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Chemical Biology Tools to Study Lipids and their Metabolism with Increased Spatial and Temporal Resolution

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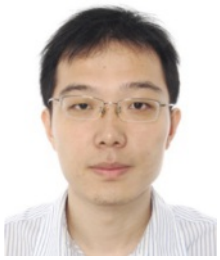
Abstract: Lipids are important cellular components providing many essential functions. To fulfill these various functions evolution has selected for a diverse set of lipids and this diversity is seen at the organismal, cellular and subcellular level. Understanding how cells maintain this complex lipid organization is a very challenging problem, which for lipids, is not easily addressed using biochemical and genetic techniques. Therefore, chemical tools have an important role to play in our quest to understand the complexities of lipid metabolism. Here we discuss new chemical tools to study lipids, their distribution and metabolism with increased spatial and temporal resolution.

Keywords: Glycerophospholipids · Lipidomics · Metabolic bias · Photocaging · Sphingolipids

Howard Riezman is Professor of Biochemistry and was Director of the NCCR Chemical Biology for 11 years. He has worked on membranes, their components and biogenesis for most of his career. His recent studies bring interdisciplinary approaches towards the study of lipid metabolism and function.



Suihan Feng worked as a postdoc in Howard Riezman lab, and now is a principal investigator at the Institut Pasteur Shanghai (IPS). In the recent years, he has proposed and developed the organelle-specific photoactivation strategy to study local lipid metabolism and functions.

Clémence Simon obtained her PhD in chemical biology under the supervision of Prof. Christophe Biot at the University of Lille and since 2020 is an HFSP postdoctoral fellow with Prof. Howard Riezman at the University of Geneva. Her research focuses on the development and use of chemical tools to follow lipid and glycan metabolism in living cells with high spatio-temporal precision.

1. Lipid Complexity Explained by their Diversity

Lipids define a broad class of biomolecules with a myriad of essential functions for the cell. They are the main component of biological membranes, forming a functional barrier between the cell and its environment and between its different organelles. They constitute a major source of energy and heat as well, allowing the cell to perform its biological functions. They can also play a vital role in cell signaling. Finally, they are also involved in the protein recruitment platforms and post-translational modification of proteins. As lipids present essential functions, errors in their metabolism can lead to severe diseases.[1] Even though mass spectrometry-based lipidomics has been developed 30 years ago to study cellular lipids, these biomolecules remain less studied than proteins and nucleic acids in part because of their intrinsic complexity. The latter can be explained in part by their diversity at two levels: chemical and compositional.[1]

1.1 *Chemical Diversity*

The wide range of functional roles of lipids can be explained by their chemical diversity. They are simply defined as molecules soluble in non-polar solvents, but they can be divided into eight categories depending on their chemical structures.[2] Among them, the most common lipid categories in eukaryotic cells are glycerophospholipids (GPLs), sphingolipids (SLs) and sterols, forming together the lipid bilayers of the biological membranes (Fig. 1). Each category is composed of a particular chemical skeleton (glycerol, sphingoid base and fused four-ring core, respectively) to which can be attached different hydrophobic fatty acids and hydrophilic headgroups. GPLs are composed of a glycerol backbone to which two fatty acids and one headgroup are linked, giving rise to different classes (Fig. 1b). The fatty acids vary in chain length, double bond number and position or hydroxylation. The sn1 fatty acid is more often saturated or monounsaturated whereas the sn2 chain is usually mono or polyunsaturated.[3] For SLs, the chemical diversity comes from the species of sphingoid base, *N*-acyl chain and headgroup (Fig 1c). Usually, they have a more saturated and longer acyl chain than GPLs.[4] Cholesterol is the main sterol in mammals whereas ergosterol is the most common one in yeast (Fig. 1d). The side chain structure and a double bond on the steroid ring is the main difference between them. Finally, other levels of chemical diversity can be found in lipids through the phosphorylation position and degree of phosphatidylinositols[5] or the glycan structures present in saccharolipids.[6]

1.2 *Compositional Diversity*

Lipid distribution is diverse at different scales. First, different mammalian tissues have characteristic lipid compositions. For ex-

