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# Examination of Mitotic and Meiotic Fission Yeast Nuclear Dynamics by Fluorescence Live-Cell Microscopy

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| **Corresponding Author:** | Susan Forsburg, PhD<br>University of Southern California<br>Los Angeles, CA UNITED STATES |
| **Corresponding Author's Institution:** | University of Southern California |
| **Corresponding Author E-Mail:** | forsburg@usc.edu |
| **Order of Authors:** | Susan Forsburg, PhD |
| | Wilber Escorcia |
| | Kuo-Fang Shen |
| | Ji-Ping Yuan |
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USC Davis School of Gerontology

3715 McClintock Ave.

Los Angeles, CA 90089-1091

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1 Alewife Center, Suite 200

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Dear editor,

We submit an article titled “Examination of mitotic and meiotic fission yeast nuclear dynamics by fluorescence live-cell microscopy” for your publication and video production consideration. We believe the simplicity of this protocol will help many yeast researchers answer important cell biology questions through live-cell fluorescence microscopy. We provide a method to successfully prepare fission yeast samples for prolonged fluorescence visualization. We also offer basic ImageJ analysis steps that researchers can follow to give meaning to their real-time microscopy observations of mitotic and meiotic events.

Although there are live-cell imaging protocols in the literature that successfully address real-time events during mitosis, few focus on the time-sensitive process of meiotic progression. In this methods paper, we present an optimized approach that has worked well in our hands and which produces abundant information from just a few live-cell videos. Provided researchers follow recommendations we stipulate in the manuscript, we believe they can study multiple aspects of meiosis that are difficult to examine through biochemical or molecular biology approaches. Some of the processes that we highlight, and which are amenable to our methods, deal with the timing, stability, and movement dynamics of nuclear events. For this reason, we ask for your serious consideration of this work.

Sincerely,

Wilber Escorcia, Ph.D

**TITLE:**

# Examination of Mitotic and Meiotic Fission Yeast Nuclear Dynamics by Fluorescence Live-Cell Microscopy

**AUTHORS AND AFFILIATIONS:**

Wilber Escorcia[1,2], Kuo-Fang Shen[1], Ji-Ping Yuan[1], Susan L. Forsburg[1]

[1]Program in Molecular and Computational Biology, University of Southern California, Los Angeles, California, USA

[2]Leonard Davis School of Gerontology, University of Southern California, Los Angeles, California, USA

Corresponding Author:

Susan L. Forsburg (forsburg@usc.edu)

Email Addresses of Co-authors:

Wilber Escorcia (escorcia@usc.edu)

Kuo-Fang Shen (kuofangs@usc.edu)

Ji-Ping Yuan (jipingyu@usc.edu)

**KEYWORDS:**

Fission yeast, mitosis, meiosis, DNA replication, chromosome segregation, cell division, live-cell microscopy

**SUMMARY:**

Here, we present live-cell imaging which is a non-toxic microscopy method that allows researchers to study protein behavior and nuclear dynamics in living fission yeast cells during mitosis and meiosis.

**ABSTRACT:**

Live-cell imaging is a microscopy technique used to examine cell and protein dynamics in living cells. This imaging method is not toxic, generally does not interfere with cell physiology, and requires minimal experimental handling. The low levels of technical interference enable researchers to study cells across multiple cycles of mitosis and to observe meiosis from beginning to end. Using fluorescent tags such as Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP), researchers can analyze different factors whose functions are important for processes like transcription, DNA replication, cohesion, and segregation. Coupled with data analysis using Fiji (a free, optimized ImageJ version), live-cell imaging offers various ways of assessing protein movement, localization, stability, and timing, as well as nuclear dynamics and chromosome segregation. However, as is the case with other microscopy methods, live-cell imaging is limited by the intrinsic properties of light, which put a limit to the resolution power at high magnifications, and is also sensitive to photobleaching or phototoxicity at high wavelength frequencies. However, with some care, investigators can bypass these physical limitations by carefully choosing the right conditions, strains, and fluorescent markers to allow for the

appropriate visualization of mitotic and meiotic events.

**INTRODUCTION:**

Live-cell microscopy allows researchers to examine nuclear dynamics without killing fission yeast cells and eliminates the need for lethal fixatives and stains. It is possible to observe the stability, movement, localization, and timing of fluorescently tagged proteins that are involved in important events such as chromosome replication, recombination, and segregation, in addition to the membrane and cytoskeletal movements. By maintaining cell viability and non-toxic signal detection, there is minimal physiological intrusion. When performed properly, this microscopy method can reduce confounding effects that arise from the extended technical handling or from spurious chemical reactivity. Furthermore, during an experiment, researchers can make pertinent observations of fission yeast nutritional and stress states, proliferative efficiency, and apoptotic status that indicate inappropriate imaging parameters or abnormal cell physiology[1-3].

Mitosis and meiosis are characterized by nuclear and cellular division. In meiosis, contrary to mitosis, genetic content is halved as parent cells give rise to daughter cells[4]. Because live-cell imaging provides a temporal dimension in addition to the spatial relationship, large numbers of cells can be examined in real-time. Live-cell microscopy has enabled researchers to examine the dynamics of nuclear division using fluorescent tags for histones[5], cohesin subunits[6], microtubules[7], centromeres[8], kinetochores[9], components of the spindle pole body (SPB)[10], and those of the chromosome passenger complex (CPC)[11]. Outside of the chromosome segregation apparatus, live-cell imaging has also captured the behavior of proteins necessary, but not directly involved in chromosome segregation. The function of these proteins has been shown to influence replication, chromosome recombination, nuclear movement, and chromosome attachment to microtubules[12-14]. These observations have contributed to a better understanding of cellular events whose functional components were not amenable to biochemical or genetic examination. With new advances in microscopy technology, limitations such as poor resolution, photobleaching, phototoxicity, and focus stability will lessen, thereby facilitating better real-time observations of mitotic and meiotic nuclear dynamics[1-3]. Meiosis is particularly challenging as it is a terminal differentiation pathway that requires close attention to timing.

The goal of this protocol is to present a relatively simple method for examining real-time fission yeast nuclear dynamics in mitosis and meiosis. To accomplish this, it is necessary to use cells that have not been exposed to environmental stress, prepare agarose pads that can withstand prolonged imaging, and mount cells at densities that facilitate visualization of single-cell dynamics. Moreover, this protocol makes use of cells carrying fluorescently tagged proteins that serve as useful markers for nuclear kinetics (Hht1-mRFP or Hht1-GFP), chromosome segregation (Sad1-DsRed), cytoskeleton dynamics (Atb2-mRFP), transcriptional activation in G1/S (Tos4-GFP), and cohesion stability in meiosis (Rec8-GFP). This method also introduces additional nuclear and cytosolic markers (**Table I**) that can be used in different combinations to address questions about specific cellular processes. Furthermore, basic Fiji tools are featured to help researchers process live-cell images and analyze different types of data. The strength of this approach stems from the use of real-time observations of protein dynamics, timing, and stability to describe processes that are integral for the proper execution of mitosis and meiosis.

