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Pulse-Chase Labeling Techniques for the Analysis of Protein Maturation and Degradation

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1. Introduction

Pulse-chase experiments have proved to be a powerful tool to study protein folding, maturation, and degradation in mammalian cells. When short pulses are applied, a fraction of the total protein pool can be followed from synthesis to degradation in its natural environment.

The technique was successfully used with a number of endogenous and viral proteins: IgA *(1)*, thyroglobulin *(2)*, Influenza hemagglutinin *(3)*, vesicular stomatitis virus G protein *(4)*, and HIV-1 Envelope glycoprotein *(5)*.

This protocol describes a basic pulse-chase assay for adherent cells and cells in suspension. With minor modifications, described in the Notes section, this protocol can be adapted to study disulfide-bond formation, folding, oligomerization, maturation and other processes as well as degradation of proteins, in both adherent and suspension cells.

Pulse chase experiments can be divided into three stages: 1) the actual pulse chase, 2) immunoprecipitation of the protein of interest, and 3) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Fig. 1**). At the end of each stage, samples in principle can be snap-frozen in liquid nitrogen and stored at –80°C.

2. Materials

2.1. Pulse-Chase

1. Adherent cell line expressing protein of interest grown in sterile 60-mm tissue-culture dishes (*see* **Note 1**).
2. 37°C humidified 5% CO_2 incubator.

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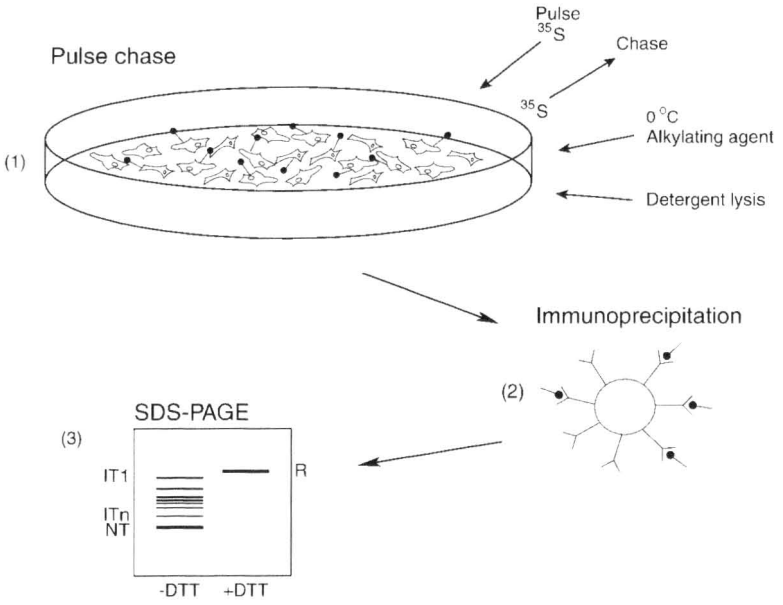

Fig. 1. Pulse-chase experiments can be divided into three stages: *(1)* the actual pulse chase, *(2)* immunoprecipitation of the protein of interest, and *(3)* SDS-PAGE analysis.

3. 37°C waterbath with nonfloating racks to hold tissue-culture dishes.
4. Aspiration flask suitable to collect liquid radioactive waste in a safe manner.
5. Flat, wide ice pan with fitted aluminium plate (e.g., VWR Scientific), covered with wet tissue paper to increase cooling of dishes.
6. HEPES (*N*-2-hydroxyethylpiperidine-*N'*-ethanesulfonic acid): 1 *M* in H_2O, pH 7.4, store at 4°C.
7. Methionine: 250 m*M* in H_2O, store at –20°C.
8. Cysteine: 500 m*M* in H_2O, store at –20°C.
9. *N*-ethylmaleimide (NEM): 1 *M* in ethanol, store at –20°C.
10. Ethylenediaminetetraacetic acid (EDTA): 200 m*M* in H_2O, pH 6.8.
11. Phenylmethylsulfonyl fluoride (PMSF): 1 *M* in dry isopropanol; store at –20°C.
12. Protease inhibitors: 10 mg/mL each of chymostatin, leupeptin, antipain, and pepstatin in dimethyl sulfoxide (DMSO), store at –20°C.
13. Wash buffer: Hank's balanced salt solution (HBSS; Invitrogen) at 37°C.
14. Depletion medium: cysteine and methionine-free tissue-culture medium (ICN) containing 10 m*M* HEPES, pH 7.4 at 37°C.
15. Labeling medium: cysteine and methionine-free tissue-culture medium containing 10 m*M* HEPES, pH 7.4, 125–250 µCi [^{35}S]-cysteine and/or methionine/mL; at 37°C; prepare fresh from stocks (*see* **Notes 2** and **3**).

