METHODS IN MOLECULAR BIOLOGY

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Site-Specific Protein Labeling

Methods and Protocols

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Cover Image Description: Confocal and STED microscopy image of methanol fixed U2OS cell expressing microtubule binding fusion protein SNAP-Cep41. SNAP-tagged protein is stained with cell permeable SiR-SNAP substrate before fixation.
Cover Courtesy: The image is courtesy of Gražvydas Lukinavičius and Kai Johnsson, École Polytechnique Fédérale de Lausanne

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Preface

Recombinant DNA technologies have revolutionized the way biologists study and manipulate proteins. The ability to produce chimeric proteins by inserting a peptide sequence before, after, or within a protein through genetic manipulation has led to the development of a multitude of techniques that render a protein of interest unique merely by adding an encoded label. Prominent examples are the introduction of small epitopes for immunolabeling, the use of affinity tags for protein purification, and the fusion to fluorescent proteins for imaging. The power of those approaches lies in the simplicity and absolute specificity of genetic encoding. However, the genetically encodable tags are a priori limited by the 20 proteogenic amino acids, which cover a very limited part of the chemical space.

This limitation is overcome by techniques that allow the covalent functionalization of a protein of interest with a synthetic probe, which includes fluorescent dyes, radiolabels, chemical cross-linkers, photoactivatable molecules, pharmacologically active compounds, toxins, synthetic biosensors, or nanoparticles [1, 2]. The application of such artificial synthetic objects in living cells or living organisms opens new avenues for studying and manipulating protein function in living systems. The issue of labeling specificity becomes critical for labeling in situ in a physiological context or in the cases where well-defined chemically modified biomolecules are desired. Classical reactive labeling techniques, however, are usually not selective enough for this purpose. This problem has been overcome over the last 15 years based on the pioneering work of Roger Y. Tsien and his group, and today various covalent labeling techniques are available that are perfectly site-specific and can be applied in the context of cells and organisms.

Today, the field as a whole is at an exciting stage: while some site-specific labeling approaches are now fully mature and well adopted by the molecular and cell biology community, new approaches and ingenious ways of applying existing approaches continue to emerge. The creative application of site-specific protein labeling techniques in cell biology beyond simple fluorescent labeling requires both a biologist's knowledge of biological problems and an organic chemist's understanding of the opportunities and problems involved in generating a custom label for the problem in question. *Methods of Site-Specific Protein Labeling* is directed at scientists from all fields that want to get a better understanding of labeling techniques. In particular, it aims at providing researchers interested in such techniques with advice on how to choose the most appropriate labeling method for their biological question and information on general considerations and problems involved in the design, the generation, and the application of the corresponding organic molecules used for the labeling step.

The first chapters deal with the background and basic considerations of site-specific protein labeling. As often, the historical perspective is insightful: In Chapter 1, B. Albert Griffin, Stephen R. Adams, and Roger Y. Tsien provide a highly interesting recollection of why and how they came to invent the FlAsH-tag. Chapter 2, written from the industrial perspective by Lukas Leder from Novartis, provides an overview of applications of labeled proteins in assays that are common in the industry, and Lukas Leder shares experiences that his laboratory made with adopting site-specific protein labeling. Chapter 3 was motivated

by a recurring issue in the site-specific labeling of intracellular proteins: whether the compound used for labeling can at all cross the cell membrane in sufficient amounts to enable intracellular reaction. A lack of permeability can render the most creative labeling molecule useless, which can be painful if it is realized only after the synthesis has been performed. The chapter, written by Nicole Yang and Marlon J. Hinner, provides a comprehensive overview of the factors that govern membrane translocation not only for small molecules and peptides but also for proteins. As the last of the overview articles, Chapter 4 by Ivan Correa provides a broad overview of general considerations for the design of labeling molecules, exemplified by SNAP-tag and CLIP-tag technology. His chapter includes a number of protocols that should be of high interest for chemists and nonchemists alike.

The chapters that follow cover the most relevant methods of site-specific protein labeling with selected applications. The techniques described include tag-based methods (which can be further subdivided), methods that rely on the incorporation of unnatural amino acids during protein translation, and methods that work specifically on native, untagged proteins.

In tag-based methods, a protein of interest is fused to a peptide sequence that acts as a genetic anchor for the attachment of the probe. This peptide sequence can contain just a few residues or be a full protein. Depending on the size of the tag and whether the tag requires an added enzyme to be linked to the label of interest, tag-based methods can be grouped into self-labeling tags, self-labeling proteins, and enzyme-mediated labeling of tags [2].

