Live-Cell Imaging

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Selective Fluorescence Labeling of Lipids in Living Cells**

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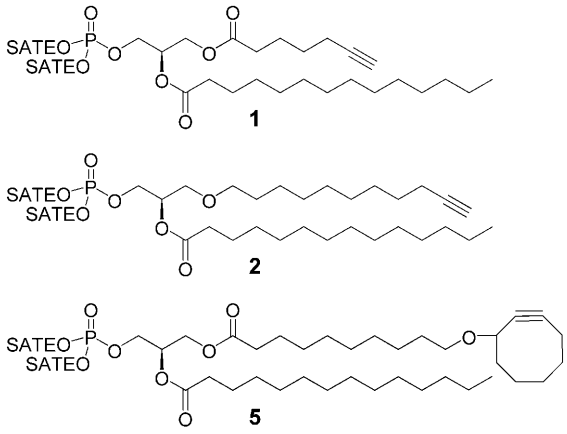
Lipids play important roles in a wide variety of cellular processes, from membrane fusion to signal transduction, and are involved in the formation and transport of microdomains.[1] However, their analysis in living cells is complicated by their extremely dynamic behavior.[2] As intermediates in metabolism, they are frequently converted into other lipid species or free fatty acids.[3] They are components of cellular membranes and quickly diffuse from one membrane compartment to another. Furthermore, the analysis of lipid fluxes is often problematic as a result of the similarity of lipid structures. A considerable amount of lipid research relies on the use of tagged lipid analogues. However, given the size of an average lipid molecule, even small probes, such as fluorophores, can have a dramatic effect on the properties of the lipid, particularly if lipid trafficking and sorting are to be investigated.[2,4] Hence, the labeled lipid should resemble its natural counterpart as closely as possible. The modification should preferably be a small hydrophobic group.

The bioorthogonal chemical reporter strategy is becoming increasingly popular for the labeling of all kinds of biomolecules in their native environment. The method comprises two steps: First, a unique chemical functionality is incorporated into the target biomolecule, preferably by the biosynthetic machinery of the cell. In the second step, the functional group is labeled with a nondisruptively delivered probe in a specific chemical reaction.[5] The most commonly used reactions are the reaction of a bisarsenite with a tetracysteine peptide motif,[5b] the Staudinger ligation, copper(I)-catalyzed azide–alkyne cycloaddition reactions, and strain-promoted azide–alkyne cycloaddition reactions.[6] Proteins,[7] polynucleotides,[8] and glycoconjugates[9] have been labeled successfully in or on living or fixed cells by using these reactions. A few studies have been concerned with the detection of lipidated proteins through the metabolic incorporation of ω-azido fatty acids.[10] However, the detection of the azidoacylated proteins through a Staudinger ligation was only possible after cell lysis. No studies on the chemical labeling of lipids of any kind in living systems have been reported to date. Herein, we report the selective fluorogenic labeling of the alkyne-containing phospholipid derivatives **1**, **2**, and **5** (SATE = *S*-acetylthioethyl) in various mammalian cells. Our approach



was based on the assumption that a terminal triple bond in a fatty acid chain should alter neither the overall structure of the lipid nor its polarity.

We chose phosphatidic acid (PA) as a model lipid. PA is a key intermediate of phospholipid metabolism and an important lipid second messenger. It is mainly generated by phospholipase D mediated hydrolysis of phosphatidylcholine in response to a variety of intracellular stimuli. Phosphatidic acid regulates a number of target proteins, such as mTOR and Raf-1, protein phosphatase-1, and cAMP-specific phosphodiesterases. It affects various cellular functions as diverse as cell proliferation, metabolism, cytoskeletal rearrangement, and exocytosis.[11] Methods for studying PA dynamics and interactions with effector proteins in living cells could reveal further roles of PA in cell signaling, or confirm findings from in vitro studies. Furthermore, any labeling approach established for PA could be extended to the investigation of other, more complex lipids by simply varying the lipid headgroup to monitor signaling events with spatial resolution.

The diacyl PA derivative **1** was synthesized in six steps and 50% yield from commercially available 6-heptynoic acid. The esterification of 6-heptynoic acid with 2,3-*O*-isopropylidene-*sn*-glycerol was followed by ketal cleavage, dimethoxytrityl (DMT) protection, ester formation with myristic acid, and DMT removal. The resulting diacyl glycerol derivative was transformed into the PA derivative **1** by phosphorylation with bis(*S*-acetyl-2-thioethyl)-*N*,*N*-diisopropylphosphoramidite (see Scheme S1 in the Supporting Information). Protection of the otherwise negatively charged phosphate headgroup with SATE groups was necessary for membrane penetration and to prevent lipid aggregation. SATE-protected phosphate groups are resistant to many conditions used in synthetic organic chemistry, but are rapidly cleaved inside living cells by cellular esterases to yield a free phosphate group. The resulting compounds cannot leak from the cell.[12]

