Journal of Visualized Experiments

Analysis of protein folding, transport and degradation in living cells by radioactive pulse chase

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58952R2
Full Title:	Analysis of protein folding, transport and degradation in living cells by radioactive pulse chase
Keywords:	Protein folding; radiolabeling; SDS-PAGE; immunoprecipitation; Pulse Chase; Conformational analysis; kinetics
Corresponding Author:	Nicholas McCaul, Ph.D Boston Children's Hospital Boston, MA UNITED STATES
Corresponding Author's Institution:	Boston Children's Hospital
Corresponding Author E-Mail:	nicholas.mccaul@childrens.harvard.edu
Order of Authors:	Nicholas McCaul, Ph.D
	Guus van Zadelhoff
	Naomi Lodder
	Ineke Braakman
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Utrecht, The Netherlands

TITLE:

Analysis of Protein Folding, Transport, and Degradation in Living Cells by Radioactive Pulse Chase

AUTHORS AND AFFILIATIONS:

Nicholas McCaul[1,2], Guus van Zadelhoff[1], Naomi Lodder[1], Ineke Braakman[1]

[1]Cellular Protein Chemistry, Utrecht University, Utrecht, The Netherlands
[2]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA

Corresponding Author:

Ineke Braakman (i.braakman@uu.nl)
Tel: (+31) 30 253 2759

E-mail Addresses of Co-authors:

Nicholas McCaul (Nicholas.mccaul@childrens.harvard.edu)
Guus van Zadelhoff (g.vanzadelhoff@uu.nl)
Naomi Lodder (n.g.lodder@uu.nl)

KEYWORDS:

Protein folding; radiolabeling; SDS-PAGE; immunoprecipitation; pulse chase; conformational analysis; kinetics

SUMMARY:

Here we describe a protocol for a general pulse-chase method that allows the kinetic analysis of folding, transport, and degradation of proteins to be followed in live cells.

ABSTRACT:

Radioactive pulse-chase labeling is a powerful tool for studying the conformational maturation, the transport to their functional cellular location, and the degradation of target proteins in live cells. By using short (pulse) radiolabeling times (<30 min) and tightly controlled chase times, it is possible to label only a small fraction of the total protein pool and follow its folding. When combined with nonreducing/reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation with (conformation-specific) antibodies, folding processes can be examined in great detail. This system has been used to analyze the folding of proteins with a huge variation in properties such as soluble proteins, single and multi-pass transmembrane proteins, heavily N- and O-glycosylated proteins, and proteins with and without extensive disulfide bonding. Pulse-chase methods are the basis of kinetic studies into a range of additional features, including co- and posttranslational modifications, oligomerization, and polymerization, essentially allowing the analysis of a protein from birth to death. Pulse-chase studies on protein folding are complementary with other biochemical and biophysical methods for studying proteins *in vitro* by providing increased temporal resolution and physiological information. The methods as described within this paper are adapted easily to study the folding of almost any protein that can be expressed in mammalian or insect-cell systems.

INTRODUCTION:

The folding of even relatively simple proteins involves many different folding enzymes, molecular chaperones, and covalent modifications[1]. A complete reconstitution of these processes *in vitro* is practically impossible, given the vast number of different components involved. It is highly desirable, therefore, to study protein folding *in vivo*, in live cells. Radioactive pulse-chase techniques prove a powerful tool for studying the synthesis, folding, transport, and degradation of proteins in their natural environment.

The metabolic labeling of proteins during a short pulse with ^{35}S-labeled methionine/cysteine, followed by a chase in the absence of a radioactive label, allows specific tracking of a population of newly synthesized proteins in the wider cellular milieu. Then, target proteins can be isolated *via* immunoprecipitation and analyzed *via* SDS-PAGE or other techniques. For many proteins, their journey through the cell is marked by modifications that are visible on SDS-PAGE gel. For example, the transport of glycosylated proteins from the endoplasmic reticulum (ER) to the Golgi complex is often accompanied by modifications of N-linked glycans or the addition of O-linked glycans[2,3]. These modifications cause large increases in the apparent molecular mass, which can be seen by mobility changes in SDS-PAGE. Maturation can also be marked by proteolytic cleavages, such as signal-peptide cleavage or the removal of pro-peptides, resulting in changes in the apparent molecular mass that can be followed easily on SDS-PAGE gel[4]. Radioactivity has considerable advantages over comparable techniques such as cycloheximide chases, where novel protein synthesis is prevented, as longer treatments are toxic to cells and do not exclude the majority of older, steady-state proteins from the analysis, as some proteins have half-lives of days. The comparison of proteins under both nonreducing and reducing conditions allows the analysis of disulfide bond formation, an important step in the folding of many secretory proteins[4-7].

Here we describe a general method for the analysis of protein folding and transport in intact cells, using a radioactive pulse-chase approach. While we have aimed to provide the method as detailed as possible, the protocol has an almost limitless potential for adaptability and will allow optimization to study each reader's specific proteins.

Two alternative pulse-chase protocols, one for adherent cells (step 1.1 of the protocol presented here) and one for suspension cells (step 1.2 of the protocol presented here) are provided. The conditions provided here are sufficient to visualize a protein expressed with medium- to high-expression levels. If the reader is working with poorly expressed proteins or various posttreatment conditions, such as multiple immunoprecipitations, it is necessary to increase the dish size or cell number appropriately.

For suspension pulse chase, the chase samples taken at each time point are all taken from a single tube of cells. The wash steps after the pulse are omitted; instead, further incorporation of ^{35}S is prevented by dilution with a high excess of unlabeled methionine and cysteine.

The presented protocols use radioactive ^{35}S-labeled cysteine and methionine to follow cellular protein-folding processes. All operations with radioactive reagents should be performed using

appropriate protective measures to minimize any exposure of the operator and the environment to radioactive radiation and be performed in a designated laboratory. As the pulse-chase labeling technique is relatively inefficient at short pulse times (<15 min), less than 1% of the starting amount of radioactivity is incorporated in the newly synthesized proteins. After the enrichment of the target protein *via* immunoprecipitation, the sample for SDS-PAGE contains less than 0.05% of the starting amount of radioactivity.

