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Caged lipids for subcellular manipulation.

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Abstract

We present recently developed strategies to manipulate lipid levels in live cells by light. We focus on photo-removable protecting groups that lead to subcellular restricted localization and activation and discuss alternative techniques. We emphasize the development of organelle targeting of caged lipids and discuss recent advances in chromatic orthogonality of caging groups for future applications.

Introduction.

Compartmentalization of cellular events is a hallmark of eukaryotic cells.[1] Classically, each compartment consists of a segregated membrane system forming a lumen, a luminal and a cytosolic membrane leaflet.[2] In addition, the plasma membrane forms the boundary to the outer world, with strikingly different lipid compositions of the inner and outer leaflet.[3] Recently, this picture was complicated by findings that the interaction of proteins with lipids on a kiss-and-run base differs strikingly from cases where the proteins are artificially transferred to a specific membrane, implying that any membrane surface might constitute its own “compartment”.[4] In this respect, it becomes relevant that lipids are transported from one membrane side to the other by a variety of flipases.[5] Further, there is constant membrane exchange between compartments via the transport of vesicles as well as lipid transport via transport proteins. For instance, COPI vesicles are released from the Golgi network and trafficked to the plasma membrane or the endoplasmic reticulum (ER) while COPII-decorated vesicles perform the retro-transport from the ER to the Golgi.[6] These transport processes are both essential and quite rapid as inhibiting COPII transport by blocking of ER exit sites will exhaust the Golgi membrane system within 30 to 60 min.[7] Apart from the exchange of entire membrane sections via vesicles, single lipid molecules such as cholesterol, phosphatidylinositols or ceramides are efficiently transferred via specific transport proteins, many of them shuttling lipids across membrane systems that are in close

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proximity, for instance at organelle-organelle contact sites.[8,9,10] Despite this fast exchange, organelles seem to maintain a specific lipid composition most likely due to site-specific metabolism.[11] The lipid composition is then not only responsible for organelle identity and function but also represents a signaling component that determines organelle transport and fusion events, for instance in the case of endosomes and their trafficking to either the plasma membrane (endosome recycling) or the lysosome (for endolysosomal destruction).[12,13]

In order to study organelle function, organelle membrane identity, lipid transport, membrane composition and the corresponding cellular functions, there is a need for tools to alter lipid compositions specifically with high spatial resolution. An elegant option is to copy nature and alter the local lipid composition enzymatically by translocating a lipid-metabolizing enzyme specifically to the membrane of interest.[14] One way to achieve this is by using chemically induced dimerization (CID) systems exploiting the formation of a tertiary protein complex (*i.e.* FRB:FKBP, eDHFR:FKBP, or SNAP:FKBP) and is induced by treatment with a small molecule (*i.e.* rapamycin, TMP-SLF, or rCD1/FK506, respectively). [15, 16, 17] Alternatively, several light-switchable genetically encoded translocation systems such as Cry2/CIB1, iLID and “Magnets” were compared as tools to switch lipid phosphatase activity.[18] However, these switching systems require the introduction of the necessary enzyme, usually by transfection or viral transduction into the cell of interest, as well as the switch system. Another less invasive method is changing the lipid composition by small molecule addition and light. For this purpose, photo-activatable lipid derivatives, usually called “caged” lipids, or photo-switchable lipids based on light-induced *cis-trans* isomerization of azobenzenes are employed.[19,20] Local release of a defined lipid species can be achieved by using highly focused light through the objective of a confocal microscope to remove a photo-sensitive protecting (“caging”) group and produce fairly well defined concentration changes.[21] A photo-switchable lipid derivative will change from an inactive to an active form or vice versa, This technique has recently been reviewed in detail.[22,23] The spatial resolution of these light-driven techniques is limited by the point-spread function of the laser light unless 2-photon excitation is used.[24,25] It is important to note that 2-photon absorption (or non-linear absorption) is generally several orders of magnitude smaller than one-photon absorption. Consequently, not all photo-removable protecting groups will be sensitive to 2-photon uncaging.[26] The second difficulty is to precisely hit a membrane or a small organelle in a 3-dimensional space. As a result, to date 2-photon uncaging was used to release glutamate in neuronal spines[24] and in deep tissue[27] but to our knowledge never for lipids in subcellular structures. A solution to these challenges might be to target caged lipids to one particular membrane system prior to the photo-release. This review covers the newest tools for achieving localized lipid release by light.

Caged lipid composition.

