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4D Microscopy of Yeast

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Phillip Steindel, PhD
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August 6, 2018

Dear Phillip,

We are pleased to submit a revised version of our JoVE manuscript entitled “4D Microscopy of Yeast”. Thanks to you and the reviewers for the many constructive suggestions. Our responses are outlined in the attached document.

We look forward to scheduling the video session.

Sincerely,

Ben Glick

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TITLE

4D Microscopy of Yeast

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KEYWORDS

Confocal microscopy, 4D imaging, fluorescence, photobleaching, deconvolution, ImageJ, yeast

SUMMARY

This protocol describes the analysis of fluorescently labeled intracellular compartments in budding yeast using multi-color 4D (time-lapse 3D) confocal microscopy. The imaging parameters are chosen to capture adequate signals while limiting photodamage. Custom ImageJ plugins allow labeled structures to be tracked and quantitatively analyzed.

ABSTRACT

The goal of this protocol is to characterize how membrane compartments form and transform in live cells of budding yeast. Many intracellular compartments in yeast are dynamic, and a full understanding of their properties requires time-lapse imaging. Multi-color 4D confocal fluorescence microscopy is a powerful method for tracking the behavior and composition of an intracellular compartment on a time scale of 5-15 minutes. Rigorous analysis of compartment dynamics requires the capture of thousands of optical sections. To achieve this aim, photobleaching and phototoxicity are minimized by scanning rapidly at very low laser power, and the pixel dimensions and Z-step intervals are set to the largest values that are compatible with sampling the image at full resolution. The resulting 4D data sets are noisy but can be smoothed by deconvolution. Even with high quality data, the analysis phase is challenging because intracellular structures are often numerous, heterogeneous, and mobile. To meet this need, custom ImageJ plugins were written to array 4D data on a computer screen, identify structures of interest, edit the data to isolate individual structures, quantify the fluorescence time courses, and make movies of the projected Z-stacks. 4D movies are particularly useful for distinguishing stable compartments from transient compartments that turn over by maturation. Such movies can also be used to characterize events such as compartment fusion, and to test the effects of specific mutations or other perturbations.

INTRODUCTION

Compartments of the endomembrane system are in constant flux, and their full characterization requires live cell imaging. Described here is a protocol that employs 4D (time-lapse 3D) confocal microscopy to visualize fluorescently labeled compartments in budding yeasts. The method was

developed to track the dynamics of secretory compartments in *Pichia pastoris* and *Saccharomyces cerevisiae*[1-3]. This protocol focuses on *S. cerevisia*e, which has a nonstacked Golgi in which the individual cisternae are optically resolvable[4]. The unusual Golgi organization in *S. cerevisiae* enabled the demonstration by 4D microscopy that a Golgi cisterna initially labels with resident early Golgi proteins, and then loses those proteins while acquiring resident late Golgi proteins[3,5]. This transition can be visualized by creating a strain in which the early Golgi protein Vrg4 is labeled with GFP while the late Golgi protein Sec7 is labeled with a monomeric red fluorescent protein. When individual cisternae are tracked, maturation is observed as a green-to-red conversion[3]. This type of analysis can provide valuable information about protein localization and compartment identity. For example, two proteins with slightly offset arrival and departure times might sometimes appear to label different compartments in static images, but can be seen in 4D movies to label the same compartment at different time points[6,7]. Thus, 4D microscopy reveals phenomena that would not otherwise be evident.

Informative 4D microscopy of yeast compartments can be achieved with appropriate procedures and equipment[2]. Whenever possible, fluorescent protein tagging is performed by gene replacement[8] to avoid overexpression artifacts. Because intracellular structures are often very dynamic, 4D imaging is needed to ensure that a structure is tracked reliably over time. The protocol described here employs a laser scanning confocal microscope equipped with high sensitivity detectors. With this device, the entire cell volume of *S. cerevisiae* can be imaged by confocal microscopy approximately every 1-3 s, with 2 s intervals being typical. Data can be collected for up to 5-15 min depending on the labeling densities of the fluorophores and their photophysical properties. The main hurdle is to minimize photobleaching. For this purpose, the laser intensities are kept as low as possible, the confocal scan speed is maximized, and the optical parameters are configured to image at the Nyquist limit in order to capture the relevant information while avoiding excessive light exposure. These settings are also expected to alleviate phototoxicity, a factor that is often overlooked during live cell imaging[9-11]. The resulting noisy data are processed with bleach correction and deconvolution algorithms to facilitate quantification of fluorescence intensities.

Even with high quality 4D movies, the analysis is tricky because yeast compartments tend to be numerous, heterogeneous, and mobile. Due to the intrinsic limitations of confocal microscopy and the non-optimal settings required for prolonged 4D imaging, fluorescent structures that are near each other are hard to resolve. This problem can be circumvented by focusing on the small number of fluorescent structures that remain optically resolvable for the duration of the labeling period, with the assumption that those structures are representative of the whole population of labeled compartments. Fluorescent compartments that can be reliably tracked are identified by viewing movies of projected Z-stacks and by creating a series of montages in which the optical sections for each time point are arrayed on a computer screen. This analysis employs custom ImageJ[12] plugins, which allow an individual structure to be tracked in isolation.

Recent methods papers covered the use of fluorescent proteins in yeast[13] as well as the theory and practice of 4D confocal imaging of yeast cells[2]. This protocol focuses on the key practical aspects of a 4D imaging experiment. It includes some enhancements to previously described

procedures, as well as updated versions of the ImageJ plugin code and documentation. The example shown focuses on Golgi dynamics, but this protocol is equally suitable for imaging other yeast compartments.

