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Definition of phosphoinositide distribution in the nanoscale

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New functionalities of phosphoinositides (PIs) are being revealed continuously, and the scale of the membrane area studied is becoming smaller, from the micrometer range like the entire surface of organelles to the nanometer range as in subdomains of organelles. Concurrently, function of less abundant PIs, such as PI(3,4)P_2 and PI(3,5)P_2, attracts increasing attention. In accordance with the progress, finer and more accurate information on PI distribution is required. The fluorescence biosensor method utilizing PI-binding domains and/or immunolabeling with anti-PI antibodies are used for this purpose in most studies but both methods are known to have caveats. In this article, we examined how PI distribution was defined in recent studies and discussed whether methodological uncertainty has any bearing on the results.

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Introduction

Phosphoinositides (PIs) are relatively minor phospholipids but they affect the distribution and function of many proteins [1,2]. PIs are often regarded as hallmarks of various organelles, but they are not static constituents of any organelle and exhibit dynamic turnover. Moreover, PIs are not uniformly distributed in an entire organelle—PI turnover occurs differentially in small subdomains, or rather, subdomains are created by the differential turnover of PIs. Thus, for deciphering PI functions, it is important to define PI distribution at the smallest-possible scale.

In this article, we reviewed how PI distribution was examined in recent works. In many of the studies, either the fluorescence biosensor method utilizing PI-binding proteins (domains), immunolabeling with anti-PI antibodies, or both were used to define subcellular distribution of PIs. The current understanding of PIs could not have been attained without these imaging methods, especially fluorescence biosensors. Both methods are known to have caveats [3–5], however, and as the dimension of the area in question gets smaller, and as the importance of minor PIs is being highlighted, further attention to the problem is required.

General consideration

For both fluorescence biosensors and immunolabeling, the authenticity of positive signals can be tested by checking whether they are specific to and dependent on the target lipid, and whether the presence of the target lipid is sufficient to give the signal [3]. In contrast, the possibility of false-negative results (i.e. signals are not observed despite the presence of target lipids) may be more difficult to exclude. False-negative results could happen, for example, if the binding of a biosensor requires a factor other than the target lipid, such as the co-presence of a protein or some properties of the membrane (e.g. lipid composition, curvature, etc.) besides the target lipid. Presence of endogenous proteins competing with the biosensor in binding to the target lipid is another possible cause of false-negative results. An example of such a case was observed in cells treated with 25-hydroxycholesterol [6•]. In this instance, although the amount of phosphatidylinositol 4-phosphate (PI4P) in the Golgi did not seem to change, the signal obtained by pleckstrin homology (PH)-domain PI4P biosensors increased whereas the immunolabeling of PI4P decreased. This contradictory result is thought to be caused by the recruitment of oxysterol-binding protein (OSBP), which probably changed the accessibility of PI4P to the biosensor and the antibody.

Another problem related to PI-binding biosensors is that their expression may affect the cellular phenomenon by abrogating the functions of endogenous proteins binding to the same target PI. In recent studies, this effect was intentionally used to demonstrate the functional importance of target PIs: overexpression of GFP-2xFYVE that binds to PI3P was shown to influence the endosome size and the autophagic flux [7•], and overexpression of either GFP-PHD3X or GFP-PH-Dok-5, both binding to PI5P, decreased autophagosome formation [8]. The same PI-

binding biosensors were also used to define endogenous PI distribution; for this purpose, what has been done conventionally is to express them at a low level. This practical caution may be good enough for most cases, but there is no gold standard to judge whether the expression of biosensors does not have any side effect.

Immunolabeling of intracellular PIs requires the removal or permeabilization of the plasma membrane, and for the purpose of preserving endogenous PI distribution, cells are chemically fixed. As discussed in the next section, however, these procedures also have problems. Caveats related to the microscopic detection of lipids, including the use of fluorescent lipid analogs, were discussed in a recent review [9].