16. Chase medium: complete tissue-culture medium appropriate for the cell line used, containing 10 m*M* HEPES, 5 m*M* cysteine, 5 m*M* methionine; at 37°C; prepare fresh from stocks (*see* **Notes 4** and **5**).
17. Stop buffer: HBSS containing 20 m*M* NEM; at 4°C (*see* **Notes 5** and **6**).
18. Iodoacetamide: 500 m*M* in H_2O, store at -20°C.
19. Iodoacetic acid: 500 m*M* in H_2O, store at -20°C.
20. Lysis buffer: phosphate-buffered saline (PBS), pH 7.4, or similar salt-containing buffer, containing 0.5% (v/v) Triton X-100, 1 m*M* EDTA, 20 m*M* NEM, 1 m*M* PMSF, protease inhibitors: 10 µg/mL each of chymostatin, leupeptin, antipain, and pepstatin (*see* **Notes 5–7**).
21. Cycloheximide: 50 m*M* in H_2O, store at –20°C.
22. 5 mL disposable pipet.
23. 1–10 mL dispenser.
24. Timer with seconds indication.
25. Cell scraper.
26. Liquid nitrogen.

2.2. Immunoprecipitation

1. 10% Protein A-Sepharose beads in PBS or similar buffer containing one-tenth of the concentration of same detergent used in lysis buffer and 0.1% bovine serum albumin (BSA) (*see* **Notes 8** and **9**).
2. Antibody against protein of interest.
3. Head over head rotator.
4. Eppendorf tube shakers.
5. Immunoprecipitation wash buffer: PBS, pH 7.4, containing 0.5% (v/v) Triton X-100 or 150 m*M* NaCl (*see* **Note 9**).
6. TE buffer: 10 m*M* Tris-HCl, pH 6.8, 1 m*M* EDTA.
7. Heat block at 95°C.
8. 200 m*M* DTT in H_2O stock; store at –20°C.
9. 2X concentrated nonreducing sample buffer: 400 m*M* Tris-HCl, pH 6.8, 6% (w/v) SDS, 20% glycerol, 2 m*M* EDTA, 0.01% (w/v) bromophenol blue.
10. 5X concentrated sample buffer: 1 *M* Tris-HCl, 7.5% (w/v) SDS, 50% glycerol, 5 m*M* EDTA, 0.02% (w/v) bromophenol blue; store at –20°C.

2.3. SDS-PAGE Analysis

1. SDS-PAGE equipment, preferably minigel systems (BioRad or Amersham-Pharmacia).
2. Coommassie stain: 0.25% (w/v) Coommassie brilliant blue in destain.
3. Destain: 30% (v/v) methanol and 10% (v/v) acetic acid in H_2O.
4. Neutralizer: 30% (v/v) methanol in PBS (*see* **Note 10**).
5. Enhancer: 1.5 *M* sodium salicylate in 30% (v/v) methanol in H_2O.
6. Schleicher & Schuell filter paper 0.4 mm.
7. Gel drying equipment.

3. Methods

3.1. Pulse-Chase (Basic Protocol)

Two alternative pulse-chase procedures, one for adherent cells (**Subheading 3.1.1.**) and one for suspension cells (**Subheading 3.1.2.**) are given.

3.1.1. Pulse-Chase for Adherent Cells

The volumes described here are based on 60 mm tissue culture dishes. When other sizes are used, volumes must be adjusted based on surface area of the dish.