Developed by Roger Y. Tsien and coworkers, the archetype of a self-labeling tag is the tetracysteine tag which can specifically react with biarsenical compounds [3]. A recently developed self-labeling tag is described in the contribution of Lina Cui and Jianghong Rao (Chapter 5), which presents how a single terminal cysteine can be exploited for site-specific labeling with cyanobenzothiazole derivatives. The contribution of Thomas K. Berger and Ehud Y. Isacoff (Chapter 6) demonstrates additionally how well positioned cysteines within a cell-membrane receptor can be functionalized with thiol-linked environment-sensitive dyes to measure protein motion in ion channels in real time.

Relying on an uncatalyzed chemical reaction can limit the kinetics of the labeling step, and using short peptides as a recognition sequence may also lead to a less-than-perfect selectivity of labeling. These limits can be overcome with self-labeling protein tags that rely on a rapid and selective, catalyzed labeling reaction. The contributions from Gražvydas Lukinavičius, Luc Reymond, and Kai Johnsson (Chapter 7) and from Hélène A. Benink and Marjeta Urh (Chapter 8) describe aspects of two self-labeling proteins that are commercially available, SNAP-tag and HaloTag. Lukinavičius et al. show in particular how the SNAP-tag technology can be exploited in the context of super-resolution microscopy. Split inteins are another example for a catalyzed reaction that can be exploited for site-specific protein labeling. In two chapters from the group of Henning Mootz, Julian Matern et al. (Chapter 9) and Anne-Lena Bachmann et al. (Chapter 10) present two different approaches that exploit split inteins for attaching a small peptide functionalized with a chemical probe to a protein of interest.

The size of the added tag sometimes being a concern, strategies combining the small size of a short peptide sequence with the speed and high specificity of protein-catalyzed labeling have also been designed. In these methods, the labeling reaction is trimolecular and involves a transferase enzyme, the molecule used for labeling, and the recognition (acceptor) peptide sequence. Here, the transferase enzyme can be added in medium or needs to be coexpressed if intracellular labeling is required. The enzyme-mediated labeling of tags is described for Sfp-mediated labeling—applied in phage display—by Bo Zhao et al. (Chapter 11), for BirA-mediated labeling by Michael Fairhead and Mark Howarth (Chapter 12), and for Sortase-mediated labeling by Max Popp (Chapter 13).

Fusing a peptide or protein tag to the protein of interest is not required in techniques relying on unnatural amino acid incorporation during protein synthesis. The inserted unnatural amino acid plays the role of the molecular anchor in this case. Since the size of the side chain of the unnatural amino acid can be limited by the cell's protein translation machinery, often a small chemical functionality is introduced to which a chemical probe can be tethered in a second step using various bioorthogonal chemical "click" reactions. Using this methodology, the contribution of Peter Landgraf, Elmer R. Antileo, Erin M. Schuman, and Daniela C. Dieterich (Chapter 14) illustrates how metabolic labeling can be used to mark newly synthesized proteomes. The contribution of Kathrin Lang, Lloyd Davis, and Jason W. Chin (Chapter 15) describes the recent development of methods to fully genetically encode these unnatural "anchor" amino acids in order to be able to selectively label a single protein at a specific residue in living mammalian cells.

The "Holy Grail" in protein labeling is to be able to specifically target any native, non-tagged protein with a chemical probe in a physiological context. The two final chapters are reserved for this topic and are written by Itaru Hamachi with coworkers Tomonori Tamura (Chapter 16) and Shinya Tsukiji (Chapter 17), respectively. They describe two related approaches to how native protein labeling can be achieved by relying on labeling probes made of three parts, (1) a recognition moiety, binding selectively to the native protein of interest, (2) the probe to be attached, and (3) a reactive group, which can react with nucleophilic residues on the protein surface. While this reactive group is in principle capable of labeling any protein in a mixture, selectivity is achieved due to close proximity of the reactive group to the protein of interest, enforced by the recognition moiety.

In putting together this edition, we have attempted to include what we perceive as the currently most relevant and best established labeling methods across the different general methodologies. A number of important techniques are not presented, however, because detailed reviews and protocols have been recently published elsewhere. This includes the tetracysteine tag [3], lipoic acid-mediated labeling [4], labeling based on the genetically encoded aldehyde tag [5], and transglutaminase-based labeling [6]. While we have not attempted to include examples for every possible application of site-specific protein labeling, the chapters are nonetheless designed to provide guidance on the limits and possibilities of each technique and references to applications that have been described in the literature. For more information on applications and a comparative analysis of the various techniques, as well as introductions to other labeling methods not included here, we invite the readers to consult recent reviews on site-specific labeling [1, 2].

Finally, we thank all the authors that have contributed to this edition of *Methods in Molecular Biology*. We hope that both authors and readers will find this compendium useful and that it will support the further development of creative ideas in the field and facilitate making site-specific protein labeling a standard, widely used lab technique.

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