The PA derivative **2** with a nonhydrolyzable ether-coupled alkyne-containing alkyl chain was synthesized from 10-

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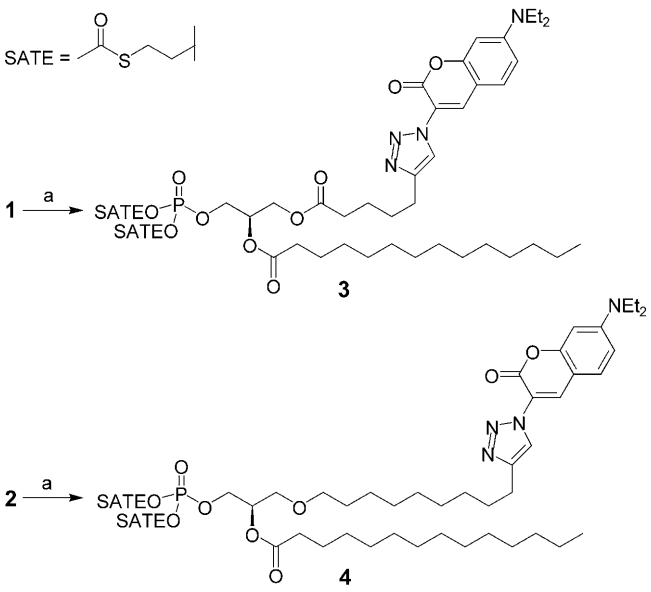
undecyn-1-ol in seven steps and 28% overall yield. Appel halogenation of 10-undecyn-1-ol and ether formation with 2,3-*O*-isopropylidene-*sn*-glycerol were followed by further steps analogous to those in the synthesis of **1** (see Scheme S2 in the Supporting Information).

To test their general suitability for copper(I)-catalyzed azide–alkyne cycloaddition, **1** and **2** were treated with fluorogenic 3-azido-7-(diethylamino)coumarin[13] in vitro. The corresponding fluorescent triazole derivatives **3** and **4** were obtained in virtually quantitative yield (Scheme 1). Compounds **3** and **4** showed excitation maxima at around 410 nm and emission maxima at around 500 nm. They were applied to living cells, and their temporal and spatial dynamics were observed (see Figure S1 in the Supporting Information). RAW macrophages turned out to be the most suitable cell type for the visualization of lipids, since their globular morphology enables individual membrane compartments to be distinguished. Shortly after addition, both **3** and **4** stained the plasma membrane; however, they quickly relocated to intracellular membranes. Especially in the case of **4**, a small, perinuclear region was exceptionally fluorescent.



Scheme 1. Synthesis of the fluorescent phosphatidic acid derivatives **3** and **4**: a) 3-azido-7-(diethylamino)coumarin, $CuSO_4$, sodium ascorbate, $EtOH/H_2O$ (1:1).

The cell-permeant, alkyne-containing PA derivatives **1** and **2** were applied to living cells in a similar way and labeled with 3-azido-7-(diethylamino)coumarin[13] in the presence of copper(II) sulfate, sodium ascorbate, and tris(benzyltriazolylmethyl)amine (TBTA). Since copper ions are toxic, cells had to be fixed prior to labeling. Paraformaldehyde fixation was chosen to restrict lateral and vesicular movement through the immobilization of lipid-binding proteins. After just ten minutes of labeling, cell membranes fluoresced brightly. Negative control cells, which were not treated with alkyne-modified lipids, but which were otherwise treated identically, remained virtually nonfluorescent even after longer labeling times (Figure 1). By using different cell types, such as HeLa and MDCK cells, the labeling method was shown to be widely applicable (see Figure S2 in the Supporting Information).