PROTOCOL

1. Preparation

1.1. Make nonfluorescent minimal NSD medium[2]. The absence of riboflavin is expected to reduce background intracellular green fluorescence and associated phototoxicity. To further suppress photodamage, add Trolox[14] to 0.5 mM during imaging.

1.2. Grow the yeast strain overnight at ~23 °C to logarithmic phase in 5 mL NSD in a 50-mL baffled flask with good aeration. About 3-4 h prior to analysis, dilute the yeast culture in fresh NSD plus Trolox so that the final OD_{600} will be 0.5-0.8 at the time of imaging.

1.3. Prepare a 2 mg/mL solution of concanavalin A (ConA). If desired, freeze aliquots of this solution in liquid nitrogen and store at -80 °C.

1.4. Spin the ConA solution for 5 min at full speed in a microcentrifuge to remove particulate matter that may interfere with imaging. Then add 250 µL of the supernatant to a clean 35-mm coverglass bottom microscopy dish. After 15 min, wash 2-3 times with 2 mL of dH_2O and let dry.

1.5. Add 250 µL of the yeast culture to the ConA-coated dish, wait 10 min to allow the cells to adhere, and gently wash 2-3 times with 2 mL NSD. Cover the cells with 2 mL fresh NSD plus Trolox.

2. Imaging

2.1. Use a 63X or 100X oil immersion lens. A numerical aperture (NA) of 1.40 is sufficient, but a higher NA lens can also be used.

2.2. Format the frame size to 256 x 128 (width x height). If a larger frame size is needed, increasing the width will not reduce the scan speed as long as the confocal microscope is equipped with a resonant scanner.

2.3. Adjust the zoom factor to make the pixel size ~80 nm.

2.4. Use the maximum scan speed, which is typically on the order of 8 kHz. Turn on bidirectional X scanning if it is available, and if control experiments confirm that the scans from the two directions are in register.

2.5. Set the line accumulation to 4 or 6. Be sure to use accumulation (summing) instead of averaging.

2.6. Set the pinhole to 1.2 Airy units. Empirically, when imaging live yeast cells, this setting captures more photons than the standard choice of 1.0 Airy unit while causing no appreciable loss of resolution.

2.7. For each fluorescence channel, set the excitation wavelength, assign a high sensitivity detector, set the emission wavelength range, and turn on photon counting mode if available. The wavelength choices will depend on the fluorophores. As an example, excite GFP fluorescence at 488 nm and collect from 495-550 nm, and excite mCherry fluorescence at 561 nm and collect from 575-750 nm.

2.8. Set the intensity of each laser to be as low as possible. This setting must be determined empirically. An appropriate intensity will result in capture of a noisy but interpretable image sequence, with the fluorescence signal bleaching no more than 50% by the end of a 5-min movie. On the confocal microscope used here (see **Table of Materials**), this intensity corresponds to a laser power setting on the order of 5%.

2.9. Use notch filters or time gating to avoid capturing reflected light from the coverslip. If time gating is available, set the gating window to 0.6 – 10.0 ns.

2.10. Turn on brightfield imaging and use a low sensitivity detector for data collection. Set the gain to a level that makes the cells clearly visible. Do not use differential interference contrast (DIC) because the prism will interfere with the capture of reliable fluorescence data.

2.11. Set the Z-step interval to 0.25-0.35 µm. Image the entire volume of the yeast cells by collecting about 20-25 optical sections per Z-stack. Specify the directionality of imaging such that "down" moves toward the coverslip.

2.12. For a typical movie, set the time interval between Z-stacks to 2 s and set the movie duration to 5 to 10 min. Depending on the compartment under study, reduce the interval to make shorter movies of relatively fast dynamics, or increase the interval to make longer movies of relatively slow dynamics.

2.13. Save the movie as an 8-bit TIFF file with the brightfield images in the last channel. Higher bit depths are unnecessary at this stage, and the processing pipeline is configured to accept 8-bit TIFF data.

3. **Deconvolution**

3.1. Launch the deconvolution software (see **Table of Materials**), using the Classic Maximum Likelihood Estimation algorithm[15].

3.2. Open the data set generated in step 2.13. Select "Deconvolution wizard". Inspect the values displayed in the "Parameter wizard". The imaging parameters should be detected and correctly

displayed. Under “Refractive indexes”, change the “Embedding med.” value to 1.40 to approximate the yeast cytoplasm. Select “Set all verified” and “Accept”.

3.3. Proceed through the “Deconvolution wizard” for each fluorescence channel. Select “Manual” as the mode for background estimation. Inspect the raw data fluorescence intensity profile to determine an estimated background value, which is typically about 5.0.

3.4. In the deconvolution setup menu, enter a “Maximum iterations” value of 40 and turn off bleach correction. Enter an estimated SNR value, which is typically about 0.1. Click “Deconvolve”.

3.5. If necessary, return to the original data file, adjust the background and SNR values, and repeat the deconvolution, until noise is sufficiently removed without eliminating genuine fluorescence from dim structures.

3.6. Merge the brightfield and deconvolved fluorescence channels. For subsequent movie editing steps in ImageJ, arrange the channels such that red is first, green (if present) is next, blue (if present) is next, and brightfield is last. Save the image sequence as an 8-bit TIFF file.

4. Bleach Correction and Movie Generation

4.1. Import the deconvolved image sequence into ImageJ and convert it into a hyperstack by choosing Image > Hyperstacks > Stack to Hyperstack. Select “xyzct” from the drop-down menu, and fill in the number of channels, Z-stack slices, and time frames.

4.2. Choose Image > Color > Split Channels.

4.3. Correct the fluorescence channels for photobleaching using the ImageJ plugin available from http://wiki.cmci.info/downloads/bleach_corrector. Once that plugin has been downloaded and installed, choose Plugins > EMBLtools > Bleach Correction. Select “Exponential Fit.”

