Journal of Chromatography B, 879 (2011) 1316–1324

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

Chemical and genetic probes for analysis of protein palmitoylation☆

Lun Li, Linjie Dong, Lisha Xia, Tingting Li, Hongying Zhong*

Key Laboratory of Pesticides and Chemical Biology, Ministry of Education, College of Chemistry, Central China Normal University, Wuhan, Hubei 430079, PR China

ARTICLE INFO

Article history:
Received 6 June 2010
Accepted 16 November 2010
Available online 23 November 2010

Keywords:
Palmitoylation
Chemical probes
Genetic probes
Mass spectrometry
Fluorescence

ABSTRACT

Reversible protein palmitoylation is one of the most important posttranslational modifications that has been implicated in the regulation of protein signaling, trafficking, localizing and enzymatic activities in cells and tissues. In order to achieve a precise understanding of mechanisms and functions of protein palmitoylation as well as its roles in physiological processes and disease progression, it is necessary to develop techniques that can qualitatively and quantitatively monitor the dynamic protein palmitoylation *in vivo* and *in vitro*. This review will highlight recent advances in both chemical and genetic encoded probes that have been developed for accurate analysis of protein palmitoylation, including identification and quantification of acyl moieties and palmitoylated proteins, localization of amino acid residues on which acyl moieties are attached, and imaging of cellular distributions of palmitoylated proteins. The role of major techniques of fluorescence microscopy and mass spectrometry in facilitating the analysis of protein palmitoylation will also be explored.

Contents

1. Introduction

Protein palmitoylation essentially refers to the covalent attachment of 16-carbon palmitic acid to the free thiol of cysteine residues [1,2]. In some cases, other saturated or unsaturated fatty acids such as myristic, stearic, oleic, and arachidonic acids can also be bonded to cysteine residues through the labile thioester linkage [3–8]. Although S-acylation is more appropriate in the nature, the term protein palmitoylation is used more frequently. Protein palmitoylation is one of the most important and versatile posttranslational modifications that contributes to protein trafficking, targeting and enzymatic activities [9,10]. Through reversible and dynamic cycles of palmitoylation and depalmitoylation, proteins are rapidly relocated among intracellular compartments in response to different extracellular signals or physiological stimuli [11–14].

It has been found that palmitoylation occurs on numerous membrane proteins and membrane associated cytosolic proteins. Although the precise functions provided by palmitoylation to such proteins are not clear so far, there are some evidences showing its roles in lipid raft and membrane microdomain targeting [15–18].

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

* Corresponding author. Tel.: +86 27 67862616.
E-mail address: hyzhong@mail.ccnu.edu.cn (H. Zhong).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.11.018

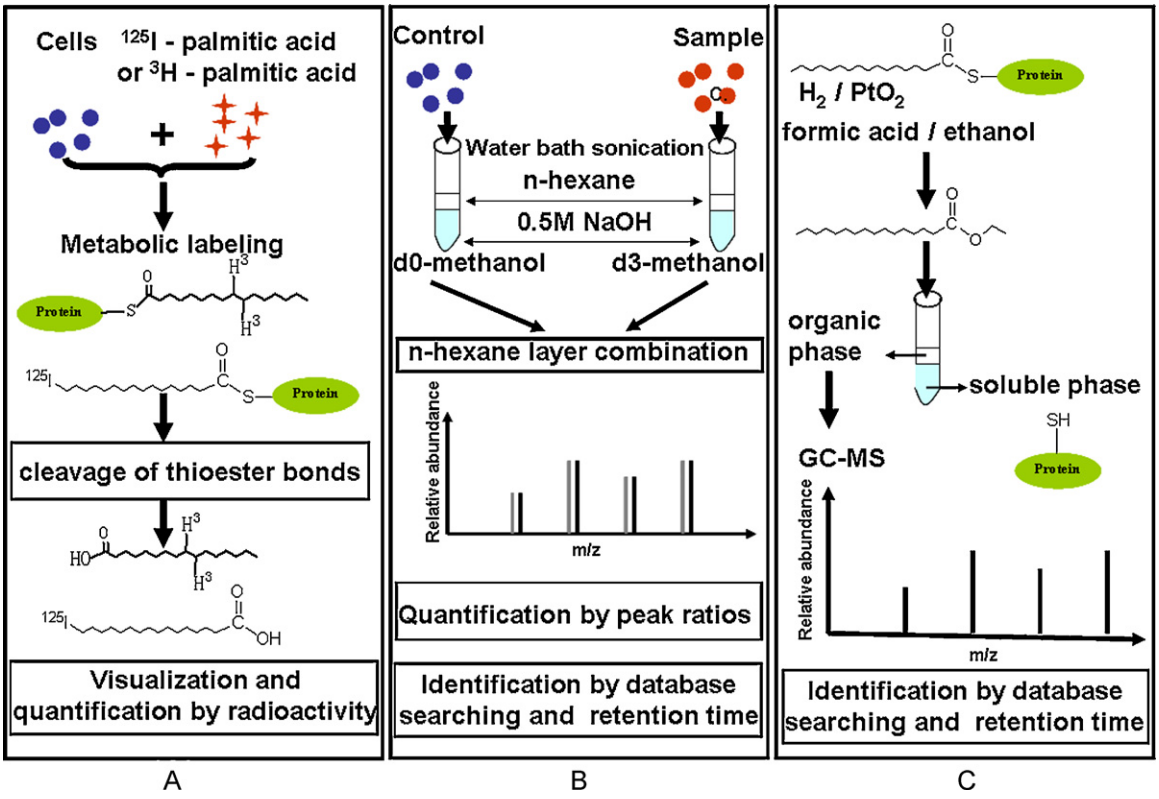

Fig. 1. Analysis of fatty acyl moieties by different methods. (A) *In vivo m*etabolic labeling by using radioactive fatty acid analogues. (B) *In vitro* fatty acid transmethylation and stable isotope labeling. (C) *In vitro* hydrogenation by platinum(IV) oxide and acid transesterification.

At present, palmitoylated proteins are mainly classified into six groups [9]. First group consists of integral membrane proteins with palmitoylation at cysteines that are close to the boundary of transmembrane domain (TMD) and cytoplasmic domain (CD). Representative such integral membrane proteins include CD8β [19], β2AR [20], CD9 [21], CD151 [22], caveolin [23], and GPCRs [24]. In contrast, a second group consists of integral membrane proteins with palmitoylation at CD cysteine residues that are distant from the TMD–CD boundary, for example, MPR [25], HIV-Env [26] and CCR5 [27]. S-palmitoylation of these proteins could efficiently increase protein hydrophobicity and promote membrane association of CD elements of these proteins. Experimental results indicate that transmembrane proteins are palmitoylated along the exocytic pathway except for the ER (Endoplasmic Reticulum) and they have been implicated in protein trafficking, recycling and stability [28–30]. As for cytosolic proteins, palmitate moieties can be attached to cysteines with or without the incorporation of other acylations such as myristoylation and prenylation [31]. The third group is featured by Src family kinases [32–34] and Gα subunits [35–37]. Seven out of the nine Src family members as well as Gα subunits are dually acylated. In such case, proteins are co-translationally myristoylated through an amide bound to glycine after the N-terminal methionine is removed. It has been experimentally demonstrated that palmitoylation of Src family kinases and Gα subunits can aid the localization of these proteins to lipid rafts and signaling [38–40]. Posttranslational palmitoylation in some cytosolic proteins can occur at more than one nearby cysteines for stable membrane association. For example, the endothelia form of nitric oxide synthase (eNOS) has dual palmitoylation sites at Cys 15 and Cys 26 [41]. The Ras family proteins are classified into the forth group that are prenylated at a C-terminal cysteine in the CAAX motif (A and X are aliphatic residues and any residues respectively). The subsequent palmitoylation occurs at the nearby cysteine to further enhance membrane association [42–46]. The fifth group consists of cytosolic proteins that are palmitoylated without the incorporation of other acylations. GAP43 [47], GAD-65 [48] and SNAP-25 [49] are representative such proteins. There is no consensus bonding motifs for those proteins. GAP-43 and SNAP-25 were found to be palmitoylated at N-terminal and C-terminal cysteines respectively but GAD-65 was palmitoylated on cysteines in the central part of the protein. All of the above five classes of palmitoylation refers to the covalent attachment of palmitates to cysteine residues through a labile thioester linkage. Few proteins such as Hedgehog and Gαs subunits were recently identified as the sixth class of palmitoylated proteins that are subjected to the covalent bonding of palmitates via amide-linkage to a cysteine or glycine residue [50,51]. The distinct difference between labile thioester linkage and stable amide-linkage is the reversibility of the labile thioester bond that provides a unique feature, “regulable”, to S-palmitoylated proteins [52,53].