Although the ^{35}S methionine and cysteine labeling mix is stabilized, some decomposition, yielding volatile radioactive compounds, will occur. To protect the researcher and the apparatus, some precautions should be taken. The researcher should always obey the local radiation safety rules and may wear a charcoal nursing mask, besides a lab coat and (double) gloves. Stock vials with ^{35}S methionine and cysteine should always be opened in a fume hood, or under a local aspiration point. Known laboratory contamination spots are centrifuges, pipettes, water baths, incubators, and shakers. The contamination of these areas is reduced by using pipette tips with a charcoal filter, positive-seal microcentrifuge tubes (see **Table of Materials**), aquarium charcoal sponges in water baths, charcoal filter papers glued in the pulse-chase dishes, charcoal guard in the aspiration system, and the placement of dishes containing charcoal grains in incubators and storage containers.

PROTOCOL:

All radioactive reagents and procedures were handled in accordance with local Utrecht University radiation rules and regulations.

1. Pulse Chase

1.1 Pulse Chase for Adherent Cells

NOTE: The volumes given here are based on 35 mm cell culture dishes. For 60 mm or 100 mm dishes, multiply the volumes by 2 or 4, respectively. This protocol uses a pulse time of 10 min and chase times of 0, 15, 30, 60, 120, and 240 min. These can be varied depending on the specific proteins being studied (discussed below).

1.1.1. Seed adherent cells (*e.g.*, HEK 293, HeLa, CHO) in six 35 mm cell culture dishes so that they will be subconfluent (80% - 90%) on the day of the pulse chase. Use at least one dish per time point and/or condition.

1.1.2. If required, transfect the cells with commercially available transfection reagents according to the manufacturer's instructions; or, virally transduce[8] the cells 1 day before the pulse-chase experiment with the appropriate construct for expression.

1.1.3. Wash the dishes with 1 mL of wash buffer (Hank's balanced salt solution [HBSS]), add 1 mL of starvation medium (normal culture medium lacking methionine and cysteine and fetal bovine serum [FBS], such as minimum essential medium [MEM] without methionine and cysteine but with 10 mM HEPES, pH 7.4), and place the dishes in a 37 °C humidified incubator with 5% CO_2 for

15 min.

1.1.4. Transfer the dishes to the racks in a prewarmed 37 °C water bath so that they are in contact with water but do not float. Start a timer.

1.1.5. At 40 s, aspirate the starvation medium, draw up 300 µL of pulse solution (starvation media + 55 µCi/35 mm dish label, see the note preceding step 1.1.1) into the pipette, and add it gently to the center of the dish at exactly 1 min. Repeat this step at 1 min intervals for the remaining dishes.

NOTE: When handling radioactive material, it is essential to follow appropriate precautions and local rules and regulations to prevent accidental exposure and/or contamination.

1.1.6. At exactly 11 min and for all following dishes, except for the 0 min chase sample, add 1 mL of chase medium directly to the dish, aspirate it, and again, add 1 mL of chase medium. Repeat this step at 1 min intervals for remaining dishes. Transfer all dishes to a 37 °C incubator.

1.1.7. At exactly 16 min, add 1 mL of chase medium (normal culture medium + 10 mM HEPES [pH 7.4], 5 mM cysteine, and 5 mM methionine) directly to the dish on top of the pulse medium to stop labeling; then, aspirate immediately, transfer the dish to a cooled aluminum plate, and add 1 mL of ice-cold stop buffer (HBSS + 20 mM *N*-ethylmaleimide [NEM]).

NOTE: This is the 0 min chase sample. For this sample, proceed directly to step 1.1.9.

1.1.8. Transfer each chase dish back to the water bath 2 min before each chase time (*e.g.*, 24 min for a 15 min chase) and, at the exact chase time (*e.g.*, 26 min for a 15 min chase), aspirate the chase media (or transfer it to a microcentrifuge tube if following protein secretion) and transfer the dish to a cooled aluminum plate. Add 1 mL of ice-cold stop buffer.

1.1.9. Incubate all dishes on ice in the stop buffer for ≥5 min; then, aspirate the stop solution and wash it with 1 mL of ice-cold stop solution. Aspirate the wash and lyse dishes with 300 µL of lysis buffer (phosphate-buffered saline [PBS] + nondenaturing detergent [see **Table of Materials**] + protease inhibitors + 20 mM NEM). Use a cell scraper to ensure that the dishes are lysed quantitatively.

1.1.10. Transfer the lysate to a 1.5 mL microcentrifuge tube and centrifuge for 10 min at 15,000 - 20,000 x *g* and 4 °C to pellet the nuclei.

1.2 Pulse Chase for Suspension Cells

NOTE: To ensure efficient labeling, cells should be pulsed at a concentration of 3 x 10^6 to 5 x 10^6 cells/mL, and chase volumes should be 4x the pulse volume. In the following example, we pulsed 5 x 10^6 cells in a volume of 1 mL for 10 min, to yield five chase time points (0, 15, 30, 60, and 120 min) of 1 mL, containing 1 x 10^6 cells per time point. All solutions are the same as in section 1.1.

1.2.1. Culture the suspension cells (*e.g.*, 3T3, Jurkat) according to a previously published protocol[9] so that there is a sufficient number of cells at the time of the experiment, at least 1 x 10^6 cells per time point and/or condition.

1.2.2. If required, transfect cells with commercially available transfection reagents according to the manufacturer's instructions, or virally transduce[8] the cells 1 day before the pulse-chase experiment with the appropriate construct for expression.

1.2.3. Pellet 5 x 10^6 cells per condition in a 50 mL tube for 5 min at 250 x *g* at room temperature, wash them 1x with 5 mL of starvation media, pellet them again, and resuspend them in 1 mL of starvation media.

