Background: Multifunctional Lipid Probes

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**This repository is still under construction, please forgive us for incomplete sections as we build features!**

Functionalized lipid probes are a toolset intended to fuse several niche biotechnologies. This overview will serve as a summary of the capabilifties, nuances, and limitations of their utility. Please find additional resources and published primary and review articles below for greater detail.

## Introduction: Why Study Lipid Interactomes?

At its core, biology is a complex network of chemical interactions. Every biological process—from metabolism to signaling—depends on the physical interactions between molecules, including proteins, nucleic acids, and lipids. While proteins have long been the primary focus of interaction studies, lipids play an equally crucial yet often under-appreciated role in cellular function. Therefore, there is a need for detailed knowledge of lipid-protein interactions (the lipid interactome). To learn about diseases and to find new therapeutic targets, in the comparison of the lipid interactomes of healthy and diseased cells and tissue is currently developing into a new field of lipid biology.

### Lipid-Lipid Interactions and Membrane Properties

Lipids are primarily organized within membrane bilayers, where their interactions define critical membrane properties such as rigidity, fluidity, and resistance to detergents. By studying lipid-lipid interactions and lipid interactomes, researchers will gain deeper insight into how these molecular interactions influence local and global membrane architecture and dynamics. Understanding these interactions and interactomes is essential for unraveling fundamental processes such as vesicle trafficking, cell division, cell signaling, and membrane compartmentalization.

### Lipids as Signaling Molecules

Beyond their structural roles, many lipids have been increasingly recognized as key signaling molecules. For example, the sphingolipid sphingosine-1-phosphate (S1P) plays a pivotal role in immune function. S1P binds to a specific family of receptors, triggering T cell migration (extravasation) and guiding their movement toward sites of infection. Rapidly turned-over phosphatidylinositol 3,4,5-*tris*phosphate triggers cell survival, growth, movement, and proliferation. These examples highlight how lipid signaling can affect cellular behavior and physiological responses.

### Lipids in Disease

Many diseases are caused by or affiliated with a lack or an accumulation of lipids, e.g. in atherosclerosis of in lipid storage diseases. However, recent evidence shows that in many if not most disease states specific lipid species are up- or down-regulated. A good example are viral and bacterial infections. If we learn about the effects of these altered lipids, we might identify new biochemical mechanisms essential for disease development and hence new therapeutic targets.

### Lipid-Protein Interactions: Unlocking Cellular Communication

A single lipid species can interact with a diverse array of proteins, influencing everything from receptor activation to intracellular signaling cascades. Mapping these lipid-protein interactions provides a powerful approach to understanding how lipids exert their wide-ranging effects on cellular processes. By deciphering lipid interactomes, researchers can uncover novel regulatory mechanisms and potentially identify new therapeutic targets in diseases where lipid signaling is dysregulated.

## Synthetic Lipid Analogs: Powerful Tools for Lipid Biology

The study of lipid biology has been greatly advanced by synthetic lipid analogs. However, it may not be immediately clear why such analogs are essential research tools. Unlike proteins and nucleic acids, lipids present unique challenges that make traditional genetic tools less effective in studying their function. In addition, synthetic lipid tools are designed to provide a defined composition of fatty acids and carbon backbones connected to a specific head group while genetic and pharmacological methods are more likely to produce a diverse group of lipid species. The lipid binding pocket of a protein might only in some cases be a great therapeutic target while the function of the lipid binding protein might well be.

### Why Lipids Require a Different Approach

Advances in genetic engineering—such as CRISPR and de novo gene synthesis—have revolutionized the study of nucleic acids and proteins. These techniques enable precise modifications, such as altering enzyme activity, redirecting proteins to different subcellular locations, modifying binding domains, and tagging proteins for visualization or isolation.

In contrast, lipids present unique challenges that make genetic approaches impractical. Unlike proteins, which are directly encoded in DNA, lipids are synthesized enzymatically. Their structures cannot be modified in living cells through genetic manipulation alone. Traditional approaches, such as gene knockouts or knock-ins, can broadly alter lipid composition but do not allow for precise control over individual lipid species.

Lipids are also much smaller than proteins, often composed of just a few dozen atoms. Even minor modifications can significantly alter their biophysical properties and biological function. Unlike proteins, which can tolerate the addition of large fluorescent or affinity tags, lipids tolerate only minimal chemical modifications to preserve their activity.