4.4. To merge the brightfield and bleach-corrected fluorescence channels into a hyperstack, choose Image > Color > Merge Channels. Save the resulting hyperstack as an 8-bit TIFF file.

4.5. Install the custom ImageJ plugins provided with this protocol. Follow the instructions in the accompanying document to view, edit, and quantify the image sequence, and to produce a final movie of the projected Z-stacks.

REPRESENTATIVE RESULTS

The example given here documents and quantifies the maturation of two yeast Golgi cisternae as visualized by dual-color 4D confocal microscopy[3]. A yeast cell contains on the order of 10-15 Golgi cisternae, each of which matures over a time course of approximately 2-4 min. Maturation can be visualized by tagging the early Golgi marker Vrg4 with GFP and by tagging the late Golgi marker Sec7 with a red fluorescent protein such as mCherry or mScarlet. An individual cisterna

labels initially with the Vrg4 marker, then undergoes a brief transition in which the markers are exchanged, and then labels with the Sec7 marker.

Because the cell contains multiple Golgi cisternae that are quite mobile, it is challenging to follow an individual cisterna throughout the entire labeling period. Cisternae are often too close together to be resolved unambiguously given the temporal and spatial limitations of the image data. Moreover, Golgi cisternae occasionally fuse[16], and late Golgi cisternae tend to cluster at sites of polarized growth[17]. As a result, a 4D movie rarely yields more than one or two cisternae that can be tracked reliably. One of the most challenging steps in the method is to examine the initial movie of the Z-stack projections and identify cisternae that are promising candidates for analysis.

The figures depict sequential steps in the procedure:

Figure 1 shows frames from movies of the Z-stack projections of either raw data or deconvolved and bleach-corrected data. **Figure 1A** compares the first frames for the raw versus deconvolved data. **Figure 1B** is from the same deconvolved movie, and shows several frames in which the two cisternae that were analyzed label first with the green Vrg4 marker and later with the red Sec7 marker. Those cisternae were chosen because they are clearly separated from other labeled structures for most of the movie. The images in this figure were generated from either raw or deconvolved and bleach-corrected 4D TIFF hyperstacks using the “Make Montage Series”, “Montage Series to Hyperstack”, and “Project Hyperstack” plugins.

Figure 2 shows part of the montage that was created for a Z-stack at one of the time points, both before and after editing to isolate the signal from one of the chosen cisternae. The images in this figure were generated from the deconvolved and bleach-corrected 4D TIFF hyperstack using the “Make Montage Series” and “Edit Montage Series” plugins.

Figure 3 shows several frames from the final movie of the projected Z-stacks (Video 1), with the original projections at the top and the edited projections at the bottom. The images in this figure were generated from the original and edited montages using the “Montage Series to Hyperstack”, “Merge Hyperstacks”, and “Project Hyperstacks” plugins.

Figure 4 shows quantification of the green and red fluorescence signals from the chosen cisternae. The data for this figure were generated from the edited hyperstacks using the “Analyze Edited Movie” plugin.

Analysis of these Golgi cisternae revealed that the Vrg4 marker arrives and persists for about 80 s, and then the Sec7 marker arrives and persists for about 60 s, with a brief period of overlap between the two markers. As illustrated by this example, 4D imaging provides both qualitative and quantitative information about the dynamics of a yeast compartment.

FIGURE LEGENDS

Figure 1. Projections of Z-stacks from a 4D movie. (A) Projections of the raw data (left) and deconvolved data (right) from the first Z-stack in a 4D movie. The green signal is from GFP-Vrg4, the red signal is from Sec7-mScarlet, and the gray signal is the brightfield images of the yeast cells. Scale bar, 2 µm. **(B)** Representative frames from the initial movie shown in (A) of the deconvolved and projected Z-stacks. Time points are indicated. The arrows mark the two cisternae that were chosen for analysis.

Figure 2. A portion of the montage from the Z-stack corresponding to the 01:20 time point. Each image in the montage is an optical section. The top panel show part of the original montage, and the bottom panel show the corresponding part of the edited montage in which the fluorescence signals from the chosen cisterna were selectively retained. In each panel, the optical sections are ordered from left to right in the first row and then from left to right in the second row. Scale bar, 2 µm.

Figure 3. Frames from the final movie **of the projected Z-stacks.** In these excerpts from Video 1, the original images are shown above the edited images. The first five frames show one cisterna that undergoes a green-to-red transition, and the next five frames show a second cisterna that undergoes a similar transition. Arrows overlaid on the original images indicate the cisternae that were tracked. Scale bar, 2 µm.

Figure 4. Quantification of the fluorescence signals from Video 1. The graphs represent the GFP-Vrg4 and Sec7-mScarlet signals from the two cisternae chosen for analysis, where the top panel represents the cisterna that becomes visible at the beginning of the movie and the bottom panel represents the cisterna that becomes visible later in the movie. Time zero is defined for each cisterna as the frame just before GFP-Vrg4 fluorescence was first detected.

Video 1. Final movie of the projected Z-stacks. The upper panel shows the original projections, and the lower panel shows the edited projections in which only the two cisternae chosen for analysis are visible.

DISCUSSION

4D confocal imaging of yeast organelles requires careful tuning of multiple parameters. The major concern is photobleaching and phototoxicity. A typical 4D movie involves collecting thousands of optical sections, so the laser illumination must be kept as low as possible. Tandem fluorescent protein tags can be used to boost the signal without increasing expression of the tagged protein[18,19]. Maximizing the scan speed helps to limit photodamage, and also allows Z-stacks to be captured at suitably short intervals. Voxel sizes that are at the Nyquist limit in both XY and Z minimize light exposure while theoretically recovering the information that is available from the optical setup[20]. In the end, each voxel in a signal-containing area of an optical section will typically receive only 1-3 photons. This amount of information is far below what is normally recommended for optimal imaging with a single Z-stack, or for deconvolution. But deconvolution still helps by smoothing the noisy signals[15], and such data sets can be analyzed and quantified.