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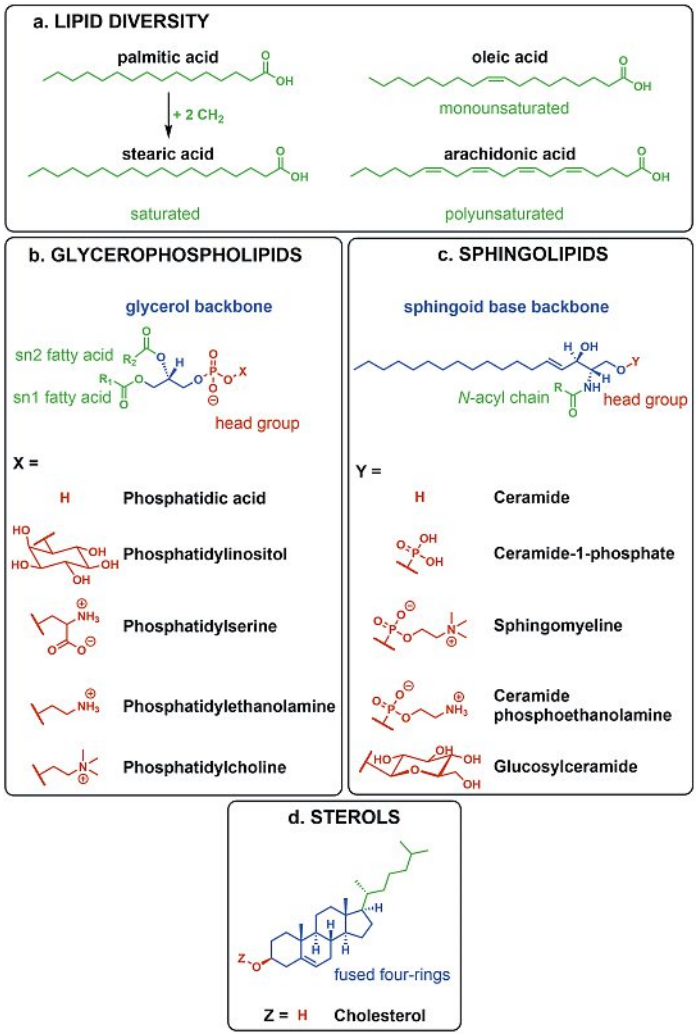

Fig. 1. Chemical structures of the major membrane lipids in mammals explaining their diversity.

ample, aminophospholipids PtdSer and PtdEth are enriched in the brain compared to other tissues.[7] Each tissue presents a different profile of GPLs composed of a specific fatty acid composition.[8] In the same way, the *N*-acyl chains of SLs vary according to the cell type and function.[4] Lipid distribution varies as well at the organelle level:[9] while the plasma membrane is enriched in SLs, PdtSer and cholesterol, the endoplasmic reticulum (ER) has less cholesterol and more unsaturated GPLs. Moreover, some lipids can be only found in one organelle such as cardiolipin which is present only in mitochondria.[10] In a single organellar membrane, the leaflets also have diverse lipid composition. For example, the plasma membrane presents an asymmetric arrangement: its exoplasmic leaflet is enriched in SLs and PdtCho whereas its cytoplasmic face leaflet is enriched in the amino-phospholipids PtdSer and PtdEth.[11]

1.3 *Metabolic Diversity*

The complexity of lipid metabolism explains the compositional diversity. First, lipid synthesis involves different organelles.[12] The ER is the major site of lipid biogenesis, producing the bulk of GPLs, cholesterol and ceramides as precursors for complex sphingolipids. These molecules are then rapidly transported to other organelles. The majority of cholesterol (60–70% of the total cell amount) is rapidly transported to the plasma membrane. The Golgi apparatus sorts and transports lipids as well as proteins. Moreover, it can synthesize SLs which are exported to the plasma membrane. The plasma membrane does not produce its own structural lipids, but is involved in synthesis and degradation reactions for lipid messengers like phosphoinositides or lipid electrophiles.[13,14] Although mitochondria import some lipids from the ER, they synthesize significant amounts of phosphatidic acid, PdtGly and all of the cardiolipins. By decarboxylation of PdtSer, they produce mitochondrial PdtEth which is exported to other organelles.[15]

While some lipid classes can be generated in different organelles (PtdEth in the ER and mitochondria), their synthesis can also be compartmentalized at the sub-organelle scale. The mitochondria-associated ER membranes (MAM) are regions of the ER that mediate communication between the ER and mitochondria through contact sites.[16] They are enriched in lipid-related enzymes, suggesting that lipid formation may be compartmentalized in the ER.[17,18] The broad specificity of some enzymes responsible for lipid metabolism can also explain some of the lipid structural diversity.[19] Indeed, several steps of lipid metabolism involve isozymes having overlapping substrate preference and different levels of expression, generating diverse lipid composition at the tissue level.