PI(4,5)P_2 domains in the plasma membrane

PI(4,5)P_2 is involved in multiple functions of the plasma membrane, but how this simple phospholipid can be so versatile has been a question. Spatial segregation through electrostatic binding with cationic proteins is thought to be a mechanism to form functional subpopulations [10–12]. The reported distribution of PI(4,5)P_2, however, is variable—from random to clustering in different degrees [11,13–15].

In many studies, the plasmalemmal distribution of lipids, including PI(4,5)P_2, has been studied using membrane sheets, or a membrane preparation mechanically detached from cultured cells [11,14,15]. The membrane sheet is usually obtained from pre-cooled cells, and then chemically fixed, and labeled by protein probes. This method is convenient in that it can be readily combined with superresolution microscopy and/or electron microscopy (EM) to obtain two-dimensional views at high resolution. But recent results suggest that each step of the procedure, that is, cooling, mechanical detachment, fixation, and probe binding, has a possibility of perturbing the endogenous lipid distribution (Figure 1).

Cooling is used to arrest the lateral mobility of membrane lipids, but phase separation is inevitable during the cooling process, in which cells are cooled slowly, for example, by being placed on ice. Change in the lipid distribution caused by cooling is evident on the nanometer scale [16], even though it may not be detectable by diffraction-limited light microscopy [17].

Preparation of the plasma membrane sheet, which often utilizes ultrasonic disruption of the cell body, may not be immune to perturbation of lipid distribution. The mechanical procedure disrupts the cytoskeleton, and this most likely affects membrane tension. In budding yeast, a decrease of membrane tension was shown to induce substantial redistribution of PI(4,5)P_2 in the plasma membrane [18••]. A similar phenomenon might be occurring during the mechanical detachment process.

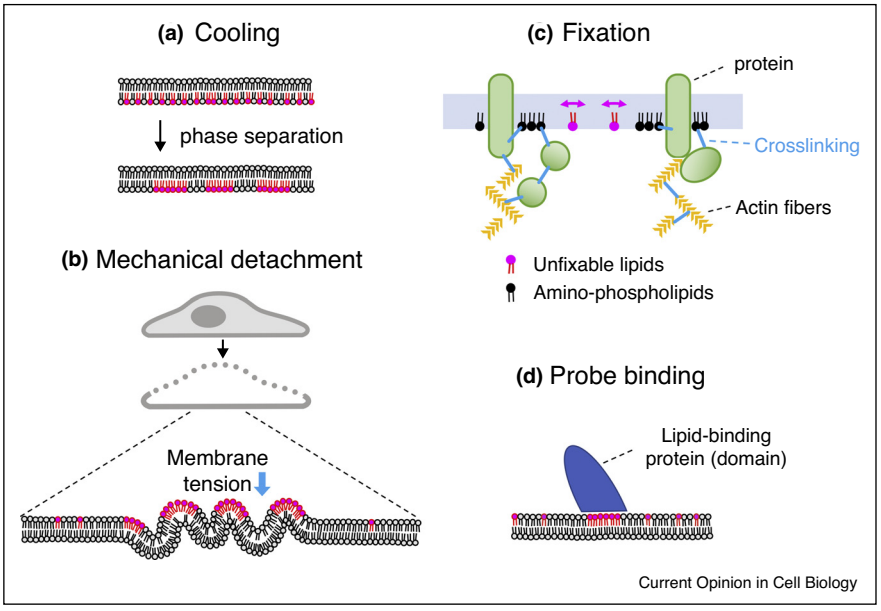
Fixation is usually performed by aldehydes. The lateral movement of lipids appears to be suppressed in cells fixed by glutaraldehyde [11,14,15], but aldehydes do not react with most lipids, and single fluorescent molecule imaging actually showed that lipids retain mobility even after glutaraldehyde fixation [19]. The latter result indicates that the apparent immobilization of lipids may be caused by the confinement of lipids within the meshwork of the membrane skeleton, which is smaller than the diffraction-limit of light [20] and is tightened by protein crosslinking by the fixative. Moreover, while most lipids do not react with aldehydes, amino-containing lipids, that is, phosphatidylserine (PS) and phosphatidylethanolamine, do react and may be crosslinked with proteins [21]. This reaction may occur only erratically, but by affecting the two major phospholipids of the cytoplasmic leaflet, it is likely to perturb the distribution of PIs existing in the same leaflet. Recently, glyoxal, a new fixative, was shown to give a higher PI(4,5)P_2 labeling than formaldehyde [22], but glyoxal is also an aldehyde and may not be exempt from the above-mentioned problems.