Additionally, lipids exist within a highly dynamic metabolic network, where they are continuously synthesized, modified, and degraded. Some signaling lipids, such as sphingosine, ceramide, and phosphatidic acid, have extremely short half-lives – sometimes less than a minute -— making them difficult to track in real-time.

To overcome these challenges, researchers have developed synthetic lipid analogs with chemical modifications that allow for targeted investigation while preserving essential biological properties. The following sections detail key modifications used in functional lipid probes.

### The Role of Synthetic Lipid Analogs

To overcome the challenges outlined above, researchers have developed synthetic analogs of native lipids. These modified lipids allow for precise investigation of lipid function while preserving their essential biological properties. The key modifications used in this approach include one to three functional modifications, namely a photo-crosslinking group (usually a diazirine), an alkyne group for click chemistry, and a photo-removable (cage) group for preventing premature probe metabolism.

#### Diazirine Group

Lipid-protein interactions are inherently transient and dynamic, making them particularly challenging to capture and study. Unlike protein-protein interactions, which often rely on extensive surface interfaces and hydrogen bonding, most lipids have minimal hydrogen bonding potential and lack a substantial structural framework for stable interactions. As a result, even biologically relevant lipid-protein contacts may be too fleeting to detect under standard biochemical conditions.

To effectively identify and analyze lipid-binding proteins, it is necessary to stabilize these interactions in a way that allows their subsequent enrichment and detection. While affinity enrichment approaches (e.g., lipid pulldown assays) can be used, they often require detergents to solubilize membrane-associated complexes, which can introduce artifacts or disrupt weak lipid-protein interactions. Covalent crosslinking, on the other hand, offers a far more reliable method to “freeze” these interactions in place, preserving their native binding state even during rigorous purification steps before mass spectrometry analysis.

##### Why Use Diazirine as a Crosslinking Motif?

Several chemical groups can be used for covalent crosslinking, but diazirine rings are particularly well-suited for lipid studies due to their small size and minimal structural interference. Other crosslinking devices are mostly aromatic and hence may alter the lipid’s physicochemical properties, potentially changing its location and interaction profile. Non-aromatic diazirine, however, is the smallest known photoactivatable crosslinking group, making it an ideal choice for studying delicate lipid-protein interactions.

##### Key Properties of Diazirine Crosslinking:

Lipid interactions are typically transient, as most lipid backbones lack opportunities for strong hydrogen bonding and are too small to provide a significant binding interface. Identifying interacting proteins requires stabilizing these interactions to allow for enrichment and analysis. While affinity-based approaches can help, covalent crosslinking is the most effective method, as it enables the removal of impurities and detergents before mass spectrometry analysis.

Among the various crosslinking motifs, the diazirine ring is the smallest, making it the least likely to interfere with a lipid’s natural interactions. Still, the introduction of diazirine into a fatty acid side chain will slightly decrease hydrophobicity and lipid ordering. Upon exposure to 355 nm UV light, the diazirine ring forms a reactive intermediate, which rapidly form covalent bonds with nearby molecules. This reaction is highly versatile, as reactive intermediates such as carbenes can insert into nearby carbon-hydrogen, sulfur-hydrogen, nitrogen-hydrogen, oxygen-hydrogen, or double bonds, allowing for broad coverage of potential interaction partners1. Despite these numerous options, reaction with glutamate seems to be modestly preferred2. The small size and high reactivity of diazirine crosslinking make it an invaluable tool for capturing lipid-protein interactions while minimizing perturbations to native lipid function.

##### The Effect of Diazirine Placement on Crosslinking Targets

The reactive carbene produced upon irradiation will bond with whatever molecule is nearest – thus, the location of the diazirine ring can have a dramatic effect on what protein interactors are stabilized via crosslinking.

The majority of lipid analogs produced to date were synthesized with the diazirine ring near the terminal end of an acyl chain. For example, [8-3 Fatty Acid](../LipidProbe/8-3_FattyAcid.qmd) has diazirine on carbon 10 of the fatty acid. These lipid analogs may be expected to preferentially crosslink to proteins which penetrate far into the hydrophobic region between the leaflets of a membrane bilayer, such as transmembrane proteins.

In comparison, moving the diazirine ring closer to the hydrophilic headgroup may increase the partiality of crosslinking towards peripheral membrane proteins in addition to transmembrane proteins. An example of this shift is [1-10 Fatty Acid](../LipidProbe/1-10_FattyAcid.qmd), in which the diazirine ring was diazirine on carbon 3 of the fatty acid.

