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Microscopic Methods to Observe the Distribution of Lipids in the Cellular Membrane

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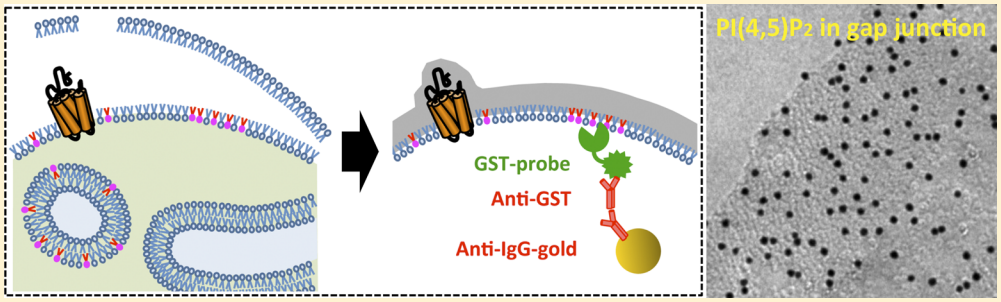

ABSTRACT: Membrane lipids not only provide the structural framework of cellular membranes but also influence protein functions in several different ways. In comparison to proteins, however, relatively little is known about distribution of membrane lipids because of the insufficiency of microscopic methods. The difficulty in studying lipid distribution results from several factors, including their unresponsiveness to chemical fixation, fast translational movement, small molecular size, and high packing density. In this Current Topic, we consider the major microscopic methods and discuss whether and to what degree of precision these methods can reveal membrane lipid distribution in situ. We highlight two fixation methods, chemical and physical, and compare the theoretical limitations to their spatial resolution. Recognizing the strengths and weaknesses of each method should help researchers interpret their microscopic results and increase our understanding of the physiological functions of lipids.

The biological membrane is a highly dynamic two-dimensional structure. Lipids provide the structural basis of the membrane by forming a bilayer, but they also serve as materials to generate bioactive substances, recruit proteins to the appropriate locations, and regulate protein functions. The protein-related functions of membrane lipids are not only exerted by direct high-affinity binding but also by a summation of low-affinity interactions through electrostatics, membrane curvature, and so on.[1] All of these lipid functions need to be exerted in the right place and at the right time. However, in comparison to proteins, relatively little is known about how lipids distribute in the membrane.

Biochemical methods have shown that each organelle has a distinct lipid composition.[2] Membrane asymmetry, or differential lipid distribution between the outer and inner leaflets of a membrane, was reported on many years ago.[3] Additionally, in recent years, microscopic studies have yielded results suggesting that some lipids may distribute nonuniformly in the plane of the membrane (Table 1). The physiological importance of such local heterogeneity in lipid distribution was highlighted by the raft hypothesis.[4] However, as membrane lipids are studied in more detail, it has become clear that most microscopic methods used for proteins are not suitable for determining lipid distribution with satisfactory precision.

With regard to microscopic visualization, many obvious differences exist between proteins and lipids. First, proteins can be tagged genetically with substances like green fluorescent protein (GFP), and this makes genome-wide screening of subcellular distribution possible.[5] In contrast, genetically encoded tags are not applicable to lipids, and the small molecular size of lipids makes it difficult to attach any tag without interfering with and/or altering their behavior. Second, proteins can be immobilized with chemical fixatives (see later sections for problems with chemical fixation) and can be examined with sophisticated immunohistochemical techniques. In contrast, most membrane lipids do not react with aldehydes and thus they remain mobile even in fixed cells.[6] This "unfixability" makes it difficult to determine lipid distribution even when specific probes are available.

In this Current Topic, we will review the methods that are currently available to study the distribution of membrane lipids. For practical reasons, we classify the methods into two categories: (1) methods used mainly for light microscopy and (2) methods used for electron microscopy (EM). We discuss these methods from the standpoint of whether and to what degree of

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Table 1. Membrane Lipids Showing Uneven Two-Dimensional Distribution[a]

lipid	cell type and/or subcelluar region	size of lipid domain (or cluster)	method[b]	probe	ref
ganglioside GM1	uropod	1−10 μm	B	cholera toxin B subunit	94
	plasma membrane	120−160 nm[c]	B	cholera toxin B subunit	95
		ca. 94 nm[c]	E	antibody	38
	polarized epithelium apical membrane, microvillus	1−10 μm	B	cholera toxin B subunit	96
	cleavage furrow (sea urchin embryo)	>10 μm	B	cholera toxin B subunit	97
	sperm plasma membrane overlying the acrosome	1−10 μm	B	cholera toxin B subunit	98
ganglioside GM3	leading edge	1−10 μm	B	antibody	94
	polarized epithelium apical membrane, primary cilium	1−10 μm	B	antibody	96
	plasma membrane	<1 μm	E	antibody	38
sphingomyelin	plasma membrane	120−160 nm[c]	B	lysenin	95
	cleavage furrow	ca. 500 nm	B	lysenin	99
	polarized epithelium apical membrane	>10 μm	B	lysenin	100
phosphatidylglucoside	plasma membrane	96.4 ± 10.8 nm (HL60 cell)[c]	E	antibody	75
		137.8 ± 54.4 nm (A549 cell)[c]			
phosphatidylserine	macrophage phagocytic cup	1−10 μm	A	lactadherin C2 domain	101
	bud (*S. cerevisiae*)	1−10 μm	A	lactadherin C2 domain	102
	plasma membrane	∼44 nm[c]	B	lactadherin C2 domain	66
phosphatidylethanolamine	cleavage furrow	1−10 μm	B	Ro09-0198 peptide (cinnamycin)	103
	polarized end (*S. cerevisiae*, *S. pompe*)	1−10 μm	B	Ro09-0198 peptide (cinnamycin)	104
diacylglycerol	leading edge	>10 μm	A	PKCβII CI domain (FRET probe)	105
phosphatidic acid	cell-free edge (non-cell-contact site)	1−10 μm	A	DOCK2 C-terminal domain (FRET probe)	106
$PI(3,4)P_2$	leading edge	>10 μm	A	TAPP1 PH domain (FRET probe)	105
	ruffling membrane	1−10 μm	B	TAPP1 PH domain (tandem)	107
$PI(4,5)P_2$	pollen tube tip (*N. tabacum*)	1−10 μm	A	PLCδ1 PH domain	108
	lamellipodium	1−10 μm	C	PLCδ1 PH domain	55
	ruffling membrane	1−10 μm	A	PLCδ1 PH domain	109
	cleavage furrow	1−10 μm	B	PLCδ1 PH domain	84
	syntaxin cluster	<1 μm	B	antibody	86
		73 ± 42 nm[c]	B	antibody, PLCδ1 PH domain	88
	cell edge	1−10 μm	A	PLCδ1 PH domain	110
	N-cadherin adhesion	>10 μm	A	PLCδ1 PH domain	111
	caveolae	60−100 nm	E	PLCδ1 PH domain	73
	immunological synapse	1−10 μm	A	PLCδ1 PH domain	112
	shmoo (*S. cerevisiae*)	1−10 μm	A	PLCδ1 PH domain	113
	bud (*S. cerevisiae*)	1−10 μm	A	PLCδ1 PH domain	85
	fungal filament (*C. albicans*)	1−10 μm	A	PLCδ1 PH domain	114
$PI(3,4,5)P_3$	leading edge	>10 μm	A	GRP1 PH domain (FRET probe)	105
	forming macropinosome	1−10 μm	A	Akt PH domain, Btk PH domain	109
	synapse (*D. melanogaster*)	<1 μm	A	GRP1 PH domain	115
$PI(3,4)P_2$/$PI(3,4,5)P_3$	leading edge	1−10 μm	A	Akt PH domain	116
	adhesion zone	>10 μm	A	Akt PH domain	117
	macrophage phagocytic cup	1−10 μm	A	Akt PH domain	118
cholesterol	filopodium	1−10 μm	C	perfringolysin O	60
	cleavage furrow (sea urchin embryo)	>10 μm	B	filipin	97
ergosterol	shmoo (*S. cerevisiae*)	1−10 μm	B	filipin	113

[a]Lipids reported to show uneven distribution in the membrane are listed. Methods used to observe the lipid distribution are classified into five categories in accordance with the description in the text. [b]For methods A−E, refer to the text for details. A, live imaging with GFP-tagged lipid-binding domains; B, labeling with lipid-binding probes; C, EM with ultrathin cryosections; D, EM with ultrathin resin sections; and E, QF-FRL. [c]Indicates the diameter shown by the marker used for labeling, which is very likely different from the size of the domain made by the target lipid per se.

precision each method can yield information on the distribution of endogenous lipids in the membrane at one time point. For this purpose, we placed a focus on fixation because it is critical to know how precisely target molecules are captured at the location where they exist at the time of fixation. However, techniques to study fast molecular dynamics, such as single-particle tracking, which have already been treated in excellent articles,[7,8] are not included. Finally, by taking ganglioside GM1 and phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] as examples, both of which have been reported to exhibit nonuniform distribution, we will discuss how much we know, or do not know, about their true distribution in cells.

