Chapter 7

Fluorescent Labeling of SNAP-Tagged Proteins in Cells

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Abstract

One of the most prominent self-labeling tags is SNAP-tag. It is an in vitro evolution product of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that reacts specifically with benzylguanine (BG) and benzylchloropyrimidine (CP) derivatives, leading to covalent labeling of SNAP-tag with a synthetic probe (Gronemeyer et al., Protein Eng Des Sel 19:309–316, 2006; Curr Opin Biotechnol 16:453–458, 2005; Keppler et al., Nat Biotechnol 21:86–89, 2003; Proc Natl Acad Sci U S A 101:9955–9959, 2004). SNAP-tag is well suited for the analysis and quantification of fused target protein using fluorescence microscopy techniques. It provides a simple, robust, and versatile approach to the imaging of fusion proteins under a wide range of experimental conditions.

Key words Snap-tag, Synthetic fluorophores, Living and fixed cells, Covalent labeling, Self-labeling tags, Fluorescence microscopy, Episomal protein expression

1 Introduction

A powerful approach for studying protein function inside living cells is their specific labeling with synthetic fluorescent reporter groups [1, 2]. One of the most frequently used methodologies is based on so-called self-labeling tags. A popular example of such a tag is SNAP-tag, an in vitro evolution product of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that reacts specifically and rapidly with benzylguanine (BG) and benzylchloropyrimidine (CP) derivatives (Fig. 1a), leading to covalent labeling of SNAP-tag with a synthetic probe [3–9]. Proteins of interest (POI) fused to SNAP-tag react only once with a single substrate molecule generating fluorescently tagged fusion proteins (Fig. 1b). The nontoxic nature of BG and CP derivatives together with the molecular specificity of SNAP-tag makes it suitable for a broad range of applications including in vivo imaging (Fig. 2). Examples of recent applications include single molecule [10, 11] and super-resolution imaging [12, 13], analysis of protein function [14], targeted protein inactivation [15], protein–protein interactions

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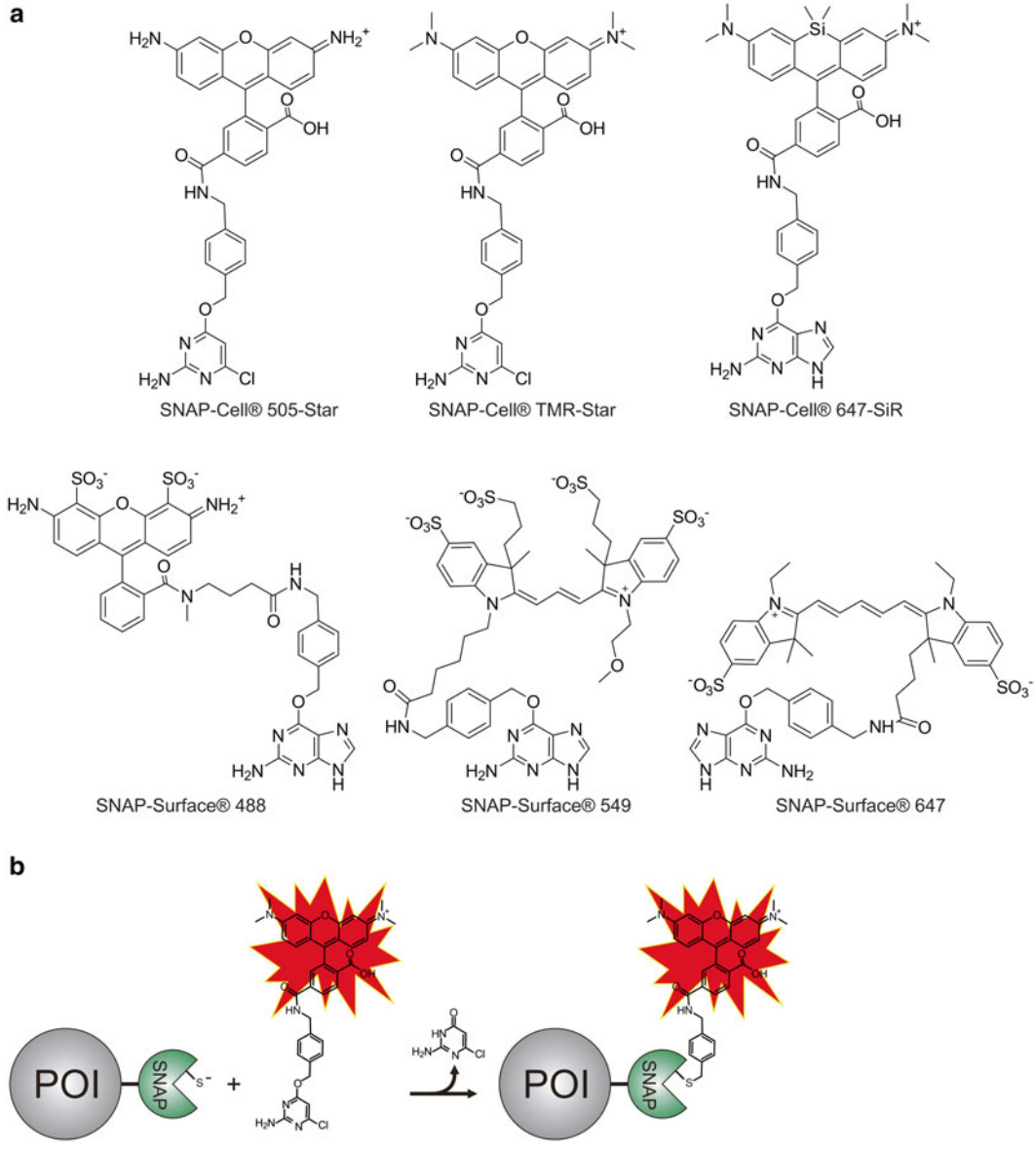