Binding of protein probes may also affect the distribution of target PIs, as long as the fluidity persists in the membrane preparation. It is conceivable that polyvalent probes, such as whole immunoglobulin antibodies, bind more than one target molecule and induce their clustering. In contrast, most PI-binding protein domains were thought to be monovalent, and thus free from such effect. Nevertheless, the binding of PH domains was shown to induce clustering of PI(4,5)P_2 [23,24]. This is probably because they have non-canonical binding sites as well as a canonical one, as indicated by molecular dynamics simulation [25••]. Interaction with multiple lipids, which may involve non-PI lipids as well as PIs, is also widespread in PI-binding proteins [26–30]. Binding of these probes may induce or augment PI clustering.

Considering the problems described above, results obtained by the membrane sheet method need to be rigorously tested by independent methods. For this purpose, the electron microscopic method using freeze-fracture replicas of quick-frozen cells is worthy of consideration, because lipids in the membrane are physically immobilized before labeling [31]. Although the method requires special equipment and has own limitations [31], it is exempt from the problems discussed above. Notably, by using this method, PI(4,5)P_2 in the plasma membrane was shown to distribute evenly except in caveolae [13].

In subsequent sections, PI distribution that was mainly studied by expression of biosensors in living cells will be discussed.

Figure 1



Potential problems in defining PI distribution using the membrane sheet method.
(a) Cooling below a phase separation temperature (e.g. cooling on ice) affects lipid distribution.
(b) Mechanical detachment is likely to decrease the membrane tension, which in yeast was shown to induce PI(4,5)P_2 clustering through phase separation.
(c) Fixation with aldehydes tightens the membrane-associated protein network through protein–protein crosslinking and also crosslinks amino-containing phospholipids to proteins.
(d) Binding of PI-binding protein domains, which have non-canonical binding sites in addition to the canonical one, induces clustering of the target PI.

Segregation of PI4P and PI(4,5)P_2 in primary cilium

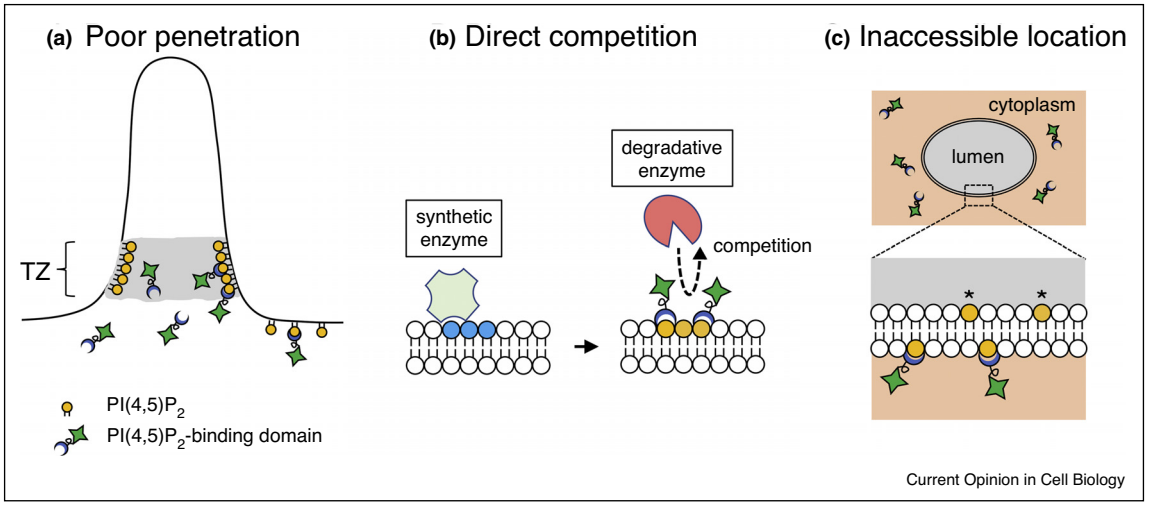
Primary cilium is a thin antenna-like structure that senses a variety of extracellular signals. Recent studies showed that PI(4,5)P_2 is largely limited to the ciliary base, whereas PI4P distributes along the ciliary membrane and that this differential distribution is generated by inositol phosphate-5-phosphatase (INPP5E) [32,33]. Upon growth stimulation, INPP5E disappears from cilia, and the resultant increase of PI(4,5)P_2 in the ciliary membrane causes actin polymerization and ciliary vesicle release by decapitation [34]. The differential distribution of PI(4,5)P_2 in the cilia is also important for Hedgehog signaling [32,33] and trafficking of integral membrane proteins to cilia [35]. Another inositol 5-phosphatase, OCRL, also appears to be involved in the PI turnover in cilia [36], and PI(3,4,5)P_3, a substrate of both INPP5E and OCRL, was shown to be critical for ciliogenesis and the functionality of the transitional zone at the ciliary base [37,38].

