Simultaneous Protein Tagging in Two Colors

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DOI 10.1016/j.chembiol.2008.02.004

The fluorescent tagging of proteins in the natural environment of the cell is an emerging technique in cell biology. In this issue of *Chemistry & Biology*, Gautier et al. introduce a fluorescent labeling procedure orthogonal to existing ones, enabling tagging of two different proteins in living cells.

Fluorescence imaging is an important tool for answering questions regarding the behavior and fate of molecules in living cells. Accordingly, fluorescently labeled molecules, in particular fluorescently labeled proteins, are now among the most used tools in cell biology. Labeled proteins can be prepared by chemically attaching a small-size fluorophore via standard protein chemistry. This procedure usually requires microinjection of the labeled protein. Generally more applicable is the fusion of an intrinsically fluorescent protein at the DNA level, resulting in expression of a fluorescently tagged protein by the cell of interest. A third approach, called in vivo labeling, is based on engineering the protein of interest in a way that allows for specific chemical labeling inside living cells. The latter is achieved by adding a small tagging motif to the protein. The only available example with little change to the molecule size so far is based on the specific recognition of a tetracysteine motif by biarsenic fluorophore derivatives (Martin et al., 2005). Alternatively, a larger protein domain might be added that exhibits the specific chemical reactivity needed for the labeling procedure. The first example of this type was published by the Kai Johnsson group and involves the fusion of a self-alkylating protein derived from the DNA repair protein alkylguanine-DNA alkyltransferase (AGT) and its membrane-permeant fluorescent substrate (Figure 1) (Keppler et al., 2003). This principle has in the meantime been extended to a variety of self-labeling protein fusions including *E. coli* dihydrofolate reductase (Calloway et al., 2007). The procedures work well, as even the addition of larger protein entities in the range of 30 kDa has in general surprisingly little effect on protein performance in living cells. The advantage of labeling proteins by small fluorescent entities is that the coupling reaction may be induced at any time during the course of an experiment. This opens up the possibility, for instance, for pulse-chase experiments to track proteins in their spatial distribution or to determine the lifetime of proteins. Lately, photoactivatable and photoswitchable fluorescent proteins have been introduced to perform similar experiments (Lippincott-Schwartz et al., 2003; Lukyanov et al., 2005).

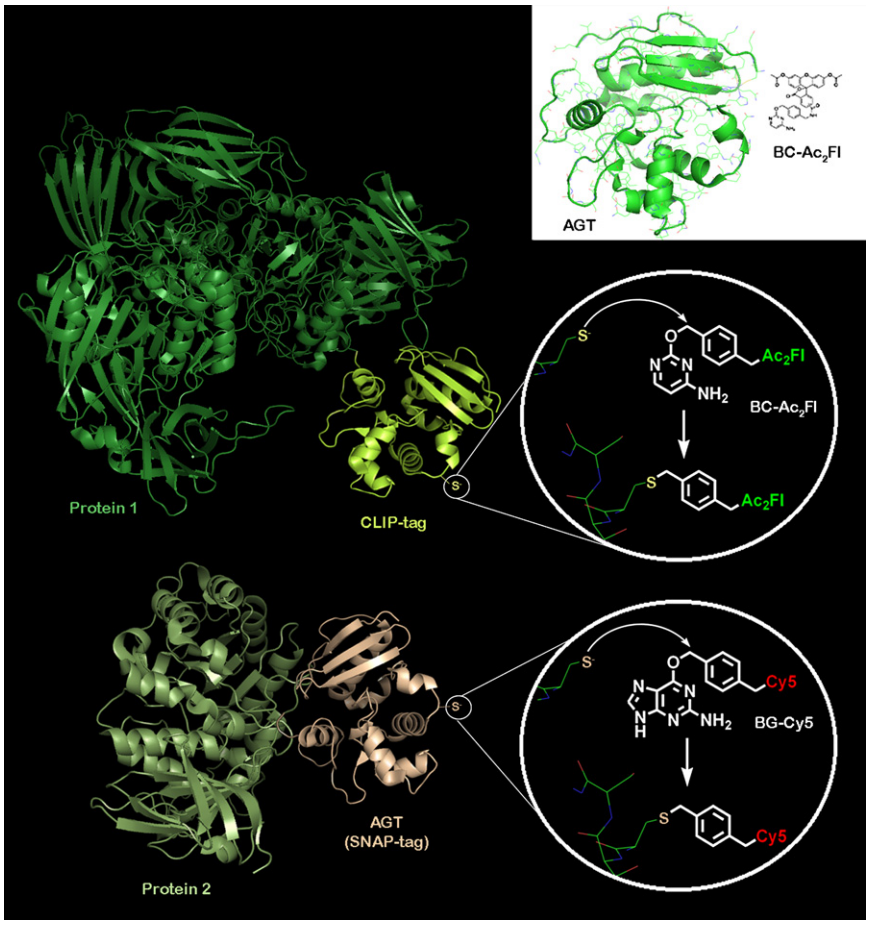

Figure 1. CLIP and SNAP Tags: Sizes and Mechanisms
Structure of AGT (PDB code: 1EH6) based CLIP and SNAP tag fusions to the putative target proteins β- (top; PDB code: 1JZ7) and α-galactosidase (bottom; PDB code: 1SZN), respectively. The upper construct was used by Gautier et al. (Gautier et al., 2008); the lower is fictive. The relative sizes of the fusion partners are roughly shown to scale, as is the AGT and substrate (insert). The dye is covalently attached via the transfer of a benzyl group to a cysteine of the alkyltransferase. Note that the location of the AGT cysteines is chosen randomly. Ac_2Fl (or DF) is a membrane-permeant variety of fluorescein that becomes green fluorescent after enzymatic acetate cleavage. Cy5 is a red fluorescent cyanine dye.