1. METHODS FOR LIGHT MICROSCOPY

Two key advantages of most light-microscopic methods are that they can be used for live imaging and that they can observe a larger number of samples with relative ease. Super-resolution microscopes have further enhanced the merit of live imaging, and

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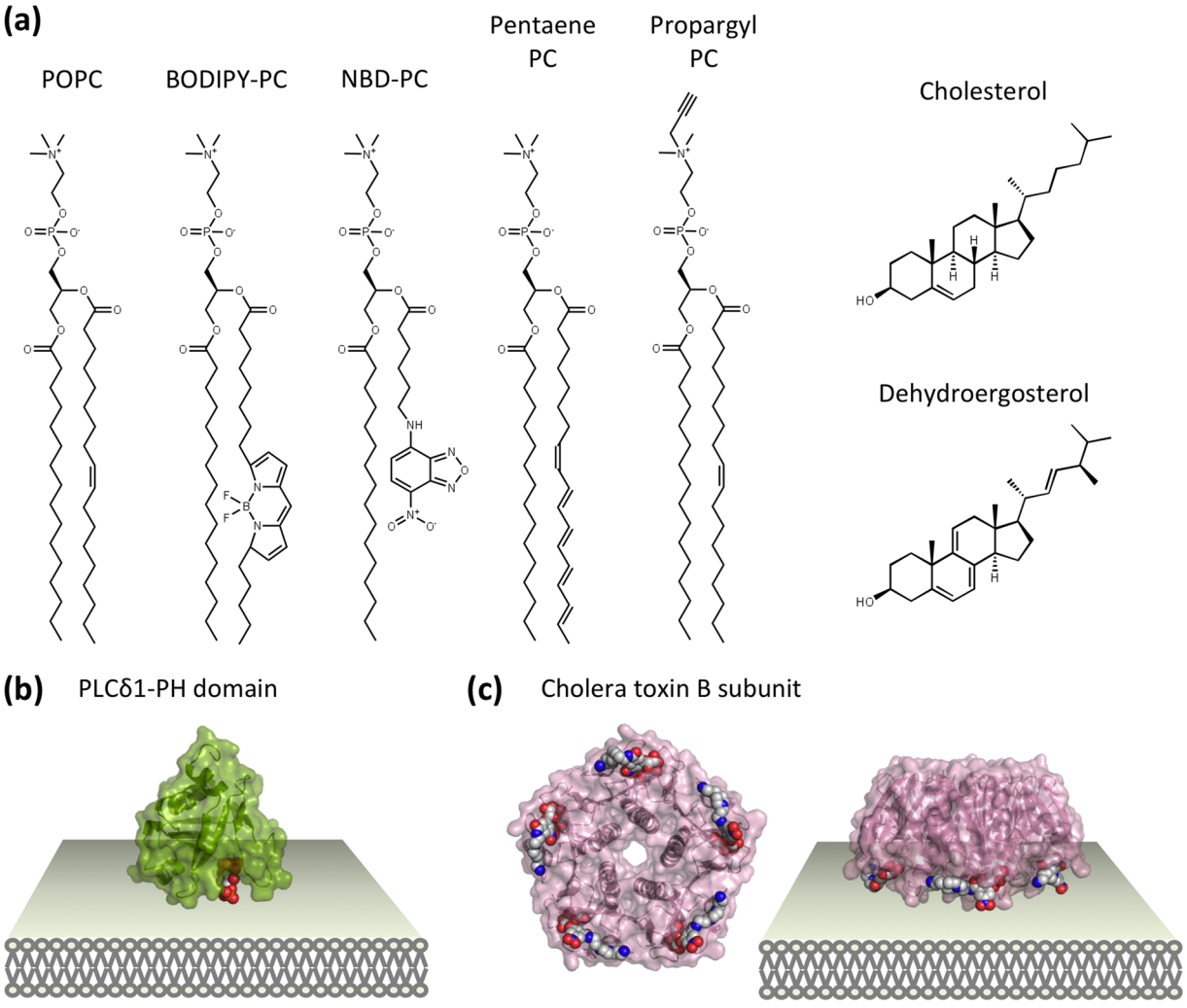

Figure 1. Methods for light microscopy. (a) Analogues of phosphatidylcholine and cholesterol. BODIPY and NBD are fluorophores that are usually conjugated to an acyl chain of phospholipids. Pentaene-fatty acid is fluorescent because of its double bonds and yet takes a linear shape, similar to natural PC. Propargylcholine incorporated into the PC headgroup is tagged with a fluorescent azide compound for imaging. Dehydroergosterol is a naturally fluorescent sterol whose chemical structure is very similar to that of cholesterol. (b) PH domain of PLCδ1 is a prototype lipid-binding protein domain used as a GFP-tagged form. The structure of PLCδ1-PH binding with Ins(1,4,5)P_3 (PDB ID: 1MAI) is shown. Note that PLCδ1-PH is much larger than the target lipid. (c) Structure of the cholera toxin B subunit (CTB) pentamer in complex. Nitrophenyl galactoside, a GM1 surrogate, is shown (PDB ID: 1PZJ). The CTB pentamer binds to five molecules of GM1 and is likely to modulate GM1 distribution.

they are especially effective in studying fast molecular dynamics.[9] As the spatial resolution of these microscopes is approaching the nanometer scale, it is becoming even more critical to label or tag lipids, with fluorescent molecules in most cases, so that the observed signal correctly reflects endogenous lipid distribution. The focus of our discussion will be on whether the results obtained by the respective methods can accurately indicate distribution of target lipids in native undisturbed cells.

1.2. Fluorescent Lipid Analogues. Lipids conjugated with fluorophores such as nitrobenzoxadiazole (NBD) and boron-dipyrromethene (BODIPY) are often used for imaging (Figure 1a). The molecular masses (M_r) of NBD and BODIPY are 160 and 190, respectively. When these fluorophores are bound to phosphatidylcholine (PC; $M_r \approx 760$ when acyl chains are 16:0 and 18:1), the relative M_r ratio of the tag and the target molecule is not much different from that of GFP and actin (GFP: $M_r \approx 27\,000$ and actin: $M_r \approx 45\,000$). Nevertheless, although GFP-actin largely mimics endogenous actin behavior (see ref 9 for a discussion of its functional deficiency), the introduction of fluorophores to an alkyl group of phospholipids was reported to grossly affect the lipids' distribution, the kinetics of transbilayer movement, and intracellular trafficking.[10,11] The effect of the fluorophores on lipids occurs probably because the intrinsic hydrophilicity of NBD (and BODIPY to a lesser extent) makes the lipids' tail portion loop back to the surface of the lipid bilayer.[12,13] This problem can be avoided by attaching the fluorophores to the headgroup of lipids, but in this case, the lipids' hydrophilic interaction with other molecules, including other membrane lipids and effector proteins, is likely to be affected. An alternative and promising option is to use polyene fatty acids, which have an overall shape similar to natural fatty acids. It has been found that phospholipids containing polyene fatty acids show natural phase partitioning in the membrane.[14]

Cholesterol ($M_r \approx 387$) is much smaller than phospholipids, and even a subtle modification is likely to change its molecular properties and impact the phase behavior of the membrane. Naturally fluorescent sterols bearing conjugated double bonds in the ring system, such as dehydroergosterol (Figure 1a) and cholestatrienol, have been shown to mimic the characteristics of