In the above studies, PI distribution in cilia was examined by both fluorescence biosensors and immunolabeling, and the overall results appear to coincide. Nevertheless, the seemingly complete lack of the PI(4,5)P_2-biosensor signal in the ciliary shaft [33,34] was divergent from the gradual decrease of PI(4,5)P_2 toward the ciliary tip by immunolabeling [37]. Here, it is not clear whether PI(4,5)P_2-biosensors can freely reach the ciliary shaft region; their infiltration may be hindered by the diffusion barrier at the transition zone and/or entrapment by PI(4,5)P_2 enriched there (Figure 2a). The difference between the results obtained by the two methods may be of note in analyzing the function of the transitional zone further.

PI4P/PS exchange at membrane contact sites

Anterograde cholesterol transport driven by OSBP at the ER–trans Golgi network membrane contact site (MCS) depends on the PI4P density gradient, which is generated by its synthesis in the Golgi and hydrolysis by Sac1 phosphatase in the ER [39]. Using a similar PI4P gradient-dependent counter-transport mechanism, ORP5 and ORP8 in mammalian cells [40–42] and Osh6 and Osh7 in budding yeast [43] were found to transport PS from the ER to the plasma membrane. The molecular function of these ORP/Osh proteins was clearly shown by *in vitro* experiments, but some uncertainties remain for their *in vivo* functionalities. Whether Sac1 works in *cis* or *trans*, that is, degrades PI4P in the ER or in the plasma membrane, is critical for the counter-transport mechanism, but the results are divided, partially depending on whether

Figure 2



Potential problems in defining PI distribution by expression of PI-binding proteins.
(a) PI(4,5)P_2-binding probes may not freely reach the ciliary shaft due to the diffusion barrier at the transition zone (TZ) and/or by entrapment by PI(4,5)P_2 enriched at TZ.
(b) PI-binding probes may directly compete with enzymes degrading the target PI, thus hindering its elimination. Such competition does not occur with enzymes synthesizing PIs, because they act on its precursors.
(c) PI-binding probes expressed in the cytoplasm cannot get access to PIs in the lumenal leaflet (*).

PI4P biosensors show an increase of PI4P in the ER upon downregulation of Sac1 or ORP/Osh [44–47]. Additionally, an increase of PS in the ER, which is expected to occur in the absence of ORP/Osh proteins, was observed in Osh6/7-deficient yeast [48], whereas the results in mammalian cells depleted with ORP5/8 were not clear [40,41]. The difference may reflect a difference between yeast and mammalian cells (e.g. difference in PS metabolism), but a methodological problem in detecting PS in the ER may also be involved.

