

Analysis of Protein Turnover by Quantitative SNAP-Based Pulse-Chase Imaging

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ABSTRACT

Assessment of protein dynamics in living cells is crucial for understanding their biological properties and functions. The SNAP-tag, a self labeling suicide enzyme, presents a tool with unique features that can be adopted for determining protein dynamics in living cells. Here we present detailed protocols for the use of SNAP in fluorescent pulse-chase and quench-chase-pulse experiments. These time-slicing methods provide powerful tools to assay and quantify the fate and turnover rate of proteins of different ages. We cover advantages and pitfalls of SNAP-tagging in fixed- and live-cell studies and evaluate the recently developed fast-acting SNAPf variant. In addition, to facilitate the analysis of protein turnover datasets, we present an automated algorithm for spot recognition and quantification. *Curr. Protoc. Cell Biol.* 55:8.8.1-8.8.34. © 2012 by John Wiley & Sons, Inc.

Keywords: SNAP • pulse-chase • quench-chase-pulse • centromeres • automated quantification • protein dynamics • protein turnover • quantitative fluorescence

INTRODUCTION

The ability to track specific populations of proteins over time in living cells is essential to gain insight into the dynamics of cellular processes. An array of methodologies exist that assess different aspects of protein dynamics in living cells. These include fluorescence recovery after photobleaching (FRAP), photoactivation, and a variety of novel techniques using innovative protein tags (see Table 8.8.1 for a more extensive list).

Here we discuss SNAP-based pulse-chase imaging, a powerful method to track protein dynamics with distinct advantages over traditional methods to assess protein dynamics. SNAP is a suicide-enzyme protein-fusion tag that catalyzes its own covalent binding to the cell-permeable molecule benzylguanine (BG), and (fluorescent) derivatives thereof (Fig. 8.8.1; Damoiseaux et al., 2001; Keppler et al., 2003, 2004). Fusion of SNAP to a protein of interest allows this protein to be (fluorescently) labeled at will in living cells. Importantly, subsequent removal of the substrate results in the specific labeling of the initial pulse-labeled pool. Changes in location and turnover of this pool can be determined and quantified. Moreover, serial labeling of SNAP-tagged proteins with different SNAP substrates distinguishes proteins synthesized at different times, such that “old” and “new” pools can be detected separately (Fig. 8.8.3A and Jansen et al., 2007).

Principal advantages of using SNAP-tagging include: (1) pools of protein synthesized at different times can be specifically visualized, which allows for determining the fate of pre-existing versus newly synthesized pools of the same protein; (2) because labeling occurs on a population basis, large numbers of cells can be analyzed in a single experiment; and (3) labeling and turnover occur in the culture chamber rather than on the microscope stage; therefore, cells are not continuously imaged, but sampled for imaging at any timepoint from hours to days post labeling. A more extensive comparison of SNAP with

Table 8.8.1 Methods to Analyze Protein Turnover

Description	Advantages	Disadvantages	Scale	Timescale	Examples	References
Methods using auto-fluorescent proteins						
Fluorescence recovery after photobleaching (FRAP)	Measures local protein turnover (time and extent) after photobleaching	Allows analysis at very short timescales	Not ideal for long timescales (hours-days); phototoxicity	Single cells	FRAP, FLIP	Reviewed in Lippincott-Schwartz et al. (2001)
Fluorescence correlation spectroscopy (FCS)	Measures diffusion of fluorescent proteins in a very small volume	Allows accurate determination of protein concentrations and diffusion rates; single molecule sensitivity	Only works for mobile proteins at low concentrations; requires highly specialized equipment	Single cells	FCS, FCCS, RICS	Reviewed in Lippincott-Schwartz et al. (2001)
Methods using inducible fluorescent proteins						
Photo-activatable fluorescent proteins (PAPFs)	Fluorescent proteins that can be “turned on” by laser activation	Allows analysis of a specific subcellular pool of protein	Fluorescence prior to activation; phototoxicity	Single cells	Dronpa, Padron, bsDronpa, PA-GFP, PAmCherry	Reviewed in Lukyanov et al. (2005)
Photo-convertible fluorescent proteins (PCFPs)	Fluorescent proteins that can “change color” upon laser activation	Allows analysis of subcellular protein pools; visualization prior to activation; low background in post-activation channel	Blocks two fluorescent channels; phototoxicity	Single cells	mEos2, Kaede, Dendra-2, KiKGR, PS-CFP2	Reviewed in Lukyanov et al. (2005)
Methods using tags that can be chemically modified						
Chelation-based tags	Short peptide tags that have affinity to chemical substances	Size (6-12 aa's)	Unspecific labeling; toxicity	Single cells/bulk	TC-tag, 6His-tag, poly-D-tag	Reviewed in O’Hare et al. (2007)
Post-translational modification (PTM)-based tags	Tags that can be specifically post-translationally modified by (artificial) groups	Size (4-80 aa's)	Requires foreign enzyme or coexpression of enzyme to catalyze reaction	Single cells/bulk	ACP/PCP, biotin, sulfatase, Q-tag	Reviewed in O’Hare et al. (2007)

continued

Table 8.8.1 Methods to Analyze Protein Turnover, *continued*

	Description	Advantages	Disadvantages	Scale	Timescale	Examples	References
Self-labeling tags	Enzymes that catalyze their covalent binding to small compounds	Allows measurements at long timescales; allows analysis of 'old' vs. 'new' protein pools	Does not allow measurements at short timescales (seconds-minutes); high fluorescent background in live cells	Single cells/bulk	Hours-days	SNAP, SNAPf, CLIP, CLIPf, Halo, TMP	Keopler et al. (2003) (SNAP); Los and Wood (2007) (Halo); Gautier et al. (2008) (CLIP); Gallagher et al. (2009) (TMP); Sun et al. (2011) (SNAPf); reviewed in O'Hare et al. (2007)
Methods using other kinds of protein tags							
Time-specific tag for age measurement of proteins (TimeSTAMP)	Self-degrading protein tag that is inhibited by drug addition	Drug is sufficiently small to allow accessibility into tissues of live animals	Only allows analysis of new protein pools	Bulk; tissues	Hours	See Lin et al. (2008)	Lin et al. (2008)
Recombination-induced tag exchange (RITE)	Floxed protein tag that is replaced by a different tag upon Cre-expression	Allows measurements of both old and new protein pools simultaneously	Analysis depends on efficiency of Cre-recombination	Single cells/bulk	Hours (days?)	See Verzijlbergen et al. (2010); Radman-Livaja et al. (2011)	Verzijlbergen et al. (2010)
Auxin-inducible degron (AID) system	Inducible degradation tag, through cell-exogenous proteasome	Allows rapid specific degradation of a tagged pool of protein	Requires introduction of multiple transgenes; only allows analysis of new protein pools	Single cells/bulk	Minutes-hours	See Nishimura et al. (2009)	Nishimura et al. (2009)
Non-fluorescent methods							
Western-blot based pulse-chase experiments	Conditionally controlled expression and inhibition of protein synthesis	Cheap, easy, no need for specialized equipment	Does not allow imaging; cell toxicity due to protein synthesis inhibitors	Bulk	Hours	Many	
Metabolic labeling of proteins	Pulse labeling using labeled amino acids or amino acid analogs	No requirement for transgenes or tags	All proteins are labeled simultaneously, thus requiring downstream techniques to purify proteins of interest	Bulk	Minutes-days	SILAC, CATCH-IT	Ong et al. (2002) (SILAC); Deal et al. (2010) (CATCH-IT)

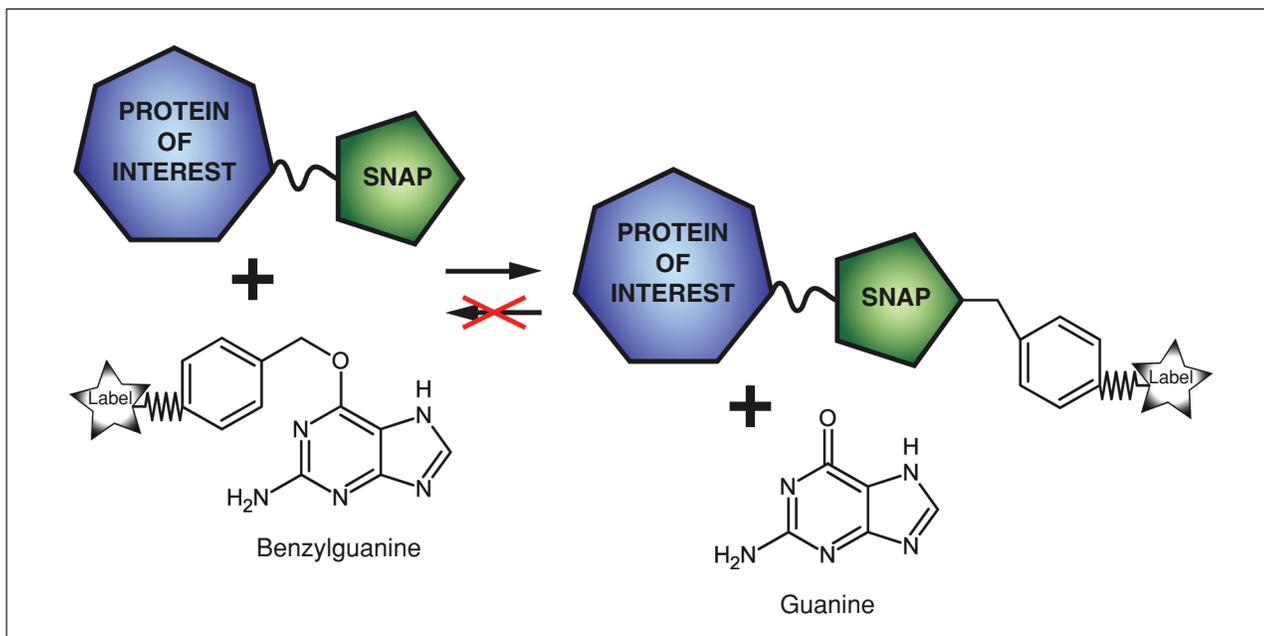


Figure 8.8.1 Principle of SNAP pulse labeling. SNAP is cloned as an epitope tag to a protein of interest. Reaction of SNAP fusion proteins with benzylguanine (or labeled derivatives) results in a covalent irreversible bond between the (labeled) benzyl moiety and a reactive cysteine in SNAP.

other pulse-labeling techniques, as well as its advantages and disadvantages, can be found in Table 8.8.1 and in Background Information.

In this unit, we explain in detail how to perform a typical SNAP pulse-labeling experiment. As an example, we will use HeLa cells that stably express a SNAP-tagged version of CENP-A, a centromere-specific histone variant (Sullivan et al., 1994; Jansen et al., 2007). Using these CENP-A-SNAP cells, we have been able to show previously that the rate of centromeric CENP-A turnover corresponds to the rate of cell division, and thus that CENP-A turns over exclusively by dilution during DNA replication (Jansen et al., 2007). Using the same technology, we demonstrated that newly synthesized pools of CENP-A assemble specifically during the G1-phase of the cell cycle (Jansen et al., 2007). The unique dynamics of CENP-A makes this an excellent illustration of the SNAP-labeling technique. However, this strategy is easily adaptable to other proteins (e.g., Fig. 8.8.3D) as well, and similar strategies have been used by us and other investigators, in a range of organisms and for different applications (Jansen et al., 2007; Erhardt et al., 2008; McMurray and Thorner, 2008; Maduzia et al., 2010; Bojkowska et al., 2011; Campos et al., 2011; Dunleavy et al., 2011; Ray-Gallet et al., 2011; Silva et al., 2012; also reviewed in O'Hare et al., 2007).

We will describe two typical SNAP-labeling strategies: pulse-chase (Basic Protocol 1) and quench-chase-pulse (Basic Protocol 2), which allow for the analysis of old and new protein pools, respectively. We also describe potential ways to combine SNAP labeling with cell synchronization and siRNA-mediated protein depletion (Basic Protocol 3). Cells can be analyzed by live imaging (Basic Protocol 4) or can be fixed and then analyzed with standard techniques such as immunofluorescence (Support Protocol 2). In addition, we present an unbiased, automated algorithm that is used for fluorescence measurements to quantify protein turnover (Basic Protocol 5). Lastly, we present an evaluation of SNAP pros and cons, as well as pitfalls and ways to troubleshoot them. Part of this evaluation is a characterization of the recently developed fast-acting variant of SNAP, called SNAPf.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

PULSE-CHASE EXPERIMENTS

This protocol will describe a general method that employs a pulse-chase strategy for analysis of a specific pool of protein in living cells. By using fluorescence pulse labeling, the fate and turnover rate of a given protein can be determined at a particular subcellular location. Specifically, SNAP-tagged protein that is present at the beginning of an experiment is fluorescently labeled (pulse), followed by removal of excess dye. After a given amount of time (chase), cells are analyzed, e.g., for localization or quantity of remaining protein by (quantitative) fluorescence microscopy (Fig. 8.8.2A). An example of a typical pulse-chase experiment of CENP-A-SNAP is shown in Figure 8.8.2B. In the approach described here, cells are fixed and analyzed at set time points following the initial pulse. As a consequence, protein dynamics can be determined at any time frame (hours, days) post labeling. However, the initial labeling and wash steps require approximately 1 hour, precluding the analysis of highly dynamic processes that occur on a timescale of seconds to minutes.

Materials

- Cells expressing SNAP-tagged fusion protein (see Support Protocol 1)
- Standard culture medium (CM; see recipe).
- TMR-Star (see recipe).
- Sterile DMSO
- Sterile 1 × PBS (cell-culture grade, Invitrogen)
- Clean, sterile, poly-lysine coated (*APPENDIX 2A*) coverslips (12 mm diameter; 1.5-mm thickness)
- 24-well plates
- Additional reagents and equipment for basic cell culture techniques including trypsinization of cells (*UNIT 1.1*)

Preparation of cells and SNAP-substrates

1. Prepare coverslips in separate wells of a 24-well plate to minimize the required incubation volumes.
2. Trypsinize cells expressing SNAP-tagged fusion protein and seed onto the coverslips in 1 ml of CM. Incubate at 37°C, 5% CO₂ (henceforth referred to as standard growth conditions).

