**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.  
2. Figure 4: Please change “sec” to “s”.  
3. Please provide an email address for each author.  
4. Please rephrase the Long Abstract to more clearly state the goal of the protocol.  
5. Please define all abbreviations before use.  
6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.  
7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.  
8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).  
9. Please revise the protocol to contain only action items that direct the reader to do something. 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.”  
10. 1.2: Please specify the growth conditions (temperature, etc.).  
11. 1.3: Please revise so that each step contains only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. What volume of dH2O is used to wash?  
12. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

The requested changes have been made.

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript summary:  
In this manuscript, Johnson and Glick described a method to track the behavior and composition of an intracellular compartment in yeast using multi-color, time-lapse imaging. The protocol includes detailed imaging parameter settings for confocal microscopy and the procedures for subsequent image processing. Additionally, they provide a useful ImageJ plugin and its detailed documents to extract the region of interest from the microscopy data. The manuscript is well written, and the protocols are clearly presented.  
  
In this reviewer's opinion, the manuscript is suitable for publication from JoVE provided that the following issues are successfully addressed.  
  
Major recommendation:  
1. (Introduction and overall) Although the protocol is written to minimize photobleaching (line 70), the protocol to reduce photobleaching should also reduce phototoxity induced by fluorescence microscopy. Thus, it should be beneficial for both authors and readers to introduce the protocol also from the perspective to reduce the phototoxity. Appropriate citations in this topic may also help navigate readers to more specialized papers, which may include, but not limited to:  
- Laissue et al, 2017 Nat Methods 14: 657-661.  
- Icha et al., 2017 Bioessays 39: 1700003.  
- Carlton et al., 2010 PNAS 107: 16016-16022.  
The last one (Carlton et al.,) analyzed phototoxity using yeast.

We agree that phototoxicity is an important issue. The text has been revised in multiple places to emphasize this point, and the suggested references have been added. In addition, the protocol was enhanced to state that omission of riboflavin is expected to reduce phototoxicity, and that inclusion of Trolox may help to reduce photodamage.

Minor recommendations:  
1. (line 50) The "Nakano group" appears abruptly and is puzzling unless the reader looks at the reference 5. Can the citations be inserted at the beginning of the sentence, i.e., "We3 and the Nakano group5 used…"?

This sentence was revised and clarified.

2. (line 85) Although ImageJ is well-recognized for the scientific community, it is still better to cite it. According to their website, there are several ways to cite ImageJ (https://imagej.nih.gov/ij/docs/faqs.html).

A citation for ImageJ has been added.

3. (line 115-116) I assume the authors use a confocal microscope equipped with a resonant scanner, that is the reason why "increasing the width will not reduce the scan speed". The description is also valid for CCD based systems, including spinning disk confocal microscopes. However, the readers of this protocol may use confocal microscopes with conventional Galvano scanners and PMT (not resonant scanners or CCD), where increasing the width may reduce the scan speed. The authors should clarify the microscope types that this description is applied to.

The protocol has been clarified by stating that this recommendation applies to confocal microscopes with resonant scanners.

4. (line 200, 228, 261 and others) The phrase of "the final projected movie" is misreading. Its usage is understandable in the "Instructions for plugins", but in the main protocol, "the projected movie at the final stage of editing" or some equivalent phrases may be appropriate.

The wording throughout the document has been changed to “movie of the projected Z‑stacks” or similar phrases.

5. (line 200) Please add "to make projections" to the list of the functions ("to view, edit and quantify") of the plugins, since the procedure for making projection is not in the main protocol, but it is very often mentioned in the "REPRESENTATIVE RESULTS".

This line has been clarified.

6. (line 261) "the complete projections" is not understandable. Does it mean "the projection without editing"?

We changed “complete” to “original”, which should be more comprehensible.

7. (Figure 2) Please clarify the order of images in montage. Labeling the indices of the Z sections in the figure may help.

The order of the images is now explained in the figure legend.

**Reviewer #2:**  
Manuscript Summary:  
-This is a well written paper.  
-The authors use a challenging system for 4D microscopy (tracking of Golgi cisternae and maturation of individual cisternae).  
-The authors reporting an imaging improvement, which is essentially low laser settings combined with self-made plug-in modules for Image J for processing/editing of noisy raw data.  
-This system enables an acquisition of z-stacks over prolonged periods of time with very limited data loss due to photobleaching.  
-The data shown are of high quality and demonstrate the maturation of individual cisternae (they get sequentially labeled with an early and a late Golgi marker).  
  
Major Concerns:  
As the authors are reporting an improvement of their protocol versus regular imaging techniques, they should demonstrate how much of an improvement this actually is. This Reviewer suggests that the authors take the same raw data set (4D movie), use similar deconvolution settings with Huygens software, but later use a conventional imaging software such as Imaris to display the movie. Can the authors still show a similar level of resolution? If not, what is the level of improvement with their protocol compared to a more conventional imaging protocol?

