane areas.¹⁷ Eggeling and colleagues demonstrated that sphingolipids and GPI-anchored proteins undergo transient confinement in the membrane of living cells. These confinement zones were less than 20 nm in size with trapping typically lasting 10–20 milliseconds and dependent on the physiological cholesterol levels. Glycerophospholipids did not undergo such transient confinement. These properties are consistent with the currently accepted definition of LRs¹² and the scales

involved are close to those detected in high-speed single particle tracking experiments.^{19,76}

Although STED offers high resolution in three dimensions, the system can be relatively complex and expensive.⁷⁷ By confining imaging to a thin section at the glass-sample interface in fixed cells, a less experimentally demanding technique, PALM, achieves super-resolution imaging in two dimensions.⁷² Here, proteins are tagged with photoswitchable fluorescent proteins and these are imaged under total internal reflection fluorescence illumination^{57,78} with low levels of converting laser energy to switch on only a few (sparse) fluorophores stochastically. Non-overlapping single-molecule spots are imaged, their precise localization is determined and the coordinates are stored digitally. These activated molecules are then bleached and a new set of fluorophores is activated and the whole process is repeated multiple times. This results in a list of coordinates that can be represented as a single image with a resolution of 10–20 nm.⁷⁹ A STORM image is generated in a similar way, but small molecule fluorophores, which can be reversibly switched many times to a dark state, conjugated to specific probes (e.g. antibodies) are used in place of photoswitchable fluorescent proteins.^{73,80}

Quantitative cluster analysis and PALM have been applied to studying the distribution of membrane proteins at the cell surface where it was observed that the membrane targeting sequence of the TCR pathway kinase Lck is heterogeneously distributed in the plasma membrane in transfected HeLa cells.^{49,50} In T cells, it is believed that a few TCR signalling pathway proteins reside in microclusters at the T-cell immune synapse.⁸¹ STORM was used to generate super-resolution maps of T-cell microclusters and it was found that, because of the resolution limit in classical microscopy, the number of these T-cell microclusters may be dramatically underestimated.⁵⁰ It also allowed the extraction of important parameters such as the fraction of molecules found to reside in such clusters. Such parameters are vital in reconciling physical data with theoretical calculations of microdomain properties.⁸²

Quantitative measurements of molecular dynamics

Traditionally, extracting information on molecular motion has been the antithesis of high-resolution microscopy. While the super-resolution techniques just discussed take a relatively long time to acquire a single image, quantitative measurements of molecular dynamics such as FCS have dispensed with spatial information entirely in favour of high-speed data collection in the form of a single-point measurement.⁸³ Several new techniques combine quantitative dynamics information with spatial information. Two other examples are raster image correlation

spectroscopy (RICS) and spatio-temporal image correlation spectroscopy (STICS). These are novel computer-based analyses of data acquired using conventional microscopes, which makes these techniques powerful and affordable.

In RICS, confocal microscopy data, which because of the scanning process contain 'hidden' temporal information, are used to extract the information on molecular dynamics of fluorescent molecules. In an image series, the pixel–pixel scan time (microseconds), the line–line time (milliseconds) and the frame–frame time (seconds) can be used, thereby generating data on molecular mobility over a wide-range of time scales.^{84,85} Unlike FCS, RICS simultaneously generates image data.

A similar technique, STICS, performs auto-correlation analysis spatially on an image (i.e. within a single frame) and temporally between image frames. An autocorrelation compares how similar the intensity value in a pixel is to other pixels at different distances from it. This allows the generation of vector maps indicating the direction and magnitude of diffusing objects on a fluorescence image.^{86,87}

Conclusions and perspective

It is likely that the questions surrounding membrane protein and lipid clustering that were raised in the previous sections can only be tested by novel molecular dynamics analytical methods combined with super-resolution microscopy and quantitative measurements applied to the same biological system. A recent example of such a study is work using PALM with quantitative image analysis, electron microscopy and FCS to investigate the clustering properties of TCR and the linker for activation of T cells during T-cell activation.⁶⁶ Previously unavailable information on membrane protein clustering in the plasma membrane was discovered using this combined approach. On the other hand, only a fraction of data presented⁶⁶ has been acquired by imaging living cells at 37°. As indicated earlier in this review, organization of molecules in lipid bilayers (membranes) is highly temperature and environment dependent. Therefore, a comprehensive analysis of a larger group of membrane molecules in living cells at 37° is required to draw a more global picture of membrane organization of lipids and proteins in resting and activated cells.

Super-resolution studies combined with quantitative measurements are limited for cell lines or *ex vivo* cells outside their natural environment. *In vivo* imaging usually requires the use of lower resolution techniques but this limitation is counterbalanced by the investigation of the sample under highly physiological conditions. An example of an *in vivo* approach is the analysis of membranes by laurdan imaging in living zebrafish (Fig. 3 and Supporting Information: Supplementary Movie), in which circulating

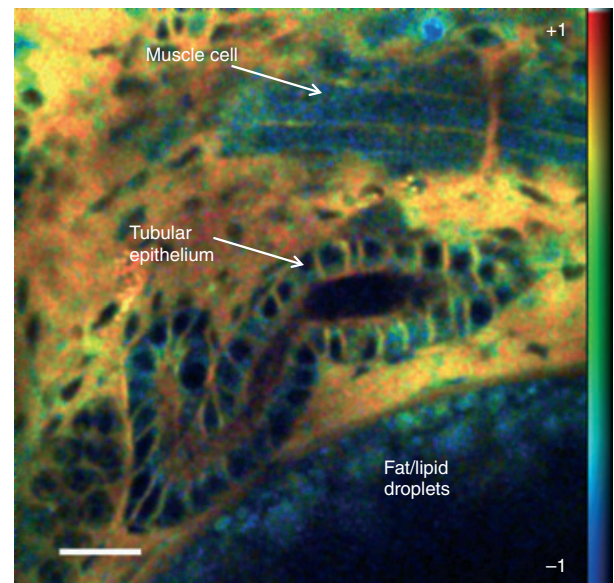


Figure 3. Generalized polarization (GP) image of a live zebrafish kidney nephron (section). A living zebrafish was incubated in Laurdan-containing imaging medium and the kidney was analysed using two-photon microscopy. The apicolateral surface of the tubular epithelium of the nephron shows high membrane lipid order (red). Low-order (blue) intracellular membranes are visible in the tubular epithelium and muscle cells. The GP values close to +1 represent high-order membranes and those close to −1 represent low-order membranes. Scale bar 20 μ m.

blood cells can be seen. For example, high order was detected in the apical membranes of the tubular epithelium of the nephron in the kidney, probably because of the highly hierarchical organization of the plasma membrane required for the water-channelling function.

We therefore suggest that *in vivo* imaging followed by the isolation of cells or tissues and application of advanced imaging and analytical techniques can uncover the differences in membrane organization between various cell types or tissues.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1. *In vivo* membrane order imaging. A living zebrafish was incubated in membrane environment-sensitive fluorescent dye (Laurdan) containing medium and analysed using two-photon microscopy. High membrane lipid order is represented in red and low order in blue (Fig. 2 in the text for colour bar). Moving blood cells can be seen in the last frames of the movie.

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