Finally, the major mammalian poly-unsaturated fatty acids (PUFAs) cannot be synthesized *de novo* and are therefore provided by diet, which affects composition of GPLs and SLs.[20]

2. Different Chemical Tools to Study Lipid Metabolism

2.1 *Main Problems to Study Lipid Metabolism*

Despite their essential function and their involvement in several human diseases, lipid metabolism remains less studied than other biomolecules such as proteins and nucleic acids. On the one hand, lipid metabolic processes are very rapid and occur in precise places, which make them difficult to detect *in vivo.* Indeed, classical techniques to study them, like pulse-chase radiolabeling or enzyme kinetic measurements, provide information about their rates of synthesis, but they cannot inform on their site of synthesis and their rates of transport in living cells. On the other hand, it is difficult to track lipids in living systems because unlike proteins, each lipid species is not directly genetically encoded and cannot be selectively reduced/overexpressed in vivo. Moreover, given the important structural diversity of lipid categories, each class necessitates an adapted method of extraction, purification, characterization and analysis.

Over the last few decades, chemical tools have been developed in order to address lipid complexity. Combined with bioanalytical techniques, they allow to obtain more information about their transport and/or metabolism.

2.2 *Metabolic Reporters*

Developed twenty years ago, the metabolic reporter strategy consists of exploiting the metabolic machinery of cells through two steps (Fig. 2a).[21] A biosynthetic precursor of lipids bearing a small and abiotic chemical tag is incorporated in living cells. This molecule-reporter is recognized by enzymes and metabolized as its natural analogue. It can be selectively linked to a probe by a bioorthogonal click chemistry reaction, allowing to detect it in cells. The chemical tag can be added either on the hydrophobic acyl chain, or on the hydrophilic head group. The probe is usually a fluorophore to localize the lipid in cell membranes after their metabolic uptake.

This two-step strategy allowed the visualization of the subcellular distribution of phospholipids,[22–24] sphingolipids[25] or cholesterol[26] in mammalian cell membranes. The most used bioorthogonal reactions for fluorophore tagging were the copper-catalysed azide-alkyne cycloaddition (CuAAC) and the strain-promoted azide-alkyne cycloaddition (SPAAC), on fixed and live cells, respectively. Developed more recently, the inverse electron demand Diels-Alder (*i.e.* DDA) reaction was also applied to a ceramide analogue bearing a trans-cyclooctene. Its localization

in the Golgi apparatus was observed by live-cell, super-resolution imaging.[27] It should be noted that fatty acid reporters can also be used, but they mainly highlight protein lipidation and not lipid metabolism.[28,29]

Stimulated Raman Scattering (SRS) was also used to detect the modified lipid analogues. It allowed to get rid of the bioorthogonal reaction of fluorophore ligation since the SRS imaging is able to selectively detect the vibrational mode of the alkyne bond. This notably allowed the visualization of alkyne-tagged PdtCho in hippocampal neurons.[30] Feeding deuterated lipids can also be used to detect them by SRS as has been done with deuterated-cholesterol in cell culture. This study revealed a diverse lipid composition of lipid droplets.[31]

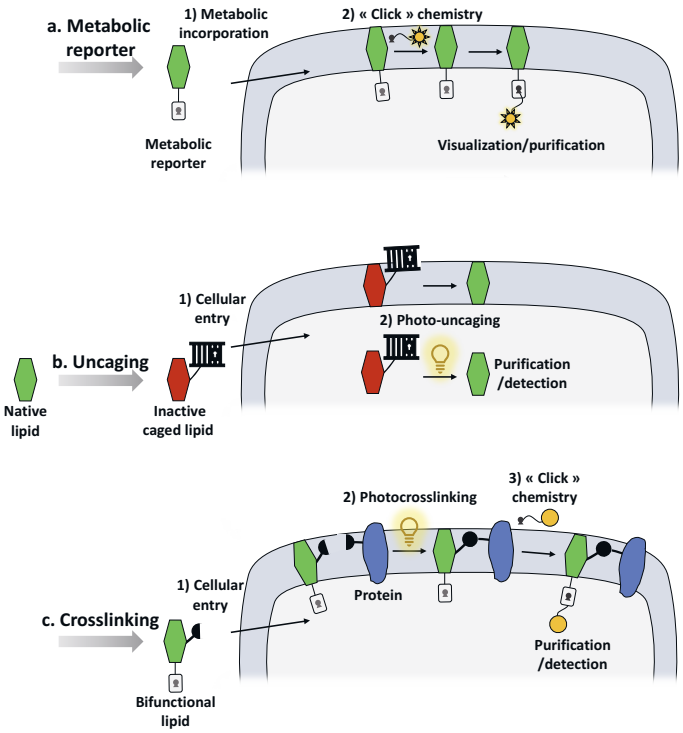

Fig. 2. Examples of chemical tools to study lipid metabolism.