1. Seed cells expressing the protein of interest in 60 mm dishes (*see* **Note 1**). On the day of the experiment the cells need to be subconfluent (90%). At least one dish per time point is needed.
2. Prepare the pulse chase set up (**Fig. 2**).
3. Wash cells with 2 mL wash buffer and add 2 mL depletion medium. Incubate cells for 15 min at 37°C in an humidified 5% CO_2 incubator (*see* **Note 11**).
4. Place the cells on the racks in the waterbath (37°C), make sure the water level is just above the racks and air bubbles do not accumulate under the dishes.
5. Pulse-label the cells, one dish at a time, by quickly aspirating the depletion medium and adding 400 µL labeling medium to the centre of the dish (for pulse times longer than 15 min a larger volume and incubation on a rocker in a 37°C humidified 5% CO_2 incubator is recommended). Swirl gently to equally divide the labeling medium over the cells, make sure no air bubbles accumulate under the dish. Incubate for the pulse period (*see* **Notes 2**, **3** and **11**).
6. For 0-min chase interval:
   a. Add 2 mL chase medium at precisely the end of the labeling interval to stop labeling instantly. Swirl gently to mix.
   b. Aspirate the chase medium as quickly as possible. Place the dish on the aluminium plate on the ice pan and immediately add 2 mL ice cold stop buffer.
7. For all other chase intervals:
   a. Add 2 mL chase medium at precisely the end of the pulse interval to stop labeling instantly. Swirl gently to mix. Aspirate chase medium and again add 2 mL chase medium. Incubate for the desired chase intervals on the 37°C waterbath for short intervals or in a 37°C humidified 5% CO_2 incubator for chase intervals over 30 min.
   b. At precisely the end of the chase interval, aspirate the chase medium. Place the dish on the aluminium plate on the ice pan and add 2 mL of ice-cold stop buffer.
8. Just before lysis, wash the cells again with 2 mL ice-cold stop buffer.
9. Aspirate dish as dry as possible and add 600 µL ice-cold lysis buffer.
10. Scrape cell remains off the dish with a cell scraper and transfer the lysate to an Eppendorf tube.

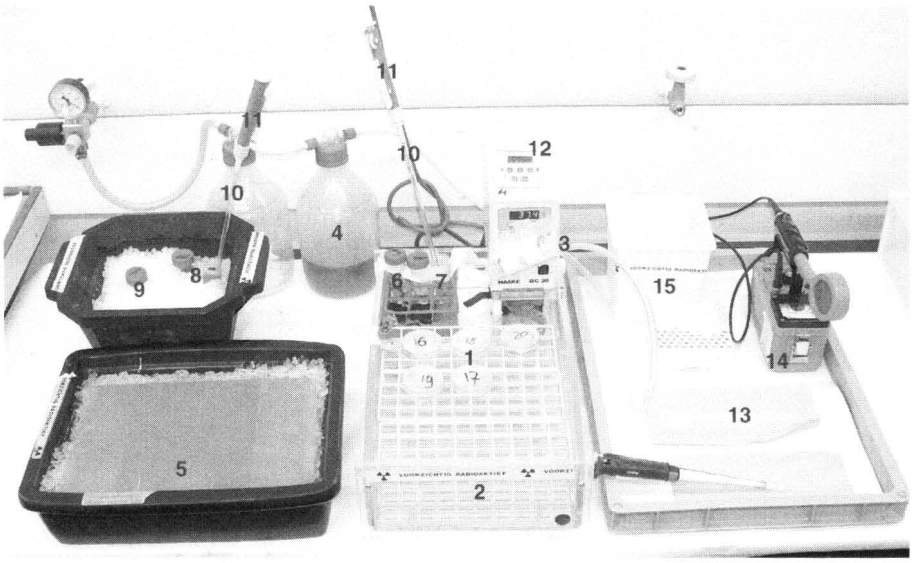

Fig. 2. Pulse-chase setup. 1) Tissue-culture dishes with adherent cells expressing the protein of interest, 2) waterbath with racks, 3) aspirating device, 4) aspiration flask with liquid radioactive waste, 5) flat ice pan with aluminium plate covered with pre-wetted tissues, 6) labeling medium, 7) chase medium, 8) stop buffer, 9) lysis buffer, 10) 5-mL pipet, 11) 1–10 mL dispenser, 12) timer, 13) cell scraper, 14) monitor for radioactivity, 15) solid radioactive waste.

11. Centrifuge the cell lysates at 16,000*g* for 10 min at 4°C to pellet the nuclei. At this point the postnuclear cell lysates can be snap-frozen in liquid nitrogen and stored at –80°C.