Fig. 1 SNAP-tag protein labeling technology. (**a**) Chemical structures of fluorescent substrates used for SNAP-tagged protein labeling: *top row*—examples of cell permeable substrates, *bottom row*—examples of cell nonpermeable substrates. (**b**) Scheme showing principle of protein of interest (POI) labeling via SNAP-tag

[16, 17], protein–drug interactions [18, 19], and the determination of protein half-life in animals [20]. Additionally, similar hAGT-based tag, named CLIP-tag, was developed recently [21]. It reacts specifically with O^2-benzylcytosine derivatives. SNAP-tag and CLIP-tag possess orthogonal substrate specificities, SNAP and CLIP fusion proteins can be labeled simultaneously and specifically

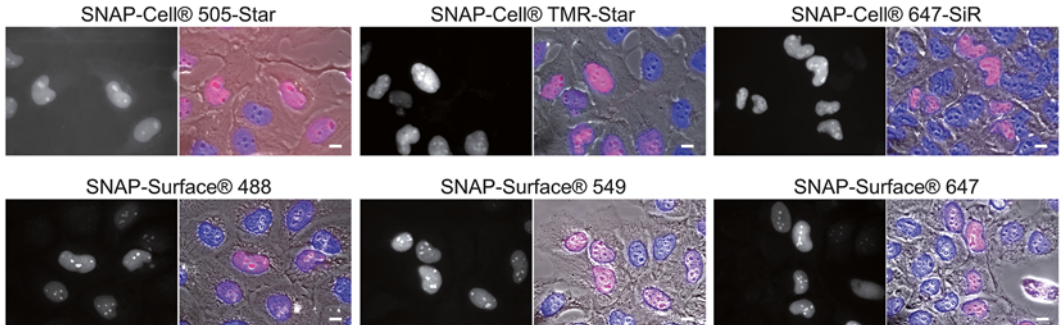

Fig. 2 Staining performance of SNAP-tag substrates. Live U2OS (*top row*) or fixed cells (*bottom row*) expressing nuclear localized SNAP-tag construct were stained with the substrates and Hoechst 33342. In the overlay image Hoechst 33342 is presented in *blue*, SNAP-tag substrate *red,* and transmission phase contrast image in *gray*. Panel to the *left* of overlay image represents SNAP-tag substrate image in *gray*. Scale bar 10 μm (color figure online)

with different molecular probes in living cells. Examples of such application include simultaneous labeling of two different fusion proteins [21], selective cross-linking (S-CROSS) of interacting proteins [16, 17, 19] and simultaneous measurement of protein SUMOylation at the single-molecule level [22].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistance of 18 MΩ cm at 25 °C) and analytical-grade reagents. All water solutions are filtered through 0.22 μm pore size membrane syringe filter directly after preparation.

