Glycolipids

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# Labeling Cell Surface GPIs and GPI-Anchored Proteins through Metabolic Engineering with Artificial Inositol Derivatives**

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***Abstract:*** *Glycosylphosphatidylinositol (GPI) anchoring of proteins to the cell surface is important for various biological processes, but GPI-anchored proteins are difficult to study. An effective strategy was developed for the metabolic engineering of cell-surface GPIs and GPI-anchored proteins by using inositol derivatives carrying an azido group. The azide-labeled GPIs and GPI-anchored proteins were then tagged with biotin on live cells through a click reaction, which allows further elaboration with streptavidin-conjugated dyes or other molecules. The strategy can be used to label GPI-anchored proteins with various tags for biological studies.*

Glycosylphosphatidylinositol (GPI) attachment to the protein C terminus is one of the most common posttranslational protein modifications in eukaryotes and helps to anchor proteins to the cell membrane.[1] Many GPIs and GPI-anchored proteins have been identified[2] and shown to play a critical role in various biological processes.[3] GPI-anchored proteins need the GPI anchor to function properly[2a,4] and losing GPI anchoring ability is lethal for mammals and conditionally lethal for yeasts.[3k] Moreover, an elevation in the levels of GPI-anchored proteins has been observed in human cancer cells, thus indicating their potential as biomarkers for cancer detection and therapy.[3n]

Regardless of their origin, all GPI-anchored proteins share a conserved construction, with the protein C terminus linked to the phosphoethanolamine [(P)-EtNH$_2$] moiety at the mannose-III 6-*O*-position of the GPI core (Figure 1).[1]

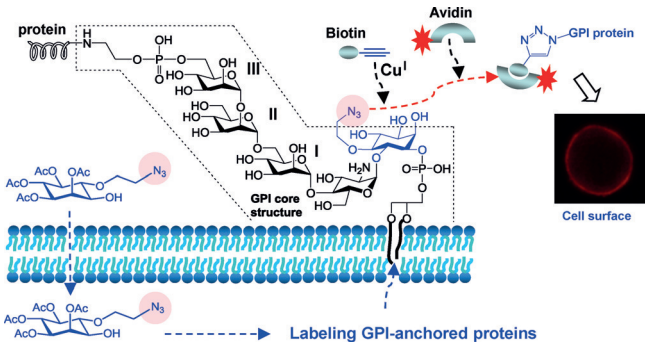


***Figure 1.*** Labeling of GPI-anchored proteins on the cell surface through metabolic engineering of the GPI core structure. Cells are incubated with an inositol derivative bearing a chemically reactive bioorthogonal functionality, such as the azido group. The unnatural inositol is taken up by the cells and incorporated into GPIs and GPI-anchored proteins. The modified GPI-anchored proteins on the cell surface can be subsequently elaborated through a specific bioorthogonal reaction for the attachment of various molecules, for example, fluorescent tags to make cell-surface GPI-anchored proteins visible, and affinity tags to facilitate the isolation of GPI-linked proteins.

This suggests a conserved biosynthetic pathway for GPIs and GPI-anchored proteins in eukaryotic cells. GPIs and GPI-anchored proteins are biosynthesized in the endoplasmic reticulum via a series of membrane-bound enzymatic transformations involving more than 20 gene products.[2a,3a,5] First, intact GPI anchors are synthesized. These are then attached to target proteins bearing a GPI attachment signal at the C terminus by GPI transamidase.[5c,6] After lipid remodeling and other subtle modifications, GPI-linked proteins are transported and anchored to the cell membrane (Figure 1).[2a]

Natural GPIs are structurally diverse. Moreover, GPI-anchored proteins are amphiphilic and are associated with the cell membrane, thus making their isolation difficult. Most importantly, GPI-anchored proteins are present in low quantities on cells. Therefore, biological studies of GPI-anchored proteins at the molecular level and on live cells are difficult. Attempts to deal with this issue have included chemical and chemoenzymatic syntheses of GPI-anchored proteins,[7] which have resulted in only unnatural products that are not applicable to live cells. Attempts to study GPI-anchored proteins through the engineering of GPIs on live cells have met with limited success.[4]