3.1.2. Pulse-Chase for Suspension Cells

The samples of the different chase intervals are taken from a single tube of labeled cells. Wash steps after the pulse and chase are not included because the centrifugation steps necessary for this procedure take too much time. Prior to starting the experiment it is necessary to determine: 1) the minimum volume for incubating the cells during the pulse (x µL), 2) the number of chase time points (y), and 3) the desired sample volume (z). For instance, we used per chase time point: $x = 100$ µL, $y = 1$, and $z = 500$ µL.

1. Transfer suspension cells to a sterile 50-mL tube with cap. Use approx 10^6 cells per time point.
2. Pellet cells at 500*g* for 4 min at 20–37°C. Resuspend cells in $2y$ mL depletion medium and pellet cells again. Resuspend cells in $2y$ mL depletion medium. Incubate cells for 15 min at 37°C in an CO_2 incubator.

3. Pellet cells at 500*g* for 4 min at 20–37°C. Resuspend cells in *x* µL depletion medium, change to appropriate tube if necessary, and place in waterbath (37°C).
4. Add 50–100 µCi ^{35}S labeled methionine and cysteine per time point and mix gently to start the pulse. Incubate for the pulse period.
5. Add ≥ 4 times *x* µL chase medium. The total volume should be slightly more than *y* times *z* µL to allow for fluid loss owing to evaporation during the experiment. Mix by gently pipetting up and down.
6. Immediately take the first sample of *z* µL. Transfer to eppendorf tube with pre-prepared *z* µL 2X concentrated lysis buffer on ice; mix well, and keep on ice.
7. After every chase interval, collect a *z* µL sample and add to *z* µL 2X concentrated lysis buffer; mix well, and keep on ice.
8. Centrifuge the cell lysates at 16,000*g* for 10 min at 4°C to pellet the nuclei. At this point the postnuclear cell lysates can be analyzed by immunoprecipitation and SDS-PAGE or snap-frozen in liquid nitrogen and stored at –80°C.

3.2. Immunoprecipitation

1. Mix 50 µL 10% Protein A-Sepharose beads and the antibody, shake or rotate head over head 1 h in an eppendorf tube shaker at 4°C (*see* **Note 8**).
2. Add 100–600 µL postnuclear cell lysate. Rotate head over head 1 h at 4°C (*see* **Note 8**).
3. Pellet Protein A-Sepharose beads by microcentrifuging 1 min at 16,000*g* at room temperature. Aspirate supernatant and resuspend beads in 1 mL immunoprecipitation wash buffer. Shake a minimum of 5 min in eppendorf tube shaker at room temperature (*see* **Note 9**). Repeat **step 3**.
4. Pellet beads and aspirate supernatant. Add 20 µL TE buffer, vortex, add 20 µL 2X sample buffer without reducing agent, vortex again.
5. Heat samples for 5 min at 95°C, vortex, and pellet Protein A-Sepharose beads. The supernatant is the nonreduced sample.
6. Transfer 18 µL supernatant to a tube containing 2 µL of 200 m*M* dithiothreitol (DTT) and vortex. Heat samples for 5 min at 95°C. Centrifuge shortly at 16,000*g* to give reduced sample (*see* **Note 12**).

3.3. SDS-PAGE

1. Prepare 1- or 0.75-mm thick polyacrylamide separating and stacking minigels (*see* **Note 13**).
2. Load 8 µL of each sample. Do not use the outer lanes of the gel and load 1X nonreducing sample buffer in empty lanes to prevent "smiling" of the bands.
3. Run each gel at 25 mA constant current until the dye front is at the bottom of the gel (approx 1 h).
4. Stain the gel with coomassie stain for 5 min and destain for at least 30 min.
5. Neutralize the gel in neutralizer for at least 5 min (0.75 mm) or 10 min (1 mm) (*see* **Note 10**).
6. Treat gel 15 min (0.75 mm) or 20 min (1 mm) with enhancer.