Protocols for the abovementioned techniques and other basic cell culture techniques are described in UNIT 1.1.

The cell density depends on a number of factors, mainly cell type and the number of days between seeding cells and fixation. Ideally, by the time of fixation, the cell density should be high enough to capture a significant amount of cells on each frame, but not so high that cells are fully confluent. Generally 60% to 80% confluency is ideal. For HeLa cells (duplication time ~1 day), we aim to have $\sim 5 \times 10^5$ cells at the time of fixation. For example, $\sim 1 \times 10^5$ cells are seeded on the afternoon of day 1, if fixation will take place on the morning of day 4.

3. Dilute TMR-Star stock to 2 μM final concentration in CM. Vortex briefly to efficiently disperse the DMSO solvent into the aqueous medium. Dilute an equal volume of DMSO (to 1% v/v) for mock labeling control. Prepare >200 μl per coverslip.

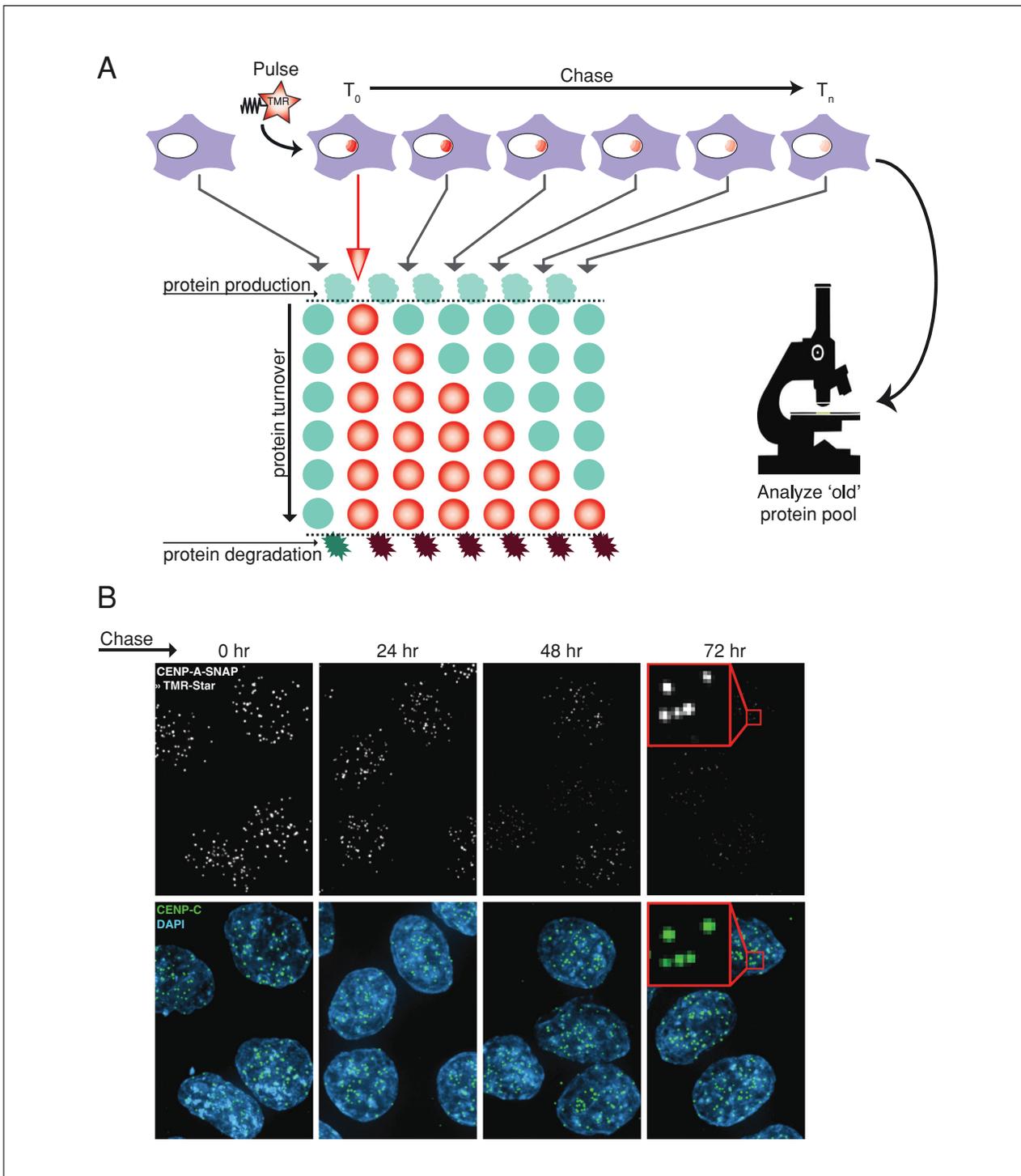


Figure 8.8.2 Pulse-chase imaging. **(A)** Schematic outlining an in vivo SNAP pulse labeling strategy (Basic Protocol 1). Cells that produce and turn over SNAP-tagged protein are incubated with the SNAP substrate TMR-Star (pulse) at time T_0 , rendering the available cellular pool of SNAP fluorescent. Following substrate washout (chase), cells continue to synthesize SNAP protein (light blue) that is not labeled, while the pulse labeled pool turns over. The remaining fluorescently pulse-labeled pool of SNAP can be visualized and quantified at various time points (T_n) during the chase by microscopy. **(B)** Example of a pulse-chase experiment using cells expressing CENP-A-SNAP. CENP-A (top panels) localizes to centromeres that are visualized as subnuclear, diffraction-limited foci. CENP-A-SNAP is pulse labeled at 0 hr with TMR-Star, after which cells are chased and the remaining pulse-labeled pool is visualized by high-magnification microscopy at indicated time points. After 72 hr, a small but detectable pool of CENP-A-SNAP is still present at centromeres (inset at 72 hr shows rescaled CENP-A::TMR-Star and CENP-C signals). Cells were counterstained with CENP-C (green) and DAPI (blue) to visualize centromeres and DNA, respectively. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0808>.

8.8.6

Prepare TMR-Star working stock only as needed and use within the hour. Although labeling is not yet saturated at this concentration, we use 2 μ M to balance signal intensities and costs per experiment (see Critical Parameters and Troubleshooting for more details). DMSO addition is an important initial control to determine background fluorescence unrelated to SNAP-labeling, as well as to determine the effect of DMSO on the cells. Once these factors have been established and an effect on cell viability, cell cycle progression, etc., are excluded for a given cell line, this control can be omitted from subsequent experiments.

4. Microcentrifuge diluted TMR-Star for 5 min at maximum speed ($\sim 16,000 \times g$) to get rid of possible insoluble fluorescent debris. Recover as much of the supernatant as possible without disturbing the pellet (which may not be visible).

Omitting this step will result in occasional but very bright fluorescent aggregates that interfere with imaging and quantification of fluorescent signals.

Pulse labeling and washes

5. Aspirate CM from cells on coverslips and add 200 μ l of CM+TMR-Star or CM+DMSO. Incubate for 15 min at standard growth conditions.

TMR-Star treatment of cells will likely result in nonspecific fluorescence (see Critical Parameters and Troubleshooting). It is therefore important to conduct pilot experiments in which the parent cells without expression of SNAP are labeled to discriminate SNAP-dependent fluorescence from nonspecific TMR-Star fluorescence.

6. Wash cells twice, each time by carefully aspirating medium/PBS and adding 1 ml sterile PBS (preheated to 37°C) to the well containing the coverslip, to wash away free substrate. Re-incubate cells in CM under standard growth conditions for an additional 30 min.

In our experience, in experiments where the cells have undergone multiple consecutive treatments prior to labeling (e.g., synchronization, RNAi, drug treatments), it is preferable to perform the washes with CM rather than PBS in this and the following steps. This enhances cell survival.

7. Wash cells twice with 1 ml sterile PBS (pre-heated to 37°C) as in step 6.

This second wash is important to remove any substrate that was retained in the cells after the initial wash. In our experience, omitting this step leads to a significant increase in background fluorescence. We calculate the chase period from the completion of this wash step, as this indicates the last time point during which SNAP-tagged proteins can be fluorescently labeled.

Chase and post processing

8. Choose among the three general options to proceed; details are presented in subsequent sections:
 - a. Pulse-fix: Fix cells immediately after the second wash and either image directly or process for immunofluorescence (Support Protocol 2).

This allows testing for SNAP-expression levels and/or serves as a control for subsequent pulse-chase experiments.
 - b. Pulse-chase: Re-add 1 ml of CM and incubate cells in standard growth conditions for a given amount of time (chase period), after which cells are fixed and treated for immunofluorescence (Support Protocol 2).
 - c. Pulse-image: Mount cells for live imaging (Basic Protocol 4).

QUENCH-CHASE-PULSE EXPERIMENTS

In this protocol, we describe a general method that allows for the analysis of a 'new' pool of protein. Specifically, the pool of SNAP-tagged protein that is present at the onset of an experiment is labeled by a nonfluorescent SNAP-substrate (quench). Subsequently, after

a given amount of time (chase), cells are labeled with a second, fluorescent substrate (pulse). In this way, only the pool of protein synthesized during the chase period is fluorescently labeled and hence will be visible by microscopy (Figure 8.8.3A), while the initial quenched pool remains undetected (Fig. 8.8.3B). This approach allows for e.g., quantitative and temporal analysis of protein translocation and/or assembly into subcellular domains. Examples of typical quench-chase-pulse experiments are shown in Figure 8.8.3C and D.

Materials

Bromothienylpteridine (BTP; see recipe)

Additional reagents and equipment for setting up the pulse-chase experiment (Basic Protocol 1)

Preparation of cells and SNAP-substrates

1. Prepare coverslips and cells as in steps 1 and 2, respectively, of Basic Protocol 1.
2. Dilute BTP to 2 μM final concentration in CM. Vortex briefly to efficiently disperse the DMSO solvent into the aqueous medium. Prepare >200 μl per coverslip. Prepare BTP working stock only as needed and use within the hour.

We have successfully used BTP at concentrations as low as 0.2 μM , resulting in fully quenched SNAP-labeling. However, because full quenching is essential for accurate interpretation of the results, we prefer using BTP at an excess of 2 μM (see step 6 for determination of quench efficiency). As opposed to TMR-Star, it is not necessary to centrifuge BTP solution prior to use.

Quench labeling and washes

Quench labeling is performed much in the same way as the pulse labeling described in Basic Protocol 1. The main difference is the time of initial incubation with BTP: 30 min, as compared to 15 min for TMR-Star (compare step 3 of this protocol with step 5 of Basic Protocol 1).

Figure 8.8.3 (appears on following page) Quench-chase-pulse imaging. **(A)** Schematic outlining an *in vivo* SNAP quench-chase-pulse labeling strategy (Basic Protocol 2). Cells that produce and turn over SNAP-tagged protein are incubated with a nonfluorescent SNAP substrate BTP (quench) at time T_0 , rendering the available cellular pool unavailable for subsequent fluorescent labeling (dark blue). Following substrate washout (chase), cells continue to synthesize SNAP protein (light blue) that is not labeled. After a set chase time, nascent protein is specifically labeled with TMR-Star (pulse). This nascent (new) fluorescent pool of SNAP can be visualized and quantified at various time points (T_n) during the subsequent chase by microscopy. **(B)** Quench-pulse control. Cells expressing CENP-A-SNAP were either pulse labeled with TMR-Star (pulse) or quenched with BTP immediately preceding the pulse-labeling step (quench-pulse), followed by immunofluorescence and imaging. While pulse labeling results in fluorescent centromeric CENP-A-SNAP, preincubation of cells with BTP (quench) renders this pool undetectable. Cells are counterstained with anti-HA, which detects the total pool of (CENP-A-) SNAP. The merged image shows TMR-Star (green) and HA (red) signals together with DAPI stain (blue). **(C)** Cells expressing CENP-A-SNAP were subjected to a quench-chase-pulse experiment as outlined in (A), processed for immunofluorescence, and imaged. Nascent CENP-A-SNAP (green) localizes to centromeres only in a subset of cells (arrow) while remaining noncentromeric in others (arrow heads), highlighting a cell-cycle dependence in nascent CENP-A-SNAP dynamics (Jansen et al., 2007). Cells are counterstained with anti-tubulin (red) and DAPI (blue) to visualize microtubules and DNA, respectively. **(D)** Experiment as in (C) except that cells expressing SNAP-tagged histone H3.1 (Ray-Gallet et al., 2011) were subjected to the quench-chase-pulse protocol. H3.1 is a canonical histone that assembles into chromatin in S-phase. Cells that either do not assemble (arrowhead) or that are in various stages of nascent histone H3.1 (red) assembly (arrows) are shown. Cells are counterstained with DAPI to visualize DNA (blue). Panels (B) and (C) are adapted from Jansen et al. (2007). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0808>.