This work aimed at developing a practical method for the labelling of GPI-anchored proteins on live cells to facilitate their identification, purification, and biological investigation. We planned to introduce a molecular handle to the GPI common core through metabolic engineering, that is, giving

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Supporting information (including experimental details and NMR and MS characterization of all of the compounds used) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201503814.

cells a modified GPI precursor for biosynthetic incorporation (Figure 1). The precursor must be accepted by the involved enzymes and the handle should be flexible to allow further elaboration. In this regard, we were interested in modified inositol because of its unique presence in GPIs, with an azido group as the molecular handle, since this group is small and can be easily elaborated through a chemoselective and bioorthogonal click reaction.[8] Although a similar strategy has been used for glycobiological studies based on cell-surface sialic acid, fucose, and *N*-acetylgalactosamine engineering,[9] it has not yet been applied to the engineering of GPIs and GPI-anchored proteins.

To find a proper precursor for cell-surface GPI engineering, we prepared inositol derivatives **1**–**3** (Figure 2 and the Supporting Information), which have an azidoethyl group

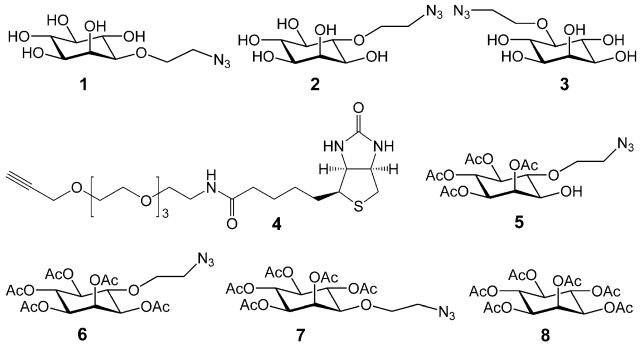


***Figure 2.*** Structures of the synthesized inositol derivatives **1**–**3** and **5**–**7**, which were used for the metabolic engineering of GPIs and GPI-anchored proteins on cells, and the biotin derivative **4**, which was used to tag modified GPIs on cells through click reaction.

linked to the inositol 3-, 4-, and 5-*O*-positions, respectively. We were interested in **1**–**3** and not derivatives with modifications at the 1-, 6- and 2-*O*-positions, since these positions are either linked to a phospholipid and glycan in GPIs or required in GPI biosynthesis. Moreover, instead of directly replacing the hydroxy groups with azides, an azidoethyl group was introduced to improve the molecular space and flexibility, which might help the click reaction later on. *Saccharomyces cerevisiae*, a fast-growing yeast that expresses abundant GPI-anchored proteins, was used to examine the efficiency of **1**–**3** incorporation in GPI-anchored proteins. The yeast cells were incubated with **1**–**3** and then subjected to click reaction with alkynylated biotin **4**. Several bioorthogonal click reactions are available, but we chose $Cu^{I}$-catalyzed azide–alkyne cycloaddition since it is convenient and fast.[10] Finally, the treated cells were stained with a streptavidin-allophycocyanin (APC) conjugate and used to analyze **1**–**3** incorporation in GPIs and GPI-anchored proteins.

Flow cytometry results for the stained cells (Figure 3a and Figure S1 in the Supporting Information) revealed that the mean fluorescence intensities (MFIs) of the cell populations treated with **1**–**3** (3 mM) were similar to that of the control, thus suggesting that **1**–**3** were not incorporated by the yeast cells. Among various potential explanations for this lack of incorporation, it was possible that **1**–**3** could not effectively penetrate the cell membrane owing to their high polarity. Inspired by the finding that acylation of free sugars could improve their efficacy in cell engineering,[11] we prepared and studied acetylated inositol derivatives **5**–**7** (Figure 2). As shown in Figure 3b, yeast cells were effectively labeled upon treatment with **5**–**7** (3 mM). The MFI for the cells treated with **5** was more than 1000-fold higher than for control cells (Figure S1), thus confirming that **5** could be used by yeast cells to label GPIs and GPI-anchored proteins.