1. U2OS cells from ATCC (HTB-96™). It is recommended to prepare multiple frozen stocks after obtaining cells. Details on cell line cultivation and cryopreservation are available on ATCC web site.
2. Growth medium: high-glucose DMEM without glutamine, pyruvate, and phenol red. Before usage, supplement with 10 % fetal bovine serum, 2 mM GlutaMAX™-I and 1 mM sodium pyruvate (*see* **Note 1**).
3. Trypsin/EDTA solution. Aliquote and store at −20 °C.
4. Opti-MEM® I reduced serum medium. Store at +4 °C.
5. Lipofectamine 2000. Store at +4 °C. Freezing might change performance.
6. 1 mg/ml Puromycin stock solution (1,000×). Aliquote and store at −20 °C.
7. 0.1 mg/ml Doxycycline stock solution (1,000×). Aliquote and store at −20 °C.

8. 1 mg/ml Hoechst 33342 stock solution (1,000×). Aliquote and store at –20 °C.
9. PBS buffer without Ca^{2+}, Mg^{2+}, or phenol red. Store in the dark at room temperature.
10. Hanks balanced salt solution (HBSS) without phenol red. Store in the dark at room temperature.
11. Lyophilized powder of albumin from bovine serum (BSA). Store at +4 °C.
12. EGTA, molecular biology grade. Store at room temperature.
13. PIPES, molecular biology grade. Store at room temperature.
14. BRB80 extraction buffer: 80 mM K-PIPES, pH 6.8, 1 mM $MgCl_2$; 1 mM EGTA, 0.2 % IGEPAL-630. Prepare freshly before experiment from the stock solutions of 0.5 M K-PIPES, pH 6.8, 1 M $MgCl_2$, 0.5 M EGTA, and IGEPAL-630 (*see* **Notes 2** and **3**).
15. Methanol, analytical reagent grade. Store at –20 °C (*see* **Note 4**).
16. 1 % w/v BSA solution in PBS. Store at +4 °C.
17. PBS-T wash buffer: PBS buffer supplemented with 0.05 % TX-100.
18. Staining buffer: PBS buffer supplemented with 1 % w/v BSA. DMSO solution of the substrate is added just before labeling procedure at concentration indicated in protocol part.
19. SNAP-tag substrates: SNAP-Cell® 505-Star (New England Biolabs), SNAP-Cell® TMR-Star (New England Biolabs), SNAP-Surface® 488 (New England Biolabs), SNAP-Surface® 549 (New England Biolabs), and SNAP-Surface® 647 (New England Biolabs). SNAP-Cell® 647-SiR (New England Biolabs) substrate synthesis is described in [23]. SNAP-tag substrates are dissolved in dry DMSO at final concentration of 1 mM and stored at –20 °C.
20. Mounting media: 90 ml of glycerol mixed with 10 ml of 10× PBS and dissolved 2–4 g of propyl gallate (*see* **Note 5**).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Generation of Cell Lines Expressing SNAP-Tagged Protein

1. U2OS cells are used for generation of cell lines expressing SNAP-tagged proteins of interest. Cells are cultured in growth medium in a humidified 5 % CO_2 incubator at 37 °C. 10 ml of medium is used for 25 cm^2 dish (*see* **Note 6**).
2. Prepare U2OS cells for transfection by splitting the cultured cells 24 h before transfection. Remove growth medium

from confluent monolayer of cells in 25 cm^2 dish. Wash cells with 5–10 ml of PBS buffer and add 1 ml of trypsin/EDTA solution. Incubate for 5 min at 37 °C and suspend detached cells in 11 ml of growth medium. Prepare six-well plate containing 2 ml of fresh growth medium in each well. Add 1 ml of cell suspension to each well and incubate overnight (*see* **Note 7**).