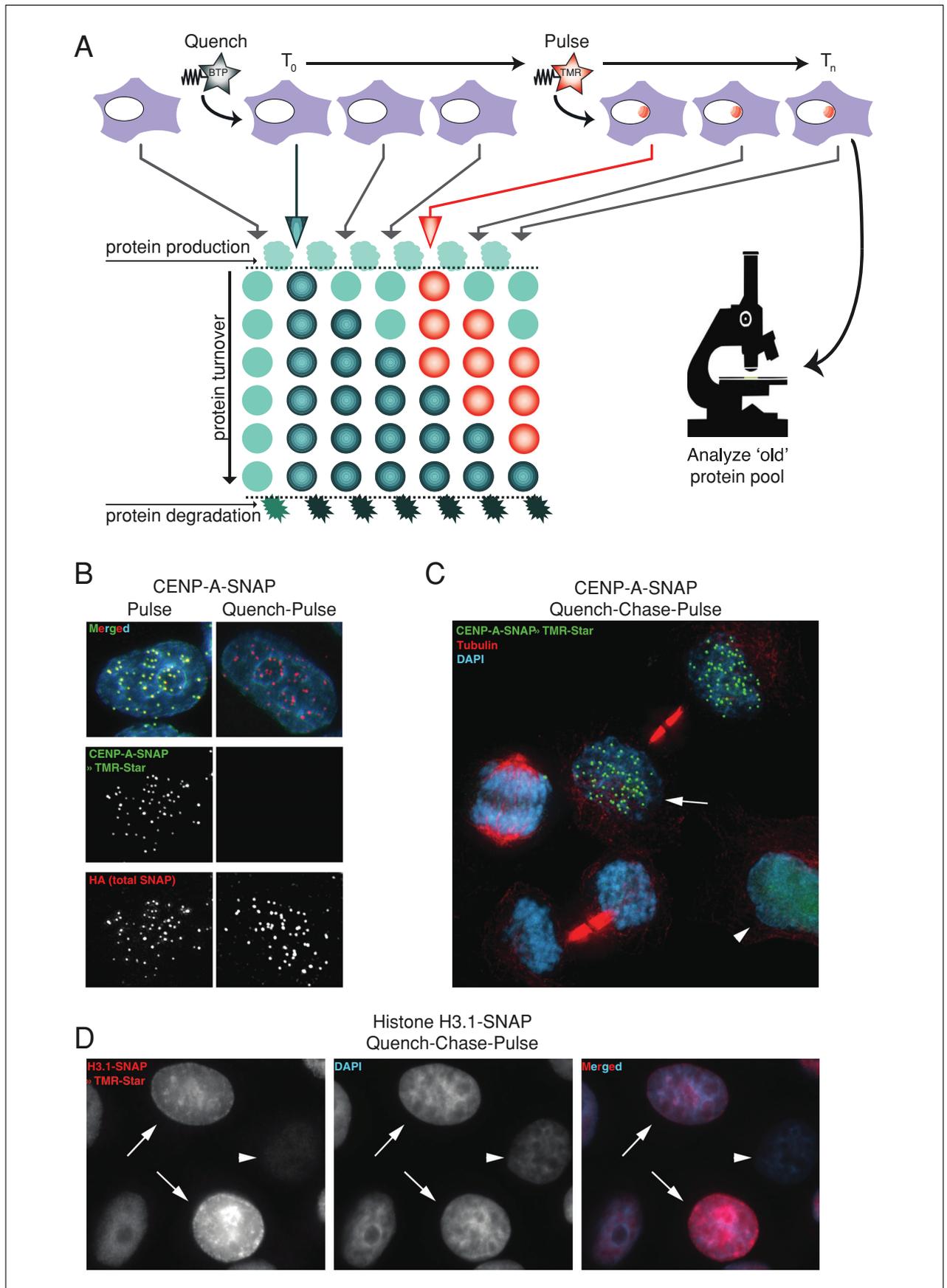


Figure 8.8.3 (legend appears on previous page)

3. Aspirate CM from cells on coverslips and add 200 μ l of CM+BTP. Incubate for 30 min under standard growth conditions.
4. Wash cells twice with 1 ml sterile PBS (pre-heated to 37°C) as in Basic Protocol 1, step 6, to wash away free substrate. Re-incubate cells in CM and standard growth conditions for an additional 30 min.

In our experience, in experiments where the cells have undergone multiple consecutive treatments prior to labeling (e.g., synchronization, RNAi, drug treatments), it is preferable to perform the washes with CM rather than PBS in this and the following steps. This enhances cell survival.

5. Wash cells twice with 1 ml sterile PBS (pre-heated to 37°C) as in Basic Protocol 1, step 6.

The second wash is important to remove all traces of free BTP. Omission of this wash will lead to continued quenching of a proportion of newly synthesized protein during the chase, resulting in smaller pool size of subsequently labeled nascent protein. We calculate the chase period from the completion of this wash step, as this indicates the last time point during which SNAP-tagged proteins can be labeled by the nonfluorescent substrate.

Quench-pulse control

6. Label at least one coverslip with TMR-Star directly following the quench step (no chase) as described in steps 2 through 7 of Basic Protocol 1.

This is a very important control, as it indicates whether or not the pre-existing SNAP-tagged protein is fully quenched by the available BTP (Fig. 8.8.3B). If this is not the case, results are very difficult, if not impossible, to interpret correctly. If BTP labeling is not complete, it may be necessary to increase the concentration of BTP and/or the incubation time. Once conditions that lead to a complete quenching of SNAP-tagged protein have been determined for a particular cell type and application, this control can be omitted in subsequent experiments.

Chase

7. Re-incubate cells in CM under standard growth conditions for the appropriate time.

Chase times will depend, among other things, on the expression levels of the protein of interest and cell type used. Typically, in human cell culture, a chase of several hours is required to create a pool size large enough for subsequent visualization by pulse labeling (e.g., for the case of CENP-A-SNAP, we found the minimum chase time required to easily detect nascent protein is 3 hr).

Pulse labeling and washes

8. For fluorescent pulse labeling and downstream applications, follow steps 3 through 8 of Basic Protocol 1.

BASIC PROTOCOL 3

INTEGRATING CELL SYNCHRONIZATION AND RNAi IN SNAP-BASED QUENCH-CHASE-PULSE LABELING EXPERIMENTS

In this section, we will describe how to combine the SNAP-labeling procedure with cell synchronization and/or siRNA-mediated protein depletions, specifically in HeLa cells. We will give a full overview of multiple synchronization and depletion steps integrated into a single quench-chase-pulse experiment (Fig. 8.8.4A). This allows for the determination of the fate of a newly synthesized pool of protein during the cell cycle and/or in response to protein depletions. It should be noted that, depending on the specific experiment, in many cases not all steps will be required. An example of a typical synchronized quench-chase-pulse experiment is shown in Figure 8.8.4B.

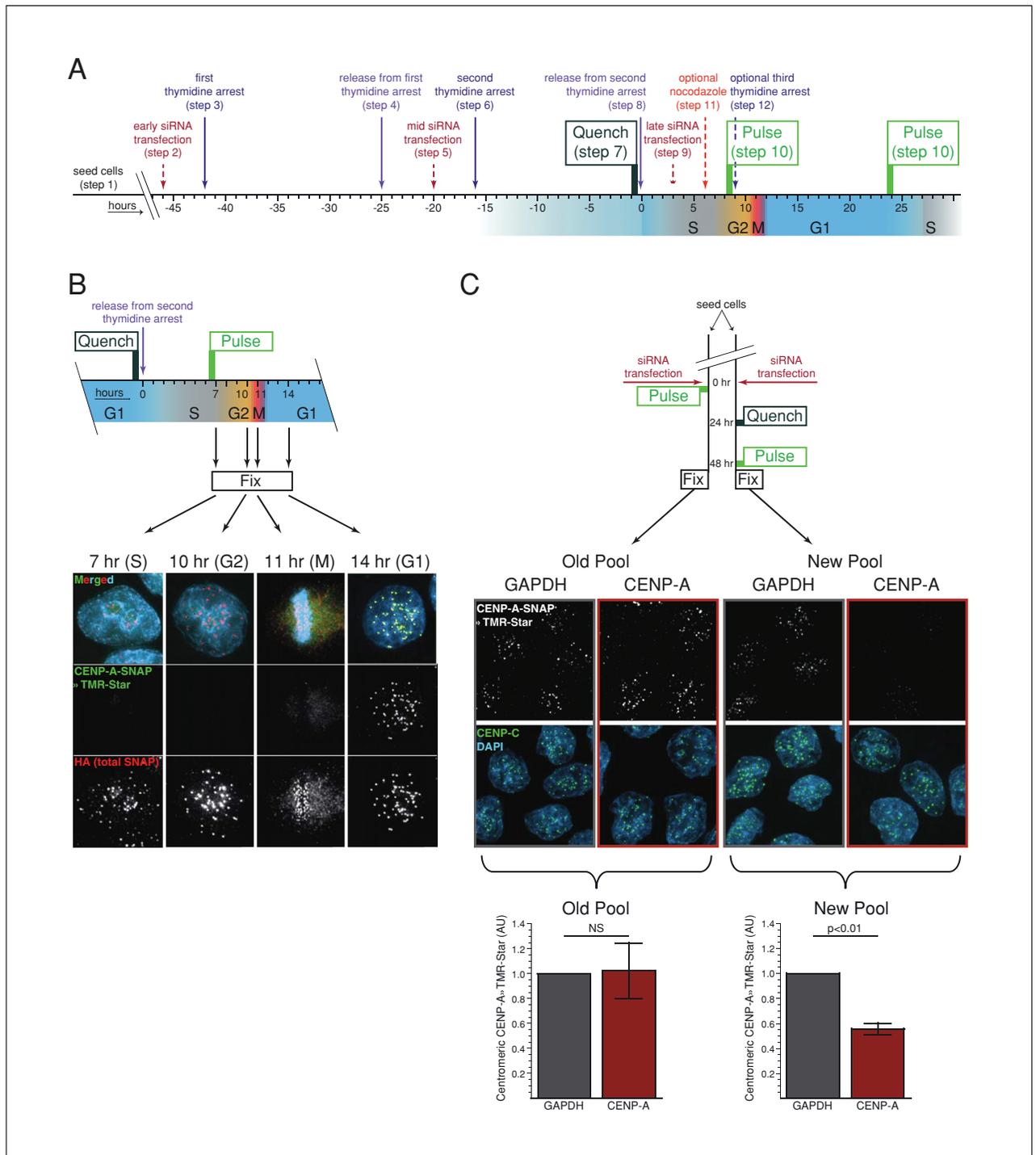


Figure 8.8.4 (legend appears on next page)

Materials

Transfection reagents for siRNAs: e.g., Oligofectamine (Invitrogen), and associated products
 siRNAs (Dharmacon)
 50 mM thymidine stock in H₂O
 24 mM deoxycytidine in H₂O
 5 mg/ml nocodazole stock in DMSO
 10 mM MG132 stock in DMSO

Cell Cycle
 Analysis

8.8.11

Additional reagents and equipment for pulse-chase and quench-pulse-chase experiments (Basic Protocols 1 and 2)

Preparation of cells and synchronization and RNAi

1. Prepare cells on coverslips as described in steps 1 and 2 of Basic Protocol 1.
2. Perform siRNA transfection for analysis of RNAi-mediated protein depletion at ~48 to 72 hr post transfection.

This step is performed as described in the manufacturer's protocol for Oligofectamine (Invitrogen). Wait at least 4 to 5 hr before proceeding to step 3. Protein depletion can only be performed at this point in the protocol (of a synchronized experiment) if the depleted proteins are not involved in cell-cycle progression. For proteins that are likely to interfere with S- or M-phase transition, siRNA transfection is best performed at a later stage in the protocol (see steps 5 and 9).

3. Add thymidine to the CM at a final concentration of 2 mM and incubate cells under standard growth conditions for 17 hr.

Cells that are in S-phase when thymidine is added will arrest immediately, while other cells progress until they enter S-phase and arrest there. Thus, after 17 hr, all cells will be arrested in S-phase, albeit at different stages of S-phase completion. Spike in thymidine, instead of replacing the CM with CM+thymidine (if RNAi was performed during step 2), as this would wash out siRNAs from the medium and reduce the efficiency of protein depletion. If siRNAs are transfected with Oligofectamine in serum-free medium in step 2, then serum can be re-added (along with thymidine) at this point to a final concentration of 10%.

4. Release cells from thymidine arrest by performing two washes with CM, followed by addition of CM+deoxycytidine (24 μ M final concentration). Incubate cells at standard growth conditions for 9 hr.

Figure 8.8.4 (appears on previous page) Combined strategies of SNAP labeling, cell synchronization, and RNAi. **(A)** Schematic outline of quench-chase-pulse protocol combined with cell synchronization using double thymidine and nocodazole arrest, and RNAi as described in Basic Protocol 3. **(B)** Combining quench-chase-pulse labeling with cell synchronization. Cells expressing CENP-A-SNAP and arrested at the G1/S boundary by double thymidine arrest (as in A) were treated with BTP to quench available SNAP pools, followed by release into S-phase during which new CENP-A-SNAP was synthesized. The nascent CENP-A-SNAP pool was pulse labeled with TMR-Star after a 7-hr chase at the end of S-phase. Cells were fixed at different time points to sample the population for centromere localization of nascent CENP-A-SNAP in S, G2, mitosis (M), and G1-phase cells. While the nascent pool is labeled at 7 hr post release, it does not localize to the centromere until cells reach G1-phase. Cells are counterstained with anti-HA, which detects the total pool of (CENP-A-) SNAP. The merged image shows TMR-Star (green) and HA (red) signals together with DAPI stain (blue). **(C)** Combining quench-chase-pulse and pulse-chase labeling with RNAi. An asynchronous population of cells expressing CENP-A-SNAP were transfected with siRNAs to block synthesis of nascent CENP-A or of a control protein (GAPDH). Cells were then either pulse-chase labeled (left) or quench-chase-pulse labeled (right) at indicated time points and assayed 48 hr after siRNA addition to determine the fate of old and new pools of protein, respectively. CENP-A-SNAP::TMR-Star signals representing old and new protein pools are shown following RNAi. Cells were counterstained with CENP-C (green) and DAPI (blue) to visualize centromeres and DNA, respectively. TMR-Star centromere intensity levels at the centromere were determined by CRaQ (Basic Protocol 5). Average centromeric CENP-A-SNAP::TMR-Star signals were determined from 3 replicate experiments. Signals after GAPDH RNAi were set to 1. Error bars indicate standard error of the mean (SEM). While CENP-A RNAi impairs the synthesis and accumulation of nascent CENP-A (new pool), the pool synthesized prior to siRNA addition is unaffected, demonstrating the ability to differentially visualize old and new protein pools. Panel (B) is adapted from Jansen et al. (2007). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0808>.

5. At 5 hr after release from the first thymidine arrest, siRNA transfection can be performed for analysis of RNAi-mediated protein depletion at ~24 to 48 hr post transfection.

This step is performed as described in the product description protocol for Oligofectamine (Invitrogen). Protein depletion can be performed at this point in the protocol for proteins that are (likely to be) required for mitotic progression, because significant levels of protein depletion are generally only observed at least 4 to 5 hr after siRNA transfection. At this point (~10 hr after release from the first thymidine arrest), most cells will have passed through mitosis already. For proteins that are not involved in cell-cycle progression, siRNA transfection can be performed at an earlier point (see step 2), while proteins that are involved in S-phase progression are best depleted at a later point (see step 9).

6. At 9 hr after the release described in step 4, add thymidine to the CM for a final concentration of 2 mM. Incubate cells at standard growth conditions for 15.5 hr.