Photo-removable protecting groups are molecular leaving groups and their departure is triggered by light. They can be categorized on the bases of their chemical structures. The most common groups are derived from arylcarbonylmethyl, nitroaryl, coumarin-4-ylmethyl, or arylmethyl groups. [28] Coumarin-based photo-removable protecting groups have become popular in the field due to high uncaging yields and faster photo-releases than classical nitrobenzene derivatives, but also due to their ability of being fluorescent under a confocal

microscope.[29] In cells, the amount of coumarin-caged lipids can easily be quantified during the microscopy experiment by directly measuring the fluorescence emission of the sample. Typically, the release efficiency of caged lipids in cells is also determined by thin-layer chromatography (TLC) but the extraction efficiency needs to be accounted for. The ability of measuring the exact concentration of released species is critical to ensure good reproducibility between experiments. This becomes even more important when comparing the effect of a signaling lipid released from two different subcellular locations. Such experiments can only be reasonably performed if it is possible to demonstrate that the amounts of photo-released lipids are comparable in both places.

The primary goal of a photo-removable protecting group is to prevent the cellular machinery to recognize and respond to the signaling molecule of interest until removed by a flash of light. To disrupt recognition of a lipid, typically the head group with its functional and chemically reactive groups serves as carrier of the often bulky cage. Examples are the phosphates of phospholipids, [30] amino groups of sphingosines, [31] and hydroxy groups of diacylglycerols (DAGs).[21] We recently developed a new chemical strategy for the caging of monoacylglycerols, which traditionally suffered from fatty acid migration and cross-activation with DAG by masking the two hydroxy groups with a photo-removable acetal.[32,33] Coumarin and nitrobenzyl cages that are connected *via* carbamates or carbonates serve well for the protection and the rapid photo-release of most lipids in living cells (Figure 1).

How the cage influences location and activity.

Due to the small size of lipids, the addition of large and aromatic fluorophores constitutes a major chemical alteration of their structure. This may dramatically affect their location and potentially the endogenous signaling properties. Fluorescently labelled lipids including those bearing photo-removable protecting groups, frequently mislocate in cells when compared to their endogenous counterparts.[34] This feature may be turned into an advantage for using caged lipids. Especially neutral coumarin cages are highly lipophilic. Therefore, most coumarin-caged lipid species tend to accumulate in membranes, mostly endomembranes. [35] This is expected for all lipid derivatives with a single charge that is masked by the cage group. A good example is caged phosphatidylinositol (PI) (Figure 2A).[36] PI is biosynthesized in ER membranes and coumarin-caged PI locates to exactly these membranes (Figure 2B). Upon uncaging, the PI derivative rapidly transferred to the plasma membrane (Figure 2C). The analysis of the PI location was made possible by two additional modifications: a diazirine group to photo-crosslink the lipid and prevent its wash-out during the cell fixation process and an alkyne group to tag the lipid after fixation via copper-catalyzed click chemistry. We refer to this combination of cage, diazirine and alkyne as trifunctional lipids. The concept is currently applied to many other lipids.[37,38] It should be mentioned that even lipid derivatives with numerous charges on the headgroup, such as the higher phosphorylated phosphoinositides, will locate to endomembranes as long as an aromatic cage group is attached.[39]

For targeting lipids to certain membranes, the lipophilicity and interaction potential of the caging group can be appropriately modulated. Tuning the chemical properties of the cage

will determine where the caged compounds will concentrate in the cellular environment. A simple introduction of charges to a coumarin cage in form of sulfonates led to an exclusive localization of caged arachidonic acid (AA) to the outer leaflet of the plasma membrane.[40] Surprisingly, the photo-release of AA at the plasma membrane was shown to have profoundly different effects on intracellular calcium oscillations in the β-cell line MIN6 than a release from internal membranes. Likely, the extracellular AA predominantly triggered the fatty acid-specific GPR40 on the cell surface while AA inside cells seem to have acted on ion channels. This example demonstrated the feasibility and relevance of local lipid release. Furthermore, it illustrated that the same lipid can exert different functions at different location in the cell. Sulfonated cages were subsequently applied to caged diacylglycerols (DAGs).[41] Schuhmacher et al successfully investigated the impact of the fatty acid composition on the interaction of DAGs with C1 domains as well as lipid trans-bilayer movement.