**PROTOCOL:**

## 1. Media preparation[15,16]

1.1. Stock solutions

1.1.2. Prepare a 50x salt stock solution by adding 52.5 g of $MgCl_2 \cdot 6H_20$, 0.735 g of $CaCl_2 \cdot 2H_20$, 50 g of KCl, and 2 g of $Na_2S0_4$ in a bottle containing 1 L of distilled water. Mix the solution thoroughly and sterilize by filtration. Place at 4 °C for long-term storage.

1.1.3. Make a 1,000x vitamin stock solution by mixing 1 g of pantothenic acid, 10 g of nicotinic acid, 10 g of inositol, and 10 mg of biotin in a bottle containing 1 L of distilled water. Mix the solution well and sterilize by filtration. Keep at 4 °C for long-term storage.

1.1.4. Prepare a 10,000x mineral stock solution by adding 5 g of boric acid, 4 g of $MnSO_4$, 4 g of $ZnSO_4 \cdot 7H_2O$, 2 g of $FeCl_2 \cdot 6H_2O$, 1 g of KI, 0.4 g of molybdic acid, 0.4 g of $CuSO_4 \cdot 5H_20$, and 10 g of citric acid in a bottle containing 1 L of distilled water. Mix the solution thoroughly and sterilize by filtration. Place at 4 °C for long-term storage.

1.1.5. Make individual nutrient stock solutions by adding 7.5 g of either adenine, leucine, histidine or lysine into a bottle containing 1 L of distilled water. Use only 3.75 g to make the uracil solution. Sterilize solutions by autoclaving.

NOTE: Over time, uracil precipitates out of solution. To bring into the solution again, warm in the microwave at 60% power in 10 s increments or place in a 55 °C water bath for 20 min. Swirl the warm solution until all uracil clumps disappear.

1.2. Yeast extract plus supplements (YES)

1.2.1. In a 2 L flask, add 1 L of distilled water, 5 g of yeast extract base, 30 g of glucose, and 225 mg each of adenine, uracil, L-histidine, L-leucine, and L-lysine. Add 20 g of agar to make the solid medium.

1.2.2. Use a magnetic stir bar to completely dissolve ingredients into the solution. Agar will melt after the medium is sterilized by autoclaving.

NOTE: For convenience, ready-to-use YES powder is also commercially available.

1.3. Edinburgh minimal medium (EMM) and pombe glutamate medium (PMG)

1.3.1. In a 2 L flask, combine 1 L of distilled water with 3 g of potassium hydrogen phthalate, 2.2 g of $Na_2HPO_4$, 5 g of $NH_4Cl$, 20 g of glucose, 20 mL of salt stock solution, 1 mL of vitamin stock solution, and 0.1 mL of mineral stock solution. Replace $NH_4Cl$ with 2.2 g of L-glutamic acid

monosodium salt to prepare PMG. To make the solid medium, add 20 g of agar.

1.3.2. Using a magnetic stir bar, dissolve ingredients thoroughly. Agar will melt after the medium is sterilized by autoclaving. Before use, add nutrient stock solutions as necessary. For every 1 L of medium, add 15 mL each of adenine, leucine, histidine, and lysine. Use 30 mL for uracil, since it is less concentrated than the other nutrient solutions.

NOTE: For convenience, ready-to-use EMM and PMG powders are also commercially available.

1.4. Malt extract (ME)

1.4.1. Use a 2 L flask to mix 1 L of distilled water with 30 g of malt extract and 225 mg each of adenine, uracil, histidine, and leucine. Adjust the pH to 5.5. Include 20 g of agar to make the solid medium.

1.4.2. Dissolve components into the solution using a magnetic stir bar. Agar will melt after the medium is sterilized by autoclaving.

1.5. Sporulation agar with supplements (SPAS)

1.5.1. In a 2 L flask, add 1 L of distilled water, 10 g of glucose, 1 g of $KH_2PO_4$, 1 mL of vitamin stock, 45 mg each of adenine, uracil, histidine, leucine, and lysine hydrochloride. Add 20 g of agar to make the solid medium.

1.5.2. Use a magnetic stir bar to thoroughly combine ingredients. Agar will melt after the medium is sterilized by autoclaving.

**2. Fission yeast culture[15,16]**

2.1.1. Use YES solid medium to wake up fission yeast strains from cryogenic preservation. Depending on the temperature requirements of each strain, incubate at either 25 °C or 32 °C for 3-5 days.

2.1.2. When colonies are visible, prepare a starter liquid culture. Pick cells from individual colonies and inoculate them into test tubes containing 3 mL YES liquid medium. Grow at the appropriate temperature to the mid- or late-log phase.

NOTE: For proper aeration, use tubes or flasks with volumes at least 5x greater than the intended liquid culture volume. Shaking speeds between 150-220 rpm are customary for routine culture growth.

2.1.3. Dispense 250-500 µL of starter culture into a 50 mL flask containing 9.5-9.75 mL of either YES, EMM or PMG plus appropriate supplements. Allow cells to grow to the desired density and check under the microscope for the proper cell morphology and nutritional state.

NOTE: To store awoken strains for up to a month, seal YES plates with paraffin tape and place at 4 °C.

## 3. Sample preparation[2]

### 3.1. Microscope slide setup

3.1.1. Add 2 g agarose in a 500 mL beaker containing 100 mL of either minimal medium plus supplements (mitosis) or liquid SPAS (meiosis). Warm the agarose solution in a microwave oven at 60% power in 10 s increments or place in a 55 °C water bath for 10 min. Swirl the solution to ensure efficient melting. Prepare agarose slide-making setup (**Figure 1A**) to ensure quick pad preparation and prevent molten agarose from solidifying prematurely.

3.1.2. Allow the molten agarose to cool for 1 min at room temperature and dispense 50-100 µL spots on microscope slides using wide-bore pipette tips. Before the agarose cools down, place a microscope slide on the top to generate a spread pad of about 1.5-2 cm in diameter (**Figure 1B-C**). The width of the agarose pad is typically proportional to the length of imaging time. In other words, thicker pads withstand longer imaging periods.

3.1.3. Use a hydrocarbon mixture as a sealant to ensure proper coverage of slides with thick agarose pads and to prevent cell death from solvent exposure at the coverslip edges. Mix equal parts (w/w) of petroleum jelly, lanolin, and paraffin in a 500 mL beaker. Heat ingredients on a hot plate at 120 °C for 5 min. As components melt, carefully swirl the molten solution to ensure appropriate mixing.