1.2.4. Transfer the cells to a 37 °C water bath and incubate them for 10 - 25 min. Agitate the tubes every 10 - 15 min to prevent the cells from settling at the bottom.

1.2.5. Start the timer. At exactly 1 min, add 275 µCi (55 µCi/1 x 10^6 cells) of undiluted label directly to the tube containing cells and swirl to mix.

NOTE: When handling radioactive material, it is essential to follow appropriate precautions and local rules and regulations to prevent accidental exposure and/or contamination.

1.2.6. At exactly 11 min, stop labeling by adding 4 mL of chase media. Mix the sample and immediately transfer 1 mL to a 15 mL tube on ice, containing 9 mL of ice-cold stop solution.

NOTE: This is the 0 min chase sample.

1.2.7. Repeat these steps for each successive time point. Once all time points are collected, pellet the cells for 5 min at 250 x *g* at 4 °C. Aspirate the medium (or transfer it to a new 15 mL tube if following protein secretion). Wash the cells with 5 mL of stop solution and pellet the cells for 5 min at 250 x *g* at 4 °C.

1.2.10. Aspirate the stop solution, completely lyse the cells with 300 µL of ice-cold lysis buffer and incubate them for 20 min on ice to ensure a complete lysis. Transfer the lysate to a 1.5 mL microcentrifuge tube and centrifuge for 10 min at 15,000 - 20,000 x *g* at 4 °C to pellet the nuclei.

2. Immunoprecipitation

2.1. Combine antibody (see the discussion) and 50 µL of immunoprecipitation beads (e.g., protein A-Sepharose) (10% suspension [v/v] in lysis buffer + 0.25% bovine serum albumin [BSA]) in a microcentrifuge tube and incubate them at 4 °C for ~30 min in a shaker.

2.2. Pellet the beads for 1 min at 12,000 x *g* at room temperature and aspirate the supernatant. Add 200 µL of lysate to the antibody-bead mixture and incubate at 4 °C in a shaker for 1 h or

head-over-head if the immunoprecipitation requires >1 h.

2.3. Pellet the beads for 1 min at 12,000 x *g* at room temperature. Aspirate the supernatant and add 1 mL of immunoprecipitation wash buffer. Place the sample in a shaker at room temperature for 5 min.

2.4. Pellet the beads as described in step 2.3 and repeat the wash 1x. Then, aspirate the supernatant and resuspend the beads in 20 µL of TE buffer, pH 6.8 (10 mM Tris-HCl, 1 mM EDTA).

2.5. Add 20 µL of 2x sample buffer without the reducing agent, vortex it, heat it for 5 min at 95 °C, and vortex again.

NOTE: If only preparing reducing samples, 2 µL of 500 mM DTT should be added at this point. Then, proceed to step 2.7.

2.6. Pellet the beads as described in step 2.3. Transfer 19 µL of the nonreduced supernatant to a fresh microcentrifuge tube containing 1 µL of 500 mM DTT, centrifuge the sample, and vortex it before heating it for 5 min at 95 °C again.

NOTE: The supernatant is the nonreduced sample.

2.7. Spin down the sample for 1 min at 12,000 x *g*; this is the reduced sample. Cool it down to room temperature and add 1.1 µL of 1 M NEM to both the reduced and the nonreduced sample.

3. SDS-PAGE

3.1. First, determine the appropriate SDS-PAGE resolving gel percentage for the protein of interest. For example, HIV-1 gp120, when deglycosylated, runs at ~60 kDa and is analyzed in a 7.5% gel.

3.2. Prepare the resolving gel mixture without TEMED according to the manufacturer's instructions (x% acrylamide, 375 mM Tris-HCl [pH 8.8,] 0.1% SDS [w/v], and 0.05% SDS ammonium persulfate [APS] [w/v]) and degas under vacuum for >15 min. While the gel mixture is degassing, thoroughly clean the gel glass plates with 70% ethanol and lint-free tissues and place them into a casting apparatus.

3.3. Add TEMED to the resolving gel mixture (at a final concentration of 0.005% [v/v]), mix thoroughly, and pipette between glass plates, leaving ~1.5 cm space for the stacking gel. Carefully overlay the gel with deionized H_2O or isopropanol and leave it to polymerize.

3.4. Once the resolving gel has polymerized, prepare the stacking gel mixture (4% acrylamide, 125 mM Tris-HCl [pH 6.8], 0.1% SDS [w/v], and 0.025% APS [w/v]).

3.5. Flush the top of the resolving gel with deionized H_2O and, then, remove all water.

Note: Use filter paper to remove the last drops.

3.6 Add TEMED to the stacking gel mixture (0.005% [v/v]), mix thoroughly, and overlay the resolving gel with stacking gel and insert a 15-well comb. Once the stacking gel has polymerized, transfer it to a running chamber and fill the upper and lower chambers with running buffer (25 mM Tris-HCl, 192 mM glycine [pH 8.3], and 0.1% SDS [w/v]).

3.7. Load 10 µL of sample per lane in a 15-lane minigel. Avoid loading the samples in the first and the last lane on the gel and load the nonreducing sample buffer in all empty lanes to prevent the smiling of bands. Run the gels at constant a 25 mA/gel until the dye front is at the bottom of the gel.

3.8. Remove the gels from the glass plates, stain the gels with protein-staining solution (10% acetic acid and 30% methanol in H_2O + 0.25% brilliant blue R250 [w/v]) for 5 min with agitation, and then, destain for 30 min with destaining solution (staining solution without brilliant blue R250).

3.9. Arrange the gels face-down on a plastic wrap and, then, place 0.4 mm chromatography paper on top of them. Place the gel sandwich chromatography paper-side down onto a gel dryer. Following the manufacturer's instructions, dry the gels for 2 h at 80 °C.