In the meantime, many more localized caged lipid tools have been introduced (Figure 3). An exciting application involved caged ceramides. [42, 43] The ceramide family of lipids have historically been quite difficult to add exogenously, due to their high hydrophobicity and toxicity. Only short-chain ceramides are readily applicable to cells. Kim et al were able to incorporate even C22 ceramide into HeLa cells by combining a coumarin cage with PEG groups for increased water solubility. [44] Interestingly, the fatty acid composition of the ceramide did affect the uncaging efficiency. Ceramide 1-phosphate (C1P) acts on several signaling pathways, both inside and at the cell surface, highlighting the importance of localization of signaling molecules. 7-Diethylaminocoumarin (DEAC)-caged C1P was compared with C1P added exogenously.[45,46] While DEAC-C1P stimulated the proliferation of macrophages and caused the phosphorylation of ERK and Akt, only exogenous C1P was able to stimulate glucose uptake and cell migration.

As shown above, neutral cages penetrate non-selectively all endomembranes and negatively charged ones accumulate at the plasma membrane. A permanently positively charged triphenylphosphonium (TPP) group on a coumarin scaffold will concentrate the caged lipid inside mitochondria. Recently applied to subcellular manipulations of sphingosine, this strategy allowed investigating the distinct metabolic fates of the signaling lipid depending of its local accumulation in living cell.[47] Stable isotope-labeled caged sphingosine precursors permitted to measure the conversion of sphingosine into ceramides and sphingomyelins after uncaging by mass spectrometry. Both caged sphingosines Mito-So and DEAC-So, once uncaged, were quickly phosphorylated to sphingosine-1-phosphate (S1P), but showed distinct patterns of incorporation into higher sphingolipids.[47] Coumarin caged Sph (DEAC-So) was quickly incorporated into ceramide, but after 20 minutes this ceramide had already begun to disappear. To the contrary, sphingosine released from Mito-So continued to incorporate into ceramide even after 20 minutes and was incorporated into sphingomyelin for longer time periods than sphingosine from DEAC-So. Here, the mitochondria-specific photo-release of the lipid showed that sphingosine metabolism was highly dependent on its subcellular localization. A cytosolic increase of sphingosine was found to release calcium from lysosomes via TCP1 channels and activate the transcription factor TFEB.[31] In contrast, a local increase of sphingosine levels in mitochondria was unable to induce calcium transients. Given the importance of lysosomal sphingosine metabolism in the

neurodegenerative disease Niemann–Pick type C1 (NPC1), a lysosomal storage disorder, targeting the lysosome with a caged sphingosine derivative was a logical strategy. Feng et al attached a morpholino group to the coumarin molecule, leading to the accumulation of caged sphingosine (but also cholesterol) inside lysosomes of living cells. [48] Combined with the nonselective as well as the mitochondria-targeting uncaging, this toolset offered a sophisticated framework to study sphingolipid metabolism with a focus on the ceramide chain length. These subcellular manipulation approaches might be useful to apply to many other lipids in living cells.

Uniting these various directing strategies, Wagner et al conceptualized a coumarin-based cage which could be modified with various organelle directing groups.[49] In this case, the amino group of DEAC was modified with a terminal alkyne group, to which a variety of azide-bearing directing groups were attached by copper-catalyzed click chemistry. The utility of this strategy was demonstrated again with both sphingosine and arachidonic acid, which were successfully targeted to the mitochondria (with the TPP group mentioned above), the lysosome (with a tertiary amino group), the plasma membrane (with a sulfonate group), or the ER (with a perfluorinated group).

The fluorescence of the coumarin molecule as a cage is often considered as an advantage, as it permits to monitor the cellular uptake and the proper subcellular localization of the caged lipid before uncaging. However, in some cases the sensitivity of the coumarin cage towards light is so high that imaging the caged compound in live cells – even at the lowest laser power – is sufficient to uncage a portion of the lipid and to perturb the experiment. To solve this limitation, Gaur et al dissociated the functions of uncaging and imaging into different units within the photo-removable group.[50] The covalent binding of a photolabile *ortho*-nitrobenzyl (ONB) cage and a rhodamine dyes delivered fluorescent cages only sensitive to high-energy wavelength light below 400 nm. Using this approach, they designed both a globally neutral cage that enters cells and endomembranes non-specifically, and another bearing negatively charged sulfonate groups that selectively localized at the plasma membrane. These caged lipids were sensitive to 365 nm light uncaging and were proven to be resistant to other wavelengths.

Conclusion and outlook.