Acetylated **5**–**7** were clearly more efficient than free **1**–**3** for GPI engineering in yeast cells. As suggested in the literature,[11] lipophilic **5**–**7** may be more effectively taken up by cells through passive diffusion. Inside the cell, the acetyl groups could be removed by esterases[11] to give free inositol derivatives that are incorporated into GPIs. Moreover, the enzymes involved in GPI biosynthesis must be able to tolerate modified inositols, especially 3/4-*O*-modified ones. It is also noteworthy that the partially acetylated **5** was more efficient for GPI engineering than the fully acetylated **6**, perhaps because **5** has a better hydrophilic/hydrophobic balance to favor its entry into cells.

This strategy was also tested in the human lung cancer cell line A549 to probe the application scope. After treatment with **5**–**7** (200 μM), the cells were subjected to click reaction with **4**, staining with streptavidin-APC, and flow cytometry. As shown in Figure 3c and Figure S2, cells treated with **5**–**7** were effectively labeled with the fluorescent tags, thus confirming the incorporation of **5**–**7**. The MFIs of the cells treated with **5** and **6** or **7** were approximately 30- and 16-fold higher than that of the control. Again, partially acetylated **5** was better than peracetylated **6**. Flow distribution analysis revealed that more than 98% of the **5**-treated cells were stained with APC (Figure S3), thus suggesting their extensive metabolic engineering. The stained cells were also analyzed by imaging flow cytometry and confocal microscopy (Figure 3d and e) and it was found that the bright red fluorescence was concentrated on the cell surface. The strong cell-surface fluorescence indicates abundant labeling of GPIs and GPI-anchored proteins and demonstrates the high efficiency of the GPI metabolic engineering.

To further verify the incorporation of **5** into GPIs, we performed a competitive inhibition analysis, in which A549 cells were treated with **5** and peracetylated inositol **8**. After staining, the cells showed decreased MFI with increased concentration of **8** (Figure 3f and Figure S4). Clearly, **8** inhibited the incorporation of **5** and GPI engineering, thus demonstrating that **5** and **8** are involved in the same biosynthetic pathway. The impact of precursor concentration and incubation time on the engineering of GPIs and GPI-anchored proteins was also studied (Figure 3g and Figure S5). A549 cells treated with 200 μM of **5** showed significantly stronger fluorescence than cells treated with 100 μM of **5**, thus confirming that the intensity of cell labeling is dependent on the concentration of **5**. In addition, cells incubated with 100 and 200 μM of **5** showed increased MFI after elongated incubation, and the cells treated with 200 μM of **5** showed the highest MFI after 2 days of incubation. However, at 200 μM, **5** inhibited cell growth by 30%, while it did not show obvious inhibition on cell growth at 100 μM.