3. Next day dissolve 3–4 μl of Lipofectamine 2000 and 2–4 μg of DNA (pEBTet plasmid) in 100 μl Opti-MEM I separately. Incubate for 5 min at room temperature. Prepare Lipofectamine 2000 and DNA complex by mixing both components and incubating for 15 min at room temperature (*see* **Notes 8** and **9**).
4. Prepare U2OS cells for transfection by replacing growth medium with 1 ml of Opti-MEM I medium. Add prepared Lipofectamine 2000 and DNA complex solution and incubate for 6 h in a humidified 5 % CO_2 incubator at 37 °C.
5. After 6 h replace Opti-MEM I medium with growth medium and incubate for additional 24–48 h in a humidified 5 % CO_2 incubator at 37 °C (*see* **Note 10**).
6. Episomal plasmid pEBTet contains gene which renders transfected U2OS cells resistant to puromycin. Select for these cells by replacing growth medium with growth medium containing 1 μg/ml of puromycin. Selective medium has to be replaced each 2 days for duration of 4–6 days (*see* **Note 11**).
7. Wash cells with 2 ml of PBS buffer and add 0.4 ml of trypsin/EDTA solution. Incubate for 5 min at 37 °C, suspend detached cells, and transfer suspension into 25 cm^2 dish with 10 ml of growth medium and 1 μg/ml puromycin. Selected cells can be passaged every 4–5 days (1:10 dilution) for about 2 months without major loss of transgene expression level (*see* **Note 12**).

3.2 Labeling of SNAP-Tagged Proteins in Living Cells

1. Prepare U2OS cells for microscopy by splitting the cultured cells 24–48 h before experiment. Remove growth medium from confluent monolayer of cells in 25 cm^2 dish. Wash cells with 5–10 ml of PBS buffer and add 1 ml of trypsin/EDTA solution. Incubate for 5 min at 37 °C and suspend detached cells in 11 ml of growth medium (*see* **Notes 13** and **14**).
2. Prepare glass bottom six-well plate containing 2 ml of fresh growth medium supplemented with 1 μg/ml puromycin and 0.1 μg/ml doxycycline in each well. Add 0.5 ml of cell suspension to each well of plate. Incubate in a humidified 5 % CO_2 incubator at 37 °C for 24–48 h (*see* **Note 15**).
3. Stain cells with cell permeable substrates by replacing growth medium with growth medium containing 0.3–5 μM substrate. 1 μg/ml Hoechst 33342 can be included in the growth

medium together with substrate (*see* **Note 16**). Incubate cells for 1 h in a humidified 5 % CO_2 incubator at 37 °C (*see* **Note 17**). Afterwards wash cells two times with 1 ml of HBSS followed by 3–5 min incubation at room temperature. Replace HBSS after last wash with growth medium and incubate for additional 1 h in a humidified 5 % CO_2 incubator at 37 °C. Samples are ready for living cell imaging after this step.

3.3 Labeling of SNAP-Tagged Proteins in Fixed Cells

1. Prepare cells for fixation, remove the growth medium, and add precooled to −20 °C methanol and incubate for 3–10 min at −20 °C in freezer (*see* **Notes 18** and **19**). Take six-well plate from freezer and wash two times cells with 2 ml PBS buffer.
2. Incubate for 60 min in 2 ml of 1 % BSA in PBS solution (*see* **Note 20**). Remove BSA solution and stain DNA by incubating with 1 ml of 1 μg/ml Hoechst 33342 PBS solution for 1 min at room temperature. Wash excess of dye three times with 2 ml PBS-T wash buffer.
3. SNAP-tagged proteins can also be labeled after methanol fixation (*see* **Note 21**). Replace PBS with 1 ml of staining buffer containing cell not permeable SNAP-tag substrate (0.5–2 μM). Incubate for 1 h at room temperature (*see* **Note 22**). Wash excess of dye 2–3 times (incubating 3–5 min each time) with 2 ml PBS-T wash buffer.
4. This step is optional and can be performed if additional antibody-based staining is needed (*see* **Note 23**). Remove PBS-T wash buffer and put 0.5 ml of primary antibodies diluted in PBS with 1 % BSA. Incubate samples overnight at 4 °C. Wash excess of antibody three times 3–5 min with 1 ml PBS-T wash buffer and add dilutions of secondary antibodies in PBS with 1 % BSA. Incubate for 4–6 h at room temperature. Wash excess of antibody 3–5 min times with 1 ml PBS-T wash buffer. Samples are ready for imaging after last wash (*see* **Note 24**).

