Simultaneous Fluorescent Labeling of Proteins in Living Cells

Introduction

Specific labeling of proteins via self-labeling SNAP-tag® and CLIP-tag™ provides an innovative tool for studying the function and localization of proteins in live and fixed cells (1,2). Covalent protein labeling brings simplicity and versatility to the imaging of mammalian proteins in living cells, as well as the ability to capture proteins *in vitro*. The creation of a single genetic construct generates a fusion protein which, when covalently attached to a variety of fluorophores, biotin or beads, provides a powerful tool for investigating proteins via fluorescent imaging, pull-down, and other biochemical analyses. Unlike some commonly used autofluorescent proteins, the fluorescent signal from a self-labeling tag can be initiated upon addition of a label, allowing time-resolved studies of protein expression, localization and degradation. A collection of non-fluorescent substrates that block SNAP- and CLIP-tag reactivity enables pulse-chase studies and assessment of the temporal dynamics of nascent protein synthesis and complex formation in live cells (3).

General Protocol

1. Seed trypsinized U2OS cells in an 8-well Lab-Tek II Chambered Coverglass (Nalgene #155409).
2. Co-transfect the cells with 0.3 µg each of $SNAP_f$ and $CLIP_f$ fusion protein construct and incubate samples for 18-24 hours at 37°C, 5% CO_2.
3. Remove transfection complex media, wash cells twice with complete media, and label cells with 3 µM SNAP-Cell® TMR-Star (NEB #S9105S) and 5 µM CLIP-Cell™ 505 (NEB #S9217S) labeling media for 30 minutes at 37°C, 5% CO_2.
4. Remove the labeling media and add media containing 5 µM Hoechst 33342 to the cells for 2-3 minutes for nuclear counterstaining (optional).
5. Wash cells 3X with complete media then incubate the samples for 30 minutes at 37°C, 5% CO_2 to allow unincorporated substrate to diffuse out of the cells.
6. Replace media one last time and proceeded to imaging.

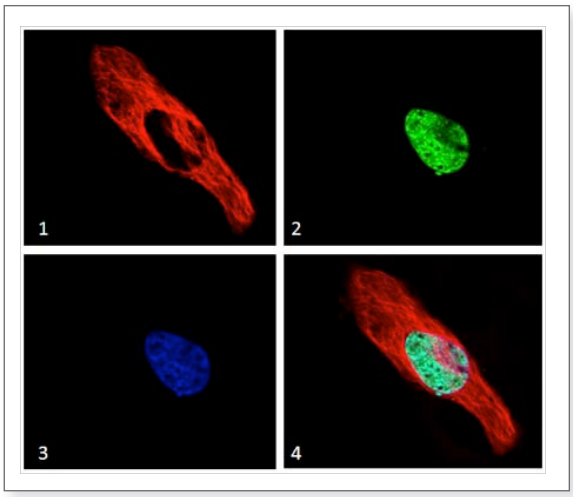

Figure 1. *Orthogonal labeling of $SNAP_f$-tubulin and $CLIP_f$-H2B fusion proteins transiently expressed in live U2OS cells. Cells were labeled with 3 µM SNAP-Cell TMR-Star (red) and 5 µM CLIP-Cell 505 (green) for 30 minutes and counterstained with Hoescht 33342 (blue) for nuclei.*

Materials

- $pCLIP_f$-H2B (NEB #N9218S)
- $pCLIP_f$-Cox8A (NEB #N9217S)
- $pSNAP_f$-tubulin (not a catalog product)
- CLIP-Cell™ 505 (NEB #S9217S)
- SNAP-Cell® TMR-Star (NEB #S9105S)
- U2OS or HeLa cells
- DMEM medium
- Hoechst 33342
- Lab-Tek II Chambered Coverglass (Nalgene)