At this time, all cells will have finished DNA replication, while none have started the next S-phase, regardless of at which point in S-phase they were arrested initially. Spike in thymidine, rather than replacing the CM with CM+thymidine (if RNAi was performed during step 5), as this would wash out siRNAs from the medium and reduce the efficiency of protein depletion. If siRNAs are transfected with Oligofectamine in serum-free medium in step 5, then serum can be re-added (along with thymidine) at this point to a final concentration of 10%.

Quench labeling and washes

7. At 15.5 hr after thymidine addition in step 6, perform quench-labeling (and first washout thereof) essentially as described in steps 3 to 6 of Basic Protocol 2, except that 2 mM thymidine is added to the CM+BTP and CM in order to maintain cells in the S-phase arrest until after the labeling is complete.
8. At 30 min after step 7, release cells from second thymidine arrest and perform second BTP washout by performing two washes with CM, followed by addition of CM+deoxycytidine (24 μ M final concentration).

This step combines the second wash of the BTP labeling and release from second thymidine arrest. Cells will now (16 hr after initiation of second thymidine arrest) all be synchronously released from early S-phase and will progress through the cell cycle largely synchronously for approximately one full cell cycle. Cells will enter mitosis at ~9 to 11 hr after release from the second thymidine arrest.

Chase

9. At ~3 hr after release from the second thymidine arrest (step 8), perform siRNA transfection for analysis of RNAi-mediated protein depletion at early time points post transfection.

This step is performed as described in the manufacturer's protocol for Oligofectamine (Invitrogen). Protein depletion can be performed at this point in the protocol for proteins that are (likely to be) required for S-phase progression, because significant levels of protein depletion are generally only observed at least 4 to 5 hr after siRNA transfection. At this point (~8 hr after release from the second thymidine arrest), most cells will have passed through S-phase already. Since maximum protein depletion is generally observed 24 to 48 hr post-transfection, for proteins that are not involved in S-phase progression, siRNA transfection is best performed at an earlier point (see steps 2 and 5).

Pulse labeling and washes

10. Perform TMR-Star pulse labeling and downstream applications as described in Basic Protocol 1, steps 3 to 8, at different time points following BTP-quench and thymidine release, depending on the application (as discussed in step 7 of Basic Protocol 2).

If siRNAs are transfected with Oligofectamine in serum-free medium in step 9, then serum can be re-added after the second washout of TMR-Star.

11. *Optional:* To gain higher synchrony in and around mitosis, arrest cells by addition of the microtubule-depolymerizing drug nocodazole to 250 ng/ml final concentration in CM.

This drug is added at ~5 to 6 hr after release from double thymidine block and will result in synchronous prometaphase arrest after ~9 to 10 hr from thymidine release. Alternatively, for metaphase arrest, nocodazole is added as described above, and replaced by MG132 (24 μ M final concentration in CM) on prometaphase-arrested cells.

Nocodazole can be added at any time to allow accumulation of cells in mitosis (optimal concentration will depend on cell type), although it is toxic and incubation times should be minimized. MG132 will arrest cells in interphase unless added in late G2-phase, in which case cells will continue to cycle until metaphase. Metaphase synchronization of cells by MG132 is therefore best combined with a double thymidine arrest, release, and nocodazole arrest and release. Arrest from these drugs is reversible, allowing the analysis of cells that are synchronously released from mitosis.

12. *Optional:* At 9 hr after release from the second thymidine arrest, re-add thymidine (final concentration of 2 mM) to collect cells synchronously at the next G1/S-phase transition, 15 hr later.

ALTERNATE PROTOCOL 1

INTEGRATING CELL SYNCHRONIZATION AND RNAi IN SNAP-BASED PULSE-CHASE LABELING EXPERIMENTS

In this alternate protocol we describe a modified version of Basic Protocol 3, where a pulse-chase strategy is employed rather than a quench-chase-pulse. This allows for tracking of a pre-existing pool of SNAP (as opposed to a newly synthesized pool) in relation to the cell cycle and in response to protein depletions. This protocol is highly similar to the Basic Protocol above, and therefore we will only describe the key steps that are different between the two protocols.

This alternate protocol can also be performed in parallel with Basic Protocol 3, e.g., to distinguish a differential effect on separate pools of the same protein (an example is given in Figure 8.8.4C).

For materials, see Basic Protocol 3.

Preparation of cells and synchronization and RNAi

1. Prepare cells, treated with siRNAs, and synchronize with thymidine as described in Basic Protocol 3, steps 1 to 6.

Pulse labeling and washes

2. At 15 hr and 15 min after thymidine addition in step 6 of Basic Protocol 3, perform TMR-Star pulse labeling (and first washout thereof), as described in steps 5 to 7 of Basic Protocol 1, except add 2 mM thymidine to the CM+TMR-Star and CM in order to maintain cells in the S-phase arrest until after the labeling is complete.
3. At 30 min after step 2 of this protocol, release cells from second thymidine arrest and perform second TMR-Star washout by performing two washes with CM, followed by addition of CM+deoxycytidine (24 μ M final concentration).

This step combines the second wash of the TMR-Star-labeling and release from second thymidine arrest.

4. Proceed to downstream applications as described in step 8 of Basic Protocol 1.

LIVE IMAGING OF PULSE-LABELED CELLS

This section will describe the basic procedure and considerations of imaging SNAP substrate signals in living cells. Live-cell imaging of SNAP-labeled proteins differs from conventional imaging of autofluorescent proteins (e.g., GFP) in that SNAP substrates generate considerable background staining, particularly in membrane compartments. This requires specific signals to be of sufficient strength to maintain an adequate signal-to-noise ratio. Despite this constraint, live cell imaging of temporally labeled SNAP-tagged proteins is a powerful approach to determine the fate of protein pools of different ages (Fig. 8.8.5).

Live-cell imaging setups for SNAP-tagged cells are similar to those of normal live cell setups. We use typically two different methods; one described in this protocol (adapted from Waterman-Storer and Salmon, 1997) and the second described in Alternate Protocol 2. For general considerations regarding the microscope setup, see Critical Parameters and Troubleshooting.

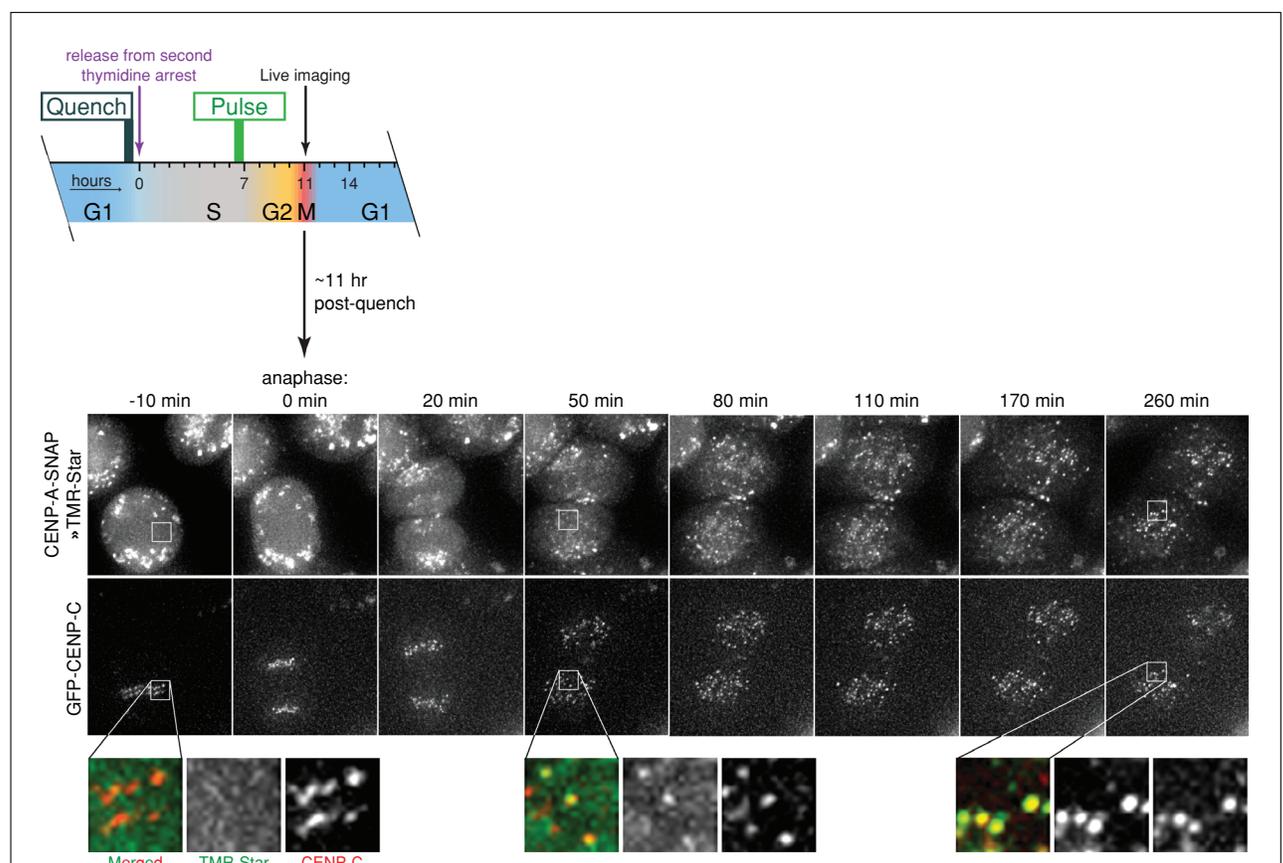


Figure 8.8.5 Live-cell imaging of SNAP-labeled cells. Schematic outlines cell synchronization and quench-chase-pulse labeling steps as shown in Figure 8.8.4B. Following pulse labeling, cells are cycled into mitosis and mounted for live cell imaging (Basic Protocol 4). Time-lapse series is shown of a cell in mitosis. At early time points, TMR-Star signals are noncentromeric, but are observed near the cell periphery, probably reflecting nonspecific retention of the fluorescent substrate in cellular membranes. As cells exit from mitosis (after anaphase, $t = 0$ min), TMR-Star signal accumulates at centromeres from $t = 50$ min onwards. Cells express GFP-CENP-C that constitutively labels centromeres throughout the experiment. Insets show colocalization of nascent CENP-A-SNAP::TMR-Star (green) with centromeres (CENP-C, red). Image is adapted from Jansen et al. (2007). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0808>.

Materials

- Cells expressing SNAP-tagged fusion protein (see Support Protocol 1)
- Live imaging medium (LM; see recipe)
- VALAP (see recipe)
- 30 U/ml Oxyrase stock (Oxyrase Inc.; <http://www.oxyrase.com/>)
- 6-well plates
- Clean, sterile, 22 × 22-mm poly-lysine coated (*APPENDIX 2A*) coverslips (1.5-mm thickness)
- Permanent double-sided tape (Scotch)
- Glass microscope slides
- Additional reagents and equipment for pulse-chase (Basic Protocol 1) and quench-pulse-chase (Basic Protocol 2) experiments

Preparation of cells and pulse labeling

1. Grow cells expressing SNAP-tag fusion proteins to 60% to 80% confluency on 22 × 22-mm square glass coverslips placed in the wells of 6-well plates, with each well containing 2 ml of culture medium.
2. Perform quench and pulse labeling steps as in Basic Protocol 1 or 2, except use labeling volumes of 600 μl in 6-well plates.

Mounting of live cell chambers

3. Glue three layers of double-sided tape, cut ~3 mm wide, along the two long edges of the glass slide such that when a coverslip is placed on top, it is sealed on two sides (along the longitudinal end of the glass slide).
4. Mount coverslips, cells facing down, onto the glass slide prepared in step 3.
5. Slowly, flow in LM under the coverslip, until the chamber is filled by capillary action (<1 ml).

Perform this step as quickly as possible after step 4 to avoid cells drying out. Phenol red is omitted from the LM to avoid background fluorescence. The use of CO₂-independent medium (e.g., buffered by HEPES) is required to maintain pH in this chamber type, as it is sealed from outside air contact. Optionally, 0.5 U/ml Oxyrase is included in the medium. Oxyrase is an oxygen-scavenging enzyme that helps reduce photobleaching and phototoxicity due to reactive oxygen species.

6. Seal the chamber on all sides with VALAP and image live cells on the microscope.

ALTERNATE PROTOCOL 2

LIVE IMAGING OF PULSE-LABELED CELLS: 8-WELL COVERGLASS METHOD

Here we describe an alternative approach to live imaging of SNAP-labeled cells. The method presented below has two main advantages over that of Basic Protocol 4. First, labeling volumes are reduced from 600 μl to 100 μl, thus reducing the amount of SNAP substrate used (and associated costs). Second, cells can be seeded into multiple wells of the 8-well coverglass and treated differently but in parallel (labeling strategies/time points, substrates used, drug treatments, RNAi conditions, etc.). Provided that a microscope with automated stage is used, different cell treatments (in separate wells) can be followed synchronously in a single experiment. The main disadvantage of the 8-well coverglass method is that it is less well suited for long time-course experiments, because sealing of wells with mineral oil here is less effective than using VALAP as in Basic Protocol 4.

Materials

Cells expressing SNAP-tagged fusion protein (see Support Protocol 1)
Live imaging medium (LM; see recipe)
Mineral oil
8-well chambered coverglass (Lab-Tek)
Additional reagents and equipment for pulse-chase (Basic Protocol 1) and
quench-pulse-chase (Basic Protocol 2) experiments

Preparation of cells and pulse labeling

1. Grow cells expressing SNAP-tag fusion proteins directly in an 8-well chambered coverglass slides to 60% to 80% confluency.
2. Perform quench and pulse labeling steps as in Basic Protocols 1 or 2, except that labeling volumes of 100 μ l are used in 8-well chambered coverglass slides.

Mounting of live cell slides

3. Following labeling and washes, replace medium with LM to a final volume of 300 μ l. Seal wells with 100 μ l mineral oil.

Due to small sample volumes, it is critical to prevent evaporation of medium during the time lapse. Sealing of the medium-air interface with mineral oil is an effective method to achieve this. The use of mineral oil is compatible with the use of DIC optics during live cell imaging. Optionally, 0.5 U/ml Oxyrase is included in the medium.