3.4 Labeling of SNAP-Tagged Proteins for Stimulated Emission Depletion (STED) Microscopy

Stimulated emission depletion (STED) microscopy [24] becomes more and more popular among the biologists who want to investigate cellular processes beyond the diffraction limit. Recent developments of fluorophores [17, 23, 25], parallelization of acquisition [26], time-resolved detection [27], and multicolor imaging [28, 29] pave the way to myriads of biological applications. Sample preparation for the STED microscopy is the same as described above (*see* Subheadings 3.2 and 3.3). However, only rhodamine-class fluorophores are recommended to be used. They display good enough photostability and brightness to be used for STED imaging (*see* **Note 25**). Image comparison of SNAP-tagged centrosomal proteins obtained with microscope operating in confocal or STED mode is provided in Fig. 3.

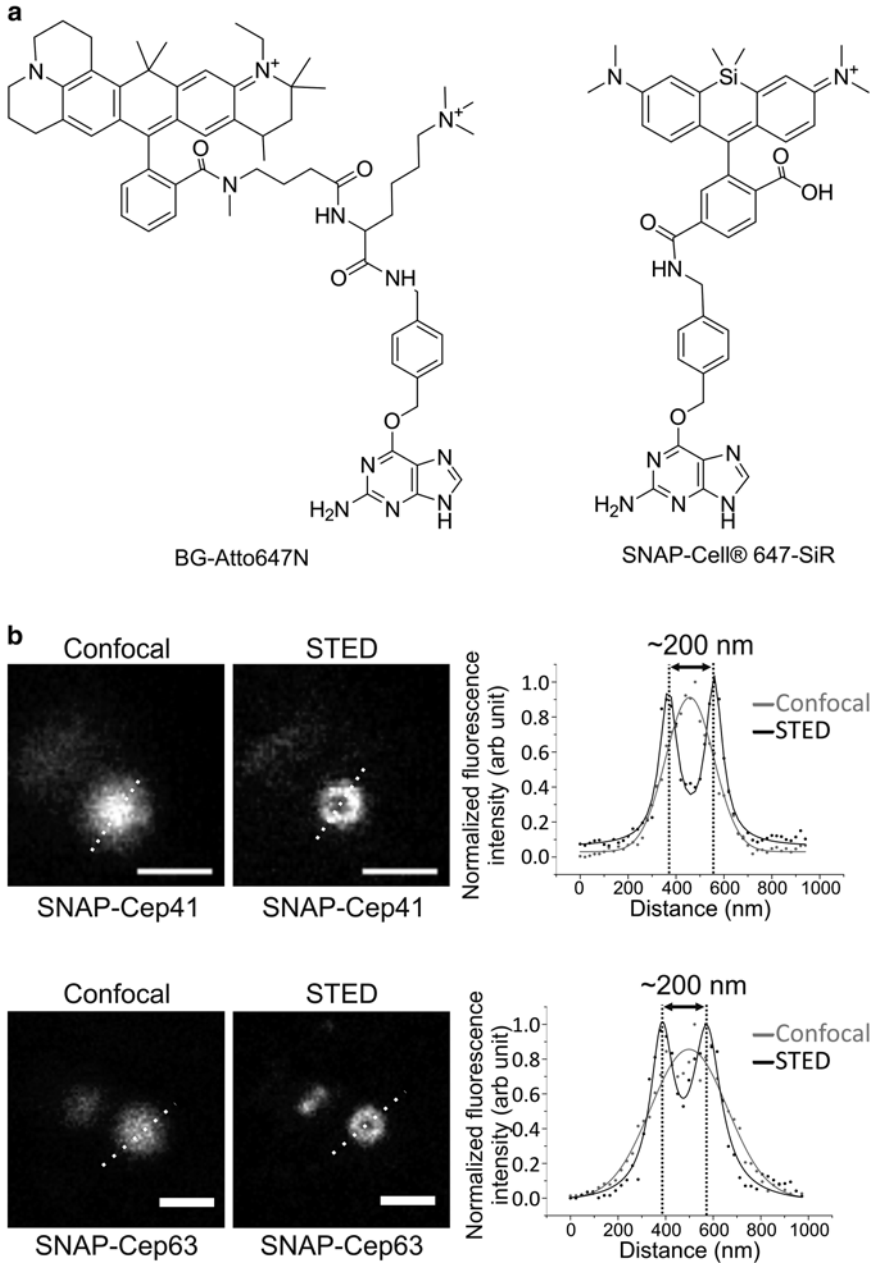

Fig. 3 STED imaging of SNAP-tagged proteins. (**a**) Chemical structures of STED compatible fluorescent substrates used for SNAP-tagged protein labeling: BG-Atto647N—example of cell nonpermeable substrate, SNAP-Cell® 647-SiR—example of cell permeable substrate. (**b**) Comparison of confocal and STED images of SNAP-tagged centrosomal proteins. SNAP-Cep41 expressing cells stained with SNAP-Cell® 647-SiR and imaged without fixing. SNAP-Cep63 expressing cells stained with BG-Atto647N after fixation. *Left panel* shows obtained images and profile line, *right panel* shows intensity profile of the line. Note, that improvement in resolutions leads to detection of doughnut shaped structure