3.10. Transfer the dried gels to a cassette and overlay them with autoradiography film or phosphor screen. If using autoradiography film, this step must be performed in a dark room.

REPRESENTATIVE RESULTS:

The folding and secretion of HIV-1 gp120 from an adherent pulse chase is shown in **Figure 2.** The nonreducing gel (Cells NR in the figure) shows the oxidative folding of gp120. Immediately after the pulse labeling of 5 min (0 min chase) gp120 appears as a diffuse band higher in the gel, and as the chase progresses, the band migrates down the gel through even more diffused folding intermediates (IT) until it accumulates in the tight band (NT) that represents natively folded gp120. This occurs as the formation of disulfide bonds increases the compactness of the protein, causing it to migrate faster than the fully reduced protein. On the reducing gel (Cells R in the figure), the disulfide bonds on all forms have been reduced such that, at all chase times, they do not affect mobility. This allows the analysis of other modifications. The shift over time from Ru to Rc represents the posttranslational signal-peptide cleavage of gp120: the mobility increases due to the loss of the signal peptide, which increases during the chase as more proteins attain the native fold and lose their signal peptide. On both the nonreducing and reducing gel, the signal begins to decrease from ~1 h onward due to the secretion of gp120. This can be monitored by analyzing the media (Medium in the figure). A comparison of the nonreducing and reducing gels uncovers disulfide bond changes and signal-peptide removal. This was only possible because another modification, N-linked glycosylation, and glycan modifications, were removed from gp120 (and the analysis) by digestion with endoglycosidase H just before SDS-PAGE.

The trafficking of the µ heavy chain of immunoglobulin M (IgM) from a suspension pulse chase is

shown in **Figure 3**. A shift over time from HC_{ER} to HC_{Golgi} represents the trafficking of the μ chain from the ER to the Golgi, which precedes secretion from the cell. This change in molecular mass is caused by the modification of N-linked glycans in the Golgi.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the protocol for pulse chase, immunoprecipitation, and SDS-PAGE.

Figure 2: Folding and secretion of HIV-1 gp120, determined by adherent pulse chase. Subconfluent 35 mm dishes of HeLa cells expressing HIV-1 gp120 were pulse labeled for 5 min and chased for the indicated times. After the immunoprecipitation of gp120, the samples were deglycosylated and analyzed using a 7.5% SDS-PAGE gel. IT = folding intermediates; NT = native gp120; Ru = reduced signal-peptide-uncleaved gp120; Rc = reduced signal-peptide-cleaved gp120; NR: = nonreducing; R = reducing.

Figure 3: Trafficking of the heavy chain of IgM, determined by suspension pulse chase. 5 x 10^6 I.29 μ^+ B cells were pulse labeled for 5 min and chased for the indicated times. After immunoprecipitation, the samples were analyzed using a 10% SDS-PAGE gel. HC = immunoglobulin μ heavy chain; LC = immunoglobulin light chain.

DISCUSSION:

Pulse-chase methods have been essential for developing scientists' understanding of protein folding in intact cells. While we have attempted to provide a method that is as general as possible, this approach has the potential for almost limitless variations to study various processes that occur during the folding, the transport, and the life of proteins inside the cell.

When performing a pulse chase using adherent cells in dishes, it is essential to treat each dish the same as much as possible, as a separate dish is used for each time point and/or condition in an experiment. Especially for short pulse and chase times (<5 min), it is essential to maintain a tight control over the pulse and chase times (with a digital timer). Pulse chases with suspension cells can help to reduce this variability as all samples are taken from a single tube. As some cell types adhere less well to culture dishes than others, care should be taken to ensure that the cells are not washed away during the various medium changes. This can be overcome by either using these cells in suspension or by coating the dishes with poly-L lysine or gelatin, for example, to adhere the cells to the culture dishes. To measure the reproducibility of labeling between dishes, a sample of lysate should be examined by SDS-PAGE to check all samples for identical total labeling and protein pattern. Alternatively, liquid scintillation counting of lysates will establish the total counts-per-minute (cpm) incorporated but will not provide information on the protein population labeled.

If a target protein labels poorly, the signal is increased by increasing the number of cells (or dishes) rather than by increasing the amount of label. Lengthening the pulse time is an option when the kinetics of the studied process will allow this. The ideal pulse time will vary with each

target owing to factors such as the expression level, the transcription rate, the number of methionines/cysteines, and the folding rate of the protein. As such, experimentation must be undertaken to determine the best balance between the above-mentioned factors in each experiment while keeping the experimental aim in mind. If intermediates during folding are being studied, for example, it is desirable to pulse the label for as short a time as possible to have the starting material as close to an unfolded state as possible, while balancing expression levels. Alternatively, if the transport or degradation of a protein is the target of study, pulse times may be lengthened to provide the highest levels of signal possible without compromising kinetic information. In general, pulse times can range from 2 - 15 min when using this protocol without modification. Previous experimentation[5] has demonstrated a lag time of ~10 s after the pulse before the incorporation of radioactivity into the total protein pool; this is important to keep in mind when deriving kinetic information from pulse-chase experiments. If extended pulse times (>1 h) are being used, it is advisable to increase the pulse volume by 1.5 times and place dishes on a rocker in a 37 °C incubator during the pulse to prevent the dishes from drying out. While labeling solutions containing individual ^{35}S-methionine/cysteine or a mix are available, the mix is preferred even when proteins that only contain methionine or cysteine are being labeled as both are needed to maintain general protein synthesis in the cell. If experimental conditions require specific methionine/cysteine labeling, the levels of nonradioactive methionine/cysteine during the pulse must be adjusted accordingly.

Chase times (and the number of time points) are determined in a similar fashion. A good starting place is to set the first chase time (after the 0 min chase) as equal to the pulse time and, then, double the length of time for each successive time point (*e.g.*, 5, 10, 20, and 40 min). However, this must be optimized for each specific protein and question.