Even with a high quality 4D dataset, tracking of labeled organelles is a complex task. With the ImageJ plugins provided here, a researcher can array each Z-stack on the computer screen and can easily move back and forth between time points and between the original and edited data. Those tools allow the labeled structures to be tracked through time and space with reasonable confidence. However, subjective judgment plays a necessary part, and bias must be avoided whenever possible. Particle tracking software could potentially help but is not yet sophisticated enough for most of the phenomena that are being studied. To offset this limitation, it is best to examine multiple markers and to test predictions of the models in different ways[3,6,7].

4D confocal imaging has played a pivotal role in characterizing the yeast secretory and endocytic pathways. This method demonstrated that ER exit sites form *de novo* and persist indefinitely[1], confirmed that Golgi cisternae mature[3,5], and clarified the role of the COPI vesicle coat in Golgi maturation[7,21]. More recently, 4D imaging provided evidence that the yeast early endosome is identical to the late Golgi, and that the yeast late endosome is a long-lived compartment[6]. While static imaging of fluorescently tagged compartments continues to be valuable, 4D imaging offers unique insights into the operating principles of yeast organelles.

An exciting recent development in the availability of self-labeling proteins for fluorescent tagging[22]. SNAP-tag behaves poorly as a fusion partner in yeast, but HaloTag behaves well. Bright and photostable membrane-permeant HaloTag substrates[23] have made it possible to perform 4D confocal imaging with far-red dyes[6]. Addition of a far-red imaging channel to the previously used green and red imaging channels allows robust three-color 4D microscopy (our unpublished data), thereby expanding the range of phenomena that can be studied in yeast by live cell imaging.

DISCLOSURES

The authors declare that they have no competing financial interests.