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Results

$SNAP_f$ and $CLIP_f$ tags for Fluorescent Labeling

$SNAP_f$ and $CLIP_f$ are improved versions of SNAP- and CLIP-tags with increased reaction rates for their fluorescent substrates (4,5). Simultaneous dual labeling of $SNAP_f$ and $CLIP_f$ fusion proteins provides researchers with a unique tool to study proteins with rapid dynamics or fast turnover rates in living cells (3). Figure 1 shows the orthogonal labeling of two proteins in live cells: $SNAP_f$-Tubulin, (a dynamic protein that polymerizes into microtubules), and $CLIP_f$-H2B (histone H2B, a nuclear protein involved in chromatin structure). Figure 2 shows the orthogonal labeling in live HeLa cells transfected with p$SNAP_f$-tubulin and p$CLIP_f$-Cox8A (mitochondrial cytochrome oxidase 8A). The data clearly demonstrate specific labeling of the appropriate target proteins, confirming that $SNAP_f$ and $CLIP_f$ can be used for orthogonal protein labeling in living cells. The fluorescent labeling can be performed simultaneously or sequentially, depending on the experimental needs.

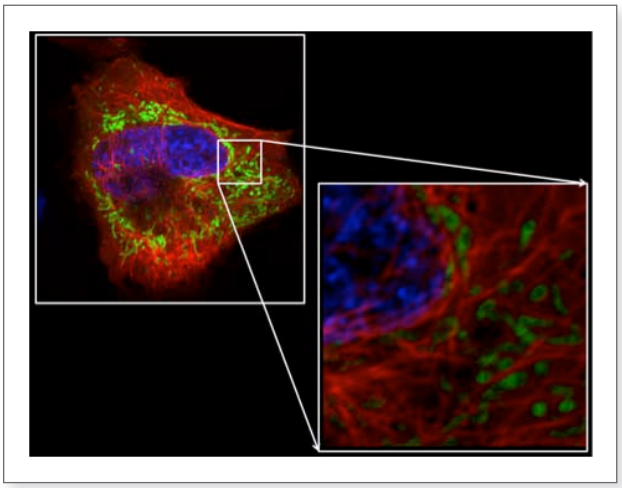

Figure 2. *Orthogonal labeling of $SNAP_f$-Tubulin and $CLIP_f$-Cox8A fusion proteins transiently expressed in live HeLa cells. Cells were labeled with 3 µM SNAP-Cell TMR-Star (red) and 5 µM CLIP-Cell 505 (green) for 30 minutes and counterstained with Hoescht 33342 (blue) for nuclei.*

Summary

Live cell imaging using fluorescent tags has been widely used by researchers to study protein expression and location. Its applications include assessing protein dynamics, cellular structures, and organogenesis.

$CLIP_f$- and $SNAP_f$-tag are valuable tools for the selective labeling of proteins in live cells. As both tags have been engineered to react with distinct classes of substrates, the system can simultaneously visualize two proteins in the same cellular environment. The dynamics of their interaction, localization and stability can be readily examined following a variety of cellular stimuli.

References:
1. Keppler, A. et al. (2003) *Nat. Biotechnol.* 21, 86–89.
2. Keppler, A. et al. (2004) *PNAS*, 101, 9955–9959.
3. Gautier, A. et al. (2009) *Chem. Biol.* 15, 128–136.
4. Sun, X. et al. (2011) *ChemBioChem*, 12, 2217–2226.
5. Pellett, P. A. et al. (2011) *Biomed. Opt. Expr.* 2, 2364–2371.

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Labeling and Imaging of Cell Surface Receptors Mediated by SNAP-tag

Introduction

Many plasma membrane receptors, such as G-protein coupled receptors (GPCR), play a vital role in cell signaling and are often the therapeutic targets for drug discovery. Fluorescent imaging of the receptor pool on the plasma membrane of live cells is made possible by extracellular expression of a SNAP-tag® fused to a receptor of interest and labeled with a cell membrane-impermeable substrate. The SNAP-tag enabled surface labeling technique provides a unique approach to monitoring localization, trafficking and turnover of a variety of cell membrane localized proteins (1-6).