### 3.2. Sample setup

3.2.1. For the examination of mitotic events, grow cells from starter cultures in either liquid EMM or PMG plus supplements to approximately mid-log phase ($OD_{595}$ of 0.4). Centrifuge 1 mL of the cell suspension at 1,375 x *g* for 1 min, remove the supernatant and resuspend the cell pellet in the minimal medium plus supplements to a final volume of 100 µL.

3.2.2. To image meiotic events, grow cells from starter cultures in the minimal medium plus supplements to late-log phase ($OD_{595}$ of 0.7-1.0). Combine equal volumes of opposite mate-type cells ($h^-$ and $h^+$) in a 1 mL cell suspension. Centrifuge cells at 1,375 x *g* for 1 min and resuspend the pellet in liquid ME. Repeat this step thrice to ensure efficient nutrient removal.

3.2.3. After the last ME wash, resuspend cells in 1 mL of ME and add it to a 50 mL flask containing 9 mL ME. Incubate for 12-16 h at 22-25 °C on minimal rotation speed (50-100 RPM). Abundant cell flocculation, which results in many round fission yeast clumps and indicates efficient mating.

3.2.4. Take 1 mL sample of the mating culture and centrifuge at 1,375 x *g* for 1 min. Eliminate the supernatant but leave 250 µL in the tube to resuspend cells. Before placing cells on agarose pads,

vortex vigorously for 5 s to disrupt clumps.

NOTE: The remaining 250 µL ME used to resuspend cells contains mating factors that help cells enter meiosis.

3.2.5. Place 20 µL of either a mitotic or meiotic cell suspension on a 2% agarose pad. Remove any excess medium by inverting the slide and putting it on the top of a lint-free paper towel for 2-3 s (**Figure 1D**). Set the slide pad-side up and gently place a glass coverslip, ensuring not to generate air bubbles (**Figure 1F**).

NOTE: Eliminating excess medium with a paper towel is more time-efficient than gravity absorption, which is particularly critical in meiosis experiments.

3.2.6. To create a cell monolayer, rotate the coverslip clockwise with the index finger for one (mitosis) or two (meiosis) full turns (**Figure 1F**). Ensure that the cell matter disperses across the agarose pad allowing for the better separation of single cells or asci. With the aid of a small wooden stick, dispense molten sealant along the edges of the coverslip to seal each agarose pad (**Figure 1G**).

3.2.7. Once the agarose pad is sealed, place it on the microscope stage and let it equilibrate for 10-15 min at the appropriate imaging conditions (e.g., temperature) (**Figure 1H**) to allow air bubbles to dissipate and let any last-minute agarose shifts to occur. Begin imaging once the slide is efficiently equilibrated and do not remove it from the stage until data collection ends.

NOTE: Control of temperature and humidity is determined by the microscope system employed and specific experimental requirements. If present, apply the temperature and humidity control to all imaging experiments. If absent, researchers must devise a way to prevent temperature fluctuations, especially during meiosis experiments.

**4. Live-cell imaging[2] and processing**

4.1. Use the 40x objective to find appropriate fields of view to the image. Switch to the 60x objective to begin data acquisition. Depending on the microscope system used, subsequent refocusing and sectioning on the sample at each time point will occur manually or through a microscope- or software-automated process.

NOTE: The images presented in this protocol were collected using a deconvolution, fluorescence microscope equipped with Sedat, RFP and GFP filter sets, nano-motion stage, 60x NA 1.4 objective lens, and 12-bit CCD camera. Built-in mechanical shutters and motorized filter wheels allowed for the reduced excitation exposure and photobleaching of samples.

4.2. Use appropriate software to acquire and process images. To deconvolve images, employ manufacturer-provided optical transfer functions.

NOTE: For details on the microscope equipment and software used in this protocol, refer to the **Table of Materials**.

4.3. To use a conventional fluorescence microscope to acquire live-cell images ensure that the microscope collects data digitally, has 40x or 60x objectives with numerical apertures (NA) greater than 1.2, and is able to perform optical sectioning.

NOTE: Without these requirements, images will show reduced resolution, compromising the quality of microscopic measurements and qualitative observations.

4.4. Use free plug-in programs (e.g., Fiji) to minimize systematic blur errors arising from the contrast loss during the image acquisition[17-20] and to ensure proper quantitative analysis of the fluorescence intensity and of other temporal and dynamic events within the cell.

NOTE: Other available commercial deconvolution programs can be found in the **Table of Materials.**

4.5. Choose the microscope filter sets that best match the fluorophores under observation. Ensure that the excitation and emission filters have the right band passes that allow for the specific detection of fluorescent proteins. For example, to detect CFP signal, an excitation and emission band pass of 430/25 and 470/30 is appropriate, but not for RFP fluorescence.

4.6. Use a minimal-effective-settings approach (MESA) when imaging fission yeast at multiple time points. In other words, avoid prolonged use of excitation wavelengths and employ the lowest excitation power and exposure time that generate acceptable, yet quantifiable and reproducible imaging data.

4.7. For prolonged imaging during mitosis or meiosis, limit the interval between acquisition time points to 5-10 min. Although 2% agarose pads can withstand 12 to 16 h imaging sessions, cell-shifting due to agarose evaporation is likely. Therefore, perform full mitosis or meiosis experiments in 4-6 h or 8-10 h windows, respectively.

4.8. Collect imaging data for 4-8 h consisting of at least 24-48 acquisition time points, respectively, one fluorescence protein, and a z-stack per time point and fluorescent channel comprised of 13 sections with 0.5-µm spacing using a 60x objective. This requires at least 0.5-1 GB of hard drive storage space. Thus, besides eliminating photobleaching and cell-shifting, create a workflow that considers computing capabilities[1-3].

NOTE: These acquisition parameters are typically set and modified in the software that controls microscope functions and which collects and processes images.

4.9. To examine specific nuclear processes in closer detail, perform image acquisition every 5-10 s, as long as the emission does not decrease substantially after frequent exposure. Carry out a preliminary fluorescence intensity analysis over different time periods to determine the point

after which the accuracy of the collected data is no longer reliable[1,3].

4.10. In the image-acquisition and image-processing software, use maximum intensity projection on the image z-stacks to observe all structures with high fluorescence density on a 2D plane regardless of their vertical location[1-3]. This facilitates a rapid examination of nuclear processes occurring in different voxels. Collect a mid-focal bright-field image to generate a reference picture of the cells under observation.

## 5. Image analysis[20,21]

NOTE: Image analysis in this protocol is performed using Fiji. For other analysis programs and Fiji plug-ins see **Table of Materials**.