Increasingly, lipid derivatives with the diazirine located on or near the lipid head group become available. Hence, many additions to the lipid interactome database now show interactions with proteins located at the membrane interface or cytosolic proteins. Future comparison of intramembrane versus membrane interface crosslinking will provide a more global view on lipid interactomes.

#### Terminal Alkyne

When studying proteins, researchers often use genetic methods to tag or modify them, making them easier to visualize or isolate. For example, adding a green fluorescent protein (GFP) domain to a protein allows it to be seen under a fluorescence microscope, whereas incorporating an HA tag enables enrichment using antibody pulldown techniques. These approaches work well for proteins because they are encoded by genes and can be modified at the DNA level.

Lipids, however, cannot be modified in the same way. Their synthesis occurs enzymatically, meaning there is no genetic sequence to alter. Large additions, like a GFP or HA domain, would drastically change a lipid’s properties, making it unrecognizable to the proteins and pathways that typically interact with it. Instead, a much smaller modification is needed—one that allows researchers to track and manipulate lipids without interfering with their biological function.

##### A Versatile Bioorthogonal Handle

One of the smallest and most effective modifications for this purpose is the terminal alkyne. This functional group consists of a simple triple bond at the end of a carbon chain, a minor structural change that does not significantly alter the lipid’s behavior. Despite its small size, the terminal alkyne serves as a powerful chemical handle, allowing researchers to attach various probes after the lipid has been introduced into a biological system.

The terminal alkyne is particularly useful because it undergoes copper-catalyzed azide-alkyne cycloaddition, the prototypical click chemistry reaction. This reaction is highly specific, rapid, and bio-orthogonal, meaning it does not produce reaction products with natural cellular molecules. . By reacting the alkyne with an azide-containing probe, researchers can introduce fluorescent markers for imaging or affinity tags for isolating lipid-protein complexes. The reaction requires catalysis by copper (I) ions and is therefore usually not suitable for intact cells and tissues but works well in fixed or lysed samples.

##### Applications in Lipid Research

By providing a simple yet effective means of modifying lipids without disrupting their native properties, the terminal alkyne has become an essential tool in lipidomics and chemical biology. One major application of the terminal alkyne is fluorescence labeling. A functionalized lipid can be introduced into cells and later reacted with a fluorophore-conjugated, ideally fluorogenic, azide, allowing researchers to visualize the lipid’s location in fixed cells. By using pulse-chase experiments, lipid movement is also trackable.

Another important use is affinity-based enrichment. Lipids containing a terminal alkyne can be captured using azide-functionalized beads (or resins), enabling the isolation of lipid-protein complexes after photo-crosslinking for analysis by mass spectrometry. This method has been instrumental in identifying proteins that interact with specific lipid species, shedding light on lipid signaling pathways and metabolic networks. An alternative is to covalently attach biotin to the lipid-protein conjugates and the use of streptavidin beads to wash and isolate them.

#### The Photo-activatable Group (Cage)

Essential roles of lipids in cellular signaling often rely on a transient abundance of lipid species, in some cases on the sub-minute scale. Hence, studying lipids in intact cells presents unique challenges due to their highly dynamic metabolism. Unlike proteins, which are relatively stable once synthesized, many bioactive lipids are continuously synthesized, modified, interconverted, and degraded as part of an extensive and tightly regulated lipid metabolic network. Even lipids so far not associated with cellular signaling show rapid turnover, which makes it difficult to study the effects of a specific lipid species in isolation, as a lipid of interest artificially introduced to cells may be enzymatically altered before it can exert its intended biological function.

One strategy to overcome this challenge is using photo-activatable (caged) lipid derivatives. These remain biologically inert until exposed to a specific wavelength of light. Irradiation releases the biologically active lipid derivative within seconds. Subcellular illumination allows researchers to control the timing and location of lipid levels, enabling targeted investigation of lipid function with high spatiotemporal resolution.

##### A Light-Activated Protection Group

There is a limited but significant number of photo-activatable protecting groups that have been used successfully in biological experiments, One of the most effective tools for photocaging lipids is based on 7-diethylamino-4-methylene coumarin, a photolabile protection group that can be removed upon exposure to 405 nm light. When attached to a lipid’s headgroup, the coumarin moiety temporarily blocks interactions with proteins, preventing the lipid from participating in its normal biological pathways. Upon photoactivation, the coumarin group is removed via a fast radical-based mechanism, restoring the lipid to its native structure and allowing it to engage with its biological targets. Another advantage are the fluorescent properties of the caging group which permits monitoring of cell entry and cell location.