The specific labeling and internalization of a GPCR protein, β2 Adrenergic Receptor (ADRβ2), is exemplified by using $SNAP_f$-tag as the fusion partner. To utilize this system, the coding region of ADRβ2 is first cloned in-frame to the C-terminus of the $pSNAP_f$ vector with a signal peptide coding sequence inserted upstream. The $SNAP_f$-ADRβ2 fusion protein is inserted in the plasma membrane with the $SNAP_f$-tag exposed to the extracellular side of the membrane. The following procedure is performed to visualize localization and internalization of $SNAP_f$-ADRβ2 using fluorescent microscopy after labeling with a SNAP-Surface fluorescent substrate. In addition, a comparative analysis of the $SNAP_f$-ADRβ2 and eGFP-ADRβ2 fusion constructs has been demonstrated.

General Protocol

1. Seed trypsinized cells in an 8-well Lab-Tek II Chambered Coverglass (Nalgene #155409).
2. Co-transfect the cells with 0.3 µg each of $SNAP_f$-ADRβ2 and eGFP-ADRβ2 fusion protein construct and incubate samples for 18-24 hours at 37°C, 5% CO_2.
3. Remove transfection complex media, wash cells twice with complete media, and label cells with 5 µM SNAP-Surface 549 (NEB #S9112S) labeling media for 30 minutes at 37°C, 5% CO_2.
4. Remove the labeling media and add media containing 5 µM Hoechst 33342 to the cells for 2-3 minutes for nuclear counterstaining (optional).
5. Wash cells 3X with complete media and proceeded to imaging.

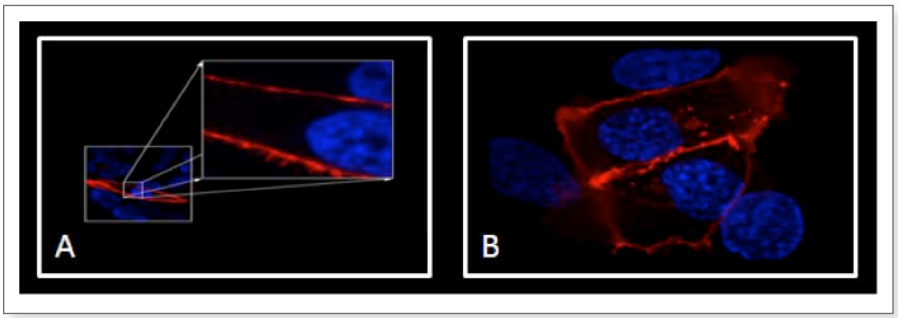

Figure 1. *Live cell imaging of surface localization and internalization of ADRβ2 receptor fused to $SNAP_f$-tag. A: Labeling of live U2OS cells transfected with $pSNAP_f$-ADRβ2 was carried out on ice for 15 minutes in the presence of 5 µM SNAP-Surface 549 (NEB #S9112S) followed by washing and imaging by a confocal fluorescence scanning microscope. B: Internalization of ADRβ2 was visualized by confocal microscopy after labeling live HEK293 cells transiently expressing $SNAP_f$-ADRβ2 with 1.7 µM of SNAP-Surface 549 (NEB #S9112S) for 15 minutes.*

Materials

- $pSNAP_f$-ADRβ2 (NEB #N9184S)
- peGFP-ADRβ2
- SNAP-Surface® 549 (NEB #S9112S)
- HEK293 or U2O cells
- DMEM medium
- Hoechst 33342
- Lab-Tek II Chambered Coverglass (Nalgene)

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Results

Localization and Internalization of Surface Receptors Fused to $SNAP_f$-tag

Live cell labeling of the $SNAP_f$-ADRβ2 pool on the plasma membrane was conducted using a cell membrane-impermeable substrate, SNAP-Surface 549 (NEB #S9112S). The fluorescent signals of the surface localized receptor population were visualized by confocal fluorescence scanning microscope. As shown in Figure 1, the receptor endocytosis in cells transfected with $SNAP_f$-ADRβ2 can be monitored without the background from the intracellular pool of the fusion proteins. Figure 2 shows fluorescent images of HEK293 cells transiently co-expressing eGFP-ADRβ2 and $SNAP_f$-ADRβ2. When labeled with a cell-impermeable SNAP-Surface substrate, HEK293 cells give a distinct cell membrane fluorescent labeling pattern. In contrast, eGFP-ADRβ2 yields fluorescent signals from both the cell surface and intracellular compartments. A higher intracellular background of eGFP-ADRβ2 makes it more difficult to specifically monitor the internalization of tagged receptor. The data demonstrate a major advantage of the SNAP-tag labeling technique over the use of auto-fluorescent protein tags for the study of receptor trafficking.