Despite the advances in genomics, proteomics and bioinformatics, precise understanding of the mechanisms involved in the regulation of diverse functions of protein palmitoylation in physiology and diseases remains unclear due to the analytical challenges [54,55]. Protein palmitoylation is often found to modify transmembrane or membrane associated proteins [56]. In addition to the intrinsic low solubility and low abundance of these membrane proteins [57,58], the attachment of long chain fatty acids further increases the hydrophobicity of proteins. And thus previous analytical methods targeted for qualitative and quantitative analysis of soluble proteins are not applicable in such situation. Moreover, unlike other posttranslational phosphorylation [59,60] and glycosylation [61,62], there are no affinitive groups for enrichment of low abundance palmitoylated proteins. The reversibility of palmitoyla-

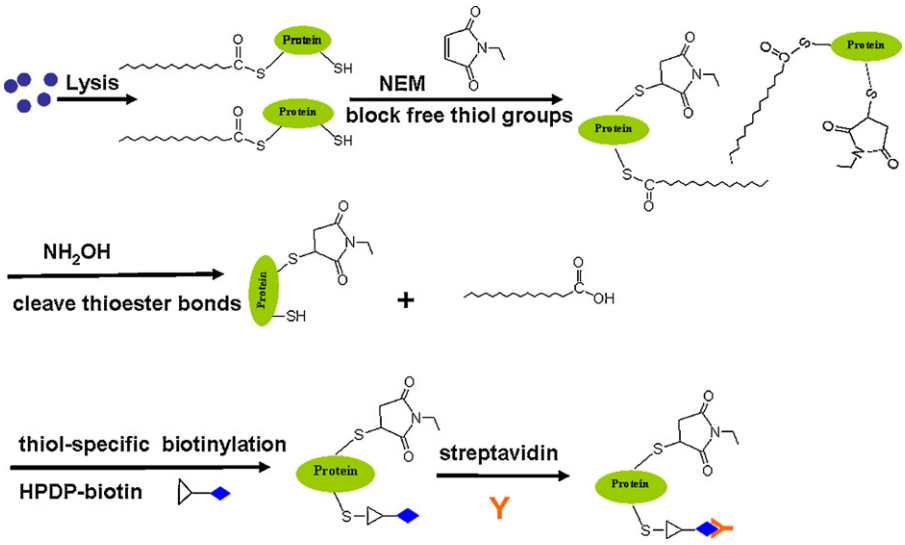

Fig. 2. Purification and detection of palmitoylated proteins by ABE method (Acyl-Biotinyl Exchange). NEM: N-ethyl maleimide.

tion makes it even difficult to monitor the dynamics of biological process [63]. Herein, we describe the analytical techniques that are aimed to enable accurate studies of fundamental biochemistry and cellular functions of protein S-palmitoylation.

2. Identification and quantification of acyl moieties

Lots of experiments has revealed that proteins can be S-palmitoylated by not only C16:0 palmitic acid but also other fatty acids including C14:0, C18:0, C18:1, C18:2, and C20:4 fatty acids [3–8]. Although the function of heterogenous S-acylation of proteins with different fatty acids was not known, it was shown that the length and composition of the transmembrane seuqences as well as the cytoplasmic sequences can influence the choice of fatty acids for acylation [64–66]. Identification and quantification of acyl moieties are essential for understanding the detailed mechanisms of protein palmitoylation.

2.1. In vivo radioactive labeling

In early days, identification of acyl moieties was mainly based on radioactive labeling by using fatty acid analogues [67–70]. As shown in Fig. 1(A), [^{3}H] palmitic acid [67–69] or [^{125}I] palmitic acid [70] was added to the cell culture for metabolic labeling. Because these radiolabeled fatty acids retain the same functions as their natural counterparts, they can be taken into metabolic pathways and incorporated into the target proteins. Cells were then harvested, lysed and isolated proteins were separated by SDS-PAGE. Following SDS-PAGE, the resolved proteins were electroblotted onto nitrocellulose paper. Interested protein bands were washed by chloroform–methanol (2:1) solution in order to remove non-covalently bonded lipids. Acyl moieties covalently attached with proteins through a labile thioester bond can be cleaved from the proteins by thiol specific hydrolysis. Resultant fatty acids were subsequently extracted by an organic solvent such as hexane and then separated with other co-existed biological molecules by using HPLC. Identification of fatty acids was achieved by comparison of chromatographic retention time with that of standard fatty acids. The ^{3}H or ^{125}I emission intensity was used for quantification. Targeted fatty acids can also be converted into p-nitrophenacyl derivatives so that they can be quantified by the absorption at 254 nm [67]. These methods are not only time-consuming for sample preparation and autoradiographic exposure but also involve the hazards of handling radioisotopes. In addition to the safety issues, radiolabeling approaches are also confronted with several technical problems: (1) the physiological effects resulting from the structural differences between natural fatty acids and radiolabeled fatty acid analogues was not clear; (2) the ratio of unlabeled and labeled palmitoylation was not known. Therefore, it is difficult to achieve accurate quantification of dynamic palmitoylation in cells and tissues; (3) due to the complexity of metabolic pathways in eukaryotic cells, fatty acid analogues maybe incorporated in different metabolic pathways and cause unexpected biological effects; (4) HPLC based approaches are difficult to avoid interferences resulting from co-existed high abundance molecules present in complex biological context.