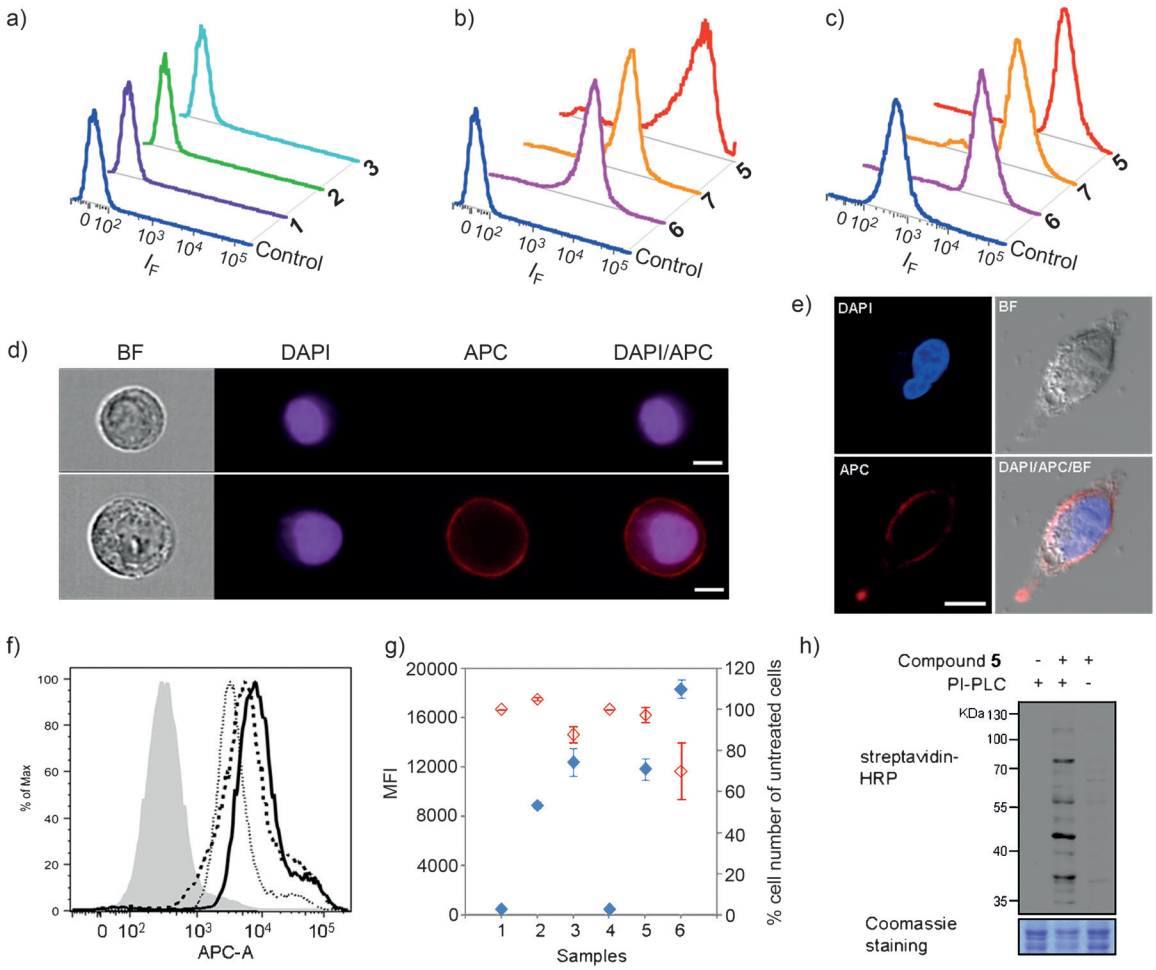


***Figure 3.*** Detection of GPIs and GPI-anchored proteins on cells treated with or without modified inositol derivatives after click reaction with **4** and staining with streptavidin-APC (for cell analysis) or streptavidin-peroxidase (for protein detection). a,b) Flow cytometry results are shown for yeast cells incubated with 3 mM **1**–**3** (a) or **5**–**7** (b). c) Flow cytometry results for A549 cells treated with **5**–**7** (200 μM). d) Imaging cytometry results for **5**-treated A549 cells suspended in PBS buffer. BF: bright field image; DAPI: a nuclear stain. The upper and lower images show untreated and treated cells, respectively (scale bar: 7 μm). e) Confocal microscopy images of **5**-treated A549 cells in a culture dish (scale bar: 10 μm). f) Flow cytometry results for A549 cells incubated with **5** (100 μM) in the presence of 0 (——), 100 (- - - -), or 200 μM (·····) of **8**. The filled histogram shows the result for cell treated with neither **5** nor **8**. g) Influence of the concentration of **5** and incubation time on metabolic engineering of GPIs and GPI-anchored proteins on A549 cells. The concentrations of **5** were 0 (samples 1 and 4; negative controls), 100 (samples 2 and 5), and 200 μM (samples 3 and 6). The cells were incubated for 1 day (samples 1–3) or 2 days (samples 4–6). Blue and red diamonds show cell labeling and growth, respectively. h) Western blot of GPI-anchored proteins expressed on **5**-treated A549 cells. The supernatants of cells treated with PI-PLC only (left), with **5** and PI-PLC (middle), and with **5** only (right) were subjected to click reaction with **4** and then probed with streptavidin-conjugated horseradish peroxidase (HRP). Bottom: Coomassie staining to show the protein loading in each lane.

Phosphatidylinositol-specific phospholipase C (PI-PLC) can be used to detach GPI-anchored proteins from the cell membrane,[3n] which was demonstrated by the detection of a GPI-anchored cancer marker, placental alkaline phosphatase, in the supernatant of PI-PLC-treated A549 cells (Figure S6). To verify that the GPI-anchored proteins on engineered cells were labeled with azides, A549 cells were incubated with PI-PLC after treatment with **5**, and the released GPI-anchored proteins in the cell supernatant were subjected to click reaction with **4** and then probed with streptavidin-conjugated peroxidase for western blot. The cells treated with both **5** and PI-PLC gave a series of protein bands that were absent or very faint when **5** or PI-PLC was missing (Figure 3h). This result confirmed the expression of azide-labeled GPI-anchored proteins on **5**-treated A549 cells. We are currently pursuing further verification and characterization of these proteins.