ACKNOWLEDGMENTS

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Figure 1

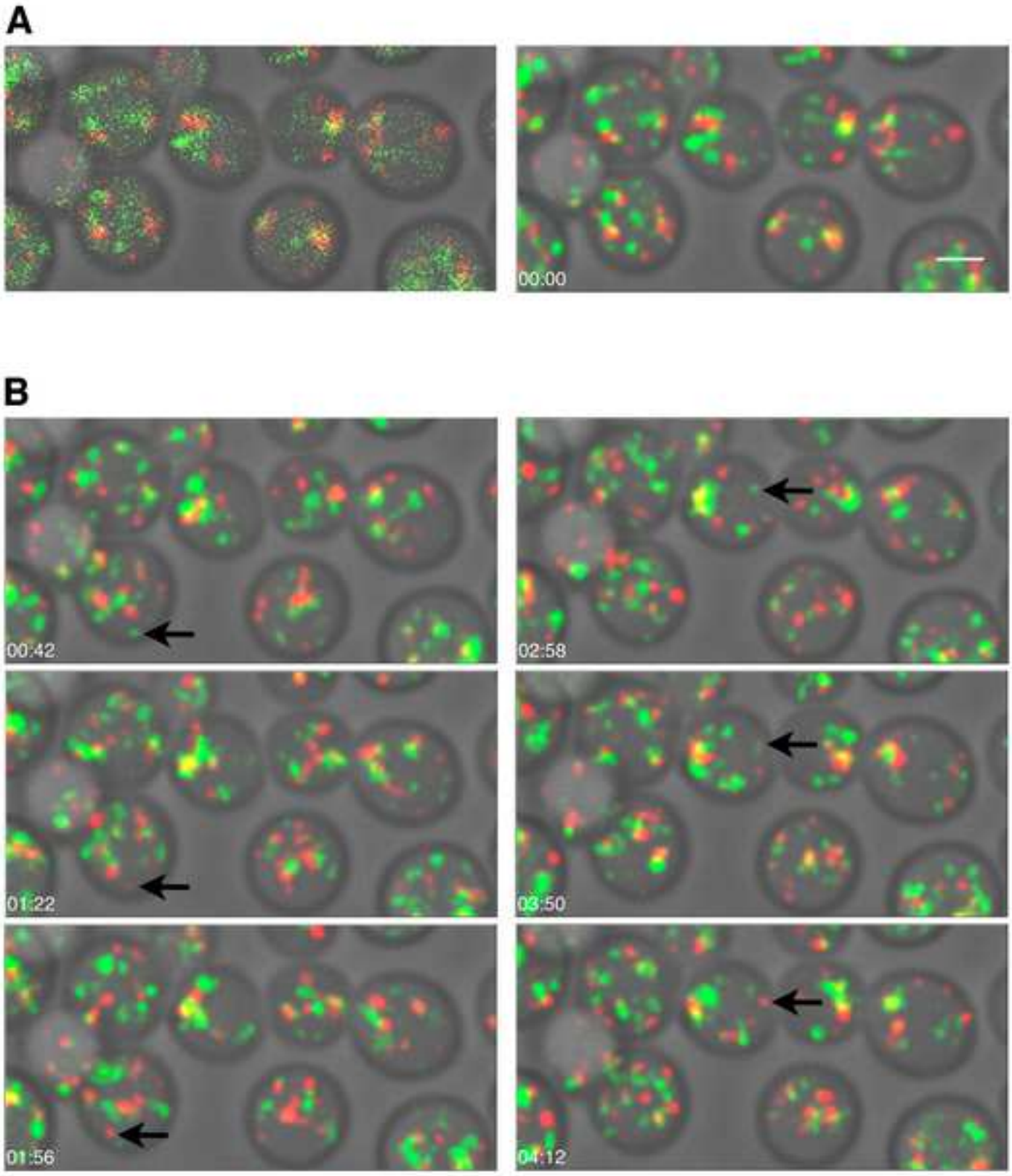

Figure 2

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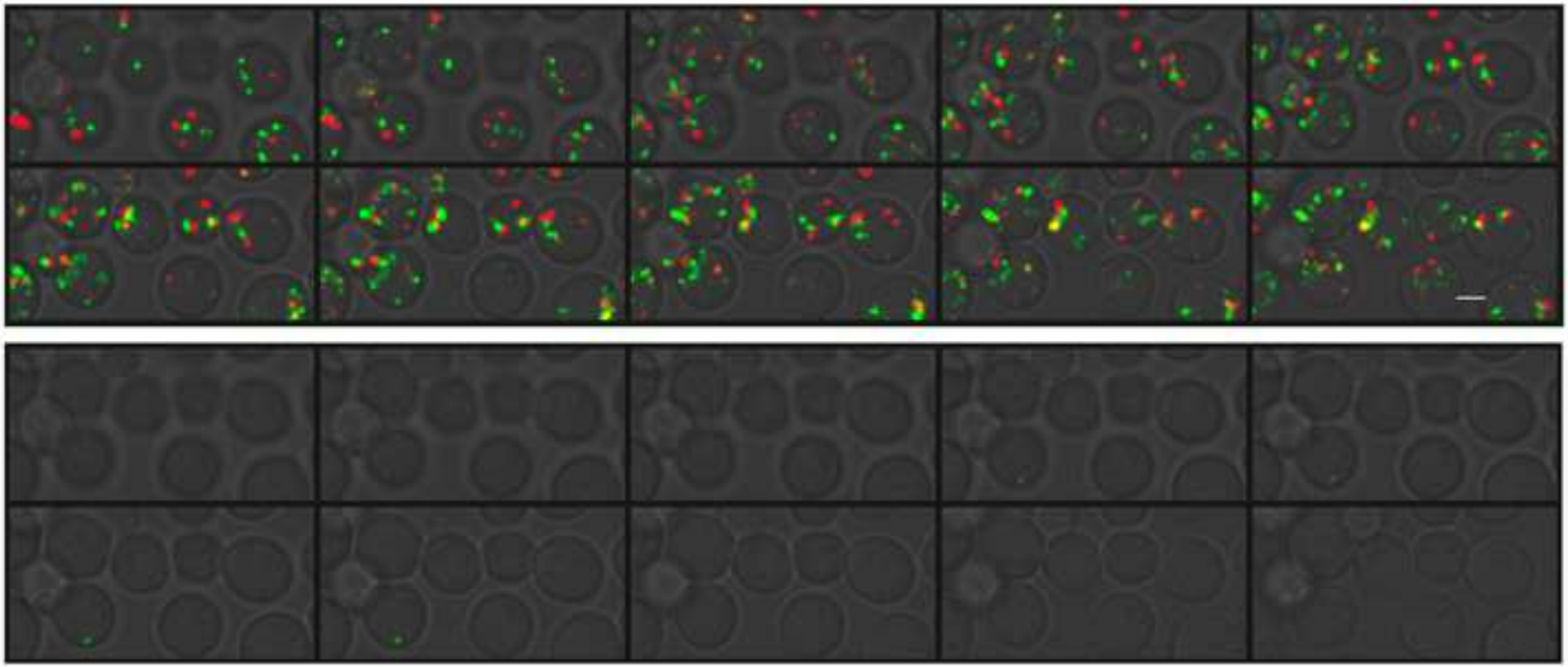

Figure 3

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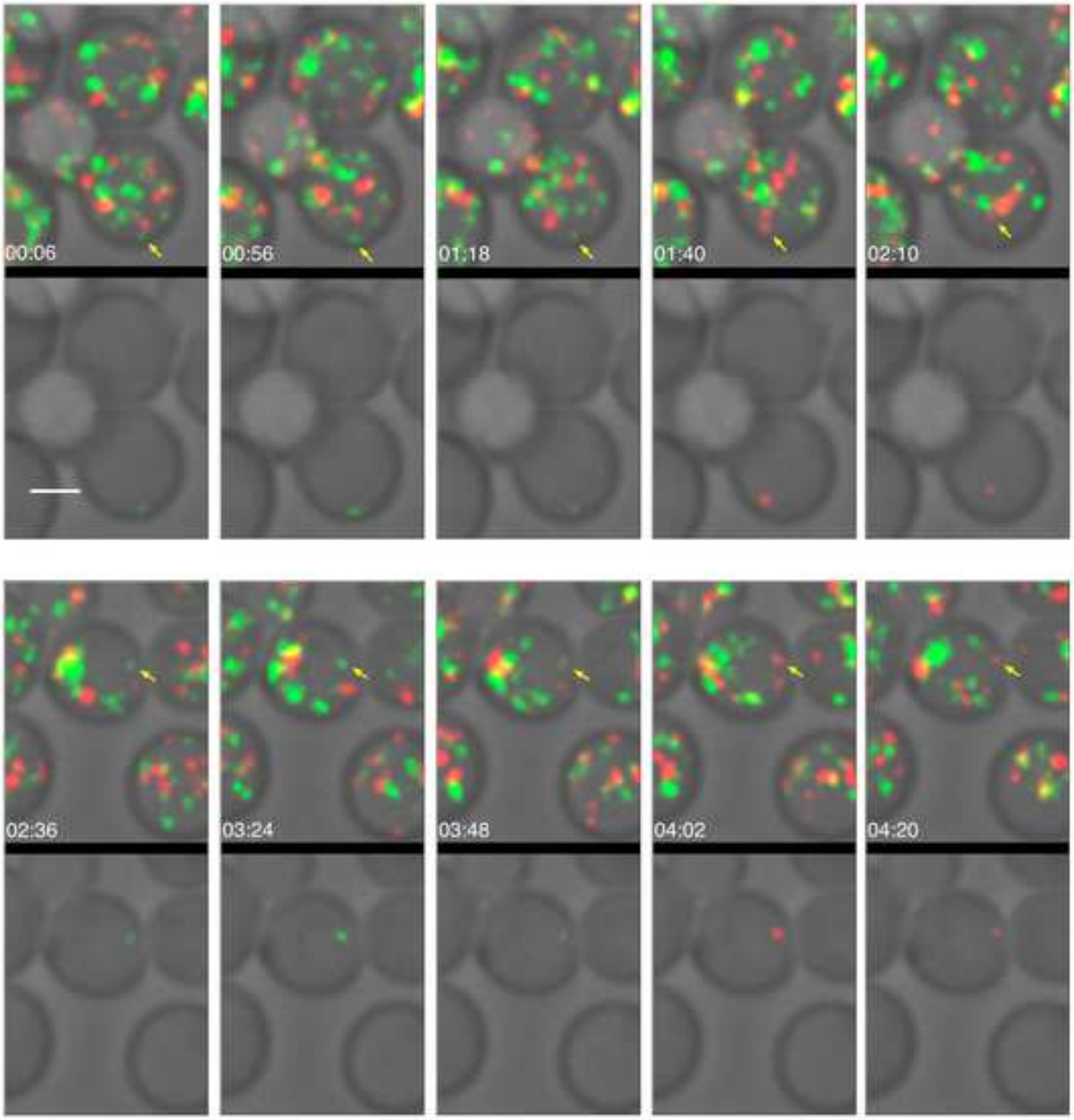