We are unsure how to respond to this comment. A number of other procedures have been used for yeast live cell imaging including Nakano’s SCLIM technology, commercial spinning disk confocal microscopy, and lattice light-sheet microscopy. We have not done an objective side-by-side comparison, and cannot claim that our method is the only one that is suitable or even the best one. This article is specifically intended to describe how to perform yeast imaging and image analysis by the method that we have optimized.

Minor Concerns:  
The authors also report self-labeling proteins (Snap-tag, Halo-tag) as a recent improvement that will facilitate 4D microscopy using three channels. However, they do not show any three channel imaging results. The authors should either include a 4D movie acquired with a three channel setting to demonstrate the feasibility of this approach, or otherwise remove the entire section on three channel 4D movies.

We recently submitted a manuscript that describes three-color 4D confocal imaging, with HaloTag linked to a far-red dye supplying the third color. That work is now cited as “unpublished data”. We will update the text with a citation if the manuscript is accepted soon enough.

**Reviewer #3:**  
Manuscript Summary:  
In this manuscript, authors describe the advantage of multicolor 4 D observation by confocal microscopy in analyzing the behaviors of endomembrane compartments in yeast cells. They show the important and concrete protocols to achieve effective 4D imaging by using confocal microscopy. In addition, publishing practical plug-ins for ImageJ is very useful for many researchers doing similar analysis not in yeast cell biology but in all the field of cell biology. The paper would be suitable for publication on the JoVE.  
  
Minor Concerns:  
It is better to describe how many watts of laser are actually irradiated from objective lens corresponding to this setting of microscopic system. It will give important information for researchers who conduct 4D imaging experiments using other confocal microscopies.

Numerical values for laser power are not always useful in our experience. As an alternative, we describe in section 2.8 how to adjust the laser intensity to a suitable level. Those guidelines should be suitable for any confocal microscopy system.

**Reviewer #4:**  
This paper nicely outlines a method for taking and analysing timelapse movies of subcellular structures in S.Cerevisiae using confocal microscopy and ImageJ. If I were a new user I would find the protocols clear and easy to follow, and the figures clearly illustrate the steps of analysis.  
  
General points:  
-Are these recommended as general imaging parameters for S.Cerevisiae or for the Golgi specifically? There could be some discussion relating to time intervals and z-section spacing when imaging other structures in 4D.

This protocol is generally suited to imaging yeast compartments, and the appropriate time interval depends on the dynamics of the compartment being visualized. To emphasize these points, we added text at the end of the Introduction and in section 2.12.

-I think the final paragraph of the results section would read more clearly if split into steps - mentioning the ImageJ plugin used in each step and the figures referenced as the expected output of each step.

We modified the Results as suggested, and agree that the new format is an improvement.

-Some discussion on the best fluorophores would be useful e.g. using newer and brighter fluorophores to allow for more intensive imaging.

This topic would require an extensive discussion, which we deem to be outside the scope of the article. Instead, we now cite a review that covers the use of fluorescent proteins for yeast imaging.

-How might you control for chromatic aberration in analysis like this? Could there be a section discussing controls such as labeling the same protein with two different fluorophores.

The reviewer makes a good suggestion. We have done tests with multi-color fluorescent beads, but dual tagging of an intracellular yeast protein would be even better. The text has not been modified because we have not yet done this control.

Specific Points:  
Protocol:  
2.1) You could talk about the best grade of oil to use at RT (or 25˚C if you're using an environment chamber)

For room temperature imaging of the type described here, the standard immersion oil provided by the manufacturer is suitable. At one point we tested the Immersion Oil Kit recommended by the Agard lab but did not see a notable benefit.

2.4) It might be useful to talk about the drawback of bidirectional scanning in some older systems.

In section 2.4, we now state that bidirectional scanning should be used only if control experiments confirm that the scans from the two directions are in register.

2.6) Why was a pinhole of 1.2 a.u. specifically chosen (rather than 1.4 or 1.6). Was the resolution judged by eye as suitable for imaging the golgi? Is 1.2 suitable for other structures?

We find empirically that using a pinhole of 1.2 Airy units rather than the conventionally recommended 1.0 Airy unit enhances photon recovery without noticeably degrading the resolution. A plausible explanation is that there is a refractive index mismatch between the oil/glass and the yeast cells, so the optics are suboptimal and the 1.0 Airy unit setting does not provide the benefit that would be obtained with no refractive index mismatch. However, increasing the pinhole above 1.2 Airy units does degrade the resolution.

An abbreviated version of this explanation has been added to section 2.6.

2.13) Why is it necessary for the images to be 8-bit? Wouldn't a higher bit depth be preferable if you want to combine this analysis with fluorescence intensity measurement?

8-bit images are more than sufficient for the number of photons that are captured with this protocol, and the processing pipeline is configured to accept 8-bit images. This point is now stated in section 2.13.

Note that the images are converted to 16-bit during the average projection, because otherwise information would be lost.

Results:  
Fig 3 - It would be useful to see an indication of the location of the segmented cisternae of the second rows indicated in the original image in this figure.

The suggested change has been made.