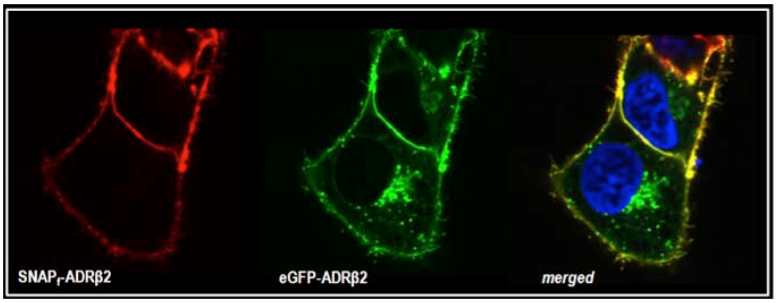

Figure 2. *Confocal microscopy of live HEK293 cells transiently co-expressing $SNAP_f$-ADRβ2 and eGFP-ADRβ2. Live cell labeling was carried out for 20 minutes on ice with 5 µM SNAP-Surface 549 (NEB #S9112S) and nuclei staining with Hoechst 33342.*

Summary

The use of SNAP-tag cell-impermeable substrates enables the fraction of a plasma membrane protein present on the surface of the cell to be visualized through specific labeling. This approach permits the discrimination of different populations of a cell surface protein: those properly translocated to the plasma membrane from those retained in the secretory pathway or already internalized, e.g., upon ligand binding. A wide range of selection of cell impermeable SNAP-Surface substrates offers excellent properties for the study of receptor localization, trafficking and protein-protein interactions. Our recent work together with the scientists at Yale University has further expanded the applications of the surface labeling approach by conducting live cell super-resolution microscopy of $SNAP_f$ tagged epidermal growth factor receptor and its ligand labeled with photostable organic dyes (5).

References:
1. Keppler, A. et al. (2003) *Nat. Biotechnol.* 21, 86–89.
2. Keppler, A. et al. (2004) *PNAS*, 101, 9955–9959.
3. Farr, G. A. et al. (2009) *J. Cell Biol.* 186, 269–282.
4. Sun, X. et al. (2011) *ChemBioChem*, 12, 2217–2226.
5. Pellett, P. A. et al. (2011) *Biomed. Opt. Expr.* 2, 2364–2371.
6. Maurel, D. et al. (2008) *Nat. Methods*, 5,561-567.

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Development of $SNAP_f$ and $CLIP_f$: Fast-labeling Variants of SNAP and CLIP tags

Introduction

The ability to monitor highly dynamic processes is integral to the understanding of how proteins function in a cellular environment. Processes such as protein synthesis, transport, and degradation can occur on a timescale measured in minutes rather than hours. Additionally, protein expression, localization, and degradation are often rapidly modulated during the course of a variety of cellular processes. In order to effectively study proteins with rapid dynamics or fast turnover rates, researchers need tools to monitor these events in real time.

Results

$SNAP_f$ and $CLIP_f$ tags Fast-labeling Systems

The SNAP- and CLIP-tag are self-labeling tags based on human O6-alkylguanine-DNA-alkyltransferase (hAGT), a DNA repair protein (1-3). New England Biolabs has developed improved versions of these tags, termed $SNAP_f$ and $CLIP_f$. $SNAP_f$ carries 19 amino acid substitutions and a C-terminal deletion compared to wild-type hAGT, and 10 extra mutations compared to SNAP26m (an earlier mutant version). The new polylinker sequences allow expression of $pSNAP_f$ vector (NEB #S9183S) on either the N- or C-terminus of the protein of interest, under control of the CMV promoter. The $CLIP_f$ expression vector $pCLIP_f$ (NEB #S9215S) bears the same backbone and compatible polylinkers. Both vectors contain a neomycin resistance (NeoR) gene for selection of stable transfectants and an IRES element for efficient expression of both the fusion protein and NeoR.