Figure 4

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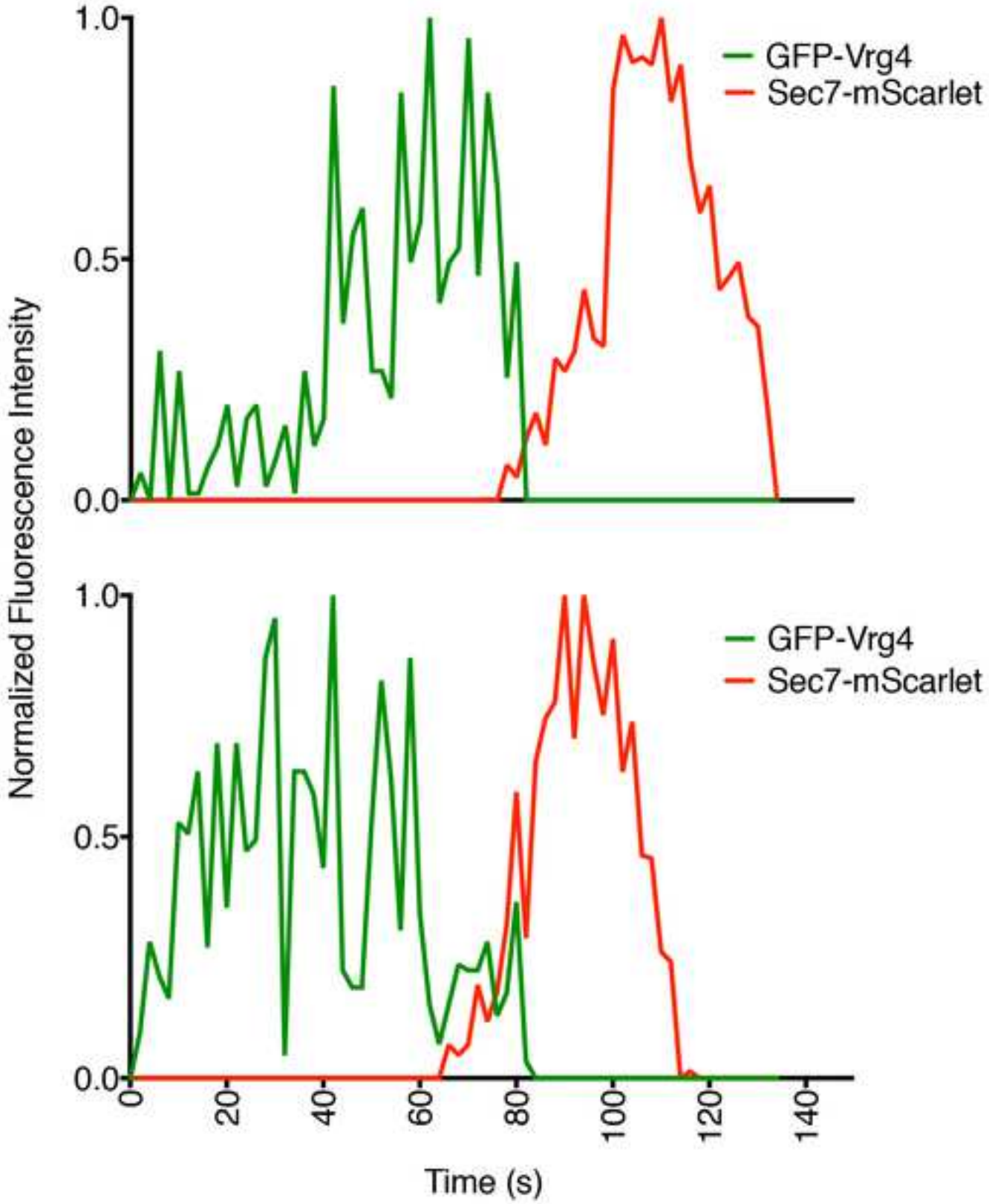
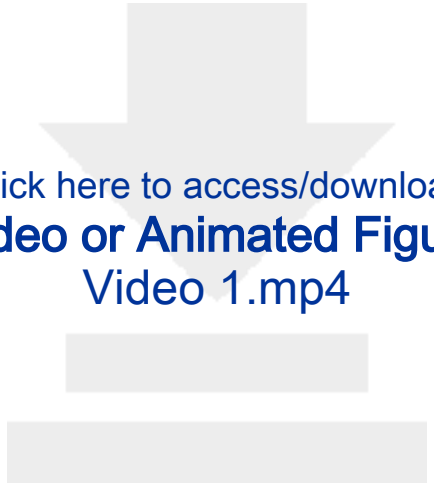