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Table 2. Probes for Labeling Membrane Lipids[a]

lipid	probe	method (ref)[b]
ganglioside GM1	cholera toxin B subunit	B (37, 119); E (38)
sphingomyelin	lysenin	B (36)
	equinatoxin-II	A (120)
phosphatidylserine	annexin V	B (33)
	lactadherin C2 domain	A, B (121); D (66)
	evectin-2 PH domain	B (122)
phosphatidylethanolamine	Ro09-0198 (cinnamycin)	B (104)
diacylglycerol	PKC CI domain	A (123)
phosphatidic acid	Raf1 PA-binding domain	A (124)
	Spo20 PA-binding domain	A (125)
	Sos PH domain	A (126)
	DOCK2 C-terminal domain	A (106)
PI(3)P	EEA1 FYVE domain	A (127)
	Hrs FYVE domain (tandem)	A, B, C (54)
	FENS-1 FYVE domain	A (128)
	p40phox PX domain	A, B (129, 130)
PI(4)P	OSBP PH domain	A (131, 132)
	FAPP1 PH domain	A, B (132, 133)
PI(5)P	ING2 PHD domain	A (134)
$PI(3,4)P_2$	TAPP1 PH domain	A (135); B (107); C (56)
$PI(3,5)P_2$	TRPML1ML1N domain	A (136)
$PI(4,5)P_2$	PLCδ1 PH domain	A (20, 21); B (107); C (55); E (73)
	Tubby C-terminal domain	A (25)
$PI(3,4,5)P_3$	GRP1 PH domain	A (137); B (107); C (138)
	ARNO PH domain	A (139)
	Btk PH domain	A (140)
$PI(3,4)P_2$/$PI(3,4,5)P_3$	AKT PH domain	A (141)
	PDK1 PH domain	B (142)
cholesterol	filipin	B (39)
	perfringolysin 0	B (143); C (60)
ergosterol	filipin	B (144)

[a]Probes that were used to label membrane lipids for microscopic observation are listed. Antibodies are available for many lipids, but they are not included in the table. [b]For methods A to E, refer to the text for details. A, live imaging with GFP-tagged lipid-binding domains; B, labeling with lipid-binding probes; C, EM with ultrathin cryosections; D, EM with ultrathin resin sections; and E, QF-FRL.

endogenous cholesterol better than NBD-cholesterol, which bears fluorophores in the side chain.[15,16]

In an attempt to minimize effects of molecular modification and expand the application possibilities, analogues containing an alkyne or an azido group have been used as precursors to label lipids metabolically. These chemical groups are small and biologically inert, and after incorporation into membrane lipids, they can be specifically conjugated with the appropriate fluorophores bearing complementary functional groups by bioorthogonal reaction.[17] Propargylcholine, a choline analogue with an alkyne group ($HC{\equiv}C{-}CH_2{-}$), has been used to label metabolically choline-containing phospholipids like PC, lysoPC, and sphingomyelin in mammalian cells, which were then visualized by reacting with fluorescent azide compounds.[18] In another study, analogues of phosphatidic acid bearing an alkyne group at the terminus of sn-2 acyl chain were incorporated into cells and imaged similarly.[19] Although rigorous verification is needed to show that these lipids behave like their natural counterparts, this kind of chemical biological approach promises to become increasingly important.

1.2. Genetically Encoded Lipid-Interacting Protein Domains. Genetically encoded, GFP-tagged lipid-binding protein domains have been used extensively to visualize target lipids by fluorescence microscopy (Figure 1b). This powerful technique enables time-resolved monitoring of lipid distribution in live cells, thereby contributing a great deal to the current understanding of lipid dynamics. The representative domains that have been used for this method are listed in Table 2. Among these, the pleckstrin homology (PH) domain of phospholipase C δ1 (PLCδ1) is the most widely used to visualize the distribution of $PI(4,5)P_2$.[20,21]

However, problems with the use of GFP-tagged domains in live cells have been pointed out repeatedly. Taking GFP-PLCδ1-PH as an example, the probe is not likely to visualize the entire population of $PI(4,5)P_2$ with a uniform efficiency because some $PI(4,5)P_2$ is likely to be sequestered with endogenous molecules (e.g., effector proteins) and may not be accessible to the probe. Furthermore, molecules that bind to the probe with high affinity may disturb the reactivity of the probe, complicating the interpretation of the obtained results. Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), a second messenger that is generated by hydrolysis of $PI(4,5)P_2$, is the most likely molecule to affect the GFP-PLCδ1-PH behavior because the dissociation constant between GFP-PLCδ1-PH and $Ins(1,4,5)P_3$ is an order lower (0.1 μM) than that between GFP-PLCδ1-PH and $PI(4,5)P_2$ (2 μM).[22] This has led to controversy about whether relocation of GFP-PLCδ1-PH from the plasma membrane to the cytosol in stimulated cells faithfully reflects a decrease of $PI(4,5)P_2$ in the plasma membrane or whether this is largely due to an increase of $Ins(1,4,5)P_3$ concentration.[23,24] To exclude the influence of

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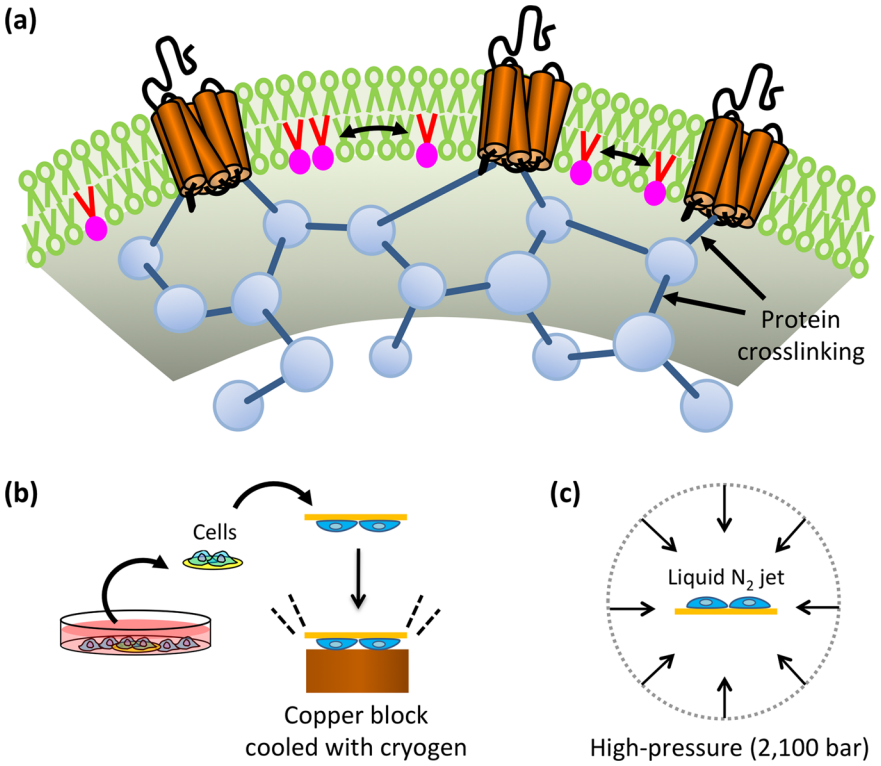

Figure 2. Chemical and physical fixation. (a) Chemical fixation. Aldehydes induce cross-linking of membrane proteins with cytosolic and other membrane proteins, whereas aldehydes do not react with most lipids. Although gross redistribution of lipids is suppressed by cross-linked proteins, lipids may remain mobile in small areas within the protein meshwork. (b) Metal-contact freezing. Specimens are quick-frozen by direct contact with a pure copper block cooled by liquid helium or liquid nitrogen. (c) High-pressure freezing. Specimens are quick-frozen with a jet of liquid nitrogen under a brief application of high pressure.

$Ins(1,4,5)P_3$, probes using Tubby, which binds to $PI(4,5)P_2$ but not $Ins(1,4,5)P_3$,[25] and a modified ENTH domain probe, which emits fluorescence only when bound to the membrane,[26] have been developed.