2.2. In vitro transmethylation and stable isotope labeling for comparative analysis of fatty acyl moieties by gas chromatography–mass spectrometry

Recently a new approach termed iFAT (Isotope-coded Fatty Acid Transmethylation) has been developed for quantitative comparison of acyl moieties in different cell states *in vitro* (Fig. 1(B)) [71,72]. The iFAT approach integrates extraction, transmethylation and stable isotope labeling into a single step with the aid of ultrasound sonication. In this approach, proteins isolated from the control and from the sample were mixed with a d0- or d3-methanol solution of 0.5 M NaOH and n-hexane solution respectively. Herein, ultrasound irradiation plays important roles in efficient transmethylation reaction and simultaneous extraction of resultant fatty acid methyl esters into the top n-hexane layer. The intense wave shocks and cavitations generated by ultrasonication not only speed-up the heterogeneous reactions but also facilitate the simultaneous mass transfer and enrichment of fatty acid methyl esters into n-hexane phase. Because of the highly efficient heterogeneous reactions provided by iFAT approach, it is thus suitable to handle detergent-resistant aggregates of hydrophobic membrane proteins. Identification of acyl moieties was achieved by database searching and comparison of chromatographic retention time with that of standard fatty acid methyl esters. The original amounts of samples were recovered in the peak ratios of d0- and d3-labeled fragment ion pairs and molecular ion pairs. Additionally, there is less interferences from the complex biological background. While the iFAT approach differentially derives fatty acyl moieties in different samples, it also volatilizes targeted molecules and thus they

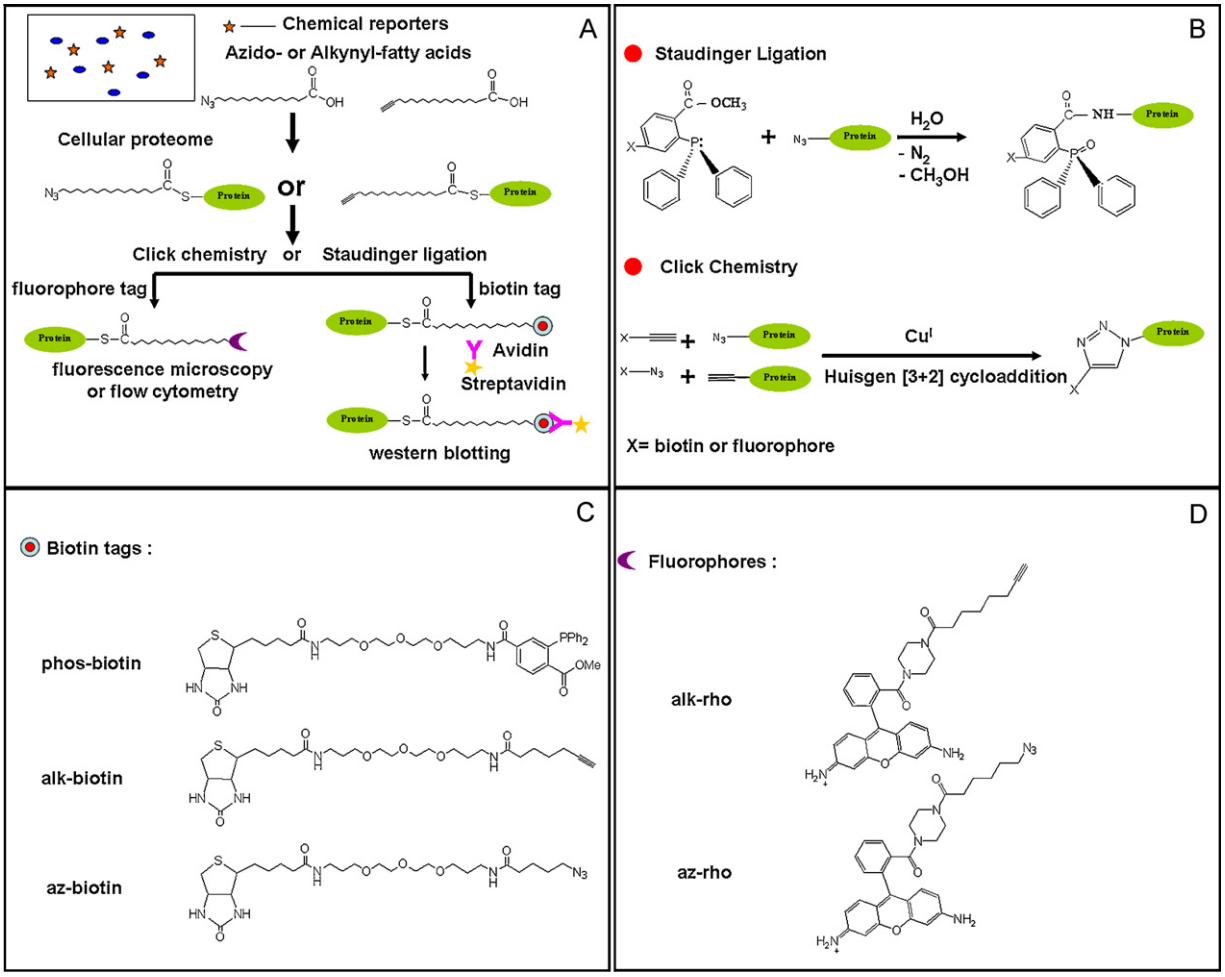

Fig. 3. Analysis of protein palmitoylation by chemical reporters. (A) Metabolic labeling of cells with chemical reporters followed by bioorthogonal ligation. Through Staudinger ligation or click chemistry, proteins that have been *in vivo* modified by chemical reporters are covalently ligated with either a fluorophore or a biotin tag for downstream fluorescence imaging or streptavidin enrichment. (B) Schemes for Staudinger ligation and click chemistry. (C) Biotin tags. (D) Fluorophores.

are isolated from the bulk background. Finally, it has also been demonstrated that the reaction rates across different acyl groups are quantitative by using this ultrasound-assisted approach. The stable isotope labeling can be used as an internal standard for accurate comparison of acyl modifications in different cell states. By using the iFAT approach, it has shown that other fatty acids in addition to C16:0 are present in cell lysates and SDS resistant pellets.

The *in vitro* iFAT approach provides a safe and accurate means for comparative analysis of fatty acyl moieties in different cell states. It is useful for dealing with hydrophobic membrane proteins or membrane associated cytosolic proteins that are present in low abundance and have poor solubility in aqueous solution.

2.3. *In vitro hydrogenation, ethyl esterification and analysis of acylated proteins by gas chromatography–mass spectrometry*

Alternatively, purified S-acylated proteins can be hydrogenated in the presence of platinum(IV) oxide so that the fatty acid moieties are released from the protein (Fig. 1(C)) [73]. The hydrogenation breaks the thioester linkage and simultaneously brings about ethyl esterification with absolute formic acid and ethanol. By using this approach, it has been confirmed that heterogenous S-acylation takes place by different acyl moieties. Furthermore, by identifying the acyl modifications, it was possible to show that S-acylation induces partitioning of ROP6 into detergent-resistant membranes and targeting of CBL1 to the plasma membrane. Hydrogenation by platnium(IV) oxide in combination with ethyl esterification efficiently derives the polar group of fatty acids. It improves the GC separation and identification. Relative quantification of S-acylation level can be achieved when protein concentrations and volumes of extraction solvents in different samples are known.

3. Detection of S-palmitoylated proteins and palmitoylation sites

S-palmitoylated proteins have been originally detected by metabolic labeling with radioactive ^{3}H, ^{14}C, and ^{125}I fatty acid analogues followed by autoradiography of acyl moieties or labeled proteins [67–70]. Although it is effective, it is tedious, hazardous and require long exposure time. More recently, nonradioactive labeling methods in combination with techniques of mass spectrometry and fluorescence microscope have been developed to improve the detection of palmitoylated proteins in cells and tissues including *in vitro* ABE (Acyl-Biotinyl Exchange) method, *in vivo* metabolic labeling by chemical reporters carrying with a functional group for subsequent bioorthogonal ligation, and *in vivo* genetic encoding GFP tag.