Caged lipids are powerful tools for investigating lipid signaling in live cell. They offer the opportunity to manipulate lipid levels without genetic or protein alterations and can therefore also be applied to primary cells. The many recent examples of targeting lipids to a distinct cellular membrane or organelle make them suitable tools for investigations at the subcellular level. The continuous development of new photo-removable protecting groups with targeting units therefore permits the precise manipulation of lipid species with exquisite spatio-temporal precision. In addition to the ability of selectively manipulating lipid concentration at subcellular levels, the diversification of the existing cages is showing premises for orthogonal, two-color uncaging (Figure 1).[27,51,52] This will allow for sequential or simultaneous manipulation of a combination of different lipids or other signaling molecules in multiple organelles of the cell. By expanding the library of protecting groups for the caging of small molecules (Figure 1), recent studies provided photosensitive

protecting groups that absorb photons in the visible part of the spectrum. Still based on a coumarin scaffold, DEAC-450 strongly absorbed blue light while being benign to ultra-violet light.[53] This range of absorption is traditionally used for many ONB and DEAC caged compounds. *Meso*-substituted BODIPY are an alternative caging tool to coumarins.[54,55] They were successfully applied to unmask carboxylic acids with green light excitation >500 nm in living cells. Using these new photo-removable protecting groups in combination with classical ONB or DEAC derivatives and with localization to different organelles in cells would pave a golden avenue for manipulating lipid levels with a unique degree of precision.

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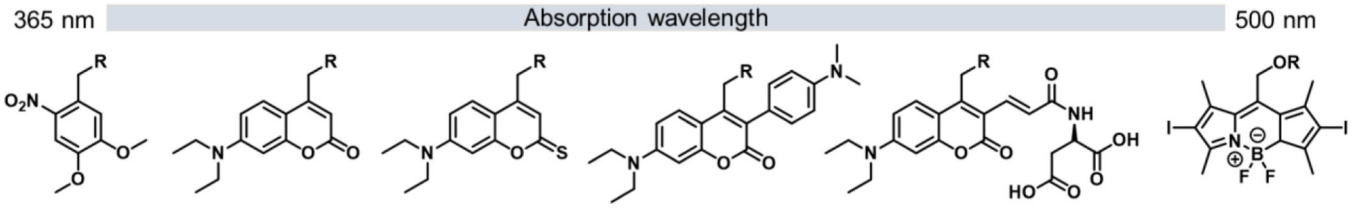

Figure 1.

Example of photo-removable protecting groups expanding the spectral range of absorption for the uncaging of small molecules from UV to visible wavelengths.

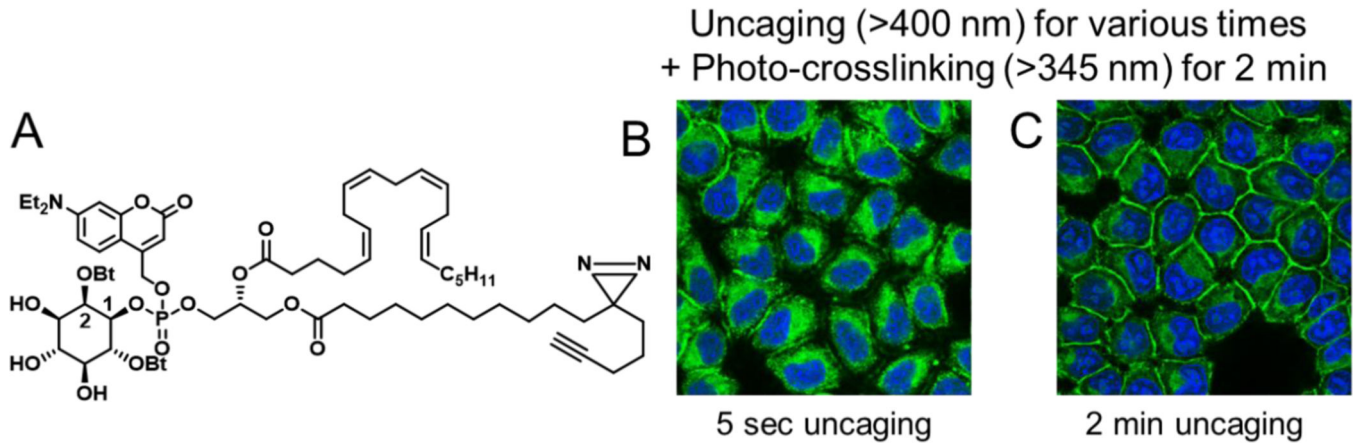

Figure 2.

