**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](http://airmail.calendar/2018-09-25%2001:00:00%20EDT), [2 h](http://airmail.calendar/2018-09-25%2002:00:00%20EDT), [4 h](http://airmail.calendar/2018-09-25%2004:00:00%20EDT), 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.