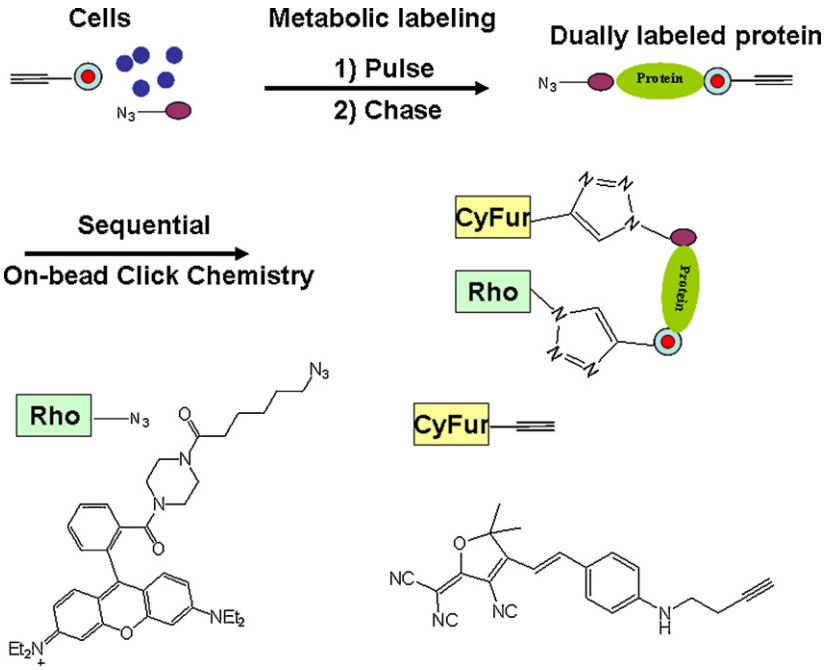

Fig. 4. Tandem fluorescence imaging of palmitoylation turnover. Cells are metabolically labeled with two chemical reporters: one for monitoring the dynamic palmitoylation and another one for protein turnover.

3.1. *In vitro Acyl-Biotinyl Exchange for detection of palmitoylated proteins*

As shown in Fig. 2 [56,74,75], detection of palmitoyl proteins based on Acyl-Biotinyl Exchange (ABE) includes the following steps: (1) free thiols present in proteins are blocked by thiol reactive reagents such as N-ethyl maleimide (NEM); (2) then hydroxylamine was used to specifically cleave palmitoyl thioester linkage; (3) the newly exposed thiol group by hydroxylamine treatment was marked with a thiol-specific biotinylation reagent HPDP-biotin (N-(6-(Biotininamido)Hexyl)-3′-(2′-Pyridylthio)Propionate); (4) the resultant biotinylated proteins were purified and enriched by avidin or streptavidin affinitive agarose. The *in vitro* labeling technique does not need living cells and thus can be performed for cell or tissues lysates. Compared with *in vivo* radioactive labeling, ABE approach not only eliminates the radioactive exposure but also improve the sensitivity of detection because of the usage of affinitive enrichment.

3.2. *In vivo metabolic labeling by chemical reporters and bioorthogonal ligation*

Protein palmitoyl modification is unique compared with other lipidation because of its reversibility. Therefore, dynamic monitoring of the cycles of palmitoylation and depalmitoylation is important for understanding the spatial and temporal regulation of complex signaling pathways. Recently developed chemical reporters carrying with a functional group for subsequent bioorthogonal ligation in combination with fluorescence microscopy or flow cytometry have enabled specific and sensitive visualization of palmitoylated proteins in cells or tissues. So far, there are mainly two kinds of chemical reporters that have been developed to enable rapid and nonradioactive detection of palmitoyl proteins [76–78]: azido-fatty acids [79–82] and alkynyl-fatty acids [63,83–85]. As shown in Fig. 3, the chemical approach involves several steps. (1) Azido or alkynyl fatty acid analogues were incubated with cells for metabolic labeling. Because these fatty acid analogues are structurally and functionally similar with their natural fatty acid counterparts, they can be incorporated into the palmitoylated proteins through metabolic pathways. The azido and alkynyl groups provide palmitoylated proteins with a specific reactivity in the follow-up processes. (2) Azido or alkynyl-modified proteins are then ligated with chemoselective detection tags such as phosphine-biotin, alk-biotin, az-biotin and alkynyl- or azido-conjugated fluorophores (e.g. alk-rho and az-rho). Staudinger ligation [86] and Cu^{I}-catalyzed Huisgen [3 + 2] cycloaddition click chemistry [87] are involved in these chemoselective reactions respectively. Copper-free click chemistry provides a means with no apparent toxicity for dyanmic monitoring of target compounds [88]. (3) Visualization of palmitoylated proteins can be achieved through either streptavidin/avidin blotting of biotinylated proteins or direct fluorescence imaging in cells. The immunoblotting methods are convenient means to visualize palmitoylated proteins but not suitable for quantifying changes in the palmitoylation level. Fluorescent detection of palmitoylated proteins with chemical reporters provides an improved means for investigating dynamic and complex biological processes. It has been shown that in-gel fluorescence scanning can reveal much more proteins compared with streptavidin blotting, particularly with the palmitic acid analogues [81]. Evenmore, studies of kinetics and specificity of labeling palmitoylated proteins in cell have also demonstrated that the click chemistry and in-gel fluorescence imaging protocol require shorter labeling time and lower concentration of fatty acid chemical reporters [81].

Fluorescent detection tags provide an opportunity to visualize the distribution of palmitoylated proteins in cells or tissues by fluorescence microscopy [89,90]. Cells or tissues can be fixed by paraformaldehyde, premeabilized with Triton X-100, and ligated with alk-rho or az-rho through click chemistry reaction. By using this method, it has been demonstrated that alk-palmitoylated proteins are not localized to one specific intracellular membrane compartment or dispersed throughout the cytoplasmic but they are distributed to several membrane-associated organelles. More recently, a tandem fluorescence imaging method (Fig. 4) [91] has

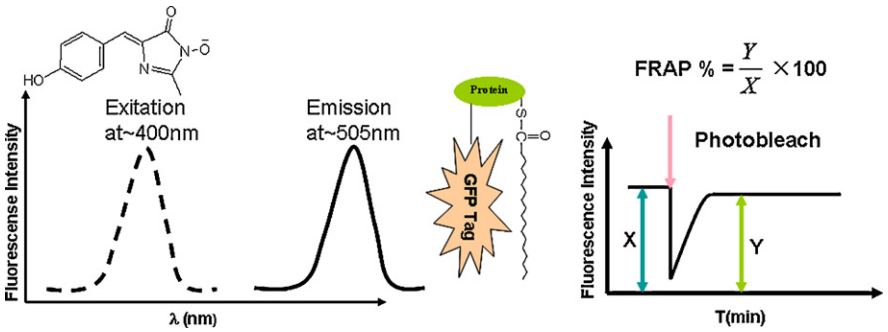

Fig. 5. Fluorescent chromophore of Green Fluorescence Protein (GFP) and the principle of FRAP (Fluorescence Recovery After Photobleach). The fluorescence recovery is defined as the final fluorescence intensity over that of the starting level of fluorescence intensity.

been developed for analyzing palmitate turnover rates in different cell states or upon the pertubation of exogenous chemicals. This method is based on the simultaneous metabolic labeling with two distinct chemical reporters followed by sequential on-bead Cu^{I}-catalyzed azide-alkyne cycloaddition (CuAAC). One of the two chemical reporters is designed for monitoring the dyanmic S-palmitoylation while the other functions as an internal control for protein turnover.