AUTOMATED QUANTIFICATION OF SNAP-TAGGED PROTEIN TURNOVER AT CENTROMERES

In this section, we present a method to perform unbiased fluorescence quantification of diffraction-limited spots. The case that we present here concerns centromeres, but this approach applies to any point-source signals in living or fixed cells. To this end, we developed an automated algorithm which we name CRaQ (Centromere Recognition and Quantification). This ImageJ-based macro detects spots in one channel and subsequently measures the fluorescence intensities in another. This allows for accurate detection and quantification of thousands of spots in a fast, unbiased, and effortless way.

In brief, centromeres are recognized and the centroid position is determined. Next, fluorescent intensities are measured for each centromere by placing a small box around the centroid position of the centromere. The peak intensity value within the box is then corrected for local background by subtraction of the minimum pixel value. This process results in an accurate measurement of centromere-specific signals.

Because this protocol is performed in an automated fashion, in this section we will first describe the steps that the researcher must take (preparation of the data, CRaQ initiation, and parameter settings, etc). Next, we will give an overview of the actual steps that the algorithm goes through for each image (Fig. 8.8.6A-E). This provides users with a good idea of how automated recognition and quantification is performed.

We have evaluated the accuracy of CRaQ by re-analyzing previously published quantifications that were performed by selecting spots (in a reference channel) by eye (Jansen et al., 2007). The results that are obtained by CRaQ are practically identical to the previously published results (Fig. 8.8.6F). In addition, we evaluated the robustness of CRaQ by analyzing replicate samples. Because CRaQ works in a deterministic fashion, re-analyzing an identical dataset without changing parameters will lead to an identical result. We show that quantification of replicate samples by CRaQ leads to result with a standard error of the mean (SEM) of \sim 5%, which is likely attributable to biological and/or experimental variation (Fig. 8.8.6G).

BASIC PROTOCOL 5

Cell Cycle Analysis

8.8.17

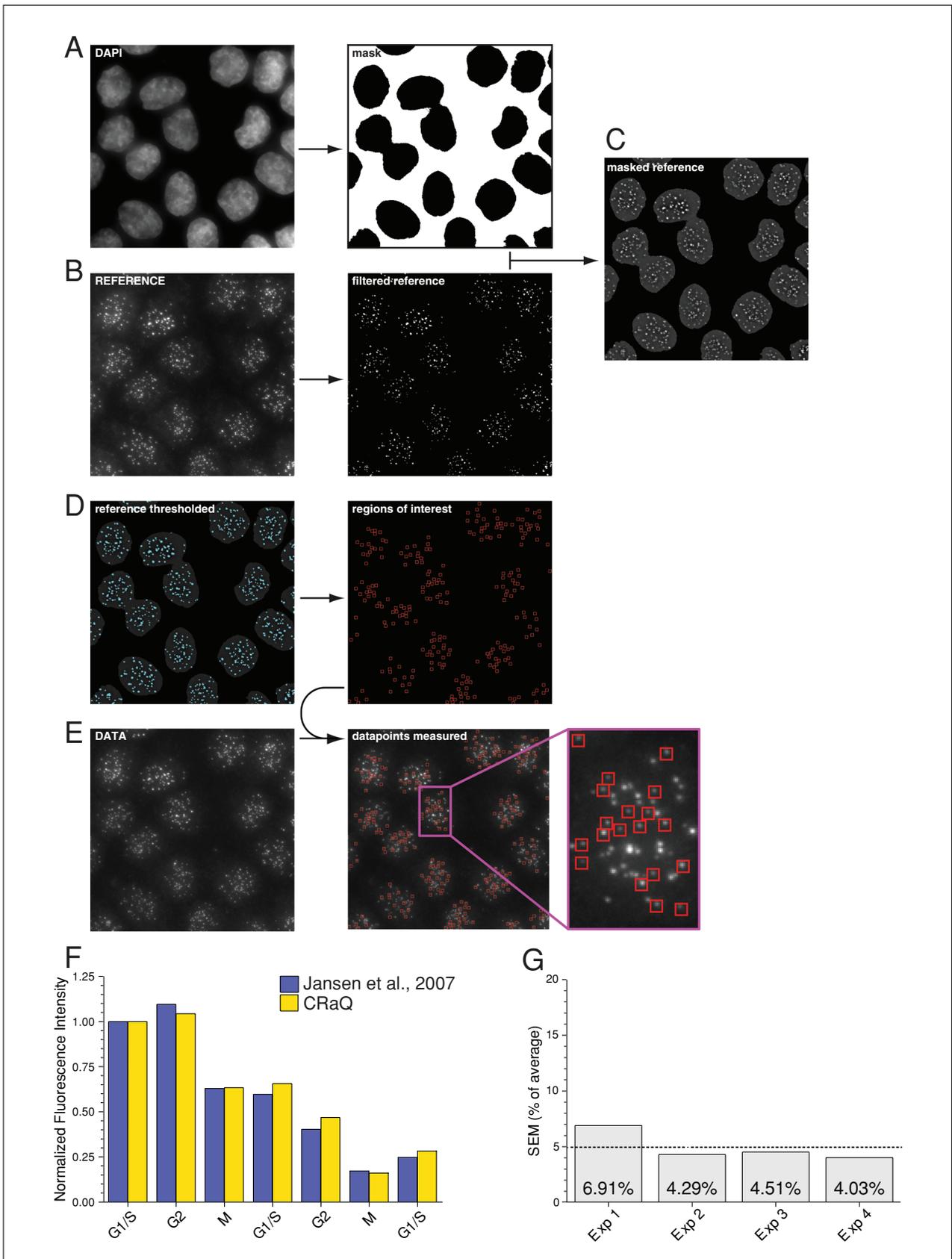


Figure 8.8.6 (legend appears on following page)

Analysis of Protein Turnover

8.8.18

Materials

A standard computer

ImageJ software (freely available from NIH, <http://rsbweb.nih.gov/ij/index.html>)

CRaQ plugin for ImageJ (go to Supplemental Files at

<http://www.currentprotocols.com/protocol/cb0808>, or download from

<http://uic.igc.gulbenkian.pt/micro-macros.htm>).

Digital images of SNAP-labeled cells, as described in Basic Protocol 1 or 2 after fixation and antibody staining as described in Support Protocol 2

Input data preparation (before running CRaQ)

1. Input files should consist of all of the channels of a single frame in one file. CRaQ can use either stacks or projected images as an input. The order of images in a file should be such that the entire image sequence of one channel is followed by the image sequence of the second channel, etc.

This is as opposed to having all channels for one frame followed by all the channels for the next frame. Additional channels that are not used during the quantification process can be stored in the same files and will be ignored by CRaQ.

2. Note the order in which the data, reference, and DAPI channels are stored in the input files.

In principle, only a data channel (the channel that will be quantified) is essential for CRaQ to run. See Critical Parameters and Troubleshooting for reasons and tips for using an independent reference channel.

3. Ideally, the order in which the images should be taken is (1) data, (2) reference, (3) DAPI, and (4) any additional channels.

In this way, potential bleaching of the data signal during reference or DAPI channel acquisition will occur only after the data have been collected.

4. Create a “base folder” with separate subfolders that contain all the images for each condition (e.g., RNAi, replicates, cell types, cell cycle stages, etc).

Any images that are located directly in the base folder will not be detected by CRaQ. If all images are to be quantified separately, they can be put into a single subfolder, as the output data file indicates which data points are derived from which image. Only files with extension .dv (produced by SoftWorx, Applied Precision) or .tif will be recognized by the macro. Thus, additional files (log files, etc.) can remain in the base folder without interfering with the macro. When rerunning CRaQ on a previously analyzed data set (e.g., using different settings), make sure to copy the previous data output prior to rerunning, as all files will be overwritten.

Figure 8.8.6 (appears on previous page) Centromere Recognition and Quantification (CRaQ). (A-E) Flow scheme outlining automated steps of centromere recognition by CRaQ (Basic Protocol 5). (A) DAPI images are thresholded and converted to binary images (mask). (B) REFERENCE images are filtered and (C) overlaid with the mask to produce a masked reference image. (D) This image is again thresholded, and spots that fit with the given parameter settings are exported as regions of interest. (E) DATA images are measured at each region of interest. The blowup shows accuracy and frequency of centromere recognition. Note that raw data images are indicated by capitals, while processed images are indicated by lowercase letters in A-E. (F) CRaQ was used to re-analyze measurements that were performed by manual selection and quantification in Jansen et al. (2007). The two methods result in practically identical quantifications, thus cross-validating both methods. (G) Replicate samples were analyzed by CRaQ, and standard error of mean (SEM) is plotted as a percentage of the average value for four independent experiments, each consisting of four replicate samples.

Installing and Running CraQ

5. Copy the CRAQ plugin into your `.../ImageJ/plugins/Analyze` folder and restart ImageJ. Run the algorithm by selecting it from the Plugins>Analyze toolbar of ImageJ.
6. In the window that appears, you can set the order in which the Data, Reference, and DAPI channels are stored in the input files, as well as the total number of channels. In addition, you can choose to change the standard parameter settings of CRAQ.

Setting the parameters

The default parameters are those that we have found to work best for most purposes. However, depending on particular experiments, this will not always be the case. What follows is an explanation of each parameter and how and why to change them.

Square size

This refers to the size of the box placed around each centromere. Square size 7 means a box of 7×7 pixels. This will generally not change the results much, as only the maximum and minimum pixel values in each box are used. However, make sure that the box is big enough to contain some background pixels, but not too large, as this will make the background signal “less local” and will decrease the number of spots identified due to exclusion of overlapping boxes.

Minimum circularity

This measure helps to exclude clustered centromeres. Circularity is a measure of how much the recognized spots resemble a circle, where 1 is a perfect circle and 0 a straight line (the most imperfect circle). Since centromeres appear as diffraction-limited spots, they should theoretically be perfectly circular, and this measure can be set very close to 1 (most single centromeres actually have a circularity of 1). Because brighter centromeres tend to be less circular, decreasing circularity will allow you to pick up more bright centromeres, but will also increase the chance of picking up doublets, clusters, or non-centromeric regions.

Maximum Feret diameter

This measure is also made to exclude doublets/clusters and is required because occasionally clusters have a very high circularity. The Feret diameter is the longest diameter of a spot. Together, stringent circularity and Feret parameters are able to exclude most doublets. Increasing the maximum Feret diameter has a similar effect to decreasing minimum circularity and vice versa.

Min/Max centromere size

This measure refers to the minimum and maximum size a centromere can have (in number of pixels). Basically, having a larger maximum size can include both brighter centromeres and more doublets. Again, a lower maximum centromere size will exclude the last few doublets, but may also exclude some of the brightest (in the reference channel) single centromeres. Increasing the minimum will discard more false positive spots, but also more truly positive (dim) spots.

Threshold offset

This parameter sets the sensitivity of recognition of spots in the thresholded image. Increasing the offset makes the threshold more sensitive to lower signals. This will both increase the number of dim spots (true and false positives) and decrease the number of bright centromeres (false negatives), as these will now appear bigger and potentially less circular.

Chromatic aberration correction

If there is a constant chromatic aberration between reference and data channels, this can be corrected by CRaQ. If the reference channel has spots shifted towards the top/right, then input positive numbers. If the reference channel has spots more to the bottom/left, input negative numbers.

Data output

All output files will be produced in an output folder inside the base folder. These are the different output files that will be produced by CRaQ:

A single file entitled: logfile.txt

This file contains the base directory and parameter settings used. Keep this file or copy the information for further reference, replicate experiments, or comparison between experiments and parameter settings.

*One *.txt file for each condition (i.e., subfolder of the base folder)*

These files contain the actual measurements made by CRaQ with a reference to the corresponding image and centromere spot number. These can be directly copied to analysis software such as Excel (Microsoft) or Prism (GraphPad) for further data processing and analysis.

*One *.zip file for each image*

This contains all the recognized spots for that image as ROI lists for ImageJ. To view spots, open the image and the corresponding *.zip file in ImageJ. An ROI Manager window will appear, and you can either see all spots by selecting “Show all” or select and display any individual spot.

If Z-stacks were used as input images, a projection of each image is saved

All channels of an image will be saved together in a single *.tif file.

Automated steps performed by CRaQ macro on each image

- a. Convert DAPI to mask (Fig. 8.8.6A).

This mask will exclude any spots that are recognized but do not overlap with DNA.

- b. Signal enhancing on reference (Fig. 8.8.6B).

This allows for more accurate spot recognition.

- c. Overlay the mask and the reference (Fig. 8.8.6C).

This excludes any non-DAPI signals.

- d. Spots that are significantly above background and fall within the restrictions given by the parameter settings are detected and exported as ROI (region of interest) lists (Fig. 8.8.6D).

Note that generally <50% of all centromeres are found. However, the recognition of centromeres does not seem to depend on the brightness of centromeres in the reference channel, much less in the data channel. Exclusion of centromeres occurs mostly based on too close proximity to other centromeres. Even though many centromeres are excluded, these measurements will always be orders of magnitude faster and less biased than doing the same by hand.

- e. Measure the centromere spots in the data channel (Fig. 8.8.6E).

A box of a set size is placed around the center of mass of an ROI. In these boxes, the maximum and minimum values of the data channel will be measured. The minimum is subtracted from the maximum, and that is represented as output. In addition, these boxes

are also saved as output. Note that no transformations or background subtractions, etc., are made to the data file before measuring. This means that you are actually measuring raw data. Alterations are only made (but not saved) in the other channels, and are used to efficiently localize centromeres. To exclude overlapping boxes, thus measuring the same spot twice, each box is made black after being measured (value = 0). The macro is programmed to exclude any box containing pixels of value 0. These black boxes are not saved to the data file, so that raw data is preserved. If there is a chromatic aberration, this can be set in the parameters (see above) and boxes are shifted accordingly before measuring. The saved output boxes are the ones that correspond to the reference channel.