To prevent the activation of stress responses by starvation (which may affect protein synthesis and folding), ideally, some unlabeled methionine and cysteine should be added to the starvation and radiolabeling solutions. The quantity will depend on the cell line, the labeling time, the quantity of the radiolabel used, and media volumes, and will need testing. A good starting point is 1% of the amount of cysteine and methionine present in the cell culture medium, which can be increased with increasing pulse times. Starvation periods, whether with or without unlabeled amino acids, should be kept in the range of 15 - 30 min to ensure adequate label incorporation and prevent the activation of stress responses. When using an extended pulse (≥1 h), starvation is not required.

The lysis buffer described here will be suitable for most purposes, but in principle, any buffer system, such as HEPES, Tris, or MES, will work. The detergent concentration needed to lyse cells will depend on the number of cells and detergent volume. The concentration should always be above the critical micelle concentration (CMC) and the quantity of detergent sufficient to lyse cells. An empirical rule of thumb is that 200 µL of 0.5% nondenaturing detergent (see **Table of Materials**) is sufficient to lyse the cell equivalent of ~1 mg of protein (~1 x 10^6 cells). The salt concentration and detergents may also be varied according to the application, but in general, conditions that break open nuclei (high salt, >0.1% SDS) should be avoided as the presence of free DNA will interfere with immunoprecipitation. If this cannot be avoided, for instance, when

complete cell lysates including nuclei or pelleted proteins need to be analyzed, DNA can be sheared by passing the cells through a small gauge needle prior to immunoprecipitation. This is preferred over sonication so as to prevent excessive foaming and the production of radioactive aerosols.

For immunoprecipitation, both the optimal quantity of antibodies and the best wash buffer for each antibody-antigen combination must be tested thoroughly to achieve a balance between the desired antigen signal and background. Antibodies should always be present in excess over antigens to ensure quantitative immunoprecipitation; a good starting point is 1 µg of antibodies per immunoprecipitation from a 35 mm dish/1 x 10^6 cells. The background can be decreased by increasing the detergent or salt concentrations. In particular, the addition of ≤0.1% SDS can help to greatly reduce the background but may also disrupt antibody-antigen interactions. To control for the specificity of antibodies during immunoprecipitation, identical cells lacking the target protein should be used when available. If this is not straightforward, such as when endogenous proteins are being analyzed, the overexpression or depletion of the antigen will work. To control for background (and specificity), either preimmune sera (where possible) or isotype controls should be used. To control for antigens binding directly to immunoprecipitation beads, beads without antibody should also be used. The conditions provided here should be considered as a starting point as each antibody-antigen pair will require an optimization of both wash buffer and wash conditions. In case of too much background, do not increase the number of washes but rather the time of washing and/or the composition of the wash buffer. If particularly sensitive interactions such as in coimmunoprecipitations have to be dealt with, it may be preferable to carry out all wash steps at 4 °C, using ice-cold buffers.

One advantage of adherent cells over suspension cells is that drug treatments can be performed at virtually any point during the pulse chase. If used with chaperone inhibitors, for example, it is possible to differentiate its effects on the co- *versus* posttranslational phases of a folding process. This can be extended by adapting the lysis and immunoprecipitation wash buffers (discussed above) to allow the coimmunoprecipitation of protein-chaperone complexes during folding[10-12].

The postlysis treatment of samples will widen the scope of questions that can be addressed. The limited protease digestion of target proteins before immunoprecipitation will provide additional conformational information and has been successfully used to monitor the folding of proteins, especially those lacking disulfide bonds[13,14]. Protein aggregation and complex formation can be monitored by sucrose density-gradient centrifugation prior to immunoprecipitation[15,16].

To take full advantage of pulse chases, the availability of high-quality reagents is required for immunoprecipitations. When examining folding intermediates, it is essential to have antibodies that are capable of pulling down all forms of the protein under analysis. If specific antibodies to the target protein are not available, this can be substituted by using affinity tags. However, this severely restricts the usefulness of postlysis methods, such as the limited proteolysis, to directly probe the protein conformation, as only tagged fragments can be recovered.

The sensitivity of pulse chases is limited by the number of methionine and cysteine residues in

the protein in question. Proteins with low numbers of methionine/cysteine residues coupled with a low expression level are undetectable in pulse-chase experiments. When performing postlysis modifications such as limited proteolysis, only methionine/cysteine-containing fragments will be detectable.

To conclude, given the kinetic detail that pulse chases provide, they are complementary to many other techniques and tools used to study molecular cell biology at the steady-state level. As such, they continue to be an invaluable component in the molecular biologist's toolbox.

ACKNOWLEDGMENTS:

The authors thank all members of the Braakman lab, past and present, for their fruitful discussions and help in developing the methods presented in this article. This project has received funding from both the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013) N° 235649 and the Netherlands Organization of Scientific Research (NWO) under the ECHO-program N° 711.012.008.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Ellgaard, L., McCaul, N., Chatsisvili, A., Braakman, I. Co- and Post-Translational Protein Folding in the ER. *Traffic.* **17** (6), 615-638 (2016).
2. Pisoni, G. B., Molinari, M. Five Questions (with their Answers) on ER-Associated Degradation. *Traffic.* **17** (4), 341-350 (2016).
3. Lamriben, L., Graham, J. B., Adams, B. M., Hebert, D. N. N-Glycan-based ER Molecular Chaperone and Protein Quality Control System: The Calnexin Binding Cycle. *Traffic.* **17** (4), 308-326 (2016).
4. Snapp, E. L. *et al.* Structure and topology around the cleavage site regulate post-translational cleavage of the HIV-1 gp160 signal peptide. *Elife.* **6**, e26067 (2017).
5. Braakman, I., Hoover-Litty, H., Wagner, K. R., Helenius, A. Folding of influenza hemagglutinin in the endoplasmic reticulum. *Journal of Cell Biology.* **114** (3), 401-411 (1991).
6. Jansens, A., van Duijn, E., Braakman, I. Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science.* **298** (5602), 2401-2403 (2002).
7. Land, A., Braakman, I. Folding of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum. *Biochimie.* **83** (8), 783-790 (2001).
8. Singh, Y., Garden, O. A., Lang, F., Cobb, B. S. Retroviral Transduction of Helper T Cells as a Genetic Approach to Study Mechanisms Controlling their Differentiation and Function. *Journal of Visualized Experiments.* (117), e54698 (2016).
9. Das, A. T., Land, A., Braakman, I., Klaver, B., Berkhout, B. HIV-1 evolves into a nonsyncytium-inducing virus upon prolonged culture *in vitro*. *Virology.* **263** (1), 55-69 (1999).
10. Chen, W., Helenius, J., Braakman, I., Helenius, A. Cotranslational folding and calnexin binding during glycoprotein synthesis. *Proceedings of the National Academy of Sciences.* **92** (14), 6229-6233 (1995).
11. Daniels, R., Kurowski, B., Johnson, A. E., Hebert, D. N. N-linked glycans direct the