3.3. FRAP (Fluorescence Recovery After Photobleach)-based assay for studies of GFP-tagged palmitoylated proteins

In addition to synthesized chemical probes, green fluorescence protein (GFP) and its variants provide alternative genetic coded probes that have also been widely used to visualize spatial-temporal processes and explore basal principles of biological pathways inside living cells [92–94]. As shown in Fig. 5, the fluorescence chromophore is spontaneously formed from a tri-peptide motif (Ser-Tyr-Gly) in the primary structure of GFP. It has a maximum excitation wavelength at about 400 nm and a maximum emission wavelength at about 505 nm [92]. Because GFP is non-toxic and can be expressed to high levels in many organisms with minor effects on their physiology, the gene for GFP has been fused with the gene of interested proteins. GFP-tagged proteins usually can retain their normal activities while the fluorescent chromophore of GFP retains its fluorescence. Therefore, GFP and its variants have been used to study the location, movement and other biological activities of palmitoylated proteins by microscopic monitoring of the GFP fluorescence [41,95,96]. FRAP is a recently developed quantitative fluorescence microscopic method that can be used for studies of the intracellular targeting and trafficking of GFP-tagged palmitoylated proteins in living cells [97,98]. In this approach, light is first focused into a small subset of the fluorescent GFP-tagged palmitoyl proteins by using a microscope. After the starting level X of fluorescence is determined for the focused area, a very strong light is flashed onto the same area and the fluorescent molecules within this area will be photobleached. Then there will be a black area filled with photobleached palmitoyl proteins but surrounded by fluorescent GFP-tagged palmitoyl protein molecules. If the palmitoylated proteins are able to diffuse, the fluorescent GFP-tagged palmitoyl protein molecules will migrate into this black area and cause the photobleached area to gradually increase in brightness. Over a period of time, the intensity of fluorescence will reach a stabilized flat line. The fluorescence recovery is defined as the ratio of the final fluorescence intensity Y over that of the starting fluorescence intensity. The lateral mobility is determined by the slope of the curve. The steeper the slope, the faster the recovery and the more mobile the palmitoylated proteins are.

3.4. Proteomics analysis of palmitoylated proteins and localization of palmitoylation sites

Lipid chemical reporters, bioorthogonal ligation as well as GFP genetic tags have provided efficient means for detection and visualization of palmitoylated proteins in cells or tissues. These methods require either metabolic labeling or gene manipulation. However, the localization of palmitoylation sites in proteins cannot be achieved through none of these methods. Tandem mass spectrometry has been proven to be very useful in the discovery of protein posttranslational modifications [18,99,100]. In this approach, cellular proteins are separated by SDS-PAGE or liquid chromatography. Then individual protein fractions are isolated and enzymatically cleaved. Resultant peptides are analyzed by a mass spectrometer that enables further gas phase isolation of interested peptides followed by collision-induced dissociation (CID) and detection of sequence specific fragment ions. The covalent modifications in proteins change the molecular weight of the amino acids. The mass differences between experimental data and theoretical masses can be detected by mass spectrometers and interpreted by computational software or manually. Mass analysis of intact proteins or peptides can reveal the type of modifications and MS/MS experiments can aid the assignment of posttranslational modifications to a specific amino acid residue. Mass spectrometry-based approaches provide several advantages for analysis of protein posttranslational modifications [101,102]: (1) high sensitivity and throughput; (2) ability to identify the amino acids that are covalently attached with modification groups; (3) ability to quantify relative changes in modification occupancy at different sites or in different cells by using stable isotope labeling.

In combination with methods of chemical reporters, cells that have been metabolically labeled with azido- or alkynyl-fatty acids can be lysed, biotinylated, enriched by streptavidin agarose beads, on-bead cleaved by proteases, and finally analyzed by LC–MS/MS proteomic analysis. By using this approach, many new candidate fatty acylated proteins have been identified with high confidence. It has been demonstrated that protein fatty acylation is heterogenuos in eukaryotic cells. Even only ODYA (alk-16) was used to target S-palmitoylated proteins, several N-myristoylated proteins have been retrieved [63].

The Acyl-Biotinyl Exchange (ABE) method has also been combined with mass spectrometry-based proteomic methods to explore the diverse S-acylation of proteins (shown in Fig. 6(A)) [56,99,103]. Cell lysates were treated by NEM (N-ethyl maleimide) or MMTS (methylmethanethiosulfonate) in order to block the free thiols. Then hydroxylamine was used to cleave thioester bonds of S-palmitoylated proteins. The newly exposed thiol groups were ligated with a cleavable thiol specific biotinylation reagent such as HPDP-biotin [56,99] or thiol-reactive ICAT [103] followed by trypsin digestion. S-palmitoylated peptides can be enriched by