SUPPORT PROTOCOL 1

EXPRESSION OF SNAP-FUSION PROTEINS

We use SNAP source vectors that include a triple HA-tag for efficient detection of SNAP-tagged proteins by immunoblotting or immunofluorescence. Maps of SNAP-3XHA, 3XHA-SNAP, and 3XHA-SNAPf vectors can be found under Supplementary Files at <http://www.currentprotocols.com/protocol/cb0808>. Fusion proteins are subsequently subcloned in transient expression vectors or in retroviral constructs (pBABE, see below) for stable expression.

For piloting SNAP fusion performance in living cells, we use standard transient transfection methods for obtaining SNAP protein expression. We transfect cells using liposome-based methods [e.g., Lipofectamine (Invitrogen) or Fugene (Roche) according to manufacturer's instructions] and assay protein expression and SNAP activity 48 hr after transfection.

For comprehensive experiments, we typically use monoclonal cell lines stably expressing SNAP fusions obtained by retroviral mediated transduction and selection. We use recombinant Moloney murine leukemia retroviral (MoMuLV) particles for the delivery of SNAP-tagged transgenes into host cell lines (e.g., HeLa or hTERT-RPE). This system is derived from a set of pBABE retroviral vectors (Morgenstern and Land, 1990), into which SNAP-tag fusion protein expression cassettes are cloned. Virus particles are assembled in HEK293-GP cells that express the essential MoMuLV gag and pol genes along with transient delivery of the vesicular stomatitis virus G protein (VSV-G), which results in a pantropic virus with a broad host cell range (Burns et al., 1993; Yee et al., 1994).

Materials

- HEK 293-GP cells (Burns et al., 1993).
- Standard culture medium (CM; see recipe)
- pBABE-Puro plasmid (Addgene, plasmid 1764), expressing SNAP-tagged protein
- pVSV-G plasmid (sold as part of Clontech kit, cat. no. 631530)
- Lipofectamine LTX (Invitrogen) and associated products
- 8 mg/ml Polybrene (hexadimethrine bromide; Sigma)
- Selection drugs (e.g., Blasticidin S, puromycin, or hygromycin; see annotation to step 12, below)
- Sterile PBS (cell culture grade; e.g., Invitrogen) containing 5% (w/v) bovine serum albumin (BSA)
- Conditioned medium (see recipe)
- 10-cm standard cell culture dishes
- 10-ml syringes
- 0.45- μ m syringe filters
- 6-well plates
- 96-well plates

Additional reagents and equipment for basic cell culture techniques including trypsinization (*UNIT 1.1*), and flow cytometry (Robinson et al., 2012)

Production of viral particles using pBABE-based retrovirus

1. Trypsinize and seed 1×10^6 HEK293-GP cells in a 10-cm dish and culture in CM using standard growth conditions.

Protocols for basic cell culture techniques including trypsinization are found in UNIT 1.1.

2. After 24 hr transfect cells with 5 μg pBABE plus 2 μg pVSV-G using 17.5 μl Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions.
3. Incubate cells using standard growth conditions and replace medium with CM after 4 hr or overnight incubation.
4. Incubate cells for 3 days for viral particle production.
5. Harvest the medium directly from the cells and filter through a 0.45- μm filter using a 10-ml syringe to avoid cellular contaminants.
6. Aliquot (1 ml) and freeze viral stocks at -80°C or use directly for infections.

Infection of target cells

7. Trypsinize and seed target cells into two wells of a 6-well plate, such that cells are at 30% to 40% confluency at time of infection.
8. Add 8 $\mu\text{g/ml}$ Polybrene immediately prior to addition of virus.
9. Add 250 μl viral stock (from step 6) to one well and 750 μl viral stock to the second well. Add CM to a final volume of 1 ml.
10. After 24 hr of infection, replace medium with fresh CM.
11. Let cells proliferate until they reach confluency (at least 24 hr after step 10).
12. Trypsinize cells, combine the two wells, and plate in a 10-cm dish containing CM with the appropriate drug selection.

We use pBABE vectors with Blastidicine S (Blast)-, puromycin-, or hygromycin-resistance cassettes. For example, HeLa cell clones are drug-selected with 5 $\mu\text{g/ml}$ Blast, 5 $\mu\text{g/ml}$ puromycin, or 250 $\mu\text{g/ml}$ hygromycin.

13. Select cells until colonies are visible to the naked eye (10 to 20 days).
14. Trypsinize (UNIT 1.1) and pool the clones and amplify for single-cell sorting.
15. To isolate monoclonal lines, wash cells in sterile PBS, resuspend in sterile PBS containing 5% BSA, and sort by standard flow sorting (using scatter to identify single cells) into 96-well plates containing conditioned medium.

Protocols for flow sorting are described in Robinson et al. (2012).

CELL FIXATION AND IMMUNOFLUORESCENCE

In this section, we describe a general method for fixation, immunofluorescence detection, and DAPI staining (of SNAP pulse-labeled cells). Immunofluorescence for detection of proteins unrelated to SNAP but localized at the same subcellular location allows for an independent measure to be used in image quantification using CraQ (see Basic Protocol 5 and Commentary). Please note that many other equally effective protocols for this purpose exist (e.g., UNIT 4.3). As this is a general protocol, we do not comment on specific antibody conditions and concentrations, as this will need to be determined for each specific application.

SUPPORT PROTOCOL 2

Cell Cycle Analysis

8.8.23

Materials

- 4% paraformaldehyde (PFA; Thermo Scientific) in PBS, 37°C
- 0.1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
- PBS-TX: 1 × PBS containing 0.1% (v/v) Triton X-100
- IF blocking buffer (see recipe)
- Primary antibodies
- Secondary fluorescently labeled antibodies (highly cross-absorbed to minimize cross reactivity between antibodies; Jackson ImmunoResearch)
- 1 mg/ml DAPI stock solution (see recipe)
- MOWIOL (see recipe)
- Clear nail polish
- Clean, sterile, poly-lysine coated (*APPENDIX 2A*) coverslips (12 mm diameter; 1.5 mm thickness)
- Fine forceps with sharp pointed ends
- Humid dark box: can be made from an empty micropipet tip-box, with a small layer of water and a thick sponge on the bottom—place a smooth glass plate, covered with a sheet of parafilm, on top of the sponge—use fresh parafilm for each experiment; any transparent surface of the box is covered with aluminum foil
- Standard glass microscope slides
- Additional reagents and equipment for pulse-chase (Basic Protocol 1), quench-chase-pulse (Basic Protocol 2), and integrating cell synchronization and RNAi in SNAP-based quench-chase pulse labeling experiments (Basic Protocol 3)

Cell fixation

1. Grow and SNAP pulse-label cells on glass coverslips in 24-well plates as described in Basic Protocols 1 to 3.
2. Wash cells twice with 1 ml sterile PBS (pre-heated to 37°C) as described in Basic Protocol 1, step 6.
Do not reincubate cells with CM, but immediately proceed to step 3 of this protocol.
3. Fix cells for exactly 10 min at room temperature in 500 µl of 4% PFA, preheated to 37°C.
4. Aspirate PFA and quench by adding 1 ml of 0.1 M Tris·Cl, pH 7.5, and incubating at room temperature for 5 min.

Cells can be stored at this point for up to a few days in PBS at 4°C, or up to 1 month in PBS containing 0.04% NaN₃ at 4°C. However, this is not the best practice after SNAP labeling, as the TMR-Star signal decreases over time, even if kept in the dark.

Antibody detection

5. Permeabilize cells by washing twice in 1 ml of PBS-TX for 5 min, as in step 2.
6. Carefully lift coverslips with a forceps and move to a parafilm-covered glass plate in a humid dark box.

Humid dark boxes prevent coverslips from drying and fluorescent dyes from photobleaching. Parafilm is a convenient receptacle for coverslips as its hydrophobic surface allows the application of small volumes to the coverslips without spilling over to neighboring coverslips.

7. Block cells for 30 min, 37°C in IF blocking buffer. Use 75 µl per coverslip.
8. Incubate cells with primary antibody diluted in IF blocking buffer for 60 min, 37°C. Use 30 µl per coverslip.

9. Wash coverslips in 75 μ l PBS-TX three times, each time for 5 min at room temperature, as in step 2.
10. Incubate cells with secondary fluorescent antibody diluted in blocking buffer for 45 min, 37°C. Use 30 μ l per coverslip.

Centrifuge diluted fluorescent antibodies for 5 min at maximum speed ($\sim 16,000 \times g$) to deplete any fluorescent aggregates that may interfere with fluorescent imaging. Use supernatant for staining.
11. Wash coverslips in 75 μ l PBS-TX three times, each time for 5 min at room temperature, as in step 2.
12. Incubate cells in 50 μ l of 500 ng/ml DAPI (prepared from 1 mg/ml stock) for 5 min at room temperature.
13. Replace DAPI solution with PBS.
14. Carefully pick up coverslips with a forceps, remove excess liquid by aspiration and/or filter paper, and mount on a glass slide (cells facing down) in ~ 5 μ l Mowiol. Allow the Mowiol to solidify overnight at 4°C in the dark.
15. Seal coverslips using nail polish to avoid air contact and micro-movements of coverslips during the imaging process.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

BTP (bromothetylpteridine)

A 2 mM stock is prepared by dissolving 100 nmol lyophilized SNAP-Cell Block (New England Biolabs, cat. no. S916S) in 50 μ l DMSO (sterile). Shake for 10 min in an Eppendorf shaker at maximum speed to dissolve. Store up to 1 month at -20°C or aliquot and store indefinitely at -80°C .

Conditioned culture medium (for HeLa)

Combine 50% (v/v) fresh standard culture medium (see recipe) with 50% (v/v) culture medium harvested from HeLa cultures in log growth phase, filtered through a 0.45- μM filter. Store up to 2 weeks at 4°C.

DAPI(4',6-diamidino-2-phenylindole dihydrochloride)

A 1 mg/ml stock is prepared in water. Dilute 2000-fold in 1 \times PBS (*APPENDIX 2A*) for working solution. Alternatively, predilute DAPI 20-fold in 1 \times PBS for a more convenient 100 \times stock solution. Either stock can be stored indefinitely at -20°C .

IF blocking buffer

1 \times PBS (*APPENDIX 2A*) containing:
2% (v/v) fetal bovine serum (FBS)
2% (w/v) bovine serum albumin (BSA)
0.1% (v/v) Triton X-100
0.04% (w/v) NaN_3
Store indefinitely at -20°C

Working stocks can be stored at 4°C for several months to minimize freeze-thaw cycles.

Live imaging medium

Phenol red-free, CO₂-independent medium (e.g., DME or Leibovitz's L-15; Invitrogen) supplemented with:

10% (v/v) fetal bovine serum (FBS; Invitrogen)

2 mM glutamine (Invitrogen)

Store up to 2 months at 4°C

MOWIOL

1. Mix Mowiol 4-88 (Calbiochem) and glycerol (Sigma) at a 2:5 (w/w) ratio.
2. Add 714 µl water per g of Mowiol/glycerol mixture and stir overnight at room temperature.
3. Add 2 volumes of 0.2 M Tris-Cl (pH 8.5) for each volume of water added and heat at 50°C for 10 min with occasional mixing.
4. Centrifuge at 5000 × g for 15 min and remove debris.
5. Add (1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) to 2.4% (w/v) and mix slowly.
6. Centrifuge at 5000 × g for 15 min and remove debris.
7. Aliquot and store indefinitely at –20°C.

Standard culture medium (for HeLa and HEK293-GP)

DMEM medium containing:

10% (v/v) NCS (newborn calf serum; Invitrogen)

1 × antibiotics (100 U/ml penicillin, 100 µg streptomycin; add from 100× stock; Invitrogen)

2 mM glutamine (Invitrogen)

Store up to 1 month at 4°C

Other cell types may require different growth media.

TMR-Star

A 200 µM stock is prepared by dissolving 30 nmol lyophilized SNAP-Cell TMR-Star (New England Biolabs, cat no. S9105S) in 150 µl DMSO (sterile). Shake for 10 min in an Eppendorf shaker at maximum speed to dissolve. Store for 1 month at –20°C or aliquot and store indefinitely at –80°C.

VALAP

Vaseline:lanolin:paraffin 1:1:1 (w/w) (adapted from: Waterman-Storer and Salmon, 1997):

1. Heat paraffin to 65°C in a large beaker in a water bath in a chemical hood.
2. When paraffin is melted, mix in vaseline and lanolin.
3. Stir to mix until the mixture has become completely homogenized.
4. Aliquot and store indefinitely at 4°C.
5. Heat to 65°C prior to use.

COMMENTARY

Background Information

Historical background

The SNAP tag is a modified version of human O⁶-alkylguanine-DNA alkyltransferase (hAGT). Endogenous hAGT is a DNA-repair enzyme that removes a broad range of alkyl adducts from the O⁶ position of guanines in

DNA. It acts as a suicide enzyme that catalyzes a covalent binding reaction between itself and the alkyl group that is removed from guanines, thereby restoring DNA integrity but inactivating its own catalytic activity (Pegg, 2000). SNAP, the modified form of hATG, has lost its affinity to DNA but efficiently

Table 8.8.2 Selection of SNAP-Substrates^a

SNAP-substrate labels	Specifications	Examples	References
Quenchers	Used to block (quench) pre-existing pools of SNAP protein to prevent their detection in subsequent labeling steps	BG, BTP	NEB ^b
<i>Fluorescent substrates</i>			
Standard fluorophores ^c	Used for most microscopy based pulse-labeling techniques	TMR-Star; BG-AF; BG-Cy3	NEB ^b , Keppler et al. (2004)
Dark-dyes (induced quenching) ^d	Reduced fluorescence prior to binding to SNAP. Allows for wash-free labeling and better signal to noise.	CBG-549-QSY7, DRBGFL	Sun et al. (2011); Komatsu et al. (2011)
Dark-dyes (natural quenching) ^e	Reduced fluorescence prior to binding to SNAP. Allows for wash-free labeling and better signal to noise.	BG-MR121	Stöhr et al. (2010)
Caged dyes ^f	Substrate that becomes fluorescent after UV-activation (analogous to photo-activatable fluorescent proteins)	^{N9} NPE-BG-FL; BG-CMNB-caged	Banala et al. (2008); Campos et al. (2011)
Photo-activatable dyes ^g	Used for PALM/STORM of SNAP-labeled proteins	BG-Cy3-Cy5	Maurel et al. (2010); Dellagiacomma et al. (2010)
<i>Protein purification substrates</i>			
Beads ^h	Used for SNAP protein extraction from cell lysates	BG-beads (agarose or magnetic)	NEB ^b
Biotinylation	Used for SNAP protein extraction from cell lysates, using streptavidin beads	BG-Biotin	NEB ^b
<i>Other types of substratesⁱ</i>			
Photosensitizer	Used to selectively sensitize cancer cells to non-hazardous light after fusing SNAP to an antibody that recognizes tumor cells	BG-Ce6	Hussain et al. (2011)
Anti-obesity drugs	Used to deliver a drug to the correct subcellular compartments for optimal activity in vivo	BG-THL	Yang et al. (2011)
Thiol	Used for generation of self-assembled-monolayers (SAM) by microcontact printing	BG-Thiol	Engin et al. (2010)

^aThis is an incomplete list; many more SNAP substrates have been described.