4 Notes

1. Phenol red is interfering with imaging of living cells and cells should be cultivated in the medium without it. Alternatively, cells can be propagated in growth medium containing phenol red, but this medium has to be replaced by fresh growth medium just before imaging experiment.
2. 0.5 M K-PIPES stock: 15.1 g of piperazine-*N*,*N*′-bis (2-ethanesulfonic acid) in 90 ml of deionized water. Adjust pH to 6.8 with 10 M potassium hydroxide. Long standing K-PIPES solution tends to develop yellow-brown color, but it is not interfering with quality of obtained results.
3. 0.5 M EGTA solution: 19.0 g of ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid in 90 ml of deionized water. Adjust pH to 7.0 using 10 M sodium hydroxide. EGTA displays low solubility in water as free acid and dissolves completely when pH is close to 7.0.
4. Cold methanol can be supplemented with 5 mM EGTA pH 7.0 solution in order to improve cytoskeleton structure preservation during fixation.
5. Dissolving propyl gallate in 90 % glycerol solution might take long time. It is recommended to sonicate solution or place tube in +50 °C water bath and periodically mix it.
6. It is recommended to use U2OS cell line for generation of SNAP-tagged protein expressing cells since there is considerable variation of expression level in-between difference cell lines [30]. For example, HEK 293 displays higher expression levels and HeLa displays lower expression levels compared to U2OS.
7. Added volume of trypsinated cells is adjusted so that ~20 % confluence is obtained if all cells are adhered to the surface of new dish.
8. Transfection efficiency is dependent on many factors. It is recommended to determine the best transfection conditions before performing this experiment.
9. Generation of episomal expression vector pEBTet encoding SNAP-tagged protein of interest (POI). Expression vector is generated by LR recombination via attL and attR sites which is a part of Gateway® Cloning Technology available commercially from Life Technologies (Fig. 4). It contains Epstein–Barr virus origin of replication (oriP), which is capable of continuous episomal propagation in the mammalian cell lines in the presence of plasmid-encoded EBNA-1 protein. Cells without episome could be easily eliminated by selection with puromycin. Protein expression is driven from inducible CMV + TetO2

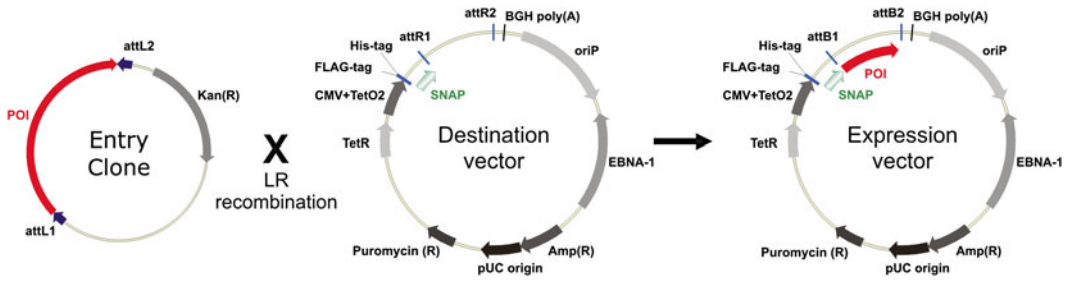

Fig. 4 Construction of the fused proteins expressing plasmids using Gateway™ cloning system

promoter [31]. Additionally, destination/expression vector can be propagated in *E. coli* cells since it contains pUC origin of replication and antibiotic resistance genes. Construction of destination vector is described in [17]. Entry clone plasmids are commercially available from Life technologies or GeneCopoeia.