5.1. Upload a deconvolved image by selecting the **Bio-Formats Importer** feature under the **Plugins** menu. In the **Import Options** pop-up window, select **Hyperstack**, **Default color mode**, and check **Autoscale** before pressing the **OK** button.

5.2. Ensure the displayed window has the correct number of fluorescence channels and time frames by scrolling sideways on the respective bars at the bottom. Save as a Tiff file, which does not compress data and prevents information loss.

5.3. Open an image that contains a scale bar generated during data processing by the microscope software. Determine the pixel length of the scale bar using the **Straight Line** tool to draw a parallel line of similar size and select **Measure** from the **Analyze** menu. Add a scale bar by setting the image pixel-to-µm ratio by selecting **Set Scale** from the **Analyze** menu.

5.3.1. In the **Set Scale** pop-up window, enter the calculated **Distance in pixels** and **Known distance in µm**, set **Pixel aspect ratio** to 1.0, put micron as the **Unit of length**, and check the **Global** box before clicking the **OK** button.

5.4. Use the **Set Measurements** option under the **Analyze** menu to choose different parameters to quantify when selecting **Measure**. For the purposes of this protocol, select the following metrics: **Area**, **Perimeter**, **Mean gray value**, **Median**, **Min & max gray value**, **Integrated density**, **Stack position**, and **Display label**.

5.5. Depending on the precision of the collected data, enter more than 1 for the **Decimal places** option and check the **Scientific notation** box if necessary. After applying the **Measure** command, a **Results** pop-up window will show the values for each pertinent parameter. Save the results as a .csv file for future analysis in a statistics program.

NOTE: It is not necessary to separate an image into its constituent fluorescent channels for determining the length, unless there is signal interference among the different colors in which case follow the step detailed below.

5.6. In the case of signal interference, select **Color** and **Channels Tool** from the Image menu and analyze each color separately. To measure the length of non-linear structures, right-click on the **Line Tool** and use the **Segmented Line** or **Freehand Line** option to trace the desired objects.

5.6.1. For linear structures or to determine the distance between two points, use the **Straight Line** feature and select **Measure** from the **Analyze** menu. Values for length in µm will be automatically added to the parameters previously selected under the **Set Measurements** option.

5.7. For measuring changes in the signal size and fluorescence intensity, first create a region of interest (**ROI**) library, which increases quantification accuracy and facilitates speedy measurements across an image stack. Select **Color** and **Channels Tool** under the **Image** menu.

5.8. In the **Channels** pop-up window, check the color channel for which intensity or area will be measured. Choose the **Adjust** and **Threshold** features under the **Image** menu. Check the **Dark background** box, select the **Default** method (unless otherwise required by the collected data), and pick **Red** to overlay the signals of interest.

NOTE: Measurement of protein stability, movement, and localization are closely dependent on the color threshold used for creating ROI libraries. It is important to choose the correct thresholding method for the type of fluorescent marker signal to be visualized[17,18].

5.9. Use the **Wand tool** to highlight each structure of interest and press the letter **T** on the keyboard to add the selected ROI to the pop-up **ROI manager** window. Repeat this process for each slice (time frame) in the image stack and store all ROIs by clicking on the **More** button and selecting **Save**.

5.10. Open the zipped **ROI folder** to load the **ROI manager** and click on each **ROI identifier** on the left side panel. Select **Measure** from the **Analyze** menu and repeat this command on each ROI identifier to quantify the objects of interest in all slices of the image stack.

5.11. If cell shifting is not an issue and the focal plane remains the same for all slices of the stack, click on **Multi measure** in the **ROI manager**. In the pop-up window, select **Measure all slices** instead of **One row per slice** to iterate measurements across the image stack. Save results as a csv file and analyze measurements in a statistics program.

5.12. For multiple nuclear signals comprising the structure of interest, press the **Shift** key while selecting objects with the **Wand tool** to create a single ROI. Alternatively, create an ROI of constant size with the **Oval tool** to encompass all the signals of interest.

NOTE: This oval approach may be necessary to measure and describe the signal behavior over an area where fluorescence signal changes in size and intensity over time. Signal intensity, in this case, is the mean signal density over a given area.

5.13. Qualitatively determine co-localization of two fluorescent signals by the change in color of

the signal overlap region. However, as the co-localization zone narrows, it is more difficult to distinguish signal overlap. In this case, follow the steps detailed below.

5.14. Using the **Channels Tool,** as described in step 5.6, draw a straight line over each signal in question, and select the **Plot Profile** command under the **Analyze** menu.

5.14.1. Repeat this step for all colors in question using the same drawn line.

5.14.2. In the **Plot of sample** pop-up window that appears after each profiling step, press the **List** button to call up a **Plot Values** window, and save the measurements for each signal as a csv file.

5.14.3. In a statistics program, create a graph that plots the **Gray Value** for each signal against its corresponding location (in µm) along the drawn line. Lack of overlap among signal profile plots generally indicates the lack of co-localization.

NOTE: Use the **Plot Profile** tool on projected images to examine signal co-localization. This is only reliable if such overlap is also observed through the image z-stack.

5.15. To monitor the dynamic behavior of fluorescent proteins over time use the **Multi Kymograph** tool found under the **Analyze** menu. The resulting image shows the fluorescent signal movement through a series of condensed snapshots in each slice of the z-stack, which represents individual time points.

5.15.1. Use the **Channels Tool** as described in step 5.6 to isolate the object of interest in the correct channel. Make sure the selected object or structure does not shift considerably through the z-stack. Place a straight line or rectangle over the object, select the **Multi Kymograph** tool from the **Analyze** menu, and enter the desired width of the line overlaying the object.

5.16. Create image panels that show nuclear dynamics over a specific time window using the **Channels Tool** as described in step 5.6 and by selecting the **Stacks** and **Make Montage** tools from the **Image** menu. In the **Make Montage** pop-up window, enter the number of **Columns** and **Rows** desired, set a **Scale Factor** of 1.0, choose the **First** and **Last** slices (time frames), and change **Border width** to at least 5 before pressing **OK**. It is possible to choose which images in the stack to show by changing **Increments** to suit the desired sequence.

5.16.1. To generate a movie, upload the desired hyperstack, select **Save** as an **avi** file from the **File** menu, choose none for **Compression**, and enter a **Frame rate** of 2-8 fps. Select the **Make Montage** command while picking **Composite** in the **Channels Tool** to merge or separate all colors comprising the image.

NOTE: Two avi files (**Movie 1-2**) are provided in this protocol. Use them in Fiji to try different features highlighted throughout step 5.