^bNew England Biolabs.

^cA broad spectrum of colors have been reported that range from ultraviolet to infrared fluorescent dyes (e.g., available from NEB and in Keppler et al., 2004, 2006, Bojkowska 2011).

^dFluorescent BG substrates are labeled to a second sidegroup that quenches the fluorescence by FRET. After protein labeling, the two sidegroups are spatially removed, leading to increased fluorescence.

^eAs above, except that fluorophores are used that are naturally quenched by guanine, alleviating the need for adding a second (bulky) sidechain.

^fCaged dyes have a photoremovable group that quenches fluorescence and is chemically removed upon irradiation with UV light.

^gOne reason to use these dyes for superresolution microscopy is their increased brightness as compared to fluorescent proteins—a limiting factor for these techniques.

^hSNAP enzyme activity must be preserved in the lysate, as binding occurs through reaction with SNAP.

ⁱBG derivatives can be readily synthesized by standard biochemical methods for essentially any kind of sidegroup, ranging from relatively small molecules (e.g., fluorophores, Keppler et al., 2004) to very large thiol molecules (e.g., matrix thiol, Engin et al., 2010). A small number of interesting examples is given.

reacts with soluble O⁶-benzylguanine (BG), of which the benzyl moiety is readily transferred to the SNAP protein (Fig. 8.8.1, Juillerat et al., 2003; Keppler et al., 2003). The benzyl rings in BG can be coupled to a large variety of molecules (Keppler et al., 2003, 2004, 2006) that include fluorescent moieties as well as nonfluorescent ones (a selection of SNAP substrates is presented in Table 8.8.2).

General considerations for SNAP-based protein turnover assays

A number of techniques exist to analyze protein turnover (Table 8.8.1). A common approach to in vivo protein turnover is the use of fluorescence recovery after photobleaching (FRAP). In this method, autofluorescent proteins are fused to proteins of interest that localize to a specific subcellular location. Local irreversible bleaching followed by repopulation of a bleached area by unbleached molecules from neighboring regions provides information on the local rate of protein turnover (see Lippincott-Schwartz et al., 2001, and references therein). A reciprocal technique utilizes inducible fluorescent proteins, which can be activated by a focused laser, which allows tracking of a specific pool of photo-activated protein (Lukyanov et al., 2005; and references therein). While widely applied, FRAP and photo-activation experiments suffer from three specific drawbacks: (1) measurement of fluorescence recovery or photoactivation typically requires continued imaging of cells, leading to problems such as photobleaching and phototoxicity, thereby restricting the time in which turnover can be measured to a few hours at most. This precludes measurement of long-term turnover rates. (2) A focused laser is required to bleach or activate fluorescence, preventing the analysis of large numbers of cells simultaneously. Lastly, (3) the turnover rates using FRAP and photo-activation are a product of the “on” and “off” rates of a protein which cannot be assessed separately. SNAP-based pulse labeling differs from traditional FRAP experiments in that a fluorescent pool is created by pulse labeling with the addition of an external dye to the culture medium. Therefore, first and foremost, imaging and quantification of fluorescence can commence at any time following labeling. This allows analysis of protein turnover at very long time scales (hours to days after pulse labeling). Secondly, because the entire cell population is treated with the dye in bulk, large numbers of cells are available for simultaneous imaging and analysis. Lastly, the combination of serial dark and fluorescent

pulse labeling strategies (“pulse-chase” and “quench-chase-pulse”) allows for the separate determination of turnover of pre-existing pools (off-rates) and turnover of newly synthesized pools of protein (on-rates) (Figs. 8.8.2-3).

Several other methods capitalize on similar advantages such as other self-labeling or destructive enzymes (see Table 8.8.1). We would like to highlight one recently developed method named “Recombination Induced Tag Exchange” (RITE), which allows for similar applications as SNAP-tagging while using a fundamentally different strategy (Verzijlbergen et al., 2010). It uses Cre-mediated recombination-induced switching of expression of differentially tagged versions of the same gene. This allows for the simultaneous visualization, tracking, and/or analysis of the original (pre-switched) pool as well as a nascent one (Radman-Livaja et al., 2011). However, this method relies on tight control over induction of Cre-mediated recombination, which is difficult to achieve in some systems (most metazoan cell lines).

The advantage of assessing long-term dynamics also implies a major disadvantage of SNAP-based pulse labeling. Labeling and washing steps require approximately 1 hr, rendering this method inappropriate to assess protein dynamics at short timescales (seconds to minutes), as pulse-labeled proteins will have reached their steady-state equilibrium before imaging can determine their dynamics. However, improvements are currently being made to both the SNAP-enzyme and the fluorescent substrates thereof, which would in principle allow labeling steps of 5 min without the need for any washes (see below and Sun et al., 2011).

Critical Parameters and Troubleshooting

SNAP Labeling

Choice of substrate: One very important parameter during the pulse-chase and quench-chase-pulse procedure in living cells is the choice of SNAP-substrate used. The limiting characteristic seems to be the ability of substrates to efficiently pass the cell membrane, as many substrates tend to strongly label the cell membrane while barely labeling intracellular SNAP proteins. In our experience, nonfluorescent benzylguanine (BG) or bromothenylpteridine (BTP) enter cells efficiently. However, addition of (bulky) side groups may impede the cell permeability.

Thus, although there are a large variety of fluorescent substrates for intra-cellular

labeling, the efficiency at which these enter the cells is not always the same. For this reason, using the optimal fluorophore for the particular microscopy and filter setup used has to be balanced with the cell permeability of the substrate. We generally obtain the best results with SNAP-Cell TMR-Star (New England Biolabs).

It is for this reason that we prefer to use BTP for quench steps in the quench-chase-pulse procedures, rather than sequentially using different fluorescent substrates (see Basic Protocol 2), because complete labeling of the initial pool is essential to ensure visualization of the subsequent newly synthesized pool only.

Of special interest are a group of recently developed SNAP substrates that display a dramatic increase in fluorescence after reaction with SNAP. These so called 'dark-dyes' are either quenched by guanine itself (Stöhr et al., 2010) or by a side-group fused to the guanine moiety of benzylguanine (Komatsu et al., 2011; Sun et al., 2011). These dark-dyes provide a number of advantages over traditional fluorescent SNAP-substrates, most importantly leading to highly reduced (unspecific) background fluorescence. Other advantages include wash-free labeling, faster downstream applications (due to shorter wash steps), and potentially more efficient live cell imaging.

In Table 8.8.2 we present a selection of (commercially available) SNAP substrates with their respective properties.

SNAP enzyme variants: Variants of SNAP have been derived by in vitro evolution. One example is the "CLIP-tag," which is derived from SNAP and reacts specifically with a variant substrate, O²-benzylcytosine (Gautier et al., 2008). Tagging of two different proteins by SNAP and CLIP allows for simultaneous labeling of two different proteins in different colors (Gautier et al., 2008; Prendergast et al., 2011). More recently, variants of SNAP and CLIP named SNAPf and CLIPf have been developed that present faster reaction kinetics (Pellett et al., 2011; Sun et al., 2011). We evaluated SNAPf and CLIPf performance in vivo by side-by-side comparison with SNAP and CLIP, using the intracellular protein CENP-A as a labeling target (data not shown and Fig. 8.8.7A). While CLIPf showed only a modest improvement over CLIP (not shown), SNAPf performed ~3- to 5-fold better across different concentrations of substrates and incubation times (Fig. 8.8.7B). The use of SNAPf therefore allows for shorter labeling times and lower dye concentrations to yield the

same signal intensity. A reduced background staining while retaining specific signals will potentially improve live cell capabilities significantly.

Dye concentration, wash steps, and pool size: Depending on the cell type, SNAP protein fusion expression levels, particular SNAP substrate, application, etc., it will be necessary to optimize the substrate concentration. More substrate is not necessarily better, because addition of substrate beyond saturation of labeling will result in more background labeling and thus poorer signal-to-noise ratios. For CENP-A-SNAP, we generally use a concentration of 2 μ M TMR-Star as a compromise between signal-to-noise and cost (although we have found that using higher concentrations up to 5 μ M increases the signal-to-noise ratio of labeling). For other purposes, it may be necessary to use saturating concentrations, or conversely, it may be sufficient to use lower concentrations.

We found that extensive washes after labeling (two quick washes, an extended wash for 30 min at 37°C, and two additional quick washes) help to remove excess unbound substrates. This results in dramatically decreased background fluorescence after pulse labeling. During quench labeling, these wash steps ensure that nascent protein synthesized during the chase is not immediately quenched, which would lower the effective pool size of the new pool and reduce specific signals in subsequent fluorescent labeling.

Chase time: A critical aspect of a successful quench-chase-pulse experiment is the chase time that the cells are given to produce new protein. Although this is largely determined by the experimental conditions, one would typically seek conditions that maximizes the time for protein synthesis prior to labeling.

Imaging and quantification

For imaging of SNAP-derived and immunofluorescent signals, any high-resolution microscope can be used as long as appropriate laser lines and filters are present. However, a detailed description of microscope setup and parameters is outside the scope of this unit. For general tips on appropriate microscope and imaging techniques and strategies, see e.g., Wolf et al. (2007) and Waters (2009).

Marker used as reference: Special care should be taken to choose the marker used as a reference for spot detection. A number of options exist. (1) The signals that require quantification can be used simultaneously as a reference of spots to measure. However, this

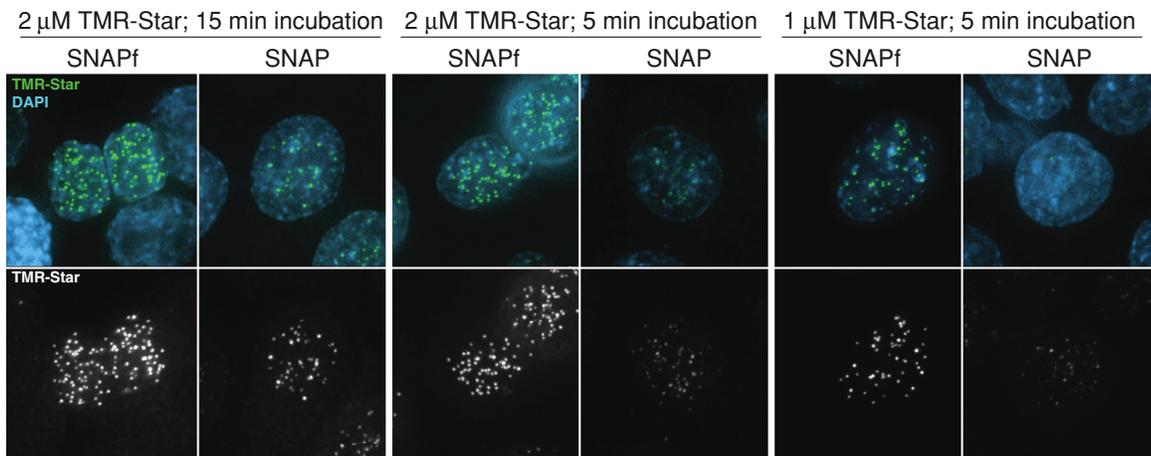
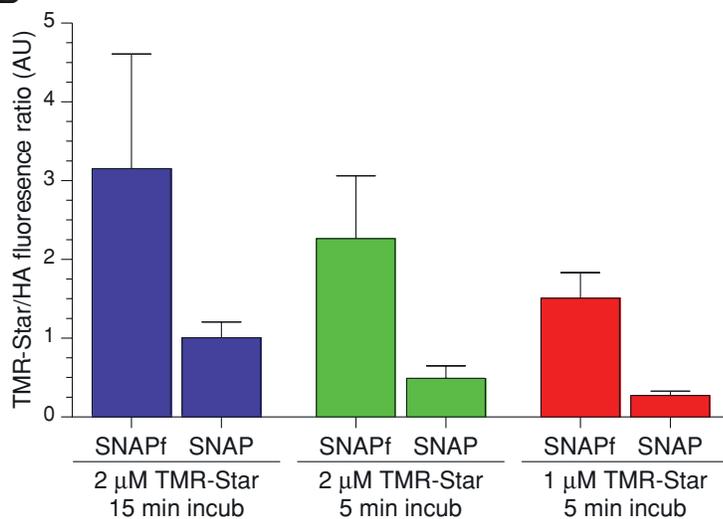
A**B**

Figure 8.8.7 Evaluation of SNAPf-tag performance. **(A)** HeLa cells were transfected with either CENP-A-SNAP or CENP-A-SNAPf fusion proteins, and labeled with TMR-Star at different concentrations and incubation times, as indicated in the figure. Representative images of cells are shown with TMR-Star signals in green and DAPI (DNA) in blue. **(B)** TMR-Star and HA fluorescence intensity were determined using CRaQ (Basic Protocol 5) and TMR-Star/HA ratios are used as a measure of SNAP or SNAPf activity. Results are plotted as fold difference, normalized to signals obtained with SNAP after incubation with 2 μ M TMR-Star for 15 min (standard conditions). SNAPf outperforms SNAP in all conditions tested (3 to 5 fold). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cb0808>.

solution suffers from the drawback that spots with very low signals will not be detected and that the detection will be inherently biased, e.g., towards bright spots. A better option is to (2) use an antibody against SNAP (available from NEB) or HA (in case an HA-tag is incorporated in the fusion protein; see Supplementary Files at <http://www.currentprotocols.com/protocol/cb0808>), which will detect the entire pool of SNAP-tagged protein independently of time-sliced signals (see e.g., Figs. 8.8.3B and 8.8.4B). However, if the protein of interest forms aggregates or has mul-

tiple possible localization patterns, these will also be quantified by automated methods such as CRaQ. Thus, whenever possible, we prefer to use (3) antibodies (or autofluorescent fusion proteins) against an independent marker for the subcellular structure (e.g., centromeres by CENP-C or CENP-T; see Figures 8.8.2B, 8.8.4C, and Silva et al., 2012). This allows for specific and unbiased detection of spots. Naturally, clean references will lead to the most accurate quantifications, and using antibodies that are highly specific and give little background staining will increase the quality of the

data. In addition, when measuring proteins that reside inside the nucleus, an additional marker such as DAPI can be used to further exclude (unspecific) reference signals outside of the nucleus.