485 cotranslational folding pathway of influenza hemagglutinin. *Molecular Cell.* **11** (1), 79-90 (2003).
486 12. Ferreira, L. R., Norris, K., Smith, T., Hebert, C., Sauk, J. J. Association of Hsp47, Grp78, and
487 Grp94 with procollagen supports the successive or coupled action of molecular chaperones.
488 *Journal of Cellular Biochemistry.* **56** (4), 518-526 (1994).
489 13. Hoelen, H. *et al.* The primary folding defect and rescue of DeltaF508 CFTR emerge during
490 translation of the mutant domain. *PLoS One.* **5** (11), e15458 (2010).
491 14. Kleizen, B., van Vlijmen, T., de Jonge, H. R., Braakman, I. Folding of CFTR is predominantly
492 cotranslational. *Molecular Cell.* **20** (2), 277-287 (2005).
493 15. Hagiwara, M., Ling, J., Koenig, P. A., Ploegh, H. L. Posttranscriptional Regulation of
494 Glycoprotein Quality Control in the Endoplasmic Reticulum Is Controlled by the E2 Ub-
495 Conjugating Enzyme UBC6e. *Molecular Cell.* **63** (5), 753-767 (2016).
496 16. Copeland, C. S., Doms, R. W., Bolzau, E. M., Webster, R. G., Helenius, A. Assembly of influenza
497 hemagglutinin trimers and its role in intracellular transport. *Journal of Cell Biology.* **103** (4), 1179-
498 1191 (1986).

Figure 1

Figure 2

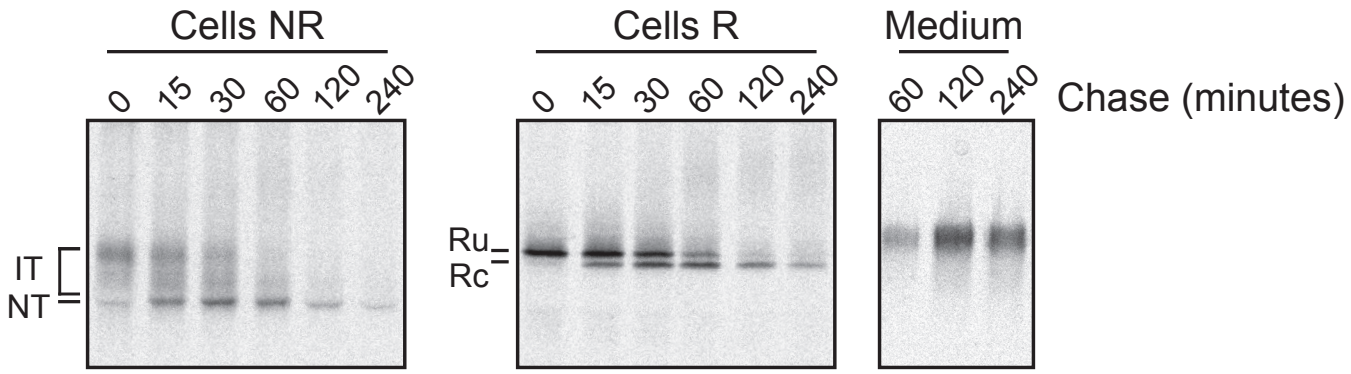
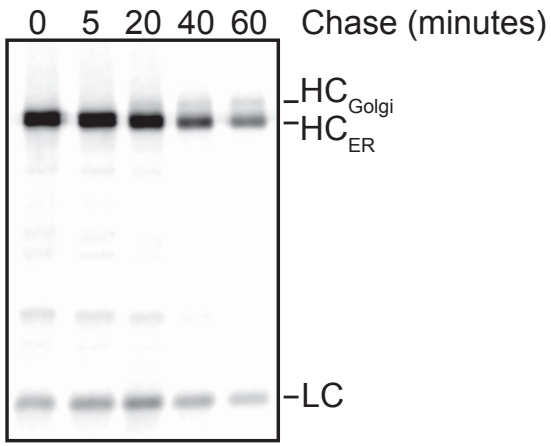


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL safeseal microcentrifuge tubes	Sarstedt	72.706.400	
Acetic Acid	Sigma	A6283	glacial acetic acid
BAS Storage phosphor screen 20x25 cm	GE Life Sciences	28956475	
Bromophenol Blue	Sigma	B8026	Molecular biology grade
Carestream Biomax MR films	Kodak	Z350370-50EA	
Cell-culture media	Various	N/A	Normal cell culture media for specific cell-lines used
Cell-culture media, no methionine/cysteine	Various	N/A	Same media formulation as normal culture media e.g DMEM/MEM/RPMI, lacking methionine and cysteine
Charcoal filter paper	Whatman	1872047	
Charcoal filtered pipette tips	Molecular bioproducts	5069B	
Charcoal vacu-guard	Whatman	67221001	
Coomassie Brilliant Blue R250	Sigma	112,553	for electrophoresis
Cysteine	Sigma	C7352	Molecular biology grade, Make 500 mM stock, store at -20
Dithiothreitol (DTT)	Sigma	10197777001	Molecular biology grade
EasyTag Express35S Protein Labeling Mix	Perkin Elmer	NEG772014MC	Other size batches of label are available depending on useage
EDTA	Sigma	E1644	Molecular biology grade
Gel-drying equipment	Various	N/A	
Glycerol	Sigma	G5516	Molecular biology grade
Grade 3 chromatography paper	GE Life Sciences	3003-917	
Hank's Balanced Salt Solution (HBSS)	Gibco	24020117	