Additionally, binding of lipid-binding probes to target lipids per se may cause some artifacts. First, binding of the probes may change the distribution and the amount of target lipids.[24,27] Second, lipid-binding probes may exert dominant-negative effects by interfering with the interaction between the target lipids and endogenous protein effectors.[21,28−30] On the basis of the expression level of GFP-PLCδ1-PH (25 μM),[29] the intracellular concentration of $PI(4,5)P_2$ (1−10 μM),[31] and the dissociation constant between the two molecules (2 μM), about 90% of $PI(4,5)P_2$ is estimated to be bound with the probe. According to a mathematical simulation, even a lower level of the probe (6 μM) was predicted to affect the kinetics and steady-state levels of $PI(4,5)P_2$ and $InsP(1,4,5)_3$.[24] This problem could be minimized by lowering the expression level of lipid-binding of probes,[32] but it is difficult to determine an expression level that does not interfere with physiological events.

1.3. Application of Lipid-Binding Probes. For lipids in the plasma membrane, lipid-binding proteins (or peptides) can be applied directly to intact cells. Annexin V has been used to label phosphatidylserine (PS) in apoptotic cells.[33] Other examples include the cholera toxin subunit B for ganglioside GM1[34] (Figure 1c), Ro09-0198 (cinnamycin) for phosphatidylethanolamine (PE),[35] and lysenin for sphingomyelin[36] (Table 2). Because these lipid-binding probes are not membrane-permeable, their binding to intact cells can be taken to indicate that their respective target lipids are present in the outer leaflet of the plasma membrane. However, because the binding of the probes is thought to disturb the dynamics of their target lipids, this method is not appropriate for examining fine lipid distribution under native conditions. Aldehyde fixation cannot prevent probe-induced disturbance because membrane lipids retain mobility even in fixed cells. Labeling at a low temperature (e.g., on ice) is also not appropriate for two reasons. First, gross energy-dependent events, such as capping in lymphocytes (i.e., accumulation of cross-linked molecule to one pole of the cell), may not occur under cold conditions, but local clustering such as patching (i.e., formation of small aggregates) persists;[37] second, lowering the temperature is likely to induce a phase shift in the membrane, thereby altering lipid distribution.[38]

To label lipids in intracellular organelles, the plasma membrane must be permeabilized to allow access of lipid-binding proteins and peptides to target membranes. Treatment with saponin and streptolysin O is often used for this purpose because these substances form pores in cholesterol-rich membranes, such as the plasma membrane and the early endosome, without disrupting overall membrane integrity. Physical methods, such as repeated freeze−thawing or brief sonication, are also used to remove the barrier for the probes. All of these methods are convenient and have contributed to revealing that lipids possess differential subcellular distributions. The possibility remains, however, that the procedures per se affect distribution of the target lipids.

Filipin is a fluorescent polyene antibiotic[39] that specifically binds sterols and induces a unique membrane deformation.[40] Because filipin penetrates the plasma membrane, treatment for permeabilization is not necessary. Nevertheless, filipin falls short as a tool for visualization of precise sterol distribution because the number of membrane deformations caused by filipin does not

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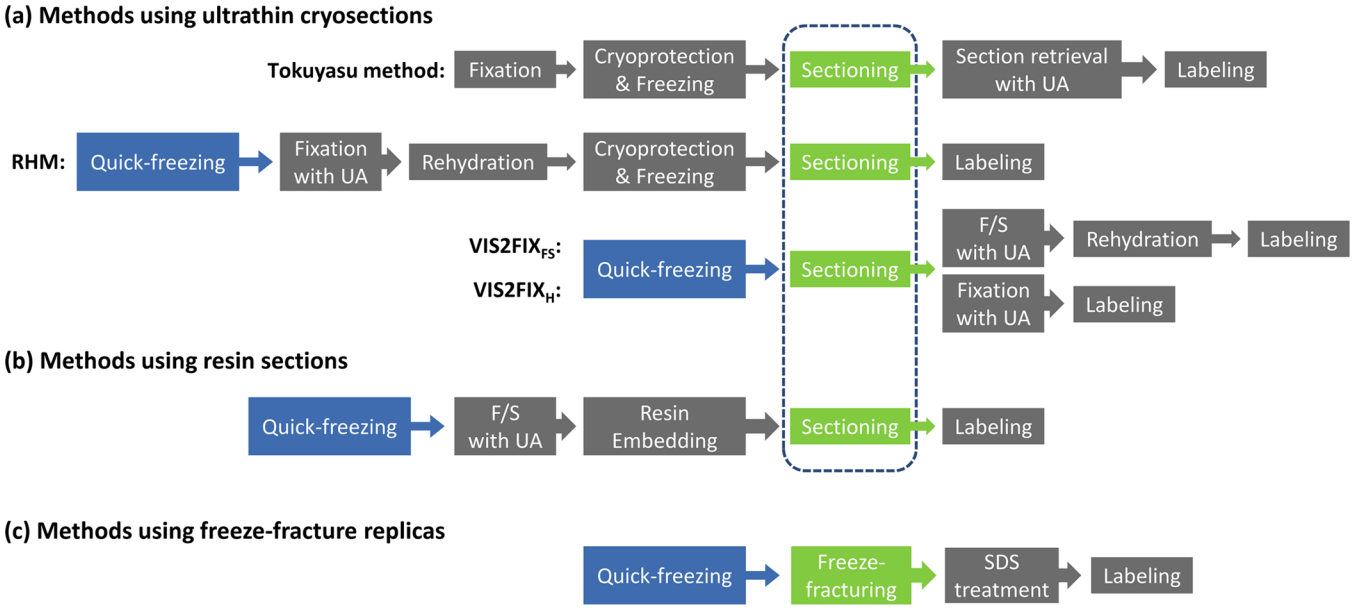

Figure 3. Flowchart of electron microscopic methods for lipids. Methods are classified according to the type of sample preparation: (a) ultrathin cryosections, (b) ultrathin resin sections, and (c) freeze-fracture replicas. F/S, freeze-substitution; UA, uranyl acetate.

necessarily reflect the membrane content of sterols.[41] Moreover, filipin cannot distinguish the two membrane leaflets, and the possibility that binding of filipin induces redistribution of sterols cannot be excluded.

2. METHODS FOR ELECTRON MICROSCOPY

EM has a much higher spatial resolution than conventional light microscopy. To harness the benefits of EM, it is important to fix target molecules at the location where they exist in living cells at the moment of interest. In this section, we will first discuss methods for fixation and then examine individual EM methods from the standpoint of how precisely the methods can determine distribution and quantities of endogenous lipids at a certain time point.

2.1. Fixation. *2.1.1. Chemical Fixation.* For immuno-EM of proteins, aldehyde fixatives (i.e., formaldehyde and glutaraldehyde) are usually used in various concentrations. Aldehydes react with thiol and amino groups in proteins and immobilize proteins by forming cross-linkages (Figure 2a). However, aldehydes do not react with most lipids except for aminophospholipids (i.e., PS and PE) that have an amino group in the headgroup.[42]

In EM samples prepared by the conventional method (i.e., fixation by aldehydes followed by osmium tetroxide, dehydration in ethanol, or acetone, series, and embedding in epoxy resin), a loss of 1% PE, 11% PC, and 57% of the neutral lipids is reported to occur.[43,44] Omission of the osmium tetroxide treatment increased the lipid loss to 47% for PE, 100% for PC, and 70% for neutral lipids. In contrast, after fixation with osmium tetroxide alone, only 2.6% PE and 2.5% PC were lost, indicating that aldehyde fixation has only a small effect on the retention of both polar and neutral lipids. As an extreme example to indicate the unfixability of lipids, gangliosides in a mouse brain specimen fixed with formaldehyde were shown to move from one sample to another over a distance of millimeters.[45]

Retention of lipids does not necessarily mean that lipid distribution in situ is preserved. That is, even when extraction is minimal, lipids can change distribution within a sample. Actually, membrane fluidity persists after aldehyde fixation and lipid molecules retain their two-dimensional mobility in the membrane[6] (Figure 2a).

Even if some chemical had the ability to immobilize lipids in situ, it would take some time for the chemical to reach and react with the lipids. The time required for chemical fixation may be on the order of a second, which is too long to define the precise localization of membrane lipids. For example, if we suppose 9×10^{-9} cm^2/s as the diffusion coefficient of phospholipids in living cells,[46] then it is estimated that the molecules can move approximately 1.9 μm in 1 s. This distance is unacceptable if we want to study membrane microdomains, which may be much smaller than 100 nm in diameter. Therefore, we think it is unlikely that any chemical fixation method can preserve the in situ distribution of membrane lipids on the submicrometer scale. Problems concerning chemical fixation will be discussed further with regards to individual EM methods.