10. Prolonged exposure to Opti-MEM I and Lipofectamine 2000 complexes result in considerable death of the cells. U2OS cell line is very well tolerating up to 6 h exposure, but it cannot be incubated overnight.
11. Puromycin is an aminonucleoside antibiotic, which causes premature chain termination during translation. Resistance gene encodes puromycin *N*-acetyltransferase which inactivates cytotoxic puromycin by acetylating it [32].
12. Expression of transgene is not stable due to silencing by various mechanisms. It is recommended not to split cells more then 10–15 times during the experiment.
13. For obtaining good quality microscopy images it is necessary that cell culture is not reaching complete confluence at the day of imaging. It is recommended to start with ~10–20 % of confluence 48 h before the imaging experiment.
14. Do not consume all the cells when setting up of an imaging experiment. The remaining cell suspension can be used for cell line propagation. This allows replication of experimental data.
15. Episomal pEBTet expression vector contains modified CMV promoter which is triggered "ON" in the presence of tetracycline or doxycycline in the medium. Keep tetracycline or doxycycline concentrations as low as possible since they display certain toxicity [33].
16. Hoechst 33342 counterstaining is recommended to include in most of the imaging experiments since it produces strong fluorescence signal, which is convenient for finding the cells and focusing.

17. It is recommended to use low concentrations (0.3–0.5 μM) of fluorescent substrates. High concentrations will result in higher background staining which is difficult to eliminate even with extensive washing. Fluorogenic SNAP-Cell® 647-SiR substrate is an exception and can be applied at concentrations up to 5 μM without need of extensive washing. Properties of cell permeable substrates are listed in Table 1.
18. SNAP-tag is highly stable and retains its activity even after fixation with cold methanol or paraformaldehyde. Fixation by cold methanol procedure is described here in more details, but paraformaldehyde or other fixatives can be used depending on which cell structure has to be preserved [35].
19. For the visualization of cell skeleton it is recommended to include preextraction step with BRB80 extraction buffer before applying cold methanol [12, 17]. It is done by replacing growth medium with 2 ml of BRB extraction buffer for 0.5 min at room temperature. Cold methanol is applied directly after this step.
20. Blocking with 1 % BSA in PBS reduces background staining in all the following steps. Hoechst 33342 staining can be combined together with incubation with 1 % of BSA in PBS.
21. SNAP-tag can be labeled with cell permeable substrates before fixation and covalent labeling survives fixation procedure very well. In general, labeling before fixation gives stronger specific signal compared to postfixation labeling [17].
22. It is recommended to use low concentrations (0.5–2 μM) of fluorescent substrates. All cell impermeable substrates are highly charged molecules with low off-target binding, but prolonged exposure to high concentrations of them will result in higher background staining which elimination requires extensive washing. Properties of cell impermeable substrates are listed in Table 2.

Table 1
Properties of common cell permeable SNAP-tag substrates

Substrate name	Excitation (nm)	Emission (nm)	QY[a]	$\varepsilon\ 10^4$ ($cm^{-1}\ M^{-1}$)	Rate constant 10^3 ($M^{-1}\ s^{-1}$)	Ref.
SNAP-Cell® 505-Star	504	532	0.92	7.4	12.4	[34]
SNAP-Cell® TMR-Star	554	580	0.68	9.1	15.5	[34]
SNAP-Cell® 647-SiR	650	670	0.39	10.0	20.0	[23]

[a]Of the unconjugated dye

Table 2
Properties of common cell impermeable SNAP-tag substrates

Substrate name	Excitation (nm)	Emission (nm)	QY[a]	ε 10^4 (cm^{-1} M^{-1})	Rate constant 10^3 (M^{-1} s^{-1})	Ref.
SNAP-Surface® 488	506	526	0.80	9.0	12.1	[36]
SNAP-Surface® 549	560	575	n.d.	15	11.1	[36]
SNAP-Surface® 647	660	673	0.25	25	n.d.	[8]
BG-Atto647N	644	669	0.65	15	2.9	[17]

[a]Of the unconjugated dye

23. SNAP-tag labeling is not interfering with antibody staining. Both techniques can be combined to obtain multicolor images [12, 17].
24. Fixed samples do not require special mounting media for wide field microscopy. Suggested fluorophores are stable enough in simple PBS solution. Samples prepared for confocal or STED microscopy should be mounted in 90 % glycerol in PBS containing 2–4 % w/v of propyl gallate.
25. SNAP-Surface® 549 and SNAP-Surface® 647 substrates are derivatives of cyanines and bleach extremely fast under STED imaging conditions.

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