5.16.2. To show a single, representative cell, use **Transform** and **Rotate** from the **Image** menu

and enter degrees of rotation. Negative numbers rotate objects to the left, while positive values rotate objects to the right. Once the cell is in the proper orientation, draw a rectangle that encompasses the entire cell through the z-stack or during the time frames of interest and select **Crop** from the **Image** menu. Proceed as mentioned in step 5.16 and 5.16.1 to generate an image panel or movie.

5.17. Add a scale bar to the image panel or movie by selecting **Tools** and **Scale bar** from the **Analyze** menu. In the **Scale Bar** pop-up window, enter 5-15 for **Width in microns** for single cells or 100-250 for entire fields of view, choose white or black for **Color**, and pick an appropriate **Location** to place the scale bar.

5.17.1. For movies, it is useful to show time progression during nuclear events. To show time change between frames, select **Image**, click on **Stacks**, use the **Time Stamper** tool, indicate the start frame in the time series, and select an appropriate location for the time display.

**REPRESENTATIVE RESULTS:**

Whether live-cell imaging is used for mitosis or meiosis, it is crucial to employ healthy fission yeast cells before making any type of observation. The quality of the resulting data relies heavily on the starting material. If cells starve due to nutrient limitation or overgrowth, they will show excess vacuoles and decreased cell size (**Starvation**, **Figure 2A**). For mitosis experiments, it is best to avoid using cells that show cellular stress (**Starvation**, **Figure 2A**). Otherwise, experimental results will be inconsistent and irreproducible. To circumvent this limitation, choose proper wild type controls and become familiar with the various phenotypes of mutants of interest. For example, mitotic cells starved for 12 h cease to actively replicate DNA. Since Tos4-GFP expression is associated with G1/S-phase activity[22,23], logarithmic cells carrying this marker and one for nuclear division (Sad1-DsRed[10]) show pan-nuclear Tos4-GFP expression, Sad1-DsRed foci separation, and ongoing septation (suggesting active DNA replication and cell division) (**Log**, **Figure 2A**), while starved cells show no such activity (**Starvation**, **Figure 2A**). This result is consistent with the effects of nutrient limitation in fission yeast, which decreases transcription in G1, activates the G1/S cell cycle checkpoint, and promotes G0 entry[5]. For meiosis experiments, cells must grow to a high density, but without reaching the stationary phase. Afterward, cells of both mate types are mixed and incubated together in low nitrogen media. Failure to mate, as is the case when cells are not sufficiently starved of nitrogen, will prevent them from entering meiosis (**Inefficient**, **Figure 2B**). Robust flocculation of the mating cell suspension increases cell-to-cell interaction and thus indicates successful mating and efficient meiotic induction (**Efficient**, **Figure 2B**).

Agarose gel pads and physical disruption of cell clumps guarantee proper visualization of single cells or asci (**Log**, **Figure 2A**; **Efficient**, **Figure 2B**). Pads must provide a rigid, yet moist platform on which cells are simultaneously fixed in place and protected from desiccation. In addition, pads must be thick enough to withstand changes due to evaporation and provide a leveled surface that permits proper focus throughout image acquisition (**Figure 1C**). Coverslip rotation is necessary to separate and spread cells within mating aggregates (**Figure 1F**; **Efficient**, **Figure 2B**). Without this step, few asci but numerous haploid cells are observed because asci become

trapped within inaccessible layers of mating clumps, while haploid cells are free to populate all available monolayer spots (**Figure 2B**). Even for mitotic experiments, where cell clumping is less problematic, coverslip rotation moves cells around the pad to create a single cell layer with sufficient space between cells to reduce crowding effects and allow for cell duplication (**Log**, **Figure 2A**).

Atb2 is an α-tubulin component involved in chromosome segregation in fission yeast mitosis and meiosis[7,14]. When fluorescently tagged, this cytoskeleton component allows for examination of the stages that characterize nuclear separation in mitosis. During prophase (**0'-20'**, **Figure 3A**), nucleation of the mitotic spindle occurs in the nuclear side of the spindle pole bodies (SPBs), as revealed by Atb2-mRFP fibers set against an Htt1-GFP[5] pan-nuclear background. During the metaphase-anaphase transition (**20'-40'**, **Figure 3A**) the mitotic spindle extends outward and the nucleus splits in two. As the cell enters telophase (**60'-80'**, **Figure 3A**), Atb2-mRFP expands bidirectionally until chromosomes are entirely separated. After this point, Atb2-mRFP fibers regroup and spread across the cell surface to regain their interphase form in each of the resulting daughter cells. Cytokinesis (**>120', Figure 3A**) occurs only after a septum forms and divides the cell by fission. Following changes in Hht1-GFP intensity over a mitotic cycle reveals three important steps in nuclear dynamics: metaphase-anaphase (medium intensity, DNA compaction: **20'-40', Figure 3A-B**), telophase (low intensity, DNA separation: **60'-80'**, **Figure 3A-B**), and G1 (increasing intensity, DNA duplication: **100'-120', Figure 3A-B**). Similarly, but using cells that only carry Hht1-mRFP and employing the **multi-kymograph tool** (see step 5.15) in Fiji, it is possible to observe mitotic division from metaphase to telophase and evaluate the nuclear movements involved during proper sister chromatid segregation (**Normal**, **Figure 3C**). Mis-segregation kymographs show signal activity between the two main nuclear paths, indicating possible mis-segregation due to chromosome fragmentation or inappropriate chromatid separation (**Lagging**, **Figure 3C**).

As mentioned previously, in budding and fission yeast, Tos4 is transcribed in G1 and functions during DNA replication in S phase[22,23]. This can be observed in a kymograph where Tos4-GFP expression increases in the nucleus after Sad1-DsRed[10] foci move to opposite directions (indicating nuclear division), but before the septum forms, which precedes cytokinesis (**39'**, **Figure 3D**; **36'**, **Figure 3E**). The phase of high Tos4-GFP activity begins at the end of the M-G1/S transition (**45'**, **Figure 3E**) and ends in G2 (**>60'**, **Figure 3E**), right after the cell division. While greatly diffused during G1, Tos4-GFP signal intensity increases post anaphase and throughout S-phase and co-localizes with segregated Sad1-DsRed foci (**45'-60'**, **Figure 3E**). This result reveals the septation period in fission yeast when genome duplication has begun in daughter cells (G1/S), but cytokinesis has not occurred yet.