2.1.2. Physical Fixation. Quick-freezing can stop molecular motion within a very short period of time. By cooling specimens at a sufficiently fast rate (e.g., > 10 000 °C/s under atmospheric pressure), water is vitrified so that the cellular ultrastructure is preserved without detectable damage by ice crystals. Among methods developed for quick-freezing, the metal-contact and high-pressure freezing methods have been widely used.

In the metal-contact method, specimens are slammed onto a mirror-finish copper block surface cooled by liquid helium or liquid nitrogen (Figure 2b). The major advantage of this method is that freezing occurs within 0.1−1 ms on the specimen surface[47] (J. Heuser, personal communication). However, a limitation of the method is that vitrification occurs only in a narrow zone no deeper than 10−20 μm from the contact surface[48] and that deformation of the ultrastructure may arise because of the compressive stress.[49]

In the high-pressure freezing method, a specimen is frozen by a jet of liquid nitrogen under a brief application of a pressure of 2100 bar (Figure 2c). Freezing by this method takes about 10 ms, but specimens can be vitrified as deep as 0.6 mm from the surface. In some model specimens, however, structural changes, probably caused by the high pressure, have been reported.[50,51]

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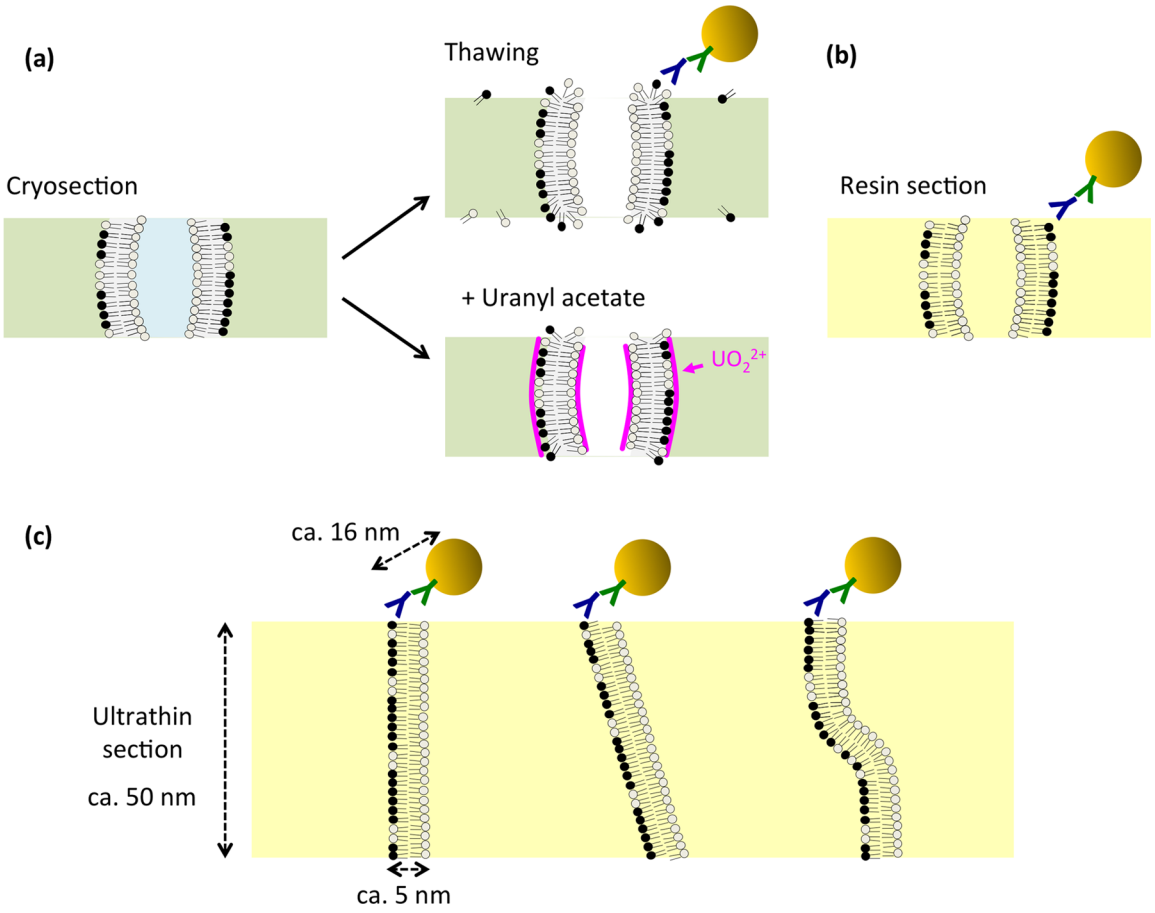

Figure 4. EM methods using ultrathin sections. (a) Ultrathin cryosections. When cryosections are thawed and the hydrophobic portion of the membrane is exposed to the aqueous environment, lipids are likely to be reorganized to re-establish a stable arrangement. Uranyl acetate is thought to prevent gross membrane reorganization by binding to phospholipids. (b) Resin-embedding method. Resin embedding physically stabilizes the membrane structure, but probes can access only the lipids at the section surface. (c) Section thickness is approximately 10 times that of the membrane thickness, and membranes often run obliquely or curve in the section. The arm distance, or the distance between the target lipid and the label (e.g., gold particle), is also larger than the membrane thickness. Because of these factors, it is difficult to define the sidedness of the target lipid accurately.

The freezing rates attained by the quick-freezing methods are sufficiently fast to capture most biological phenomena. For example, phase transition of liposomal membranes, which occurs when the temperature is lowered slowly, is not observed in quick-frozen specimens.[52] It needs to be remembered, however, that phospholipids with a diffusion coefficient of 9×10^{-9} cm^2/s move 19 nm in 0.1 ms and 190 nm in 10 ms. Although the diffusion coefficient decreases as the temperature is lowered, the distance lipids move may not be trivial in the study of microdomains. Nevertheless, the quick-freezing methods can provide a much better spatial resolution in defining membrane lipid distribution than methods that use chemical fixation.

2.2. Immunoelectron Microscopy Using Ultrathin Cryosections (Tokuyasu Method). The method of cryosectioning developed by Dr. Kiyoteru Tokuyasu in the 1970's has been used successfully in many immuno-EM studies.[53] Cells and tissues are first fixed with aldehydes, infiltrated with a dense sucrose solution, and frozen by immersion in liquid nitrogen (Figure 3a). Sucrose works as a cryoprotectant to prevent ice-crystal formation and as an appropriate matrix for ultrathin sectioning. Cryosections thus prepared are thawed and incubated with various probes to label target molecules for electron microscopy. Using the Tokuyasu method, the subcellular distributions of PI3P,[54] $PI(4,5)P_2$,[55] and $PI(3,4)P_2$[56] have been observed.

Data on lipid retention in Tokuyasu cryosections are not available, but some estimation can be made using results obtained with cryostat sections. Roozemond measured the release of lipids from cryostat sections of unfixed rat brain into water or 4% formaldehyde with or without 1% $CaCl_2$.[57] In water, phospholipid extraction was measured to be about 7.1%, with the different phospholipid classes extracted in proportion to their concentration. The extraction was reduced to 2.7% in 4% formaldehyde, and because this fixative reacts mainly with PS and PE, the degree of extraction varied among phospholipids. Addition of 1% $CaCl_2$ to the fixative further decreased the lipid extraction to 1.3% and also changed the ratio of extraction. This is probably because Ca^{2+} binds to negatively charged and zwitterionic phospholipids[58] and alters their polarity.[59] Lipid extraction from Tokuyasu cryosections is most likely comparable to, or lower than, that from cryostat sections because the sample is cryoprotected to avoid ice-crystal formation, which damages the membrane and contributes to extraction.