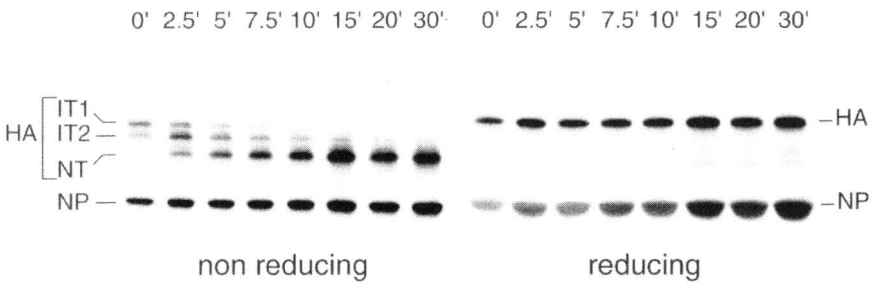

Fig. 3. Folding of influenza hemagglutinin (HA) in HeLa cells at 27°C. Cells were pulse labeled for 2 min with 50 µCi ^{35}S-labeled cysteine and 50 µCi tran[^{35}S] label. Chase medium contained 0.5 m*M* cycloheximide. Stop buffer and lysis buffer contained 20 m*M* NEM. HA was immunoprecipitated with a polyclonal antiserum directed against influenza virus and 10% heat-killed, fixed *Staphylococcus aureus* cells. HA folds into its native form (NT) via two less compact folding intermediates, IT1 and IT2 (nonreducing SDS-PAGE). When the same samples are reduced, one HA band is detected (reducing SDS-PAGE). In addition to HA, the polyclonal antiserum immunoprecipitates nucleoprotein (NP).

7. Dry gel on 0.4 mm Schleicher & Schuell filter paper. Expose to film (for example, Kodak BioMax MR-1) at –80°C or to a Phosphor imaging screen.

With minor modifications, the basic protocol can be extended to study conformational changes (*see* **Note 14**); aggregation and oligomerization of proteins (*see* **Note 15**); degradation (*see* **Note 16**); protein localization (*see* **Note 17**); or the effect of various conditions on folding, maturation, and degradation (*see* **Note 18**). **Figure 3** illustrates a typical folding assay for influenza hemagglutinin.

4. Notes

1. When (endogenous) protein expression is very low, cells can be transiently transfected with a lipid mixture and a plasmid encoding the protein of interest behind an appropriate promotor (*see* Chapter 16). Alternatively, virus-based expression systems can be used, such as Vaccinia T7 ***(6)*** or adenovirus ***(7)***.
2. Labeling medium should contain 50–100 µCi [^{35}]S-methionine and/or cysteine per 60-mm tissue-culture dish with subconfluent cells (1×10^6 cells). This amount is sufficient to visualize a highly expressed average protein in a 1- to 2-min pulse. Depending on the number of cysteines and methionines in the protein, ^{35}S-labeled methionine or a mixture of the two can be used. The stabilized form of methionine and cysteine (e.g., Redivue Promix ^{35}S cell labeling mix, Amersham Pharmacia) is less volatile and is preferred to minimize radioactive contamination of air, pipets, and equipment.

3. When the effects of certain conditions, such as ATP depletion, are tested it may be necessary to separate the folding process from translation. Disulfide-bond formation, and thereby folding of disulfide-bonded proteins, can be prevented by incubating the cells in a reducing agent. When the reducing agent is removed after pulse-labeling, disulfide bond formation and folding may proceed *(8)*. At **step 5** of **Subheading 3.1.1.**, use pulse-labeling medium with 5–10 m*M* DTT, then proceed as described.
   Labeling efficiency may be slightly diminished by the presence of DTT; the duration of the pulse labeling therefore may be increased. During long incubations, DTT may affect cellular ATP levels, depending on the cell line. The efficiency, rate, and outcome of completely posttranslational folding should be compared to the regular folding assay before the effects of different conditions are tested.
4. When the kinetics of a process, e.g., disulfide-bond formation or degradation, are studied, cycloheximide (final concentration 1 m*M*) should be added to the chase medium. Cycloheximide will stop elongation of unfinished nascent polypeptide chains and will prevent incorporation of label in full-length protein after the pulse period.
5. Prepare pulse-chase media freshly before use. With a half-life of approx 30 min at 37°C and a few hours on ice, PMSF is highly unstable in water. Add just before use or as an alternative use the more stable Pefabloc (Boehringer).
6. The basic protocol can be used to study folding and disulfide-bond formation in proteins. An alkylating agent, (e.g., NEM) iodoacetamide, or iodoacetic acid) to block free SH-groups of cysteines must be included in the stop buffer and lysis buffer to prevent disulfide-bond formation to occur after the chase interval. In theory, alkylating agents can be omitted when only reduced samples are analyzed. Free SH-groups of cysteines, however, can promote aggregation, which may diminish detection of the protein of interest. Add NEM, iodoacetamide, or iodoacetic acid to a final concentration of 20 m*M*. In principle, these alkylating agents should give similar results except that electrophoretic mobilities may change *(9)*.
7. During lysis it is crucial to keep the nuclei intact. A lysis buffer should contain a buffer with buffering capacity of approx pH 7.4 such as PBS, HEPES, or MES. Salt concentration should be chosen such that the nuclei are not disrupted owing to osmosis, preferably iso-osmotic. The amount and choice of detergent depends on the protein analyzed and the purpose of the experiments. When noncovalent interactions are studied, Triton X-100 often is not preferred. Instead, detergents with different characteristics can be tested, such as CHAPS ((3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), deoxycholate, octylglucoside, digitonin, or a mixture of detergent and lipid.
8. The amount of antibody needed is dependent on the antigen, the antibody, and its concentration. For each antibody-protein combination, the optimal coupling time and conditions need to be determined. During coupling, head over head rotation is preferred over shaking to minimize damage to Protein A-Sepharose beads.