Hht1 is histone H3 in fission yeast and is commonly modified with fluorescent tags to visualize nuclear DNA[5,14]. In mitosis, as cells transition from metaphase to telophase (**20'-120'**, **Figure 3A**), two distinguishable changes to nuclear mass are observed. The first change involves a contraction of nuclear size in metaphase (**20', Figure 3A**), while the second shows nucleus splitting during anaphase (**40'**, **Figure 3A**). Besides sharing these changes with mitotic cells, meiotic cells exhibit nuclear oscillation (i.e., horse-tailing) (**-100' to -50'**, **Figure 4A-B**) during homologous

recombination and further reduction of nucleus size at the end of anaphase II (**70'-90'**, **Figure 4A**). Hht1-mRFP is used to examine mis-segregation phenotypes such as lagging or fragmented chromosomes (**Lagging**, **Figure 3C**). It is also employed to observe and describe the nuclear dynamics in each of the phases of meiosis (**Figure 4A-B**). Nuclear mass is large and fluctuates in size during much of horse-tailing (**HT**: **-100' to -50'**, **Figure 4A-B**)). As cells enter metaphase (**MT**: **-40' to 0'**, **Figure 4A-B**), nuclear size and oscillation decrease, consistent with increased condensation activity at this stage. The onset of both anaphase I (**MI**: **10'-60'**, **Figure 4A-B**) and II (**MII**: **70'-90'**, **Figure 4A-B**) is associated with further nuclear reduction and lack of nuclear movement, which correlates well with the two nuclear divisions that characterize meiosis I and II (MI & MII). Thus, meiosis is associated with nuclear size fluctuations when homologous chromosomes align and recombine and with nuclear size reductions following two rounds of chromosome separation. Alleles that change these nuclear dynamics are likely associated with genome stability regulation in meiosis[12,13].

Rec8 is the α-kleisin subunit of meiotic cohesin in fission yeast. It is loaded onto the chromatin after DNA replication (mei-S) and keeps sister chromatids tethered to each other until anaphase II. After metaphase I, Rec8 is removed from chromosome arms by separase and is protected at the centromere by Sgo1-PP2A. At the end of homolog segregation, Rec8 is also eliminated at the centromere, thereby allowing for sister chromatid separation (**Figure 5A**)[6,24]. Rec8-GFP along with markers of chromosome segregation such as Sad1-DsRed or Hht1-mRFP allow for the visualization of cohesion dynamics during meiosis. Rec8-GFP signal is pan-nuclear during mei-S (**<<-90'**, **Figure 5A-B**), prophase I (**-90' to -50**', **Figure 5A-B**), and metaphase I (**-40' to 0'**, **Figure 5A-B**). This observation reveals the association of Rec8-GFP along chromosomes axes that follows DNA duplication. As cells enter anaphase I (**10'**, **Figure 5A-B**), Rec8-GFP intensity decreases throughout the nucleus but remains strong at the centromere, where it forms a focus in each nuclear mass (**20'-70'**, **Figure 5A-B**). This result is consistent with Rec8-GFP degradation along chromosome arms but not at the centromere, that ensues after homolog segregation. Before anaphase II, the Rec8 focus disappears, freeing sister chromatids for separation in MII (**70'-80'**, **Figure 5A-B**). The Rec8-GFP marker is, thus, useful to examine conditions that disrupt the establishment, removal, and overall stability of meiotic cohesion. Paired with a marker of nuclear division such as Hht1-mRFP[5,13], Rec8-GFP can be employed to address questions of how cohesion timing and stability prior to and during meiosis affect proper chromosome segregation[12,13]. This protocol does not address proteins whose dynamics occur primarily in the cytosol. However, **Table I** shows a few cytosolic proteins that are commonly used to examine cytoskeleton and membrane processes necessary for endocytosis, exocytosis, and cytokinesis among other processes.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Preparation of agarose pads for live-cell imaging.** (**A**) Slide preparation set-up assembled using a pipette tip holder, lab tape, and microscope slides. Placing slides perpendicular to each other creates a pocket where the agarose pad forms. Addition of lab tape pieces at the sides adjusts the agarose pad width to the required specifications. (**B**) Molten agarose is let to cool down before setting it on microscope slides to make pads. In this step, it is necessary to dispense the agarose volume slowly to avoid creating air bubbles. (**C**) The top slide is placed on

the dispensed agarose spot to form a circular pad with a straight surface, even edges, and no visible agarose cracks. (**D**) After putting a sample of cell suspension on the agarose pad, invert slide and place atop a lint-free paper towel to remove extra liquid medium. (**E**) Carefully place a coverslip on the agarose pad, making sure not to trap any air bubbles and checking that the focus plane is flat to avoid cell shifting and focusing issues. (**F**) Slowly and cautiously, rotate coverslip clockwise to disrupt cell clumps and to generate a cell monolayer that allows for cell expansion and better cell visualization. (**G**,**H**) Use a heated hydrocarbon mixture to seal agarose pads and allow them to equilibrate to the desired temperature before imaging.

**Figure 2: Picking the right cells.** (**A**) Top panels show fission yeast cells left to starve in EMM plus supplements for 72 h and bottom panel shows cells undergoing logarithmic growth. In cells that are left to starve, small cell morphologies can be appreciated. The red open arrow shows vacuolar granules that are characteristic of starvation. In logarithmic cultures, cells showing elongated morphologies and septa (red arrow) abound, indicating active cycling dynamics. The right-side panels show cells expressing Tos4-GFP and Sad1-DsRed signals during starvation and proliferation. Pan-nuclear Tos4-GFP expression and Sad1-DsRed signals that localize to opposite cell poles are seen during active proliferation, but not in starvation. (**B**) Top panel shows cells of the same mate-type (h+) failing to mate efficiently. The red open arrow shows a pair of vacuolar cells undergoing karyogamy. This is not unusual in cultures of h+ cells, where 10% of cells can switch mate-type to h-. Bottom panel shows asci resulting from efficient mating. Zygotic asci take multiple forms including the zig-zag (red arrow) and banana cell shapes (red open arrow). Scale bar represents 5 µm in length.

**Figure 3: Mitotic nuclear dynamics.** (**A**) Histone H3 (Hht1-GFP) and microtubule (Atb2-mRFP) proteins were followed during one mitosis cycle (120 min). Individual signals for Hht1-GFP and Atb2-mRFP are shown in black and white panels, while the BF merge shows them in their respective colors. (**B**) Quantification of Hht1-mGFP intensity over a mitotic cycle. The change in Hht1 levels is indicative of nuclear division during mitosis and DNA duplication in G1. (**C**) Kymographs showing normal or abnormal segregation in mitosis where the resulting nuclear paths either bifurcate and retain DNA integrity or deviate and promote chromosome fragmentation or premature chromatid separation, respectively. (**D**,**E**) Kymograph and micrograph panels showing the duration of M-to-G1/S revealed by the segregation of Sad1-DsRed and appearance of nuclear Tos4-GFP signals. Note the middle panels in **E** which were generated using the FIRE LUT in Fiji to create an intensity-graduated thermal map that facilitates the identification of spots with high Tos4-GFP expression; in this case, localized to the nucleus and overlapping Sad1-DsRed signals, as expected. Scale bar represents 5 µm in length.