Because the thickness of Tokuyasu cryosections is in the range of 50−100 nm, most organelles are likely to be cut transversely in the limiting membrane. This leads to the exposure of the hydrophobic portion of polar membrane lipids at the section surface (Figure 4a). Upon thawing, these lipids are reorganized in an unpredictable manner, with the hydrophobic portion oriented away from water, to reestablish a stable structure in the aqueous

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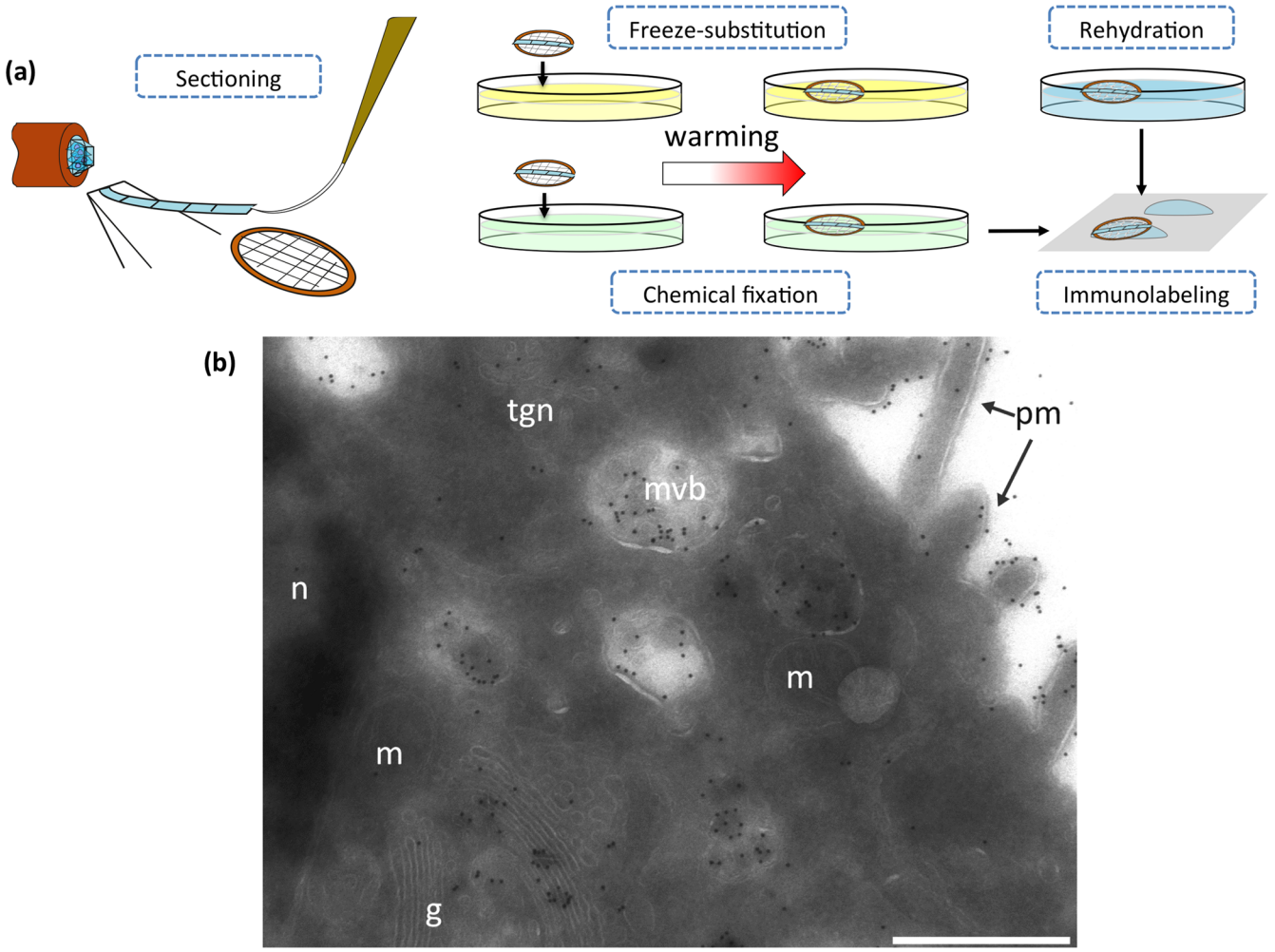

Figure 5. VIS2FIX method. (a) Outline of the method. Frozen hydrated sections prepared from high-pressure frozen cells are statically adhered to an EM grid and transferred to either a freeze-substitution mix at −90 °C (VIS2FIX$_{FS}$) or placed on frozen fixative at −90 °C (VIS2FIX$_{H}$). For VIS2FIX$_{FS}$, sections are rapidly freeze-substituted, rehydrated to buffer, and subsequently immunolabeled. For VIS2FIX$_{h}$, sections on frozen fixative are thawed, fixed on ice, and subsequently immunolabeled. (b) VIS2FIX$_{H}$ section of high-pressure frozen MDCK-II cells labeled for Forssman glycolipids. Gold labels are observed from the Golgi to the plasma membrane and in the endocytic pathway. n; nucleus, M; mitochondria, g; golgi, tgn; trans-golgi network, mvb; multivesicular body, and pm; plasma membrane. The scale bar is 500 nm. (Image courtesy of M. A. Karreman.)

environment. It is likely that because of this inevitable structural disturbance and reorganization of membrane lipids cholesterol is released from membranes and adheres all over the cryosection.[60]

The membrane disturbance in thawing cryosections may be alleviated with uranyl acetate, which can stabilize phospholipids by binding to the phosphate group.[61,62] The simplest method is to add uranyl acetate to the solution that is used to retrieve cryosections[63] (Figure 3a). By this method, labeling of cholesterol is confined to the membrane.[60]

More elaborate methods that use cryosections and uranyl acetate have been developed that harness the strength of quick-freezing methods to capture molecules in situ and stabilize phospholipids by utilizing uranyl acetate (Figure 3a). In one of the methods, called the rehydration method (RHM), quick-frozen samples are first freeze-substituted at a subzero temperature (e.g., −90 °C) to replace water with an organic solvent containing uranyl acetate. The samples are then rehydrated and subjected to Tokuyasu cryosectioning.[64] The other method is called VIS2FIX, in which VIS stands for vitreous sections (Figure 5a). In the VIS2FIX method, quick-frozen samples are directly subjected to ultrathin sectioning, and cryosections electrostatically adhering to EM grids are processed in two different ways. In VIS2FIX$_{FS}$, cryosections on grids are subjected to freeze-substitution, rehydration, and labeling.[65] VIS2FIX$_{FS}$ is essentially a rapid freeze-substitution procedure using ultrathin sections instead of cell/tissue blocks so that the level of lipid extraction is theoretically comparable to that of conventional freeze-substitution (see later for lipid extraction during freeze-substitution). In contrast, in VIS2FIX$_{H}$, cryosections on grids are placed on a frozen fixative, warmed to 0 °C, and labeled. Addition of uranyl acetate to the fixative helps to preserve the membrane structure. Forssman antigen has been labeled successfully with VIS2FIX$_{FS}$ and VIS2FIX$_{H}$ (Figure 5b),[65] but it remains to be seen to what extent these methods can be applied to other membrane lipids.

2.3. Immunoelectron Microscopy of Ultrathin Resin Sections Using Quick-Frozen, Freeze-Substituted, and Low-Temperature-Embedded Specimens. In this method, quick-frozen specimens are embedded in resins for ultrathin sectioning (Figure 3b). Specimens are freeze-substituted at a subzero temperature (e.g., −90 °C) using an organic solvent and then infiltrated with embedding resin solution also at a subzero temperature. After polymerization, the specimens are allowed to warm to ambient temperature, and ultrathin sections are labeled

with appropriate probes, as in conventional immuno-EM. This technique was used successfully to examine the distribution of PS.[66]

Even at very low temperatures, lipid extraction occurs during freeze-substitution and resin embedding, but it can be reduced by adding uranyl acetate to the freeze-substitution medium. For example, a loss of 9% of total phospholipids was reported for erythrocyte ghosts when they were freeze-substituted in methanol and embedded at −70 °C, whereas 15% was extracted when embedding took place at −30 °C. Lipid extraction was reduced to 2 and 4%, respectively, when 0.5% uranyl acetate was added to methanol during freeze-substitution.[67]

The effect of uranyl acetate is based on the binding of uranyl ions to the phosphorus groups of phospholipids[61,68] (with the exception of phosphatidic acid[68]). One uranyl ion can bind up to four lipid phosphorus groups,[62] thereby effectively cross-linking membrane phospholipids and inhibiting lateral lipid diffusion. Additionally, binding of the positively charged uranyl ions to the membrane affects the polarity and extractability of the lipids. Because uranyl acetate does not react with glycosphingolipids or cholesterol,[68] improvement of cholesterol retention in cryosections after treatment with uranyl acetate may be caused by the synergistic effect of increased sphingomyelin retention.[60]

With the use of uranyl acetate, distribution of target lipids at the moment of quick-freezing is thought to be largely preserved in the solidified resin sample. Because freeze-substitution is done at a temperature (e.g., −90 °C) far below the phase-transition temperature of almost all lipids, the lateral movement of lipids is expected to be small. Using an embedding resin that can be polymerized at the lowest-possible temperature (e.g., Lowicryl HM23 at −80 °C), the chance of redistribution during the sample preparation process should be further reduced. In this method, distribution of membrane lipids can be mapped in direct correlation to the subcellular structures, whose morphology is observed in a similar manner to conventional EM using ultrathin sections, allowing for the qualitative analysis of lipid distribution.