Other photo-activatable groups include nitrobenzyl groups and Bodipy cages. The former often need shorter wavelengths for the (somewhat slower) photo-reaction. In addition, nitrobenzyl groups are not fluorescent and hence cannot not be used for following lipid derivative entry into cells. Bodipy cages have great photo-chemical properties and permit uncaging at longer wavelengths but add significantly more bulk to the lipid derivative.

This uncaging approach is very useful for studying lipid-protein interactions in intact cells and tissue where longer incubation times are needed, as uncaging the lipid in a controlled manner ensures that protein binders recognize the native lipid structure only when and where the researcher intends.

##### Applications in Cellular Signaling Studies

The power of coumarin-caged lipids has been demonstrated in studies examining intracellular lipid signaling. For example, in 2015, Höglinger et al. used a caged sphingosine analog to investigate the role of sphingosine in calcium signaling. They found that upon photoactivation of the caged sphingosine, lysosomal calcium release was triggered, revealing a crucial link between sphingosine metabolism and intracellular calcium dynamics3. This discovery significantly expanded the understanding of the sphingosine signaling cascade and its downstream effects.

Beyond biochemical studies, coumarin-caged lipids enable live-cell imaging approaches. Selectively irradiating specific subcellular regions allows the researcher to uncage lipids in a highly localized manner and assess their effects in real time. While this approach is not well-suited for large-scale proteomic studies, it provides a powerful method for investigating lipid function via fluorescence microscopy.

## Limitations of multi-functionalized lipid probes

* While significant effort has minimized the effect that each modification has on the biophysical properties of the lipid analogs, it remains the case that adding a diazirine and a terminal alkyne will necessarily affect their physical properties – perhaps affecting sub-cellular localization and binding.
* The coumarin cage has been repeatedly shown to protect lipid analogs from premature metabolism, but it should be recognized that this comes with some distinct changes to the behavior of the native lipid.
  + For example, when a cell is exposed to a photocaged lipid probe, the probe intercalates predominantly to endomembranes even if the native lipid is known to reside in the plasma membrane, as was shown for PIP3. This may lead to the identification of non-biologically-relevant hits or require enough time for lipid translocation after uncaging.
* Similarly, the diazirine and alkyne groups may have minor effects on the biophysical properties of the lipid probe as it interacts with other lipids and proteins within the membrane bilayer. This may be more relevant for proteins which preferentially bind to the acyl chain.
  + For example, some work has been done to demonstrate that the placement of the diazirine ring along the length of the acyl chain can have impacts on the interactome of the lipid probe4. Farley et al (2024) demonstrated this using trifunctional fatty acid probes by placing the diazirine either 3 carbons from the terminus (8-3 FA) or 10 carbons from the terminus (1-10 FA). They identified several proteins with distinct enrichment profiles, including VDAC1 and VDAC2 (which are preferentially enriched to the 8-3 FA probe), and ACAD9 and UBB (which are preferentially enriched to the 1-10 FA probe).
* Quantification of +UV versus -UV
  + The details of the quantitative mass spectrometry are described in our Overview of Proteomics Methods. However, we hope to succinctly describe that quantitative mass spectrometry techniques necessarily require normalization at sample input level – thereby exaggerating the abundance of proteins in the -UV control samples.
  + It should be noted, therefore, that there is little meaning to a protein which is “enriched” toward the -UV condition. For this reason, all proteins which are “depleted” following irradiation with UV have been designated as “no hit” in most of the analyses.

1. Haberkant, P. & Holthuis, J. C. M. [Fat & fabulous: Bifunctional lipids in the spotlight](https://doi.org/10.1016/j.bbalip.2014.01.003). *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1841**, 1022–1030 (2014).

2. West, A. V., Amako, Y. & Woo, C. M. [Design and Evaluation of a Cyclobutane Diazirine Alkyne Tag for Photoaffinity Labeling in Cells](https://doi.org/10.1021/jacs.2c08257). *Journal of the American Chemical Society* **144**, 21174–21183 (2022).

3. Höglinger, D. *et al.* [Intracellular sphingosine releases calcium from lysosomes](https://doi.org/10.7554/eLife.10616). *eLife* **4**, (2015).

4. Farley, S. E. *et al.* [Trifunctional fatty acid derivatives: The impact of diazirine placement](https://doi.org/10.1039/D4CC00974F). *Chemical Communications* **60**, 6651–6654 (2024).