PI(3,4)P_2 in endocytosis

The dynamic conversion of PIs in clathrin-mediated endocytosis was captured using coincidence-detecting fluorescence biosensors [49•]. In order to clearly visualize PI(4,5)P_2, PI4P, PI3P, and PI(3,4)P_2 in the coated pit region, coincidence-detecting biosensors were constructed by fusing the clathrin-binding domain of auxilin-1 and PI-binding domains. The biosensor for PI(3,4)P_2 detected a signal in coated vesicles, but not in coated pits, suggesting that PI(3,4)P_2 increases after scission from the plasma membrane [49•]. This result appears to differ from that of a previous study using immunolabeling in fixed cells, which suggested that PI(3,4)P_2 increases during the maturation of coated pits [50], and regulates constriction of the neck by recruiting SNX9, a PX-BAR protein [51]. The discrepancy might result because the neck region of coated pits is too distant from the auxilin-1-binding site of the clathrin cage for the coincidence-detecting biosensor to bind [52], but this point needs to be explored further. PI(3,4)P_2 is also related to fast endophilin-mediated endocytosis [53], and the definition of its distribution at the small scale is critical to understand the functional importance.

PI(3,5)P_2 and endosomal tubular domains

PI(3,5)P_2 as well as PI3P are involved in the endosomal recruitment of BAR domain-containing sorting nexins (SNX-BAR), which coordinate tubular membrane formation and cargo selection [54]. By using freeze-fracture replica labeling and the biosensor method, PI(3,5)P_2 in endosomes was found to be deficient in the tubular domain in comparison to the vesicular domain [55•]. One possible cause of the local PI(3,5)P_2 deficiency may be hydrolysis by myotubularin 1, which was shown to be recruited to the tubular portion by a Rab11-dependent mechanism [56], and PI5P generated by hydrolysis of PI(3,5)P_2 activates myotubularin 1, further promoting the decrease of PI(3,5)P_2 [57]. Downregulation of PI(3,5)P_2 should decrease recruitment of SNX-BARs and is also likely to facilitate branched actin network formation [58], leading to endosomal fission [59], suggesting its importance in the regulation of endosomal tubular extension.

Theoretically, PI hydrolytic processes are likely to be more susceptible to expression of PI-binding biosensors than PI production processes, because the biosensors and eliminating enzyme(s) may directly compete in binding to target PI (Figure 2b). This is especially so for minor PIs like PI(3,5)P_2. Possible effects of biosensors on physiological processes may need to be evaluated more carefully when a decrease of the target PI is involved.

Concluding remarks

We discussed methods that were used in recent studies to examine PI distribution in various dimensions—the nanometer range in the plasma membrane to the micrometer range as in the primary cilium. It is noteworthy again that PI-binding probes play critical roles in almost all the works. The possibility that PI-binding biosensors perturbs cellular phenomena may not be high if their expression level is kept low, but the margin of safety for minor PIs, such as PI(3,4)P_2 and PI(3,5)P_2, may be smaller in comparison to that for much more abundant PI(4,5)P_2 and PI4P, especially when their functionalities in small areas are examined. Information obtained by methods based on different principles, such as freeze-fracture replica labeling, should be particularly important in studying PIs of low abundance [55•].

One terrain that is difficult to approach by conventional PI-biosensors and has thus been left largely unexplored is the lumenal leaflet of intracellular organelles (Figure 2c). PIs are generally confined to the cytoplasmic leaflet, but PI3P and PI(4,5)P_2 were shown to exist in the extracellular leaflet of the plasma membrane [60•,61]. The results suggest that organelles may also harbor PIs in the lumenal leaflet under some conditions, but little is known, except for PI3P in yeast autophagosomal membranes [62]. How PIs are moved to the lumenal/extracellular leaflet is largely unknown, except for involvement of ABCA1 in exposing PI(4,5)P_2 to the cell surface [60•]. In this context, it is notable that scramblases can translocate lipids with large headgroups probably by deforming the membrane lipid bilayer [63]. It will be interesting to study whether such scramblase activity causes translocation of PIs to the lumenal/extracellular leaflet and what functions PIs have in that location.

Conflict of interest statement

Nothing declared.

Author contribution statement

Draft and funding acquisition: T.T., S.T., and T.F., Conceptualization and writing: T.F.

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