$SNAP_f$ and $CLIP_f$ exhibit faster labeling kinetics than their predecessors in both *in vitro* and in live cell assays (4,5). When comparing the reactivity of $SNAP_f$ and SNAP26m towards several fluorophore conjugates, $SNAP_f$ shows up to 10-fold increased *in vitro* activity relative to SNAP26m (Table 1). The time required for 50% labeling of $SNAP_f$ at 1 µM protein concentration with 5 µM SNAP-Surface 488 (NEB #S9124S), SNAP-Cell TMR-Star (NEB #S9105S), SNAP-Surface 549 (NEB #S9112S), and SNAP-Surface Alexa Fluor 647 (NEB #S9136S) was calculated to be 11, 12, 13, and 34 s, respectively. $SNAP_f$ reacts with a wide range of benzylguanine substrates with efficiencies greater than 90% after a 5 min incubation and greater than 95% and after a 30 min incubation (Table 2).

General Protocol

1. Seed trypsinized U2OS cells stably transfected with $pSNAP_f$-H2B (or pSNAP26m-H2B) in an 8-well Lab-Tek II Chambered Coverglass (Nalgene #155409).
2. Incubate sample for 18-24 hours at 37°C, 5% CO_2.
3. Remove transfection complex media, wash cells twice with complete media, and label cells with 3 µM SNAP-Cell® TMR-Star (NEB #S9105S) labeling media from 0 to 30 minutes at 37°C, 5% CO_2.
4. Lyse cells in 1X SDS loading buffer.
5. Analyze total protein from ~10^5 cells per lane by SDS-PAGE.
6. Transfer samples onto a NC membrane.
7. Scan and visualize using Typhoon 9400 Imager (Ex./Em. filter set: 532/580 nm).
8. Perform Western blot analysis with anti-Tubulin antibody (1:2000) to assess protein level.

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Materials

- $pSNAP_f$-H2B (NEB #N9186S)
- SNAP-Cell® TMR-Star (NEB #S9105S)
- U2OS cells
- DMEM medium
- Anti-Tubulin antibody
- Lab-Tek II Chambered Coverglass (Nalgene)

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Table 1. Kinetics properties of $SNAP_f$ and SNAP26m

SUBSTRATE	MUTANT	$T_{½}$[S]	K_{OBS} [$M^{-1}S^{-1}$]	REL. RATE
SNAP-Surface 488 (NEB #S9124S)	SNAP26m	88	1583	1
	$SNAP_f$	11	12183	7.6
SNAP-Cell 505 (NEB #S9103S)	SNAP26m	28	4945	1
	$SNAP_f$	11	12447	2.5
SNAP-Cell Oregon Green (NEB #S9104S)	SNAP26m	654	212	1
	$SNAP_f$	111	1249	5.9
SNAP-Cell TMR-Star (NEB #S9105S)	SNAP26m	90	1536	1
	$SNAP_f$	12	12033	7.8
SNAP-Surface 549 (NEB #S9112S)	SNAP26m	129	1074	1
	$SNAP_f$	13	11138	10.4
SNAP-Surface Alexa Fluor 647 (NEB #S9136S)	SNAP26m	219	634	1
	$SNAP_f$	34	4768	7.5

Activities of $SNAP_f$ and SNAP26m mutants were measured using proteins isolated with the IMPACT protein expression and purification system. The relative rates are calculated by comparing the k_{obs} value of $SNAP_f$ to that of SNAP26m labeled with the same substrate at 25ºC.