A. Chemical structure of tri-functional phosphatidylinositol (PI). Accumulation of PI in endo membranes after uncaging for 5 sec (B) or 2 min (C) followed by 2 min of photo-crosslinking. The location of the lipid was monitored by confocal microscopy after fixation and labelling the terminal alkyne of the lipid tail with an azide-bearing fluorophore *via* copper click chemistry.

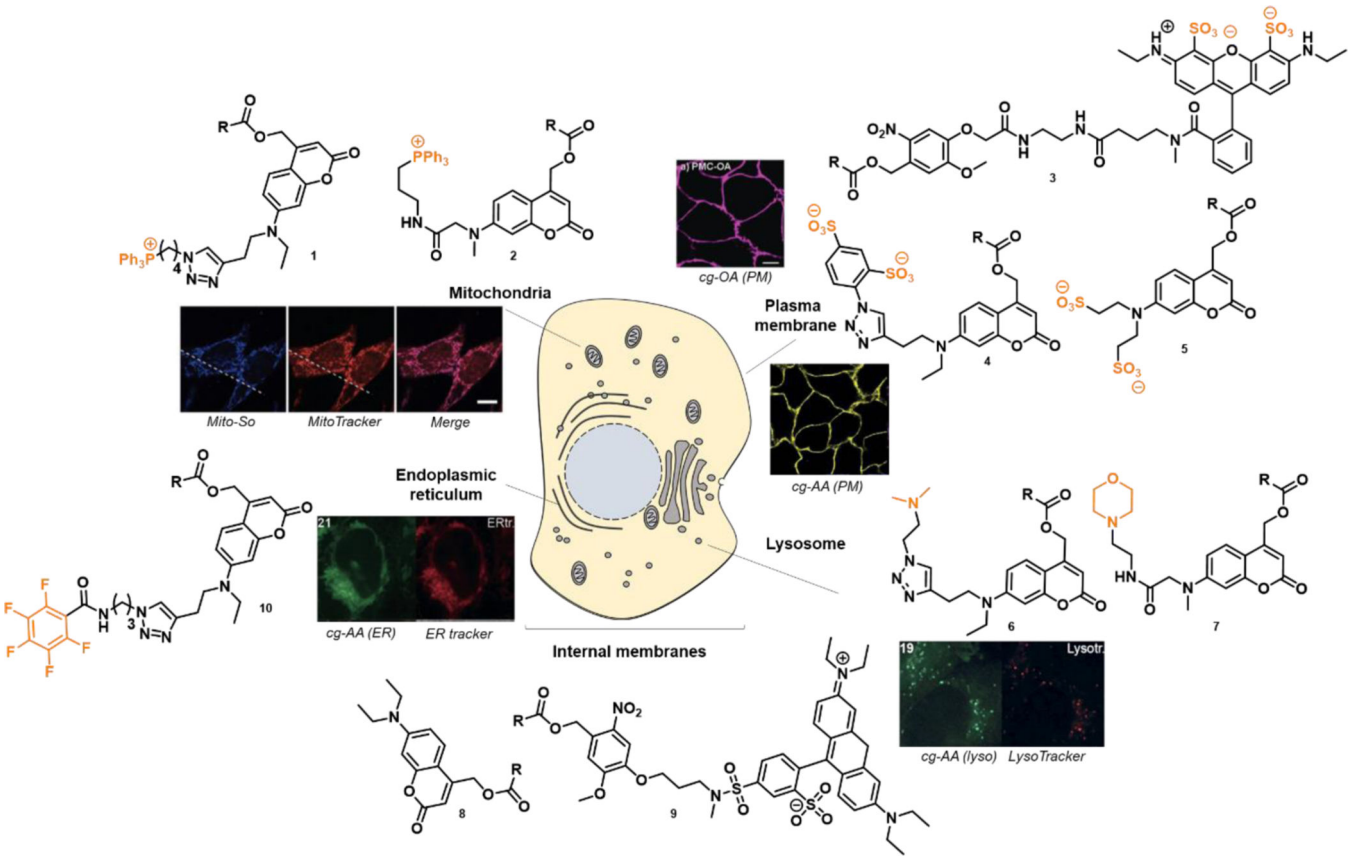

Figure 3.

Chemical structures of photo-removable protecting groups targeting distinct cellular membranes. This allows local photo-release of signaling lipids in live cells. R refers to the lipid moiety.