HEPES	Sigma	H4034	Molecular biology grade, Make 1M stock pH 7.4, store at 4˚C
Kimwipes delicate task wipes	VWR	21905-026	
MES	Sigma	M3671	Molecular biology grade
Methanol	Sigma	MX0490	
Methionine	Sigma	M5308	Molecular biology grade, Make 250 mM stock, store at -20
Minigel casting/running equipment	Various	N/A	
NaCl	Sigma	S7653	Molecular biology grade
N -ethylmaleimide	Sigma	E3876	Molecular biology grade, Make 1M stock in 100% ethanol, store at -20
PBS	Sigma	P5368	Molecular biology grade
Protein-A Sepharose fastflow beads	GE health-care	17-5280-04	
Sodium Dodecyl Sulfate (SDS)	Sigma	L3771	Molecular biology grade
Triton X-100	Sigma	T8787	Molecular biology grade
Trizma base (Tris)	Sigma	T6066	Molecular biology grade
Typhoon IP Biomolecular imager	Amersham	29187194	
Unwire Test Tube Rack 20 mm for waterbath	Nalgene	5970-0320PK	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Analysis of protein folding, transport and degradation in living cells by radioactive pulse chase
Author(s):	Nicholas McCaul, Guus van Zadelhoff, Ineke Braakman

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Ineke Braakman
Department:	Cellular Protein Chemistry, Department of Chemistry, Faculty of Science
Institution:	Utrecht University
Title:	Professor
Signature:	
Date:	15 Aug 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:
Changes to be made by the author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Line 51: Please note the reference number starts with 3, not 1. Please update the reference numbers in the text and in the reference list.

This was due to the accidental inclusion of two references in the abstract. They have been removed and the numbering updated.

3. Please define all abbreviations before use.

All abbreviations have been defined.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Numbering has been adjusted and bullets, dashes and indentation have been removed throughout the manuscript.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Roche, Triton X-100, Carestream BioMax, Whatman, etc.

Done, all commercial language has been moved to the table of materials.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed from the protocol text.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

All protocol steps have been modified to contain only directives in the imperative tense.

8. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. Please move notes to the corresponding steps to which they apply. Please do not number notes.
9. Lines 82-123: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.), or move the solutions, materials and equipment information to the Materials Table.

Done. The majority of details contained in the notes section have been relocated to the discussion.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Manuscript has been updated to provide additional details where requested.

11. Line 135: Please specify the type of cells used in this protocol.

Practically all mammalian cells can be used with this protocol, as we aim to give a general overview of how to adapt the method to the given needs of any user we did not find it appropriate to specify a single cell type. We have updated the manuscript at lines 104 and 153 to include examples of cell lines that are appropriate to use.

12. Lines 137-138: Please describe how to transfect or virally transduce cells.

Transfection and/or viral transduction may not be necessary depending upon the needs of the specific end user. Additionally, transfections/transductions can also be complicated protocols requiring their own optimizations. To keep the manuscript on aim, instead of providing detailed protocols for transfection/transduction, we have included references to appropriate articles that deal with these topics in depth.

13. Line 146: Please specify incubation conditions.

This refers to leaving the dishes on the waterbath for the duration of the pulse. The text has been updated to make this clearer.

14. Lines 167-175: The Protocol should contain only action items that direct the reader to do something. Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”

The protocol has been updated to include only directions in the imperative text.

15. Lines 177-180: We cannot film such generalized steps; please provide specific details.

Specific details have been provided to aid with filming.

16. Line 191: Does 5’ refer to 5 minutes?

Yes, this has been changed throughout the manuscript to be clearer.

17. Lines 200, 223: What amount is considered to be appropriate?

For the immunoprecipitation described on line 200, it is difficult to directly describe an appropriate quantity of antibody to use as this varies greatly with each antibody, dependent upon the strength of binding. This is discussed in the critical steps/troubleshooting section of the discussion on page 8 and the step now refers to this section. Likewise, gel percentage needs to be adjusted based on specific protein analyzed, a suggestion has been indicated based on the protein analyzed in the figure.

18. Line 222: Please describe how.

Done, we have provided instructions in steps 1-4.

19. Line 227: Please describe how to stain and destain gel.

Done, we have added detailed instructions at step 8.

20. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Done, the protocol now contains less single-instruction steps.

21. Please include single-line spaces between all paragraphs, headings, steps, etc.

Done, single line spaces have been inserted between all paragraphs, headings and steps.

22. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
23. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
24. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Points 22 to 24: Done, the essential steps in the protocol have been highlighted in the way requested.

25. Figure 1: Please include a space between the numbers and their corresponding time units (1 h, 2 h, 4 h, etc.).
Done

26. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

The discussion of the figure has been moved to the representative results.

27. Discussion: Please discuss any limitations of the technique.

A section has been placed in the discussion on page 6

28. Reference 10: Please do not abbreviate journal titles.

All journal titles have been written out in full.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript provides a step by step protocol for performing analysis of protein folding, transport and degradation in living cells using the method of radioactive pulse chase. This method is widely used and a video demonstrating it visually will be very helpful.

Minor Concerns:
Parts of the text are highlighted in yellow !

This is for the editors at JoVE so that they know which parts should be made into a video.