Table 2. Labeling Efficiency of $SNAP_f$ tag

SUBSTRATE	LABELING TIME	
	5 MIN	30 MIN
SNAP-Cell TMR-star (NEB #S9104S)	95%	95%
SNAP-Cell 505 (NEB #S9103S)	93%	95%
SNAP-Cell Fluorescein (NEB #S9107S)	95%	95%
SNAP-Cell Block (NEB #S9106S)	95%	95%
SNAP-Surface 488 (NEB #S9124S)	94%	95%
SNAP-Surface Alexa Fluor 488 (NEB #S9129S)	95%	95%
SNAP-Surface 549 (NEB #S9112S)	92%	95%
SNAP-Surface Alexa Fluor 546 (NEB #S9132S)	95%	95%
SNAP-Surface 647 (NEB #S9137S)	93%	95%
SNAP-Surface Alexa Fluor 647 (NEB #S9136S)	95%	95%
SNAP-Surface Block (NEB #S9143S)	90%	95%
SNAP-Vista Green (NEB #S9147S)	95%	95%
SNAP-Biotin (NEB #S9110S)	95%	95%

The labeling reactions were performed in the presence of 5 µM $SNAP_f$ protein and 10 µM substrate at 25ºC. The reactions were analyzed by mass spectrometry to detect the unlabeled and labeled $SNAP_f$ species.

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Results

$SNAP_f$ Protein Labeling in Living Cells

Time course labeling using SNAP-Cell TMR-Star (NEB #S9105S) exemplifies the superior rate of reaction of $SNAP_f$ over SNAP26m (Fig. 1). The results demonstrate that labeling of $SNAP_f$-H2B can be visualized after an incubation time as short as 5 minutes using 1 µM SNAP-Cell TMR-Star, whereas at least 15 minutes are necessary for the detection of a distinct signal for labeled SNAP26m-H2B. The significantly enhanced labeling of $SNAP_f$ fusion is consistent at every labeling time point from 5–30 minutes.

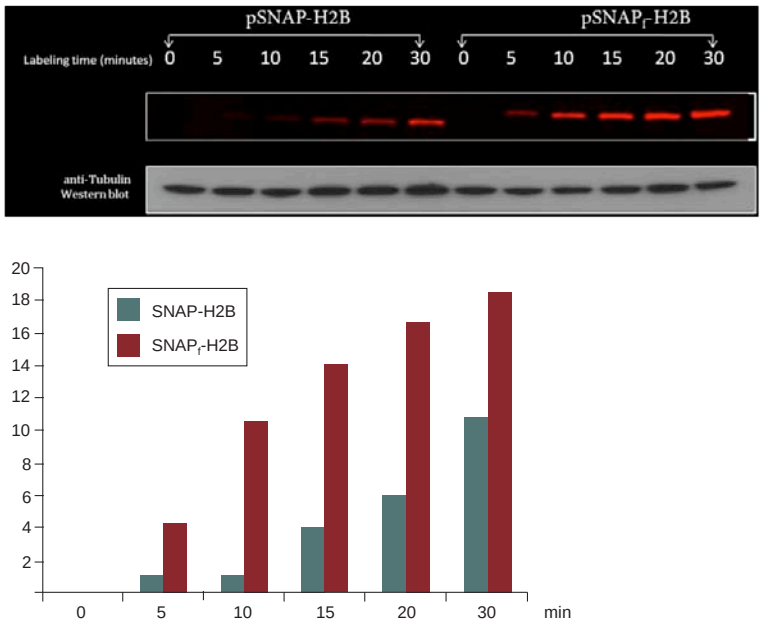

Figure 1. *Improved labeling efficiency of $SNAP_f$ fusion proteins in live U2OS cells. Stable U2OS cells expressing $SNAP_f$-H2B and SNAP-H2B were labeled with 1 µM SNAP-Cell TMR-Star from 0 to 30 min. Labeling efficiency was evaluated by SDS-PAGE and in-gel fluorescence scanning after cell lysis. Tubulin was used as protein expression control.*

Summary

The new generation of fast self-labeling $SNAP_f$ and $CLIP_f$ systems opens new avenues for analysis of highly dynamic processes in living cells with high spatial-temporal resolution. The improved kinetics of $SNAP_f$ and $CLIP_f$ associated with their unique molecular specificity enables the investigation of proteins with regard to their dynamic behavior, including cellular trafficking, stability and interactions in complex biosystems.

References:
1. Keppler, A. et al. (2003) *Nat. Biotechnol.* 21, 86–89.
2. Keppler, A. et al. (2004) *PNAS*, 101, 9955–9959.
3. Gautier, A. et al. (2009) *Chem. Biol.* 15, 128–136.
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