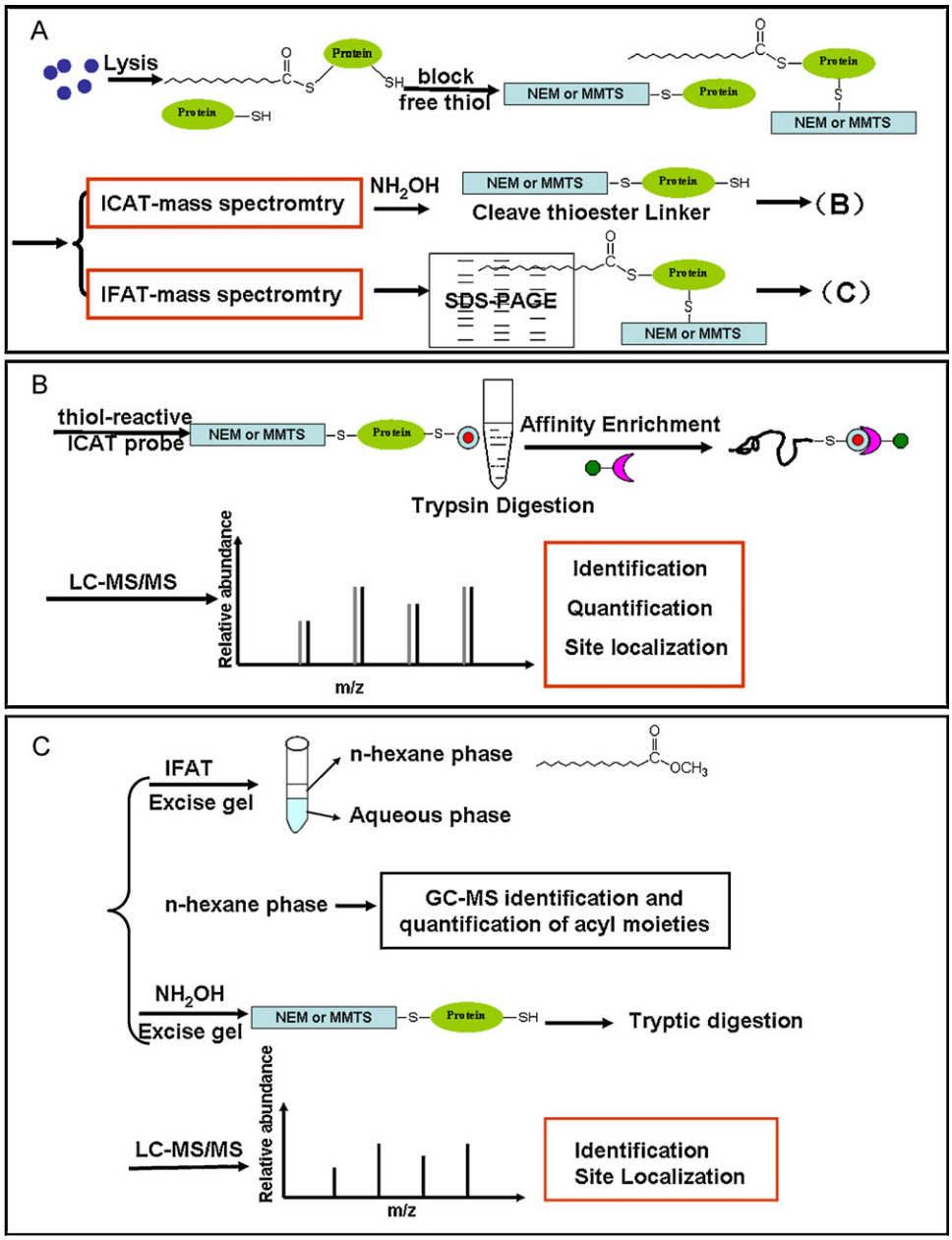

Fig. 6. Mass spectrometry based proteomic approaches for analysis of protein palmitoylation. (A) Free thiols were blocked by NEM or MMTS. NEM: N-ethyl maleimide; MMTS: methylmethanethiosulfonate. (B) ICAT approach for identification and quantification of palmitoylated proteins as well as localization of amino acid residues that are covalently bonded with fatty acyl moieties. (C) iFAT approach in combination with mass spectrometry for identification and quantitation of fatty acyl moieties, identification of palmitoylated proteins and localization of amino acids that are covalently bonded with fatty acyl moieties.

streptavidin and analyzed by LC–MS/MS. Interpretation of the masses of molecular ions and fragment ions reveals the identity of palmitoylated proteins and the specific amino acid residues that are covalently bonded with the palmitate. Quantitative comparison of palmitoylated proteins in different cell states can be achieved by the intensity ratios of light and heavy ICAT reagent derived molecular ions and fragment ions [103] (Fig. 6(B)). As shown in Fig. 6(C), we developed an alternative way for global profiling of protein palmitoylation (Hongying Zhong et al., unpublished data). After cell lysates were treated with NEM or MMTS, proteins were separated by SDS-PAGE and visualized by dye staining. Interested bands were excised and subjected to in-gel alkaline-catalyzed transmethylation and stable isotope labeling by using d0- or d3-methanol with the aid of ultrasonication. Resultant d0- or d3-derived fatty acid methyl esters were analyzed by GC–MS. The original amounts of palmitoylated proteins in different cell states can be recovered from the ratios of differentially labeled fatty acid methyl esters. Identity of acyl moieties can be achieved by database searching and comparison of chromatographic retention time with that of standard fatty acid methyl esters [71]. In parallel, interested gel bands can be tryptically digested and identified by MS/MS spectra. Another mass spectrometric method has been developed recently in which the spectral count was used for protein quantification. It provides a label free approach for proteome scale enrichment, protein identification and site characterization [104].

Mass spectrometry-based proteomic approaches have provided efficient means for large scale profiling of palmitoylated proteins. Radioactive labeling methods or genetic tags can only detect or image palmitoylated proteins. With the aid of mass spectrometry, it is possible to resolve protein palmitoylation at the level

of amino acid sequencing. Not only proteins but also acyl moieties can be qualitatively and quantitatively identified, even for unknown modifications. Thus, it is possible to explore the diverse protein acylations beyond palmitoylation [105,106]. In fact, development of these new proteomic and imaging methods has revealed the important roles of protein palmitoylation in various aspects of biology and biomedical fields such as the mechanism of innate or adaptive immunity to microbes [107], neuronal development and synaptic plasticity [53], as well as spatially organizing for peripheral membrane proteins [108]. Further, the emerging ability of mass spectrometry to perform tissue imaging [109–111] provides a perspective tool for dynamic monitoring of complex palmtoylation.

4. Conclusions

Protein palmitoylation is unique among the diverse posttranslational lipid modifications because of its reversibility and the special roles in protein trafficking and targeting. There is continuously increasing interest in protein palmitoylation. More and more experimental evidences have shown that protein palmitoylation is closely related with cancer and other diseases of public health importance. Accurate understanding of the mechanism and regulation of protein palmitoylation or depalmitoylation highly depends on the methods we used to test our hypothesis. The continued development of methods with improved sensitivity, selectivity, accuracy and throughput should push the present studies forward. There are major three important issues needed to be continuously addressed in the future: (1) due to the structural diversity of fatty acids, identifications of new or unknown palmitoylation remains challenging. So far, either *in vivo* metabolic labeling followed by bioorthogonal ligation, or genetic GFP tagging is hypothesis-directed approaches in which known probes were used to demonstrate the unknown functions. And it has also been assumed that chemical reporters or GFP tags have no effects on the native activities of modified proteins. *In vitro* Acyl-Biotinyl Exchange (ABE) can provide useful insight into the S-palmitoylation based on labile thioester bonds. But N-linkage or even other unknown linkages are stable and resistant to hydroxylamine treatment. In such situation, acyl moieties cannot be released. (2) Protein palmitoylation is often found to modify transmembrane proteins that are very hydrophobic and low abundant. The attachment of long chain fatty acids further dramatically increases the hydrophobicity. Additionally, unlike phosphorylation and glycosylation, there is a lack of functional groups for affinity enrichment. Therefore it is difficult to use present methods that are useful for soluble proteins to tackle with palmitoylated proteins. (3) Palmitoylation distinguishes with other lipid modifications because it is reversible and thus regulable. The dynamic cycling of palmitoylation and depalmitoylation states of proteins directly determines the trafficking and targeting of proteins to specific cellular sites. Thus the development of new methods with high spatial-temporal resolution is critical to such venture. Chemical probes or genetic probes in combination with quantitative microscopy have been widely used to visualize the distribution and dynamics of palmitoylated proteins. They are effective but require either metabolic labeling or gene manipulation. The emerging approach of mass spectrometry imaging provides a label-free *in vitro* method that can directly work on the cells or tissues.

Collectively, protein palmitoylation and depalmitoylation are critical mechanisms that regulate protein trafficking and activities in cells and tissues. The aberrant regulation of protein palmitoylation or depalmitoylation has been implicated in disease progression. Development of accurate methods that can identify the palmitoylated proteins and acyl moieties, localize the amino acid residues on which the palmitoylate are attached, monitor the cellular distribution as well as quantify the changes in the level of palmitoylation is the key to explore the diverse kingdom of protein palmitoylation.

Acknowledgments

Authors greatly appreciate the support from CCNU, HBNSFC(2009CDA001), PCSIRT(No. IRT0953) and Research Platform of Hubei Province for Monitoring of Pesticide Residues and Agricultural Products Safety.