Reviewer #2:
Manuscript Summary: This is a methods paper describing protocols for labeling mammalian cells in suspension or in plates with 35S methionine/cysteine, in order to follow kinetics of modification or degradation of individual proteins in vivo.
Overall this is a useful contribution that will help researchers that are novices in the field to set up such experiments.
Following is a list of comments for the consideration of the authors.

Abstract: The abstract concludes with "In adapted form, its use expands successfully to yeast and bacterial cells." "Its use" presumably refers to "the methods as described", so it should be "Their use". But beyond grammar, I do not think that this statement is appropriate in a methods paper that describes exclusively work with mammalian cells. The protocols for yeast or bacteria are substantially different.

We have removed the sentence.

What is the control for the specificity of the immunoprecipitation? A Protein A sepharose beads-only i.p., without antibody?

Specificity of immunoprecipitation can and should be controlled by performing immunoprecipitations against lysate from cells lacking the target antigen (untransfected or knock-out cells) or where this is impossible, a lysate that expresses the antigen at a different level (e.g. overexpression). A control antibody is also required; pre-immune sera from the immunized animal is always ideal but a different negative control (e.g. isotype control) is often practical and usually sufficient. If it is thought that the background comes from binding to protein-A sepharose beads then immunoprecipitation should be performed without the antibody. The discussion text on page 8 has been updated to indicate this.

Is it known how long it takes for the intracellular methionine pools to equilibrate with the extracellular methionine? This is an important theoretical consideration in order to estimate the accuracy of the kinetics derived from the labeling experiment.

Previous work (Braakman et al JCB (1991)) demonstrated that after the pulse there is a 10 s lag before incorporation of radioactive label into the total protein pool, accounting for the time that it takes radiolabel to enter the cell and be used during translation. It is reasonable to assume that the addition of unlabeled amino acids during the chase follow similar kinetics. Text has been added on page xx to address this point.

The labeling precursor reagents that can be purchased are either 35S-methionine or a 35S-methionine/35S-cysteine mix. Which is recommended, if any (the precursor listed, Easytag express, is, I believe, the mix)? Why?

In general, the met/cys mix is preferable as it allows maximal labeling of proteins and therefore maximal sensitivity during experiments. Both methionine and cysteine are required to sustain general protein synthesis of all proteins during the pulse, so if only methionine or cysteine labeling is performed, then levels of cold methionine/cysteine during the pulse must be adjusted. The discussion on page 8 has been updated to address this point.

L. 298, comment 11: why must some antibodies be pre-coupled to the protein A sepharose beads and not others? How is this determined?

Unfortunately, this can only be established empirically by testing various conditions. Due to editorial comments the corresponding comment was moved, and this specific text removed. Pre-coupling of antibody to sepharose beads before addition of lysate always works and as such this is the protocol that we have kept in the protocol.

l. 259: what is "volume sed"?

This was a typo and has been corrected.

lines 365-372: The importance of having "identical" cell densities in the different plates when performing pulse-chase on adherent cells is correctly emphasized, however how does one know that the dishes and manipulations are reproducible? Recommending duplicate plates for each time point might be helpful here. An additional way to normalize the protein amounts between samples is to measure incorporated radioactivity and immunoprecipitating identical amounts of cpm.

As an internal control in each experiment we normally take a sample of lysate to analyze total-protein labeling in each sample by SDS-PAGE. This allows us to control both for the intensity (cpm) but also the banding pattern between samples which controls for many additional factors such as degradation, infection etc. The reviewer's suggestion of normalizing immunoprecipitations via cpm is also a valid one. We have updated the manuscript to include both suggestions.

l. 372: recommending a digital timer is somewhat quaint (are there labs that still use hourglasses??). Besides, the need for a timer should be obvious to anyone planning a kinetics experiment.

We agree with the reviewer but prefer not to make any assumptions.

Reviewer #3:

Manuscript Summary:

The manuscript describes in detail various variants of the pulse-chase protocol to monitor the life and the various stages of proteins after their synthesis. Particular emphasis is posed in the monitoring the emergence of the folded state. Nevertheless, the method is more general and is not restricted to this process. The manuscript is easy to read and follow.

Major Concerns:

As a non-experimentalist, I cannot judge the influence of buffer types, concentrations, temperatures and I cannot judge how difficult it is to perform things "fast" so that the cell population can be considered homogeneous at each time point of pulse and/or chase. Nevertheless, the timing of the pulse length and the time intervals (quantity, and intervals) in the chase phase have been superficially discussed.

The manuscripts main feature and weakness is to describe the protocol regardless of how the data will be analyzed afterwards. In the world of what we like to call "experimental design" this should not be done. The length of the pulse and so also the timing of the chase depend on which question one would like to answer, which competing hypotheses are going to be tested, how many intermediate states from birth to death of the protein should be detected. An example of how complex the analysis can go, can be obtained from the paper McShane et al Cell (2016).

It is correct to say that a short pulse allow seeing more structure (states) during the lifespan of the protein (provided there is enough material) but it is not possible to know that a priori and it is not possible to estimate the correct pulse length by knowing only the synthesis rate. In the paper cited above, for proteins with a complex life (such as proteins that are part of a complex or that undergo several stages of folding) the rate by which the various states are visited is independent of the synthesis rate.

This comment boils down to the idea that the first step is to formulate an hypothesis, then a first experiment to have a rough idea of the rate constants and the a second round where the timing of pulse and chase is decided in such a way that other hypothesis (or the null model) can be ruled out.

The reviewer is correct that it is difficult to determine the ideal pulse or chase times for any given protein *a priori* given only length or other sequence information. When beginning to work with any new protein there is no other option than moving forwards with the experiment and trying several different pulse and chase times and adapting each experiment iteratively until one has a setup to move forwards with. As we originally stated in the discussion, the conditions that we have provided should be considered a starting point and extensive optimization will be required for each protein that one wishes to study. We have updated the discussion on page 7 to include additional guidance on considerations when determining ideal pulse and chase times.