The above studies with different cell types indicate that this new strategy for the engineering of GPIs and GPI-anchored proteins may be widely useful. To verify this, four other human cancer cell lines, MCF-7, Hela, K562, and SKM28, were treated with **5**, subjected to click reaction and fluorescent staining, and then analyzed. Flow cytometry showed that all four cell lines were labeled with the fluorescent tag, and imaging cytometry showed that the fluorescence was focused on the cell surface (Figure S7–S10). These results confirm the efficiency, flexibility, and broad applicability of **5** for the engineering of GPIs and GPI-

anchored proteins. It is also noteworthy that, except for with the MCF-7 cells, **5** did not have a significant impact on cell growth at concentrations up to 100 μM.

In summary, we have developed a facile and effective method for the labeling of cell-surface GPIs and GPI-anchored proteins. This was realized by feeding the cells an azide-modified inositol derivative and allowing the cells to incorporate it into GPIs. The azide-labeled GPIs and GPI-anchored proteins on the cell surface were then elaborated through a bioorthogonal click reaction to enable their visualization. This could potentially be used to investigate the trafficking and organization of GPIs and GPI-anchored proteins on cells by quantitative fluorescence microscopy[12] and other methods. The new GPI labeling strategy was effective and applicable to different cells. Moreover, we envision that this technique could be used for the capture and analysis of GPI-anchored proteins, as well as the discovery of new GPI-anchored protein markers. It could also be used for targeted drug delivery and for the diagnosis and tailored treatment of diseases via monitoring of cell-surface GPI-anchored proteins.

**Keywords:** carbohydrates · glycolipids · GPI anchor · inositol · metabolic engineering

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[1] M. A. J. Ferguson, A. F. Williams, *Annu. Rev. Biochem.* **1988**, *57*, 285–320.

[2] a) P. Orlean, A. K. Menon, *J. Lipid Res.* **2007**, *48*, 993–1011; b) M. G. Paulick, C. R. Bertozzi, *Biochemistry* **2008**, *47*, 6991–7000; c) Y. Tsai, X. Liu, P. H. Seeberger, *Angew. Chem. Int. Ed.* **2012**, *51*, 11438–11456; *Angew. Chem.* **2012**, *124*, 11604–11623.

[3] a) J. R. Thomas, R. A. Dwek, T. W. Rademacher, *Biochemistry* **1990**, *29*, 5413–5422; b) P. J. Robinson, *Cell Biol. Int. Rep.* **1991**, *15*, 761–767; c) M. J. McConville, M. A. J. Ferguson, *Biochem. J.* **1993**, *294*, 305–324; d) M. A. J. Ferguson, *Parasitol. Today* **1994**, *10*, 48–52; e) G. N. Gaulton, J. C. Pratt, *Semin. Immunol.* **1994**, *6*, 97–104; f) M. A. J. Ferguson, *J. Cell Sci.* **1999**, *112*, 2799–2809; g) K. Kasahara, Y. Sanai, *Glycoconjugate J.* **2000**, *17*, 153–162; h) M. D. Marmor, M. Julius, *J. Biol. Regul. Homeostatic Agents* **2000**, *14*, 99–115; i) T. Zhou, L. Xu, B. Dey, A. J. Hessell, D. Van Ryk, S.-H. Xiang, X. Yang, M.-Y. Zhang, M. B. Zwick, J. Arthos, D. R. Burton, D. S. Dimitrov, J. Sodroski, R. Wyatt, G. J. Nabel, P. D. Kwong, *Nature* **2007**, *445*, 732–737; j) C. Chan, C. Stanners, *Curr. Oncol.* **2007**, *14*, 70–73; k) Y. Varma, T. Hendrickson, *ChemBioChem* **2010**, *11*, 623–636; l) J. E. Bradley, J. M. Chan, J. S. Hagood, *Lab Invest.* **2013**, *93*, 365–374; m) S. Park, C. Lee, P. Sabharwal, M. Zhang, C. L. F. Meyers, S. Sockanathan, *Science* **2013**, *339*, 324–328; n) S. Dolezal, S. Hester, P. S. Kirby, A. Nairn, M. Pierce, K. L. Abbott, *Cancer Biomarkers* **2014**, *44*, 55–62; o) T. Kinoshita, *Proc. Jpn. Acad. Ser. B* **2014**, *90*, 130–143.