References

[1] M.D. Resh, Biochim. Biophys. Acta 1451 (1999) 1.
[2] P.J. Casey, Science 268 (1995) 221.
[3] J.C. Demar, R.E. Anderson, J. Biol. Chem. 272 (1997) 31362.
[4] H. Hallak, L. Muszbek, M. Laposata, E. Belmonte, L.F. Brass, D.R. Manning, J. Biol. Chem. 269 (1994) 4713.
[5] O.A. Bizzozero, J.F. McGarry, M.B. Lees, J. Biol. Chem. 262 (1987) 2138.
[6] T. Fujimoto, E. Stroud, R.E. Whatley, S.M. Prescott, L. Muszbek, M. Laposata, R.P. McEver, J. Biol. Chem. 268 (1993) 11394.
[7] F.Y. Zeng, B.S. Kaphalia, G.A.S. Ansari, P.H. Weigel, J. Biol. Chem. 270 (1995) 21382.
[8] M. Laposata, L. Muszbek, Lipids 31 (1996) S217.
[9] M.J. Bijlmakers, M. Marsh, Trends Cell Biol. 13 (2003) 32.
[10] M.E. Linder, R.J. Deschenes, Nat. Rev. Mol. Cell Biol. 8 (2007) 74.
[11] O. Rocks, A. Peyker, M. Kahms, P.J. Verveer, C. Koerner, M. Lumbierres, J. Kuhlmann, H. Waldmann, A. Wittinghofer, P.I.H. Bastiaens, Science 307 (2005) 1746.
[12] N. LeBrasseur, J. Cell Biol. 168 (2005) 850.
[13] S. Baekkeskov, J. Kanaani, Mol. Membr. Biol. 26 (2009) 42.
[14] M.B. Dalva, J. Cell Biol. 186 (2009) 7.
[15] D.A. Brown, E. London, Annu. Rev. Cell Dev. Biol. 14 (1998) 111.
[16] D.A. Zacharias, J.D. Violin, A.C. Newton, R.Y. Tsien, Science 296 (2002) 913.
[17] D. Lingwood, K. Simons, Science 327 (2010) 46.
[18] W. Yang, D.D. Vizio, M. Kirchner, H. Steen, M.R. Freeman, Mol. Cell. Proteomics 9 (2010) 54.
[19] A. Arcaro, C. Gregoire, N. Boucheron, S. Stotz, E. Palmer, B. Malissen, I.F. Luescher, J. Immunol. 165 (2000) 2068.
[20] C. Bélanger, H. Ansanay, R. Qanbar, M. Bouvier, FEBS Lett. 499 (2001) 59.
[21] X. Yang, O.V. Kovalenko, T.V. Kolesnikova, M.M. Andzelm, E. Rubinstein, J.L. Strominger, M.E. Hemler, J. Biol. Chem. 281 (2006) 12976.
[22] X. Yang, C. Claas, S.K. Kraeft, L.B. Chen, Z. Wang, J.A. Kreidberg, M.E. Hemler, Mol. Biol. Cell 13 (2002) 767.
[23] D.J. Dietzen, W.R. Hastings, D.M. Lublin, J. Biol. Chem. 270 (1995) 6838.
[24] R. Qanbar, M. Bouvier, Pharmacol. Ther. 97 (2003) 1.
[25] A. Schweizer, S. Kornfeld, J. Rohrer, J. Cell Biol. 132 (1996) 577.
[26] C. Yang, C.P. Spies, R.W. Compans, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 9871.
[27] C. Blanpain, V. Wittamer, J.M. Vanderwinden, A. Boom, B. Rennboog, B. Lee, E.L. Poul, L.E. Asmar, C. Govaerts, G. Vassart, R.W. Doms, M. Parmentier, J. Biol. Chem. 276 (2001) 23795.
[28] S. Takida, P.B. Wedegaertner, FEBS Lett. 567 (2004) 209.
[29] S. Bonatti, G. Migliacclo, K. Simons, J. Biol. Chem. 264 (1989) 12590.
[30] H. Sprong, P. van der Sluijs, G. Van Meer, Nat. Rev. Mol. Cell Biol. 2 (2001) 504.
[31] T. Magee, M.C. Seabra, Curr. Opin. Cell Biol. 17 (2005) 190.
[32] P.S. Kabouridis, A.I. Magee, S.C. Ley, EMBO J. 16 (1995) 4983.
[33] L.K. Yurchak, B.M. Sefton, Mol. Cell. Biol. 15 (1995) 6914.
[34] I. Sato, Y. Obata, K. Kasahara, Y. Nakayama, Y. Fukamoto, T. Yamasaki, K.K. Yokoyama, T. Saito, N. Yamagucchi, J. Cell Sci. 122 (2009) 965.
[35] M.Y. Degtyarev, A.M. Spiegel, T.L. Jones, J. Biol. Chem. 269 (1994) 30898.
[36] J. Morales, C.S. Fishburn, P.T. Wilson, H.R. Bourne, Mol. Biol. Cell 9 (1998) 1.
[37] P. Tsutsumi, Y. Fukata, J. Nontake, J. Iwanaga, F. Perez, M. Fukata, Mol. Cell. Biol. 29 (2009) 435.
[38] A.M. Shenoy-Scaria, D.J. Dietzen, J. Kwong, D.C. Link, D.M. Lublin, J. Cell Biol. 126 (1994) 353.
[39] S.M. Robbins, N.A. Quintrell, J.M. Bishop, Mol. Cell. Biol. 15 (1995) 3507.
[40] J. Greaves, G.R. Prescott, O.A. Gorleka, L.H. Chamberlain, Mol. Membr. Biol. 26 (2009) 67.
[41] J. Liu, T.E. Hughes, W.C. Sessa, J. Cell Biol. 137 (1997) 1525.
[42] E. Choy, V.K. Chiu, J. Silletti, M. Feoktistov, T. Morimoto, D. Michaelson, I.E. Ivanov, M.R. Phillips, Cell 98 (1999) 69.
[43] A. Apolloni, I.A. Prior, M. Lindsay, R.G. Parton, J.F. Hancock, Mol. Cell. Biol. 20 (2000) 2475.
[44] J.F. Hancock, Nat. Rev. Mol. Cell Biol. 4 (2003) 373.
[45] L.P. Wright, M.R. Philips, J. Lipid Res. 47 (2006) 883.
[46] Y.I. Henis, J.F. Hancock, I.A. Prior, Mol. Membr. Biol. 26 (2009) 80.
[47] J.H. Skene, I. Virag, J. Cell Biol. 108 (1989) 613.
[48] M. Solimena, R. Dirkx Jr., M. Radzynski, O. Mundigl, P. De Camilli, J. Cell Biol. 126 (1994) 331.
[49] J. Greaves, G.K. Prescott, O.A. Gorleku, L.H. Chamberlain, Biochem. Soc. Trans. 38 (2010) 163.
[50] J.A. Buglino, M.D. Resh, J. Biol. Chem. 283 (2008) 22076.