When small volumes are used, however, shaking may give better results. Coupling times can vary between 30 min and overnight incubations. For some antibodies, the preincubation of antibody and sepharose beads can be omitted. Instead of Protein A-Sepharose beads, a solution of 10% heat-killed, fixed *Staphylococcus aureus* cells can be used. Some antibodies only bind protein G-sepharose beads. If coupling of the antibody to sepharose beads or *S. aureus* cells is poor, a linker of an anti-mouse IgG can be used.

9. The optimal wash buffer needs to be determined for every antibody-antigen combination. The wash buffers mentioned in the protocol are extremely mild and will maintain most of the antibody-antigen interaction but may lead to high background. To decrease background many variables can be changed. Detergents with different characteristics such as Triton X-100, CHAPS, deoxycholate, octylglucoside, digitonin, or a mixture of detergent and lipid can be used with increasing concentrations. The addition of SDS at a concentration $\geq 0.05\%$ (w/v) may be especially helpful to reduce background. Also, salt concentration may be increased. In addition, the time of shaking during the washes can be increased, rather than the number of washes. The temperature of the wash can also affect the level of background: the higher the temperature, the lower the background. Wash temperatures may range from 4°C to room temperature. Different buffers may be tested, but their pH optimally should be above 7.0. Another method to reduce background is to preclear the antibody with nonlabeled lysates of cells lacking the protein of interest, or to preclear the lysate by a 1-h incubation with *S. aureus* cells. Protein A-sepharose beads should be washed and taken up in a buffer containing of 0.1% BSA to reduce nonspecific binding to the beads.
10. The incubation period with neutralizer should be minimally 5 min to ensure the pH > 6 (pH can be checked with pH paper) to prevent precipitation of salicylate in the enhancer solution. Prolonged incubations should be avoided to prevent diffusion of bands.
11. Variation in labeling between different dishes is a problem often encountered during pulse-chase experiments. The reason usually is insufficient accuracy during the pulse labeling. In this case, a digital timer with seconds indication is indispensable. The starvation incubation should be 15 min for the first pulsed sample and not exceed 30 min for later pulsed samples, to ensure optimal labeling. Starvation periods outside this time frame may lead to less labeling of proteins. As a control for variation in labeling, 5 µL total lysate of each sample can be analyzed by SDS-PAGE.
12. In reducing SDS-PAGE the protein ideally forms one band, although modifications of oligosaccharides can change the electrophoretic mobility, resulting in a fuzzy smear. In gel re-oxidation can also occur and is prevented by adding at least 2 times the molar DTT concentration of NEM to the samples prior to loading samples on gel. Samples containing excess NEM or another alkylating agent should not be heated to 95°C, to prevent nonspecific NEM binding to other amino acids than cysteine.