[4] D. R. Taylor, N. M. Hooper in *Post-Translational Modifications in Health and Disease*, Springer, New York, **2011**, pp. 39–55.

[5] a) P. T. Englund, *Annu. Rev. Biochem.* **1993**, *62*, 121–138; b) J. Takeda, T. Kinoshita, *Trends Biochem. Sci.* **1995**, *20*, 367–371; c) S. Udenfriend, K. Kodukula, *Annu. Rev. Biochem.* **1995**, *64*, 563–591.

[6] a) C. A. Bailey, L. Gerber, A. D. Howard, S. Udenfriend, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 22–26; b) S. E. Maxwell, S. Ramalingam, L. D. Gerber, L. Brink, S. Udenfriend, *J. Biol. Chem.* **1995**, *270*, 19576–19582.

[7] a) Z. Guo, *Curr. Org. Synth.* **2013**, *10*, 366–383; b) S. Yu, Z. Guo, C. Johnson, G. Gu, Q. Wu, *Curr. Opin. Chem. Biol.* **2013**, *17*, 1006–1013; c) M. Wehle, I. Vilotijevic, R. Lipowsky, P. H. Seeberger, D. Varon Silva, M. Santer, *J. Am. Chem. Soc.* **2012**, *134*, 18964–18972; d) Z. Wu, X. Guo, Q. Wang, B. M. Swarts, Z. Guo, *J. Am. Chem. Soc.* **2010**, *132*, 1567–1571; e) M. A. Ferguson, *Nat. Chem. Biol.* **2008**, *4*, 223–224; f) C. F. W. Becker, X. Liu, D. Olschewski, R. Castelli, R. Seidel, P. H. Seeberger, *Angew. Chem. Int. Ed.* **2008**, *47*, 8215–8219; *Angew. Chem.* **2008**, *120*, 8338–8343; g) M. G. Paulick, A. R. Wise, M. B. Forstner, J. T. Groves, C. R. Bertozzi, *J. Am. Chem. Soc.* **2007**, *129*, 11543–11550.

[8] a) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021; *Angew. Chem.* **2001**, *113*, 2056–2075; b) J. A. Prescher, C. R. Bertozzi, *Cell* **2006**, *126*, 851–854.

[9] a) C. T. Campbell, S. G. Sampathkumar, K. J. Yarema, *Mol. BioSyst.* **2007**, *3*, 187–194; b) S. T. Laughlin, N. J. Agard, J. M. Baskin, I. S. Carrico, P. V. Chang, A. S. Ganguli, M. J. Hangauer, A. Lo, J. A. Prescher, C. R. Bertozzi, *Methods Enzymol.* **2006**, *415*, 230–250; c) G. A. Lemieux, C. R. Bertozzi, *Chem. Biol.* **2001**, *8*, 265–275; d) C. R. Bertozzi, L. L. Kiessling, *Science* **2001**, *291*, 2357–2364.

[10] H. Jiang, T. Zheng, A. Lopez-Aguilar, L. Feng, F. Kopp, F. L. Marlow, P. Wu, *Bioconjugate Chem.* **2014**, *25*, 698–706.

[11] a) J. A. Prescher, D. H. Dube, C. R. Bertozzi, *Nature* **2004**, *430*, 873–877; b) G. A. Lemieux, K. J. Yarema, C. L. Jacobs, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 4278–4279; c) C. L. Jacobs, S. Goon, K. J. Yarema, S. Hinderlich, H. C. Hang, D. H. Chai, C. R. Bertozzi, *Biochemistry* **2001**, *40*, 12864–12874.

[12] a) A. Esposito, S. Schlachter, G. S. K. Schierle, A. D. Elder, A. Diaspro, F. S. Wouters, C. F. Kaminski, A. I. Iliev in *Cytoskeleton Methods and Protocols*, Springer, New York, **2010**, pp. 117–142; b) S. Mayor, H. Riezman, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 110–120.

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