CRAQ: There are a number of critical aspects to take into account when using CRAQ. First and foremost, as this is an automated algorithm, the results should be validated by the user. After initiating the macro, one can follow the screen shots that pop up to monitor which spots are recognized as reference points. If the macro is poorly tuned, it may already be obvious at this early stage (e.g., recognition of the entire image). Next, after completion of the macro, data output files should be checked to validate whether the correct spots are detected (e.g., by doing this manually for a small, random subset of pictures and comparing this to the spots recognized automatically). If automated spot recognition is not accurate, the parameters should be optimized as described in Basic Protocol 5. Parameter optimization and testing is best done on a small subset of pictures to save time.

Evidently, using a high-end microscope with appropriate filter combinations and a sensitive camera is instrumental to obtain good fluorescence quantifications. In addition, potential chromatic aberrations between reference and data channels must be corrected for in the quantification (this can also be set as a parameter of CRAQ). One way to determine the chromatic aberration is to use beads that are fluorescent in the two channels used and determine whether and by how many pixels the center of mass is shifted between the colors.

In principle, a flatfield correction should be performed on all images prior to quantification, to correct for unequal illumination of the sample. Flatfield corrections can be performed directly while imaging in SoftWorX imaging software (Applied Precision), or post-acquisition. Protocols can be found e.g., in Wolf et al. (2007), or, alternatively, using the “shading corrector” plugin for ImageJ (freely available from <http://rsb.info.nih.gov/ij/plugins/shading-corrector.html>).

Finally, although inorganic dyes are generally very photostable, we have observed that imaging TMR-Star labeled cells as soon as possible after fixation (1 to 2 days) facilitates obtaining the most optimal signals.

Live-cell imaging

Typically, for live-cell imaging of mammalian cells, a heated chamber on the microscope setup is required to maintain both the

cells and the microscope stage at the appropriate temperature.

Fluorescent SNAP substrates are based on organic dyes (e.g., TMR). Bleaching is therefore not as big a concern as with live imaging of autofluorescent proteins such as GFP or RFP. However, due to nonspecific labeling (of membranes), background signals are relatively high as compared to autofluorescent proteins. Exposure times, laser strength, neutral-density filter settings, and choice of temporal resolution largely depend on signal strength and considerations of cellular phototoxicity.

Anticipated Results

SNAP-labeling

Because SNAP substrates are added to the culture medium, virtually all SNAP-expressing cells are labeled in any given experiment. The ability to detect SNAP-tagged proteins depends on the expression level of the protein and the efficiency of SNAP substrate entry into the cells. In quench-chase-pulse experiments, the chase time during which cells synthesize and assemble new protein will determine which cells will become labeled during the second, fluorescent labeling step. In the case of CENP-A-SNAP, the appearance of centromeric signals will largely depend on cell-cycle position (Figs. 8.8.4B and 8.8.5). The expected results for other proteins will depend on the biological properties of the protein of interest.

Many SNAP substrates have difficulty passing through the cell membrane. For this reason, it is normal to see relatively high background fluorescence, as compared to, e.g., antibody or fluorescent protein detection. We try to minimize this background fluorescence by extensive washes of the fluorescent substrate after labeling is completed (e.g., steps 6 to 7 of Basic Protocol 1).

Image quantification

Using CRAQ, we generally have very low false-positive rates, where off-target sites or doublets comprise $<<1\%$ of all spots detected. In addition, this macro is generally able to detect a good proportion of the correct spots to be analyzed ($\sim 50\%$), although this largely depends on the quality of the reference signal. Using a generic present-day desktop computer, we can readily collect hundreds to thousands of data points in 15 to 20 min. The rate-limiting steps are testing parameter settings (although generic parameter settings usually work very well) and analyzing the data generated.

Time Considerations

The time that is required for the experiments outlined above is highly variable and depends on the precise setup of the experiment. Quench and pulse labeling each take about 1 to 1.5 hr to perform. However, the chase time can be anywhere between a few hours and a few days. Furthermore, adding sequential steps, such as synchronization and/or RNAi procedures, can increase the total time of the experiment to more than a week. Fixation and antibody labeling require approximately 4 to 5 hr to perform, and cells are preferentially imaged on the following day. Imaging requires roughly 30 min per coverslip used, although this again depends on many factors, including the microscopy system, signal intensity (i.e., exposure times needed), cell density (i.e., number of images required), sample thickness (i.e., number of slices required), etc. Running CRaQ generally takes no more than 20 min, even for large datasets, and validation of the output takes about the same time. Finally, processing of the output data into comprehensible tables/graphs takes about 30 min to 1 hr, depending on the size of the dataset.

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Key References

Jansen et al., 2007. See above.

First description of use of SNAP-tag for determination of protein turnover and assembly using pulse-chase and quench-chase-pulse experiments.

Kepler et al., 2003. See above.

First description of use of SNAP-tag in living cells for fluorescent labeling of proteins.

Internet Resources

<http://rsbweb.nih.gov/ij/index.html>

ImageJ software and Grouped_ZProjector plugin.

<http://uic.igc.gulbenkian.pt/micro-macros.htm>

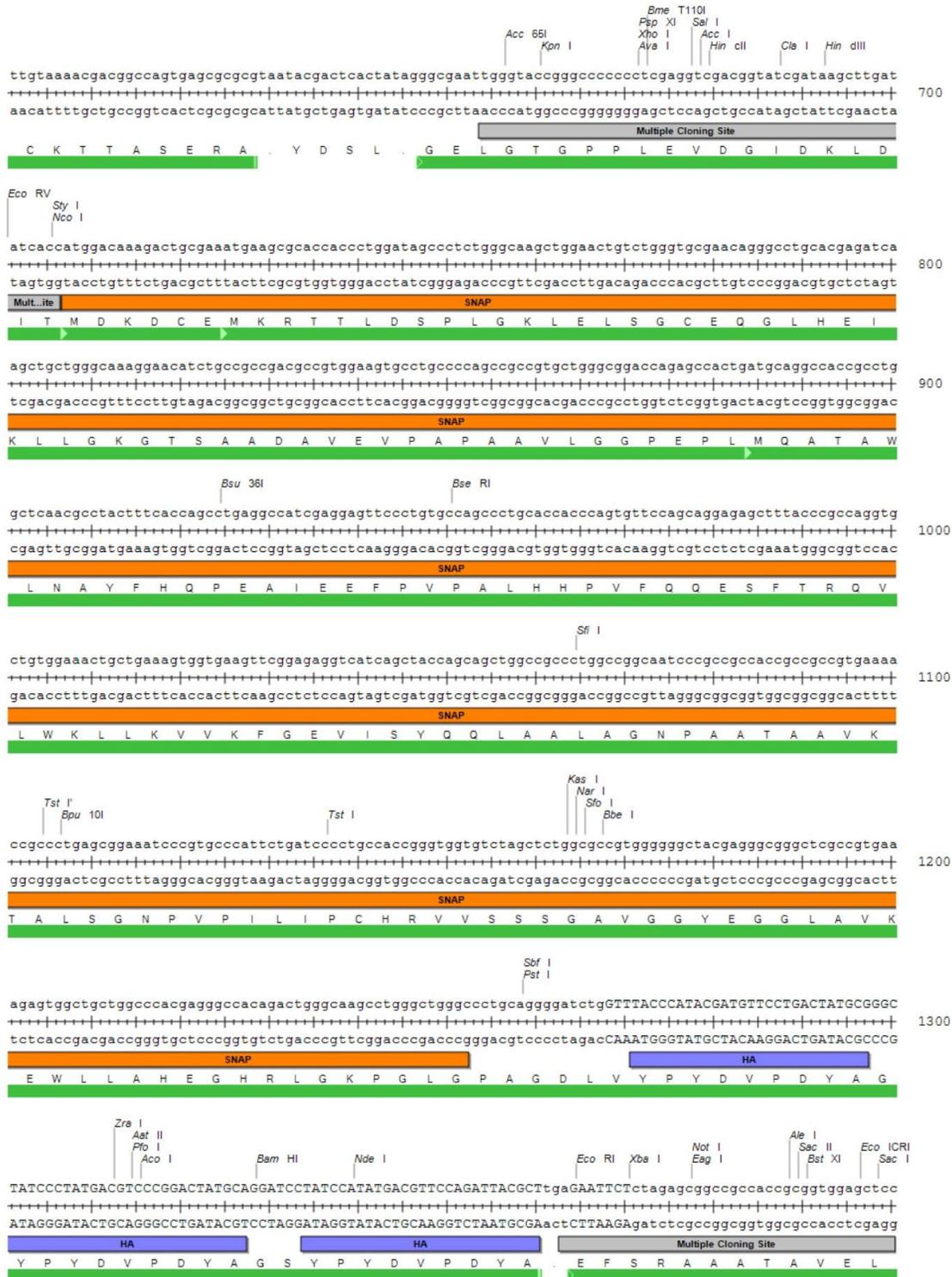
CRaQ macro for ImageJ.

<http://rsb.info.nih.gov/ij/plugins/shading-corrector.html>

Shading corrector plugin for ImageJ.

C-terminal SNAP-3XHA tag

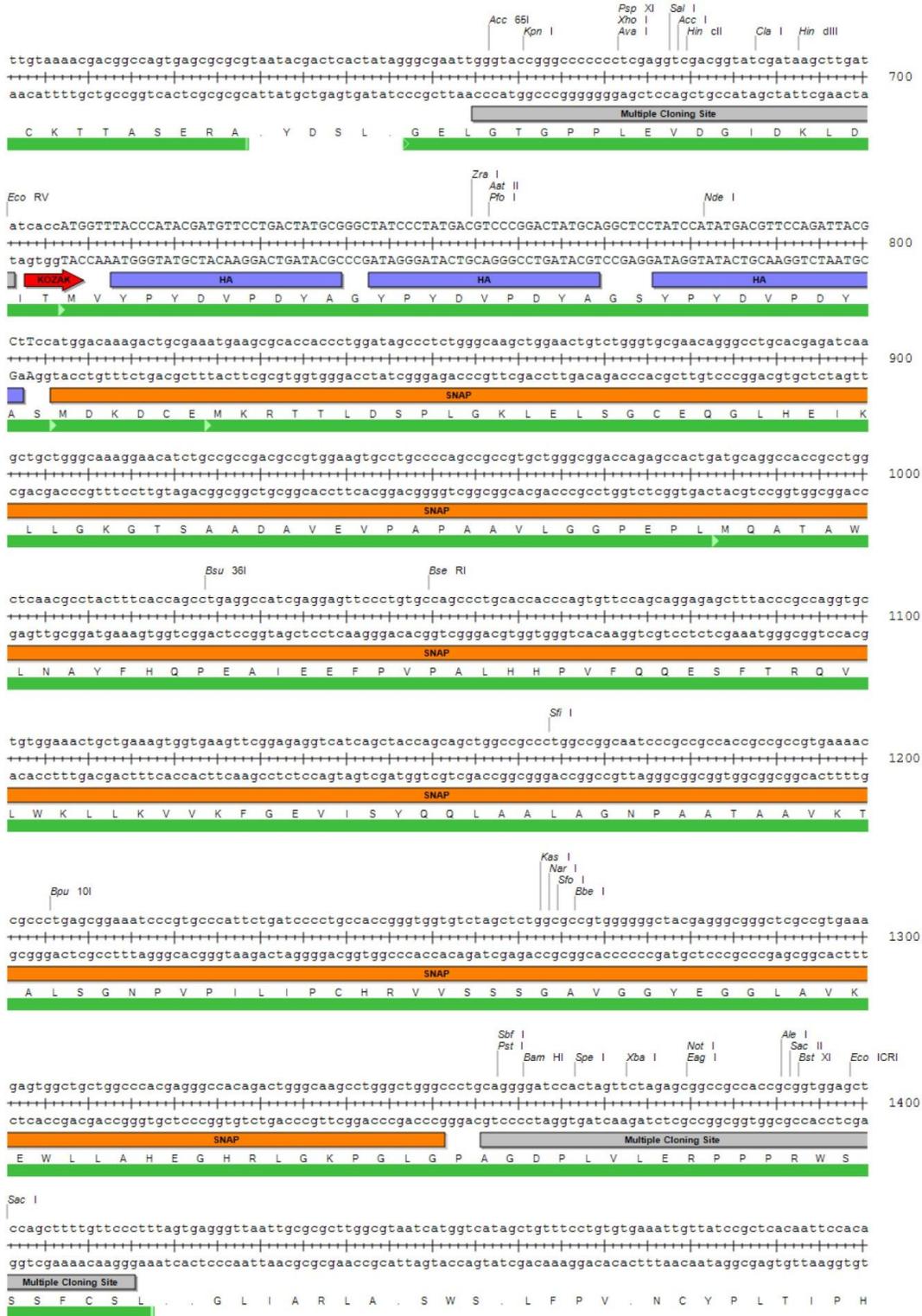
pLJ222 – constructed into the pSS26m backbone; a promoter-less source vector (Amp^r) (Jansen et al., 2007)
unique sites are indicated



N-terminal 3XHA-SNAP tag

pLJ223 – constructed into the pSS26m backbone; a promoter-less source vector (Amp^r) (Jansen et al., 2007)

unique sites are indicated



N-terminal 3XHA-SNAPf-2XPreScission tag

pLJ470 – constructed into the pSS26m backbone; a promoter-less source vector (Amp^r) (Jansen et al., 2007)
unique sites are indicated