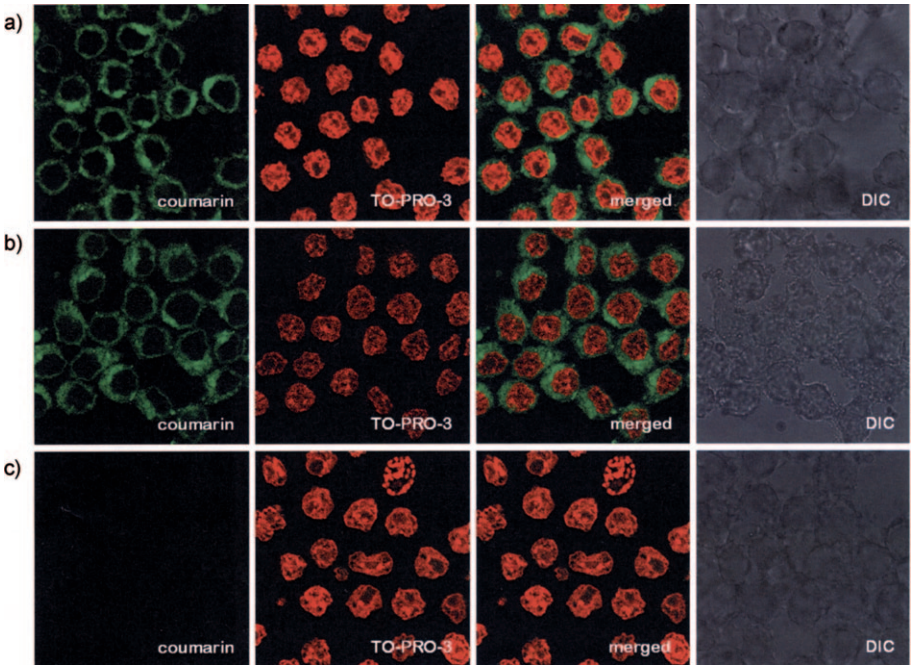


Figure 1. Fluorescence microscopic images of fixed RAW macrophages treated with the PA derivatives **1** or **2** for 1 h and with 3-azido-7-(diethylamino)coumarin, $CuSO_4$, sodium ascorbate, and TBTA for 30 min. a) Cells treated with **1** and the nuclear marker TO-PRO-3 iodide. b) Cells treated with **2** and the nuclear marker TO-PRO-3 iodide. c) Negative control cells treated with 3-azido-7-(diethylamino)coumarin, $CuSO_4$, sodium ascorbate, and TBTA only. DIC = differential interference contrast.

In contrast to the results obtained with the prelabeled lipids **3** and **4**, the labeling of **1** and **2** in living cells led to more or less equal staining of all cellular membranes. Whereas the *sn*-1 ether derivative **4** stained the perinuclear region exceptionally brightly, the *sn*-1 ether derivative **2** appeared to be distributed evenly, which indicates that the localization of fluorophore-tagged lipid analogues is often assumed wrongly. We also showed by fluorescence recovery after photobleaching (FRAP) that paraformaldehyde fixation resulted in lipid immobilization, as no movement of unbleached, fluorescent molecules into bleached regions of cells was observed even after 30 min (see Figure S3 in the Supporting Information). The results indicated that the intracellular localization of the labeled PA derivatives was

not directed by the fluorophore but reflected the position of the alkyne lipid at the moment of fixation. It cannot be completely ruled out that the ester functionality of **1** is cleaved prior to fixation and labeling. However, in vivo labeling of hexynoic acid resulted in a slightly different fluorescence distribution (see Figure S4 in the Supporting Information).

Owing to the toxicity of copper ions, cells needed to be fixed prior to labeling. However, fixation prevents the investigation of dynamic cellular processes. We therefore designed the cyclooctyne-containing PA derivative **5**, to label lipids in living cells through strain-promoted azide–alkyne cycloaddition.[14] A cyclooctyne-containing fatty acid was synthesized from commercially available cycloheptene in three steps and 39% yield. Further synthetic steps according to the procedure described for **1** gave **5** in 46% yield after six steps (see Scheme S3 in the Supporting Information). Compound **5** was then applied to living RAW macrophages. Fixed (see Figure S5 in the Supporting Information) and living cells were labeled successfully with 3-azido-7-(diethylamino)coumarin[13] within 3 h in the absence of auxiliaries (Figure 2). The staining pattern was very similar in both cases. No cell toxicity was observed even after several hours, and mitosis continued to occur.

In conclusion, we have described herein the first successful labeling of lipids by azide–alkyne cycloaddition in fixed and living cells. The localization differences associated with a simple ester-to-ether modification and, more importantly, the differences in the distribution of prelabeled lipids and those labeled in vivo demonstrate the relevance of the technique. We assume that the effect of a terminal triple bond on the biophysical properties of a membrane lipid is negligible, but the extent to which the cyclooctynyl group influences lipid diffusion and location remains to be shown. In the future, more reactive triple bonds will be beneficial for staining procedures in the minute range.[14b,c] The labeling technique developed herein should enable the monitoring of lipid dynamics in fixed and living cells and give new insight into cellular distribution, lipid-interaction partners, and behavior in response to endogenous or environmental stimuli.

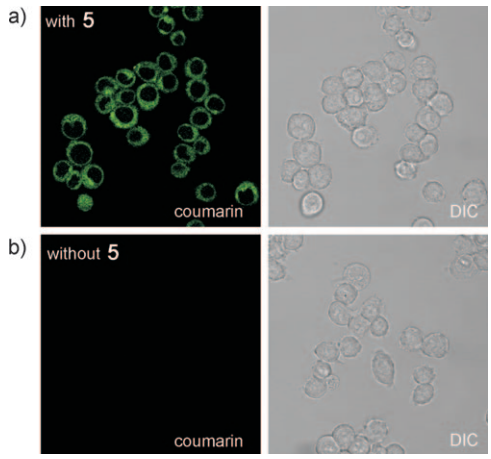


Figure 2. Fluorescence microscopic images of living RAW macrophages. a) Cells treated with **5** for 1 h and 3-azido-7-(diethylamino)-coumarin for 3 h. b) Negative control cells treated with 3-azido-7-(diethylamino)coumarin for 3 h only.

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