All these tools are instrumental in increasing our knowledge of dynamic molecule behavior in cells. Fluorophores permit observation by a microscope and therefore experiments in single cells. However, often there is large cell-to-cell variability observed and the timing and intensity of events may vary significantly, even in neighboring cells of the same dish. Therefore, it is very important to observe sets of events in each cell simultaneously to receive a concise data set for individual cells. This is also essential to eventually draw general conclusions of event progression in cell populations.

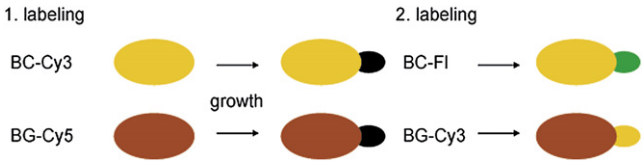

Figure 2. Differential Visualization of Protein Age
Simultaneous labeling of two yeast strains expressing either the original AGT fusion (SNAP tag) or the newly generated AGT variety (CLIP tag) demonstrated that the benzylguanine (BG) and the benzylcytosine (BC) linked labels can be selectively incorporated within one experiment, respectively. The membrane proteins Aga2p of the mother cells are stained with the first label. After a growth phase, the resulting buds are selective labeled with the second label offered, showing the different age of Aga2p and the destiny of the newly synthesized protein.

In this issue of *Chemistry & Biology*, the Johnsson group introduces a different labeling procedure orthogonal to the previously described AGT-based technique (Gautier et al., 2008). This technique makes use of the self-alkylating enzyme fused to the protein of interest for selective tagging of this protein (SNAP tagging). The SNAP-tag recognizes O^6-benzylguanine (BG) carrying a fluorophore probe and transfers the benzyl group to itself, thereby labeling the protein that it is fused to. The idea is to have a method sufficiently similar to AGT labeling that cell treatments can be performed with two tags under identical conditions to be able to directly compare imaging results of simultaneously tagged proteins. For successful probe application, it is obviously important to have high specificity but also similar reaction rates for the labeling procedure. Otherwise, the first protein of interest is already redistributing while the second protein is not yet sufficiently labeled.

The authors searched for a mutant of the self-alkylating protein AGT that would selectively accept a substrate other than guanine (Figure 1). As a nucleobase substrate, they chose O^6-benzylcytosine (BC), which is sufficiently similar to BG. BC derivatives were not substrates for the original AGT, thus exhibiting the necessary specificity for orthogonal labeling. For generating the new self-labeling fusion protein, seven critical residues were randomly exchanged. Directed evolution using three iterative rounds of mutagenesis and fluorescence-activated cell sorting of yeast cells was employed to detect self-labeled surface proteins. The eventually selected clone showed a 100-fold preference for BC but a low reaction rate. Error-prone PCR was employed to introduce further mutations, and improved mutants were selected using phage display. The fastest reacting mutant, named CLIP-tag, showed increased reactivity toward BC while retaining stability and specificity. Finally, double in vivo labeling was demonstrated by using both AGT-based tags simultaneously on various mammalian targets.

The number of potential applications for double in vivo labeling is quite large. Most obviously, protein localizations of two species can be followed simultaneously, as presented by the authors; the two tags were expressed with a terminal farnesylation motif, fused to a globular cytosolic protein and with a nuclear location sequence (NLS), respectively. Double pulse-chase experiments were performed in yeast, demonstrating that the orthogonal labeling segregates old from new proteins as was evident during bud formation (Figure 2). It will be exiting to apply this technique to complex and not fully understood events, such as the internalization, recycling, and digestion of different growth factor receptor isoforms. Orthogonal pulse-chase labeling might show differences in receptor turn-over and transport behavior. Time-dependent expression profiles of different proteins in cancer cells could be used to better understand their roles in disease progression. Monitoring new production of different viral proteins during viral translation would be useful when looking for inhibition of viral translation in real-time experiments without the necessity of fixing and staining cells, thereby reducing the number of artifacts. In general, transport events and applications where the age of proteins is relevant will be among the most obvious applications. Also, the labeling of two proteins permits crosslinking of both by one ligand containing a benzylguanine and a benzylcytosine moiety. It will therefore be possible to determine relative copy numbers of the proteins of interest or to irreversibly translocate one of the proteins. Thereby, the technique presented here may be developed into a new "chemical dimerizer" strategy.

In the future, it will be interesting to find out whether additional in vivo labeling procedures such as the use of the tetracysteine tag or other novel methods will give rise to triple- or even quadruple-event experiments. This would increase the versatility of the approach and may give us a more complete picture of the timing of cellular events. It may also be desirable and feasible to eventually transfer in vivo labeling into living animals. Alkyl-nucleotide-coupled dyes are probably suitable for being administered to animals. With fluorescently tagged proteins, we are still waiting for the demonstration of FRET imaging between two different proteins in animals. With in vivo labeling, we might be able to make defined areas of cells visible, such as the apical membrane of epithelial cells, which would allow us to perform locally restricted FRET experiments with minimal background fluorescence from other areas.

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