**Figure 4: Meiotic nuclear dynamics.** (**A**) Panel series showing movement, compaction, and division of the nucleus (Hht1-mRFP) during meiosis. Horse-tailing (HT) shows extensive side-to-side nuclear oscillations followed by Metaphase (MT), where nuclear lateral movement decreases and entirely halts right before anaphase I. In meiosis I (MI), the main nuclear mass splits into two, while in meiosis II (MII) four nuclei are generated during the process of sister chromatid separation. (**B**) Quantification of nuclear area change (Hht1-mRFP) in prophase, metaphase, MI & MII. Nuclear area is dynamic during HT oscillations, becomes condensed in MT,

and further decreases in size during MI & MII, where little nuclear movement is observed (Long Nuclear Axis). Measuring the longest nuclear axis (Long Nuclear Axis) is based on the idea that as a circle stretches, it ceases to have a constant diameter and generates at least two main axes: long and short. Thus, when monitoring changes in the nucleus, changes in either the long or short axis provide indications of nuclear movement. Scale bar represents 5 µm in length.

**Figure 5: Meiotic cohesin dynamics.** (**A**) Panel series showing how Rec8-GFP signal change correlates with meiotic nuclear dynamics. During HT and MT, Rec8-GFP signal is pan-nuclear and follows nuclear movement (Hht1-mFRP). In MI, Rec8-GFP dissipates from the nucleus, leaving only a pair of foci, which is consistent with Rec8 removal from chromosome arms and establishment of Sgo1-PP2A protection at the centromere. As the cell approaches MII, Rec8-GFP foci begin to disappear and are no longer seen right before anaphase II. (**B**) Quantification of Rec8-GFP intensity from HT to MII. Similar to what is seen in **A**, GFP signal is high through HT and MT, substantially decreases during MI and completely disappear in MII. This pattern corroborates cohesin dynamics at chromosome arms and the centromere, where it keeps sister chromatids tethered until ready to be separated in MII. Scale bar represents 5 µm in length.

**Table 1: Markers of nuclear and cytosolic dynamics.**

**Movie 1: M-to-G1/S transition showing spindle pole body (SPB; Sad1-DsRed) separation preceding septation and accumulation of Tos4-GFP nuclear signal.**

**Movie 2: Completion of the mitotic cycle showing the microtubule (Atb2-mRFP) extension correlating with nuclear (Hht1-GFP) division.**

**DISCUSSION:**

Live-cell imaging during mitosis and meiosis offers the opportunity to examine nuclear dynamics without substantially disrupting fission yeast physiology during data acquisition. However, caution must be exercised to ensure cells grow to the desired densities free of environmental stresses; and for meiosis, that the cells are imaged during the time of terminal differentiation and not too early or late. Excess cellular crowding, starvation, and inappropriate growth temperatures are common factors that contribute to irreproducible observations. In addition, during strain construction, it is crucial to test that fluorescent tags do not interfere with the functions of target genes nor impact overall cell fitness. Failure to closely inspect the phenotypes of strains carrying fluorescent markers may result in inconsistent and incomparable results across similar genotypes.

Though microscope agarose pads are simple to assemble, they can also pose a hurdle in obtaining valuable imaging data. Creating agarose pads is as simple as placing three microscope slides parallel to each other, dispensing molten agarose on the middle slide, and putting a slide that transverses the top of the other slides. It is useful, however, to assemble a slide-maker apparatus that allows for width modifications to manufacture resistant, situation-specific agarose pads. A simple slide-maker that gives pad width flexibility consists of a platform (pipette tips holder or the bench), two microscope slides, and lab tape acting as the pad-width adjusting mechanism

(**Figure 1A**). Exact pad thickness will depend on imaging time requirements, agarose brand, temperature, coverslip sealant, evaporation rate, etc. Thus, it is important to calibrate the imaging system before data acquisition to increase technical reproducibility and to enhance the quality of live-cell imaging[1-3]. Pad surface, rigidity, and composition are essential during prolonged image acquisition. Agarose has low background fluorescence, can be easily molded, and at the appropriate concentration, withstands structural deformation due to evaporation. Moreover, combining fission yeast media with agarose does not compromise image quality and allows for extended observation of multiple mitoses and meiosis cycles. Also, it is important to avoid any air bubbles for time-lapse microscopy. Inside agarose pads and within the sample, air pockets expand over time, causing cell-shifting and disrupting focal planes. Provided cells are efficiently spread apart by coverslip rotation, researchers can follow mitotic processes across several generations and meiotic events from nuclear fusion to sporulation. Making and choosing the right pad for imaging experiments takes time to master, but once achieved, can contribute to highly informative microscope observations.

As is the case with other methods, live-cell microscopy is not free of technical limitations. The use of fluorescently-tagged proteins introduces boundaries to experiments that limit the scope of what can be studied. Photo-bleaching and photo-toxicity are problems typical of prolonged image acquisition. They can be counteracted by not exposing cells to short wavelengths for too long or too frequently. For experiments involving GFP, CFP, YFP, mRFP, and DsRed, typical fluorescent proteins used in fission yeast live-cell imaging, it is common to employ 5-15% excitation light and 100-500 ms exposure time for routine experiments. These parameters change, however, according to the specific experiment requirements of the researcher. In addition, z-stacks comprised of 9-13 sections with 0.5 µm spacing using a 60x objective is enough to resolve the thickness of a fission yeast cell. Moreover, it is important to confirm that fluorescent markers do not disrupt proper regulation or function of target proteins or generate spurious phenotypes. Thus, it is advisable to construct strains containing different fluorescent and affinity tags for the same target gene to corroborate observations by different means. Furthermore, it is the responsibility of researchers to ensure that experimental parameters can be reproduced across experiments and that the collected data is fit for downstream analysis[1,3]. No technology can replace the time-tested practice of meticulously validating experimental systems by careful observation and rigorous examination of linearity in detecting fluorescent signals.