Video 1



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Video or Animated Figure

Video 1.mp4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
35 mm glass bottom dishes No. 1.5	MatTek	P35G-0.170-14-C	Imaging dishes
Concanavalin A powder	Sigma-Aldrich	C2010	
Trolox	Vector Laboratories	CB-1000	
Leica SP8 confocal microscope	Leica Microsystems		
Leica Application Suite X	Leica Microsystems		Microscope software
Huygens Essential software, version 17.04	Scientific Volume Imaging		Deconvolution software
ImageJ	NIH		Image processing and analysis software

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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.

Editing 4D Movies with ImageJ Plugins

The goal is to edit a 4D movie to display only one or a few spots. An edited movie can be analyzed to quantify the fluorescence time courses.

Typical Workflow

This procedure assumes that you are starting with a deconvolved and bleach corrected movie in the form of a 4D TIFF hyperstack. The description is for a movie with three fluorescence channels plus a fourth channel for the cell images, but if there are fewer fluorescence channels, the procedure is essentially the same.

The custom plugins for 4D movies should be placed in a folder within the ImageJ "plugins" folder. Detailed instructions for using these plugins are provided in later sections of this document.

1. Run the "Make Montage Series" plugin, and choose the 4D TIFF hyperstack. The result will be a scaled montage with adjusted gray values for the cells. In addition, the blue channel color will be adjusted to make the signal easier to see. Save this montage series.

2. Run the "Montage Series to Hyperstack" plugin. The result will be a 4D hyperstack that includes the adjustments described above. Save this hyperstack.

3. Run the "Project Hyperstack" plugin. The result will be an average projected composite TIFF movie showing all of fluorescence signals. Save this original movie.

4. View the original movie, and identify one or more structures that can potentially be followed cleanly in all three fluorescence channels. Note the positions and times at which those structures are present.

5. Run the "Edit Montage Series" plugin. Choose the previously saved montage series, and create a copy for editing.

6. Edit each fluorescence channel of this montage series so that it displays only the structure of interest in all three channels. Erase all of the extraneous fluorescence signals. Save this edited montage series.

7. If additional structures of interest were identified, repeat steps #5 and #6, creating an additional edited montage series for each structure.

8. Run the "Montage Series to Hyperstack" plugin for each edited montage series. Save these edited hyperstacks.

9. To quantify the fluorescence signals from each isolated structure, run the "Analyze Edited Movie" plugin for the appropriate edited hyperstack. Save the results.

10. If you wish to show more than one isolated structure in a combined edited movie, run the "Merge Two Hyperstacks" plugin and merge the first two edited hyperstacks.

The same plugin can be run again to merge in additional edited hyperstacks. Save the merged edited hyperstack, which can be projected to make the edited movie.

11. If you wish to show the original movie above the edited movie, run the "Merge Hyperstacks" plugin again, but this time choose the option of placing the original hyperstack above the edited one.

12. Once you have a fully processed hyperstack, run the "Project Hyperstack" plugin. Save the resulting final movie.

13. If you wish to make changes in how the fluorescence signals were edited, go back and edit the appropriate montage series, then regenerate the final movie.

14. As described below, use ImageJ to adjust the display parameters for the final movie if desired, and then generate an MP4 version of the final movie.

Plugin: Make Montage Series

1. Run the plugin, and choose a 4D 8-bit TIFF hyperstack from a confocal. This data set should already be deconvolved, bleach corrected, and cropped if desired.

2. Choose a scale factor for magnifying the individual slice images. The default value is 4.0, but you can choose another number as long as the montage will fit on the screen.

3. Choose the slices that will be used to create the montage. You can omit slices that have no fluorescence data. (Alternatively, such slices can be removed ahead of time by editing the hyperstack in ImageJ.)

4. Allow the montage series to be created. The plugin cannot be aborted during scaling, but can be aborted using Esc during assembly of the montage series.

5. Save the montage series, which will serve as the "original" montage series during the editing phase.

Plugin: Edit Montage Series

1. Run the plugin, and open the original montage series.

2. The first time you work with a given montage series, choose the option "Create New Montage" to duplicate the original montage series.

   If you have already begun editing the montage series, use "Open Existing Montage", and choose the edited montage series.

   In either case, the edited version of the montage series will be displayed on top of the original version.

3. Use the keyboard commands listed below to work with the original and edited montage series. You can also use the sliders at the bottom of the window. It is recommended to use these controls whenever possible rather than the standard ImageJ menu commands and image adjustment dialogs.

   a) Use Tab to switch between the original and edited montage series windows. These two windows should stay synchronized with respect to channel, time point, and display parameters.

   b) Use the Left Arrow and Right Arrow keys to move between time points. You can also drag the lower slider.

   c) Use Opt together with the Left Arrow and Right Arrow keys to switch between channels. You can also drag the upper slider.

   d) Use the Up Arrow and Down Arrow keys to increase or decrease the display brightness of the currently selected channel.