[51] C. Kleuss, E. Kranse, EMBO J. 22 (2003) 826.
[52] T. Iwanaga, R. Tsutsumi, J. Noritake, Y. Fukata, M. Fukata, Prog. Lipid Res. 48 (2009) 117.
[53] Y. Fukata, M. Fukata, Nat. Rev. Neurosci. 11 (2010) 161.
[54] E.W. Tate, J. Chem. Biol. 1 (2008) 17.
[55] S. Miersch, B. Mutus, Curr. Anal. Chem. 3 (2007) 81.
[56] A.F. Roth, J. Wan, A.O. Bailey, B. Sun, J.A. Kuchar, W.N. Green, B.S. Phinney, J.R. Yates III, N.G. Davis, Cell 125 (2006) 1003.
[57] H. Zhong, S.L. Marcus, L. Li, J. Am. Soc. Mass Spectrom. 16 (2005) 471.
[58] C.C. Wu, M.J. MacCoss, K.E. Howell, J.R. Yates III, Nat. Biotech. 21 (2003) 532.
[59] T.E. Thingholm, T.J.D. Jorgensey, O.N. Jensen, M.R. Larsen, Nat. Protoc. 1 (2006) 1929.
[60] L.J. Holt, B.B. Tuch, J. Villen, A.D. Johnson, S.P. Gygi, D.O. Morgan, Science 325 (2009) 1682.
[61] H. Kaji, Y. Yamauchi, N. Takahashi, T. Isobe, Nat. Protoc. 1 (2007) 3019.
[62] B. Wollscheid, D. Bausch-Fluck, C. Henderson, R. O'Brien, M. Bibel, R. Schiess, R. Aebersold, J.D. Watts, Nat. Biotech. 27 (2007) 378.
[63] B.R. Martin, B.F. Cravatt, Nat. Methods 6 (2009) 135.
[64] M. Veit, H. Reverey, M.F.G. Schmidt, Biochem. J. 318 (1996) 163.
[65] L.V. Kordyukova, M.V. Serebryakova, L.A. Baratova, M. Veit, J. Virol. 82 (2008) 9288.
[66] L.V. Kordyukova, M.V. Serebryakova, L.A. Baratova, M. Veit, Virology 398 (2010) 49.
[67] F.E. Callahan, H.A. Norman, T. Srinath, J.B. St. John, R. Dhar, A.K. Mattoo, Anal. Biochem. 183 (1989) 220.
[68] R.C. Drisdel, J.K. Alexander, A. Sayeed, W.N. Green, Methods 40 (2006) 127.
[69] M.D. Resh, Methods 40 (2006) 191.
[70] S.M. Peseckis, I. Deichaite, M.D. Resh, J. Biol. Chem. 268 (1993) 5107.
[71] J. Li, Y. Yue, X. Hu, H. Zhong, Anal. Chem. 81 (2009) 5080.
[72] J. Li, Y. Yue, T. Li, X. Hu, H. Zhong, Anal. Chim. Acta 650 (2009) 221.
[73] N. Sorek, S. Yalovsky, Nat. Protoc. 5 (2010) 842.
[74] R.C. Drisdel, W.N. Green, Biotechniques 36 (2004) 276.
[75] J. Wan, A.F. Roth, A.O. Bailey, N.G. Davis, Nat. Protoc. 2 (2007) 1573.
[76] J.A. Prescher, C.R. Bertozzi, Nat. Chem. Biol. 1 (2005) 13.
[77] G. Charron, J. Wilson, H.C. Hang, Curr. Opin. Chem. Biol. 13 (2009) 382.
[78] C.R. Bertozzi, E.M. Sletten, Angew. Chem. Int. Ed. 48 (2009) 6974.
[79] H.C. Hang, E.J. Geutjes, G. Grotenbreg, A.M. Pollington, M.J. Bijlmakers, H.L. Ploegh, J. Am. Chem. Soc. 129 (2007) 2744.
[80] M.A. Kostiuk, M.M. Corvi, B.O. Keller, G. Plummer, J.A. Prescher, M.J. Hangauer, C.R. Bertozzi, G. Rajaiah, J.R. Falck, L.G. Berthiaume, FASEB J. 22 (2008) 721.
[81] G. CHarron, M.M. Zhang, J.S. Yount, J. Wilson, A.S. Raghavan, E. Shamir, H.C. Hang, J. Am. Chem. Soc. 131 (2009) 4967.
[82] M.A. Kostiuk, B.O. Keller, L.G. Berthiaume, Methods Enzymol. 457 (2009) 149.
[83] R.H. Hannoush, N. Arenas-Ramirez, ACS Chem. Biol. 4 (2009) 581.
[84] P. Cintas, A. Barge, S. Tagliapietra, C. Boffa, G. Cravotto, Nat. Protoc. 5 (2010) 607.
[85] M.C. Yap, M.A. Kostiuk, D.D.O. Martin, M.A. Perinpanayagam, P.C. Hak, A. Siddam, J.R. Majjigapu, G. Rajaiah, B.O. Keller, J.A. Prescher, P. Wu, C.R. Bertozzi, J.R. Falck, L.G. Berthiaume, J. Lipid Res. 51 (2010) 1566.
[86] M. Kohn, R. Breinbauer, Angew. Chem. Int. Ed. 43 (2004) 3106.
[87] H.C. Kolb, M.G. Finn, K.B. Sharpless, Angew. Chem. Int. Ed. 40 (2001) 2004.
[88] P.V. Chang, J.A. Prescher, E.M. Sletten, J.M. Baskin, I.A. Miller, N.J. Agard, A. Lo, C.R. Bertozzi, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 1821.
[89] S.J. Moench, D.H. Stewart, T.G. Dewey, Biochemistry 33 (1994) 5791.
[90] I. Mikic, S. Planey, J. Zhang, C. Ceballos, T. Seron, B. Von Massenbach, R. Watson, S. Callaway, P.M. McDonough, J.H. Price, E. Hunter, D. Zacharias, Methods Enzymol. 414 (2006) 150.
[91] M.M. Zhang, L.K. Tsou, G. Charron, A.S. Raghavan, H.C. Hang, Proc. Natl. Acad. Sci. U.S.A. (2010), doi:10.1073/pnas.0912306107.
[92] R.Y. Tsien, Ann. Rev. Biochem. 67 (1998) 509.
[93] B.N.G. Giepmans, S.R. Adams, M.H. Ellisman, R.Y. Tsien, Science 312 (2006) 217.
[94] S.G. Megason, S.E. Fraser, Cell 130 (2007) 784.
[95] T. Nakata, S. Terada, N. Hirokawa, J. Cell Biol. 140 (1998) 659.
[96] H. Kameda, T. Furuta, W. Matsuda, K. Ohira, K. Nakamura, H. Hioki, T. Kaneko, Neurosci. Res. 61 (2008) 79.
[97] A.K. Kenworthy, Methods 40 (2006) 198.
[98] J. Kanaani, G. Patterson, F. Schaufele, J. Lippincott-Schwartz, S. Baekkeskov, J. Cell Sci. 121 (2008) 437.
[99] R. Kang, J. Wan, P. Arstikaitis, H. Takahashi, K. Huang, A.O. Bailey, J.X. Thompson, A.F. Roth, R.C. Drisdel, R. Mastro, W.N. Green, J.R. Yates III, N.G. Davis, A. El-Husseini, Nature 456 (2008) 904.
[100] D. Vellucci, A. Kao, R.M. Kaake, S.D. Rychnovsky, L. Huang, J. Am. Soc. Mass Spectrom. (2010), doi:10.1016/j.jasms.2010.04.004.
[101] M. Mann, O.N. Jensen, Nat. Biotech. 21 (2003) 255.
[102] E.S. Witze, W.M. Old, K.A. Resing, N.G. Ahn, Nat. Methods 4 (2007) 798.
[103] J. Zhang, S.L. Planey, C. CEballos, S.M. Stevens Jr., S.K. Keay, D.A. Zacharias, Mol. Cell. Proteomics 7 (2008) 1378.
[104] W. Yang, V.D. Di, M. Kirchner, H. Steen, M.R. Freeman, Mol. Cell. Proteomics 9 (2010) 54.
[105] B. Martin, B. Cravatt, FASEB J. 24 (2010) 905.
[106] R.N. Hannousch, Nat. Chem. Biol. 6 (2010) 498.
[107] J.S. Yount, B. Moltedo, Y. Yang, G. Charron, T.M. Moran, C.B. Lopez, H.H. Hang, Nat. Chem. Biol. 6 (2010) 610.
[108] O. Rocks, M. Gerauer, N. Vartak, S. Koch, Z.P. Huang, M. Pechlivanis, J. Kuhlmann, L. Brunsveld, A. Chandra, B. Ellinger, H. Waldmann, P.I. Bastiaens, Cell 141 (2010) 458.
[109] M. Stoeckli, P. Chaurand, D.E. Hallahan, R.M. Caprioli, Nat. Med. 7 (2001) 493.
[110] D.S. Cornett, M.L. Reyzer, P. Chaurand, R.M. Caprioli, Nat. Methods 4 (2007) 828.
[111] L.A. McDonnell, R.M.A. Heeren, Mass Spectrom. Rev. 26 (2007) 606.