2.3 *Caged Lipids*

Although the chemical reporter strategy allows to visualize lipid distribution in biological cells, the precursor is usually incorporated into different lipid species and does not enable only one particular species to be labelled. Moreover, lipid movement is rapid, making it difficult to study metabolism at a precise time. Photocaged tools allow to study lipids with high temporal resolution based on light control (Fig. 2b).[32] A caged lipid is composed of the natural lipid of interest to which a photosensitive protecting group (the cage) is added. It masks a key part of the lipid necessary for its function (usually its headgroup) in order to inactivate it. After delivering it into living cells, a flash of light of suitable wavelength is sufficient to cleave the covalent linkage between the lipid and the protecting group, releasing the lipid in its bioactive form. The cage is usually a fluorescent coumarin, enabling the delivery of the inactive lipid in the cell to be followed. But it can also be a *o*-nitrobenzyl group.

Delivery of a native species with a high temporal resolution constitutes the main advantage of this photocaging technique. It was mainly used to study signaling lipids as it enables to artificially increase concentration of a specific species in the cells at a given time and in a non-invasive way.[33] Caged analogues were developed for phosphoinositides,[34] lysophosphatidic acid,[35] glycerides,[36] ceramides,[37] or arachidonic acid,[38] and allowed their role in calcium signaling or translocation of proteins of specific lipid-binding domains to be studied.

However, only a few examples were reported in the field of lipid metabolism and intracellular transport. For this, introduction of additional spatial resolution was very useful. The main application involves sphingosine.[39,40]

Although the current uncaging methods present a high temporal resolution due to the light control, the spatial resolution remains limited by the random distribution of molecules in cells. It is generally too difficult to focus light only in one subcellular compartment with current lasers and there is certainly insufficient resolution in the z-axis. However, lipid metabolism, transport and function strongly depend on their subcellular location. Efforts were therefore made in recent years to target the caged lipid to a specific organelle/subcellular place by adding a location tag on the protecting group.[33] Interestingly, Wagner *et al.* designed a coumarinyl cage which could be decorated with different tags using a bioorthogonal click chemistry reaction, allowing to target either mitochondria, lysosomes, plasma membrane or the ER.[41]

2.4 *Other Light-controlled Lipids*

Lipids with photocrosslinking groups are another class of photoactivatable tools (Fig. 2c). Here, the amphiphile molecule is modified with a photoreactive group such as a diazirine or a benzophenone, which will form a covalent cross-linkage with the protein-binding partner under irradiation. This photocrosslinking tool allows the detection of native interactions between bilayer lipids and membrane proteins.[42,43] Photocrosslinking lipids are often combined with at least one other chemical tool, allowing an acute profiling of lipid–protein interactions in biological membranes.[38,44,45] For example, Schultz *et al.* designed tri-functional phosphoinositides bearing a photocrosslinkable diazirine to identify lipid–protein interactions, a photolabile coumarin for a high temporal control, and an alkyne tag for the enrichment and live cell imaging. They showed that the putative binding protein ATP11A and MPP6 were involved in the transport of phosphatidylinositol-3,4,5-triphosphate to the plasma membrane.[34]

Although they are not used to study lipid metabolism, photoswitchable lipids as a light-controlled tool should be mentioned. Contrary to caged lipids for which the light activation is irreversible, photoswitchable lipids can be in an active or inactive state depending on the conformation of photosensitive azobenzene introduced in one of the acyl chains. They are mainly used to modulate artificial membrane biophysical properties or to control cellular processes like ion channels, G protein-coupled receptors or nuclear hormone receptors.[46]

3. Tracking Lipid Metabolism

Historically, radioactively labelled tracers have been widely used to track lipid conversion in living systems, which, upon combining with thin-layer chromatograph (TLC), offers a convenient way to quantify lipid metabolism with high sensitivity. Over the last two decades, the rapid development of mass spectrometry provides an alternative experimental means and reshaped lipid research. Nowadays, more and more people use stable-isotope lipids to monitor lipid conversion, which are safer than radioactive lipids. More importantly, the systematic lipidomic analysis ('shotgun' approach),[47] when coupled to high sensitive mass spectrometry, is able to profile the metabolic footprints of the lipid tracers in great detail, thus gaining more and more attention. Furthermore, the 'pulse-chase' type of experiments allows not only to obtain a static profile, but also to get dynamic information of the metabolic flow. This so-called fluoxomics is very powerful when applied to study lipid biogenesis and lipid metabolic reprogramming *in*

vivo. Stable-isotope tracing and the relevant technical details are covered by some recently published reviews.[48,49]