However, there are several shortcomings to immuno-EM using resin sections. One of these is a low capture ratio (i.e., only a small percentage of existing target lipids are labeled). This is partly because probes can bind only to target molecules exposed to the resin section surface (Figure 4b).[69] It is difficult to estimate the capture ratio of this method, but the reported label density of PS in the plasma membrane (e.g., 4.03 gold labels/μm^2 section in a rat liver cell) may represent far less than 10% of existing PS molecules considering the fact that PS generally comprises 10−20 mol % of phospholipids.[2] Additionally, it is not easy to distinguish which leaflet of the membrane is labeled for target lipids because membranes, which are about 5 nm in thickness, run and curve in random directions in sections of 50−100 nm in thickness. This is more of a problem in resin sections than in cryosections because the membrane structure is less distinct in resins. Moreover, the gold probe is separated from its target molecule by the length of at least one antibody. It is possible that the probe, which is located on the surface of the section, folds to the other side of the membrane (Figure 4c).

2.4. Immunoelectron Microscopy of Freeze-Fracture Replicas Using Quick-Frozen Specimens. In freeze-fracture, quick-frozen specimens can be processed without any chemical treatment (Figure 3c). In this method, membranes in the frozen specimen are split between the two leaflets, and thin layers of carbon and platinum are deposited on the revealed hydrophobic interface by vacuum evaporation[70] (Figure 6a). Membrane molecules are thus physically stabilized by the carbon and platinum backing, which is called a replica. The replica is treated with an SDS solution to dissolve extramembranous molecules. This treatment exposes the true membrane surface and makes the headgroup of membrane lipids accessible to probes. Using the quick-freezing and freeze-fracture labeling (QF-FRL) method, PC,[71] gangliosides GM1 and GM3,[38,72] PI(4,5)P_2[73,74] (Figure 6b), and phosphatidylglucoside[75] have been labeled specifically.

In ultrathin sections, the membrane is observed as a linear section, whereas in the freeze-fracture replica, the membrane is presented as a two-dimensional sheet. Therefore, probes can easily access the headgroup of membrane lipids. Because of this advantage, the capture ratio of QF-FRL was estimated to be 19−28% for GM1[38] and may be in the range of 30−50% for PI(4,5)P_2.[73] Another strength of this method is that the two leaflets of the membrane are split by freeze-fracture so that the asymmetric distribution of membrane lipids can be clearly analyzed.

Membrane molecules backed up by the replica are unlikely to move laterally. With regard to extraction, the amount of PC in the replica after the SDS treatment is no less than 80% of the amount in the replica treated with Triton X-100 alone.[71,76] The difference of 20% probably reflects the inability of Triton X-100 to remove extraneous PC (i.e., liposomes that were not freeze-fractured), but the possibility cannot be excluded that some extraction of PC from the replica occurs by the SDS treatment.

Although the membrane surface is widely exposed in the replica, steric hindrance between probes is unavoidable.[73] This is because membrane lipids are densely packed and the diameter of their head groups is much smaller than the size of protein probes (Figure 1b). For example, phospholipid head groups are about 1 nm in diameter.[77] Use of smaller markers mitigates this problem: the labeling density with 5 nm colloidal gold particles is approximately twice that of 10 nm colloidal gold particles (J. Cheng and T. Fujimoto, unpublished observation). The smaller probes are also important in decreasing the distance from the target lipid to the observable marker.

In analyzing membrane asymmetry, carbohydrates that exist only in the extracellular (and luminal) leaflets (i.e., glycolipids and glycoproteins) might preclude a uniform access of probes to phospholipid head groups. The effect of carbohydrate chains on the labeling efficiency is not significant, however, because in an experiment using liposomes containing different concentrations of glycolipids, the labeling of phospholipids was not reduced even when the content of glycolipids was as much as 30 mol % (J. Cheng and T. Fujimoto, manuscript in preparation).

Theoretically, the QF-FRL method appears to be superior to methods that use chemical fixatives in terms of defining endogenous lipid distribution at the nanometer resolution level, but more detailed evaluation is necessary to determine whether membrane lipids are invariably retained in the replica and to ensure that they are accessed evenly with labeling probes.

3. HOW MUCH WE KNOW (OR DO NOT KNOW) ABOUT TWO-DIMENSIONAL DISTRIBUTION OF MEMBRANE LIPIDS

In this section, we consider two membrane lipids, GM1 and PI(4,5)P_2, the distribution of which has been the subject of many studies. On the basis of the methodological considerations in the previous sections, we will discuss how much we know about their endogenous distribution.

3.1. GM1. Ganglioside GM1 is a glycolipid that is expressed in a wide range of cells and has been used as a raft marker in many

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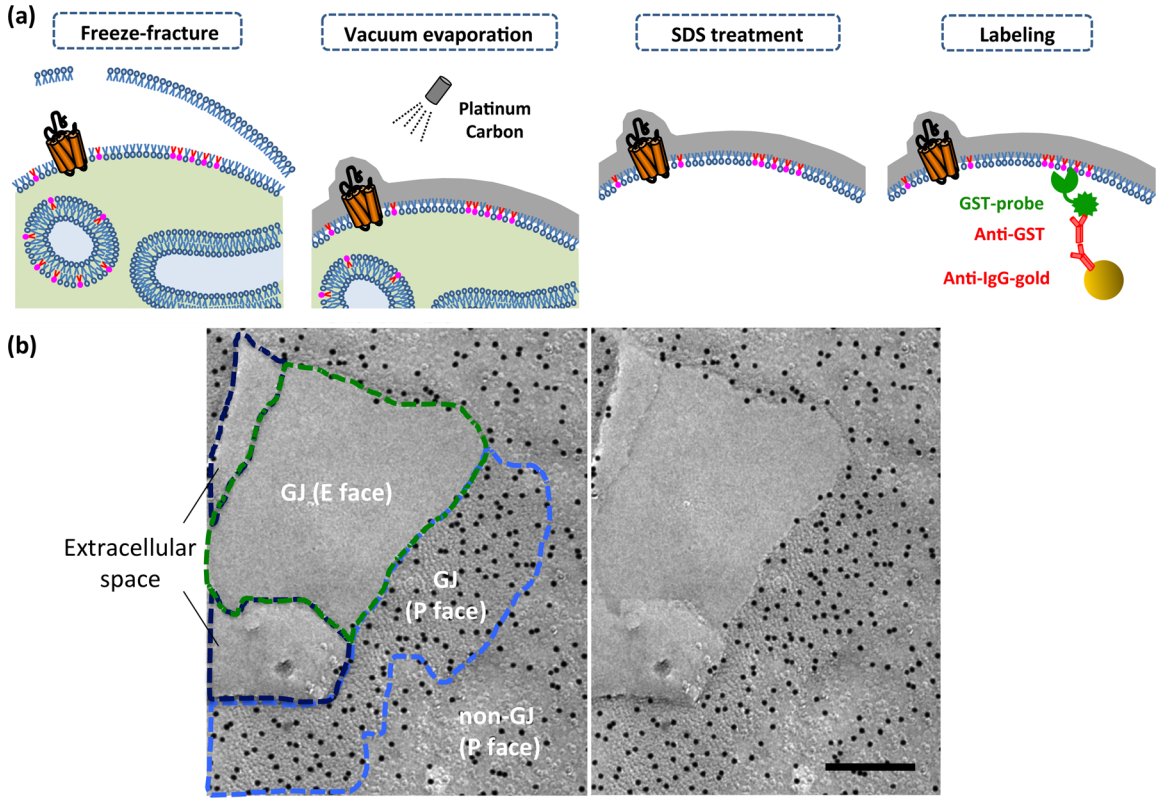