13. Adjust the acrylamide percentage to your protein. For instance, influenza HA (84 kD) folding is analyzed on 7.5% SDS-PAGE.
14. Because disulfide-bond formation is not identical to folding, it can be necessary to monitor conformational changes during folding of the protein. Combinations of the following modifications on the basic pulse-chase protocol are especially informative:
    a. By using an array of conformation-specific antibodies for immunoprecipitation, conformational changes can be monitored during folding and maturation *(10)*.
    b. In general, a protein will become more compact during the folding process; this can lead to DTT resistance of particular disulfide bonds in a protein in the intact cell *(11)*. DTT resistance can be analyzed by performing an additional 10 min chase in the presence of 10 m*M* DTT after the normal chase interval (**Subheading 3.1., steps 6a**, and **7a**) of the basic pulse chase protocol.
    c. Changes in conformational compactness can also lead to protease resistance of certain parts of the protein *(12)*. Follow the basic protocol until **Subheading 3.1., step 11**. Omit EDTA, PMSF, and other protease inhibitors in the lysis buffer, and incubate part of the postnuclear lysate with the desired protease on ice. Optimal concentration and incubation time should be determined for each protease. Add PMSF and (specific) protease inhibitors and proceed with immunoprecipitation as in **Subheading 3.2., step 1** of the basic protocol. With time, one or more protease resistant bands can appear. With the use of conformation-specific antibodies, specific resistant domains can be identified.
15. Aggregation and oligomerization can be detected by the appearance of large molecular complexes in the gel. Noncovalent complexes need to be detected by velocity sucrose gradients *(13)* or can be stabilized by chemical cross linking *(14,15)*.
16. To study protein degradation, proteasome inhibitors and inhibitors of lysomal and autophagic pathways *(16,17)* can be used in the pulse-chase protocol.
17. Protein localization can be indicative for the maturation state of proteins. The following adaptations on the basic protocol allow the detection of different maturation states:
    a. Endo H sensitivity. Because Endo H specifically cleaves *N*-linked glycans in their pre-Golgi-state, Endo H sensitivity is a measure for pre-Golgi localization of an *N*-glycosylated protein *(18)*. Follow the basic protocol until **step 2** in **Subheading 3.2.**, then pellet beads and aspirate supernatant. Resuspend beads in 15 µL 0.2% SDS in 100 m*M* sodium acetate, pH 5.5, and heat at 95°C for 5 min. Cool, add 15 µL 100 m*M* sodium acetate, pH 5.5, containing 0.025 U EndoH (Boehringer) and protease inhibitors: 10 µg/mL each of chymostatin, leupeptin, antipain, and pepstatin, and incubate 1.5 h at 37°C. Add 7.5 µL 5X sample buffer and mix. Continue basic protocol with **step 5** in **Subheading 3.2.**
    b. Surface expression. Surface expression can be monitored by protease digestion on intact cells *(19)*. Follow the basic protocol until **Subheading 3.1.1., step 7b**, wash the cells two times with 2 mL stop buffer. Add 0.5 mL PBS

containing 100 µg/mL trypsin or another protease and 2 m*M* $CaCl_2$ at 4°C to the cells. Incubate 30 min on ice. Collect fluid from cells and add (in final concentration) 100 µg/mL soy bean trypsin inhibitor, 1 m*M* PMSF, and other protease inhibitors: 10 µg/mL each of chymostatin, leupeptin, antipain, and pepstatin. Use for immunoprecipitation. Add to the cells 0.5 mL PBS containing 100 µg/mL soy bean trypsin inhibitor or other protease inhibitors and incubate 5 min on ice and repeat. Lyse the cells in 600 µL lysis buffer and continue with the basic protocol **step 11** in **Subheading 3.1.1.** When the protein of interest is on the cell surface, the protein (fragments) can be found in the fluid taken off the cells after digestion and a decrease in signal should be observed in the cell fraction. However, if the protein cannot be detected in the fluid collected from the cells, the protein might be totally degraded by the trypsin. Protein fragments might be present on the cell surface. For instance, Influenza hemagglutinin trimers are digested into two subunits that stay associated with the plasma membrane and can be detected by 12% SDS-PAGE ***(12)***.

Other methods to monitor surface expression include biotinylation and antibody recognition of surface proteins ***(20)***.

In accordance, secretion of the protein in the medium can be monitored by collecting the chase medium after the chase interval and using this for immunoprecipitation.

18. The effect of various conditions, such as the influence of *N*-glycosylation ***(21)***, ATP ***(22)***, calcium ***(23,24)*** and temperature (**Fig. 3**), can be tested on folding, maturation, and degradation.

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