While being a powerful technique, stable-isotope tracing cannot be automatically applied to study intracellular lipid transport. In fact, numerous lipids are synthesized in one organelle and transported to another organelle for use or further modification. Various studies have indicated that the major lipid trafficking routes are mediated by non-vesicular transport through a diverse and large group of lipid transfer proteins (LTP).[50] A critical feature of LTPs is that the majority is localized to membranes where two organelles are in close proximity (≤ 30 nm), known as membrane contact sites (MCS). However, we still lack a fundamental understanding how lipids, particularly the major species, are transported within the intracellular space. The fact that most lipids can be transported using more than one route[50] makes it difficult to obtain a clear phenotype when genetic knockout is performed, further restricting our access to a global picture of intracellular lipid transport and metabolism.

In the past years, our group has developed a series of photocaged lipid probes capable of localizing to different subcellular compartments including lysosomes and mitochondria. As explained above, the photolabile coumarin molecule was chemically attached to functional tags to ensure the accumulation of caged lipid in specific subcellular localization as designed. Using stable-isotope labelled sphingosine as a precursor, we applied this strategy to monitor sphingosine metabolism in different organelles, and found that its metabolic conversion to ceramide, a major metabolic product of sphingosine, is greatly affected by its original location to subcellular compartments (Fig. 3).[39] The different fate of the same lipid precursor depending on its subcellular location is a demonstration that there is a metabolic bias for lipid metabolism based on subcellular location. This implies that lipids do not freely diffuse in cells, but follow precise pathways. These results demonstrated a new approach of tracking lipid metabolism with subcellular resolution and have the potential of being extending to other lipid molecules in the future.

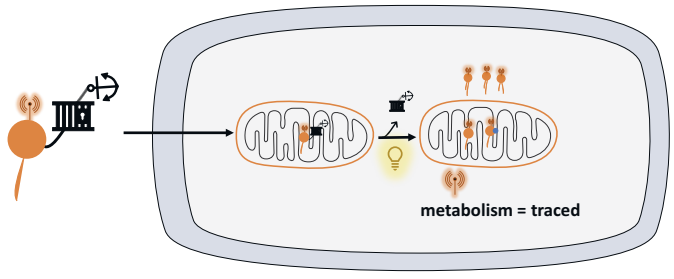

Fig. 3. Combination of isotopic labelling and selective photocaging to monitor sphingosine metabolism mitochondria.

Very recently, we further developed this technique to release sphingoid long chain bases (LCB) in the vacuole of *S. cerevisiae*, and monitored real-time sphingolipid metabolic flux out of vacuole by mass spectrometry. In order to differentiate the photo-released LCB from the endogenous ones, we used C17 phytosphinganine (C17-PHS) as a lipid tracer. We have found that the ER-vacuole tethering protein Mdm1 facilitated the metabolism of sphingoid bases into ceramides. Mdm1 protein is a tethering protein that localized to the ER but forms contacts with vacuoles and lipid droplets in yeast. Our results show that Mdm1 facilitation of conversion to ceramide cannot be explained uniquely by its tethering function.[51] Using the localized-uncaging approach, we provided novel insights into sphingolipid metabolism from vacuole to the ER, helping to define a pathway of lipid recycling and highlighting the potential of using caged chemical tools to identify lipid transporter/mediator in the intracellular space.

4. Discussion and Concluding Remarks

Lipids are fundamental building blocks in all life forms, but unlike proteins and DNA, we still lack a fundamental understanding how lipid homeostasis is organized and maintained. One major reason is due to the scarcity of techniques that are capable of modulating or tracking lipid activities with great precision. Genetic techniques are powerful in controlling levels of proteins including lipid metabolic enzymes, but they cannot be used to control lipids at the single molecule level. On the other hand, the relatively small size of lipids, in comparison to DNA and proteins, means that any chemical modification on the lipid molecule may risk altering its functions, and thus special care has to be taken.

This lack of technical availability brings both challenges and opportunities to chemical biologists who are working in the lipid field. In recent years, a number of new chemical tools have been introduced for studying lipid–protein interactions and exploring signaling functions of lipid messengers. More attention needs to be paid to lipid metabolism studies. Spatial and temporal control are clearly two future directions in lipid metabolism studies, which should help to achieve more precision, discover mechanisms and make this field flourish.

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