      Use Opt together with the Up Arrow and Down Arrow keys to jump to either the default brightness, or the maximum or minimum brightness, depending on the current brightness setting of the currently selected channel.

   e) Use the R, G, and B keys (uppercase or lowercase) to toggle visibility of the red, green, and blue channels, respectively. Use the Y key to toggle visibility of the gray channel showing the cells.

   f) With the edited montage series visible, use the Z key to replace the edited contents of the currently displayed channel and time point with the original contents.

      Note that Z is not a generic Undo key. This replacement function is irreversible.

   g) Use the A key to select the entire image.

   h) Use the E key to erase all of the fluorescence in the selected channel going forward or back in time. You will be prompted to choose the direction.

i) To delete everything except the desired spot, first select the spot in each of the relevant slices by drawing regions of interest (ROIs). A fast method for drawing ROIs is described below in (j).

You can use Shift to add pixels to an existing ROI, or Opt to trim pixels from an existing ROI.

When you are satisfied with the ROI, press the Delete key. For the currently selected channel, the nonselected pixels outside the ROI will be deleted.

If you make a mistake, reverse the procedure by pressing Z.

If you want to delete the selected pixels inside the ROI, use Opt together with the Delete key. If nothing is selected, the entire image will be deleted.

j) To automatically trace a spot in all of the relevant slices, do the following. First, select the spot by drawing a crude ROI. Make sure the appropriate channel is selected. Then press Return. The spot will be outlined in the original slice and in the adjacent slices.

If you change the brightness using the Up Arrow and Down Arrow keys, and then repeat the automatic tracing, the borders will expand or contract accordingly.

If the automatic tracing captures too much or too little, you can add to the selection using the Shift key or remove part of the selection using the Opt key.

4. Periodically save the edited montage series. The S key will work for this purpose. You can return to the edited file later if you wish to make additional edits.

Plugin: Montage Series to Hyperstack

1. Open an edited montage series.

2. Run the plugin. Choose the desired time points to generate an edited hyperstack.

3. This edited hyperstack can be merged with other hyperstacks as described below, or it can be projected to make a 4D movie. In addition, it can be quantified using the Analyze Edited Movie plugin described below.

Plugin: Analyze Edited Movie

1. Open a hyperstack generated from an edited montage series.

2. Run the plugin. You will need to enter the time interval between Z-stacks.

3. The results will be displayed in a window.

   "Red", "Green", and "Blue" are the total signals from the Z-stacks at each of the indicated time points.

   "Red Integrated", "Green Integrated", and "Blue Integrated" are the time-dependent numerical integrals of the signals.

4. If you click on this window and then save, the result will be a file that can be opened by Excel. You can then plot the data to show the primary or integrated fluorescence signals over time.

Plugin: Merge Two Hyperstacks

1. Run this plugin, and choose two hyperstacks generated by the "Montage Series to Hyperstack" plugin.

2. If you want to merge two edited hyperstacks to show two isolated structures in the same movie, choose the default "Merge fluorescence signals" option.

   If desired, the resulting hyperstack can be used as the input for another iteration to merge in data for a third isolated structure, and so on.

3. If you want to show the original movie above the edited movie, choose the "Place first above second" option.

   First choose the hyperstack corresponding to the original movie. Then choose the hyperstack corresponding to the edited movie, which may show two or more isolated structures as described in step #2.

Plugin: Project Hyperstack

1. Open a hyperstack that was either generated directly by the "Montage Series to Hyperstack" plugin, or further processed using the "Merge Two Hyperstacks" plugin.

   If desired, you can examine this hyperstack to see if there are slices at the top or bottom that contain either no fluorescence throughout the time course, or extraneous background fluorescence.

2. Run the "Project Hyperstack" plugin. When prompted, choose whether to project all of the slices, or to remove undesired slices from the top or bottom of the stack.

   Make sure that "Average intensity" is chosen for the projection. You can choose to project all of the time points, or just a subset if desired.

Adjusting and Converting the Final Movie

1. The result of the "Project Hyperstack" plugin should be a movie that is easy to view and appropriately scaled. However, you can adjust the output for each channel as follows.

    a) Choose Image > Adjust > Brightness/Contrast. In the projected movie, use the "c" slider to choose the channel (red, green, blue, or gray) that will be adjusted.

    b) Adjust the "Maximum" slider as desired to set the output of a given fluorescence channel.

    Alternatively, click "Auto" and then drag the "Minimum" slider back to "0".

    Alternatively, click "Set" and specify the values manually. This approach is particularly useful if you wish to have identical display values for two movies, e.g., an original and an edited movie.

    c) Another way to adjust a channel is by choosing Process > Enhance Contrast. A value of 0.1% saturated pixels will give the same result as the "Project Hyperstack" plugin. You can vary this number as desired.

2. To add a time stamp, choose Image > Stacks > Label. Use the format 00:00, with the appropriate time interval in seconds.

    To place the label in the lower left corner, set the Y location to be 1 less than the Y value of the lowest row of pixels.

    To place the label in the lower right corner, also set the X location to be 55 less than the X value of the rightmost column of pixels.

3. To make a movie for publication, choose File > Save As > AVI. For the compression, choose PNG. A frame rate of 10 fps is typically suitable.

    Convert the AVI file to MP4 format at https://video.online-convert.com/convert-to-mp4.

"Analyze Edited Movie" Plugin

"Edit Montage Series" Plugin

"Make Montage Series" Plugin

"Merge Two Hyperstacks" Plugin

"Montage Series to Hyperstack" Plugin

Click here to access/download

Supplemental Coding Files

Montage_Series_to_Hyperstack.java

"Project Hyperstack" Plugin