Figure 6. QF-FRL method. (a) Outline of the method. Quick-frozen cells are freeze-fractured to reveal the hydrophobic interface between the two membrane leaflets. A platinum/carbon replica made by vacuum evaporation is treated with SDS to remove extramembranous molecules and labeled with lipid-binding probes or antibodies. Membrane samples thus prepared can be observed as a two-dimensional sheet. (b) QF-FRL showing enrichment of $PI(4,5)P_2$ at the P face (representing the cytoplasmic leaflet) of the gap junction (GP) membrane. Labeling density of $PI(4,5)P_2$ at the P face of non-GP membrane is significantly less than that of the GP membrane, whereas the E face (representing the extracellular leaflet) of the GP membrane is not labeled at all. The scale bar is 200 nm. (Modified from ref 74.)

studies. GM1 in the extracellular leaflet of the plasma membrane can be labeled by use of fluorescence-tagged cholera toxin B subunit (CTB) without any special pretreatment.

CTB is a pentamer that can bind five molecules of GM1 head groups[78] (Figure 1c). Because of its multivalency, CTB induces clustering of GM1, which eventually leads to cap formation when cells like lymphocytes are incubated at 37 °C.[37] Fixation with formaldehyde is thought to suppress gross CTB-induced redistribution like capping, but local clustering or patching may still occur.[79,80] Therefore, it is difficult to examine the innate distribution of GM1 by use of CTB.

Two methods that appear to preserve endogenous GM1 distribution are (1) the use of glutaraldehyde as a fixative and (2) cooling to a low temperature. Glutaraldehyde causes extensive cross-linking of proteins, thereby inducing gelation of the cytosol and immobilization of membrane proteins. Because of these effects, the long-range movement of membrane lipids appears to be suppressed.[79,80] However, because glutaraldehyde does not directly react with most lipids, lipids remain mobile on a small scale[6] (Figure 2a). Additionally, fixation with glutaraldehyde takes some time to complete and may not proceed evenly across the membrane. These factors compromise the spatial resolution of the glutaraldehyde method and, more importantly, make it difficult to define the spatial resolution of the method accurately.

Use of a low temperature is problematic for two reasons. First, because membrane molecules can move even at a low temperature (e.g., 4 °C), which allows local clustering of CTB.[37] Second, because it takes time to cool specimens (i.e., there is no quick-cooling method), phase transition is likely to occur in the membrane during the cooling process (e.g., from 37 to 4 °C).[81] This change is likely to affect the distribution of membrane lipids significantly. For example, lipids showing preference to the liquid-ordered phase, like GM1, may be confined to a small liquid-ordered phase area at 37 °C, but as the temperature goes down and the area of the liquid-ordered phase increases, these lipids are likely to redistribute and show less clustering. Therefore, distribution of the probe observed at a low temperature may not indicate the distribution of the target lipid at the initial condition.

On the basis of the above considerations, we think that distribution of GM1 observed by QF-FRL is likely to reflect its endogenous distribution more faithfully than that obtained by other methods. With QF-FRL, GM1 in fibroblasts was observed to make clusters of 94 nm in diameter, and clustering decreased significantly when free cholesterol was extracted from the cell or the cell was cooled on ice before quick-freezing.[38]

Ganglioside GM3 was also shown by QF-FRL to distribute in clusters that decrease upon cholesterol depletion. However, labels for GM1 and GM3 rarely showed coclusters, and their mutual relationship changed significantly when actin was depolymerized.[72] This result indicates that distribution of endogenous GM1 and GM3 is not determined only by their propensity to partition in the liquid-ordered phase but is affected significantly by other factors, including the cytoskeleton.

3.2. $PI(4,5)P_2$. $PI(4,5)P_2$ is the most abundant phosphoinositide, and it is mainly distributed in the inner leaflet of the plasma

membrane. $PI(4,5)P_2$ has a wide variety of physiological regulatory functions in endocytic membrane trafficking, membrane-cytoskeletal interaction, and activity of ion channels and transporters. The question has been asked of how a simple phospholipid can perform so many different functions, and the most likely answer to this question is thought to be the presence of independent $PI(4,5)P_2$ populations that are compartmentalized in different membrane domains. It is important, therefore, to know the distribution of $PI(4,5)P_2$ on the smallest-possible scale and as quantitatively as possible.[82,83]

Seemingly in accordance with the above speculation, concentration of $PI(4,5)P_2$ in a small membrane region has been suggested. By live imaging, GFP-PLCδ1-PH, a sensor probe for $PI(4,5)P_2$, was found to be distributed densely in the phagocytic cup of macrophages,[29] the cleavage furrow of dividing cells,[84] and the shmoo tip of yeast.[85] These examples are relatively large membrane areas, up to about 10 μm in width, but clustering of $PI(4,5)P_2$ in sub-micrometer-sized domains has also been reported.[86−88] The latter results were obtained using membrane lawns that were prepared by ripping the upper part of culture cells by brief ultrasonication. The preparation, either fixed or unfixed, was then incubated with antibodies or lipid-binding protein probes, and the label was observed by fluorescence microscopy.

Considering the insufficiency and pitfalls of chemical fixation and lipid-binding protein probes as discussed in previous sections, it is arguable whether and to what extent the signals obtained using the above procedures accurately indicate the endogenous $PI(4,5)P_2$ distribution. For example, it appears difficult to exclude the possibility that the binding of the probe induced clustering of $PI(4,5)P_2$. As a matter of fact, in a separate experiment, when cells fixed with formaldehyde were labeled with antibodies to GAP43, CAP23, or MARCKS, which are known to interact with $PI(4,5)P_2$, the labeling for the proteins showed sub-micrometer-sized clusters that overlap precisely with $PI(4,5)P_2$ labels; in contrast, in cells fixed with glutaraldehyde, both the protein and $PI(4,5)P_2$ labels distributed homogenously without clustering.[27] These result suggest that binding of multivalent antibodies to $PI(4,5)P_2$-binding proteins caused clustering of the proteins as well as $PI(4,5)P_2$ and that glutaraldehyde is effective in preventing gross molecular redistribution. As already mentioned in previous sections, however, the use of glutaraldehyde does not preclude lipid movement on a smaller scale.

Inclusion of $PI(4,5)P_2$ in a detergent-resistant membrane fraction suggests that $PI(4,5)P_2$ may be clustered in raft microdomains.[89] However, $PI(4,5)P_2$ contains abundant polyunsaturated acyl chains,[90] which may not be compatible with raft-philic lipids enriched with saturated acyl chains. Actually, with QF-FRL, labels for $PI(4,5)P_2$ were not significantly clustered in the fibroblast plasma membrane but showed marked concentration at the caveolar orifice.[73] We speculate that the recovery of $PI(4,5)P_2$ in the detergent-resistant membrane is caused by binding to proteins such as caveolin-1, which can sequester $PI(4,5)P_2$ by electrostatic interaction.[91]

4. OUTLOOK

In this Current Topic, we have discussed various microscopic methods from the standpoint of how precisely they can define the distribution of membrane lipids at the time of interest. Although some methods are not appropriate to determine lipid distribution on the nanometer scale, they may be useful to observe lipid distribution on a larger scale and/or to keep track of lipids in live cells. Differences in spatial resolution among the various methods also occurs for membrane proteins, but these differences are more drastic for membrane lipids because lipids diffuse much faster than proteins and lipids are not reactive with chemical fixatives.

Many microscopic methods that are available today were basically developed to observe the distribution of proteins, and some of the protein-oriented methods are not suitable for lipid studies. However, other methods, such as the chemical biological approaches described in this Current Topic, may be used more readily for lipids than for proteins. The application of super-resolution microscopy to membrane lipids in combination with the least-perturbing fluorescence tags (or labels) should also be further expanded.[92,93] We need to exploit these possibilities and create new methods that are better suited to studying lipid distribution in situ. Just as GFP technology revolutionized studies on proteins, such new methods should dramatically deepen our understanding of the physiological roles of membrane lipids.

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Notes

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