Finally, it is crucial to analyze microscopy data in formats that do not modify raw images. Fiji provides excellent documentation tools to keep track of raw data measurements and allows researchers to examine different cell parameters in a single session. These features, as well as its wealth of online tutorials and extensive literature coverage, make Fiji an important tool for measuring, analyzing, and presenting live cell imaging data that describe the nuclear dynamics of fission yeast in mitosis and meiosis.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1

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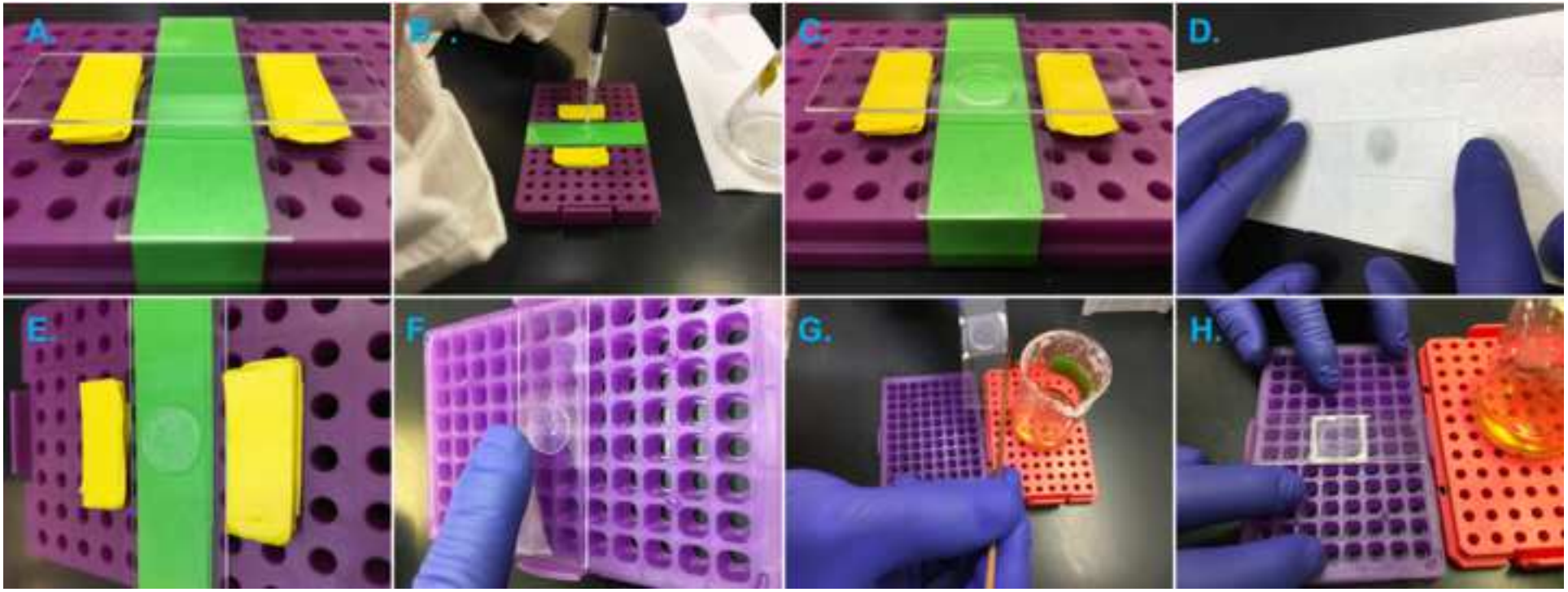

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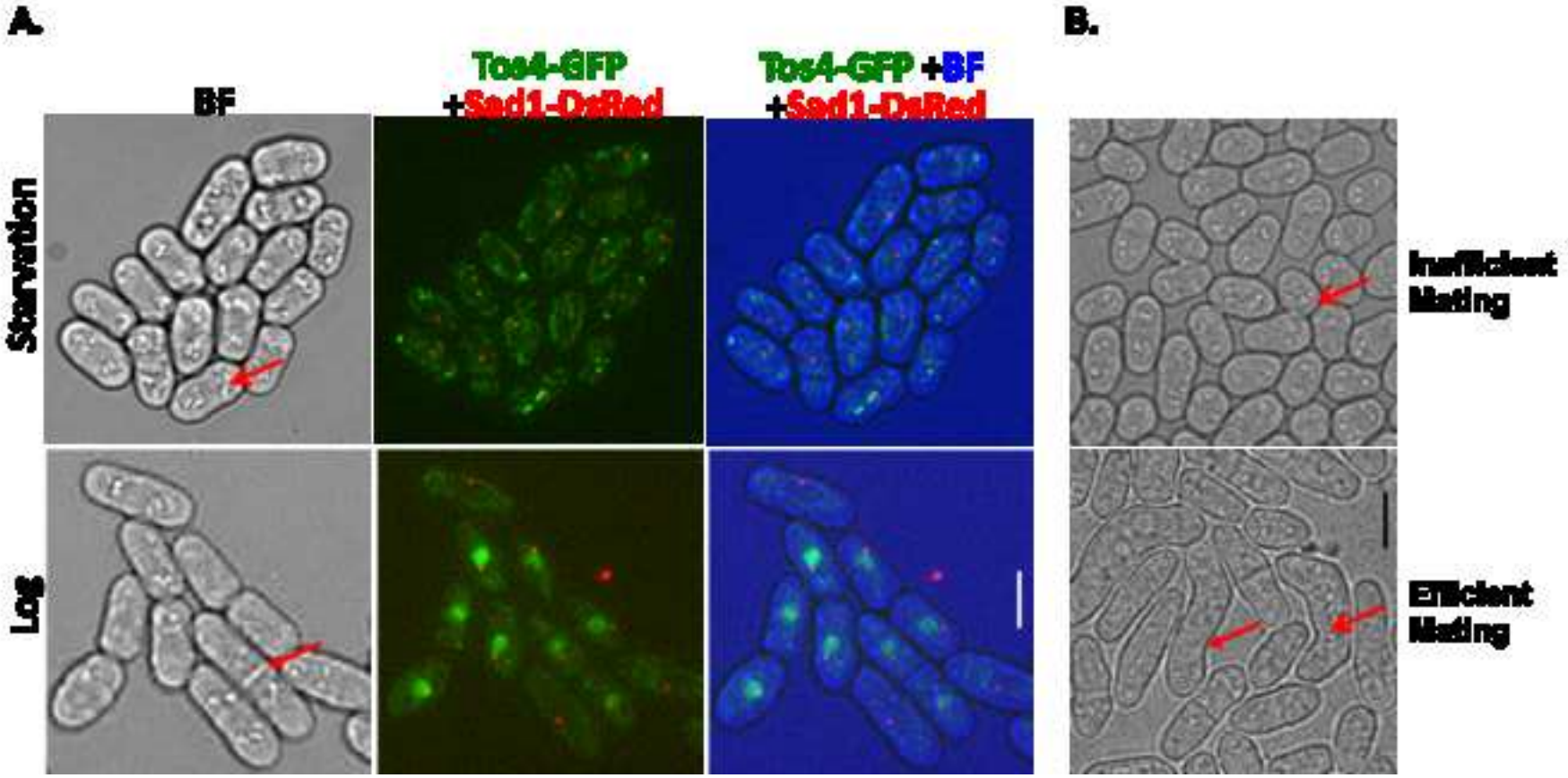

Figure 3

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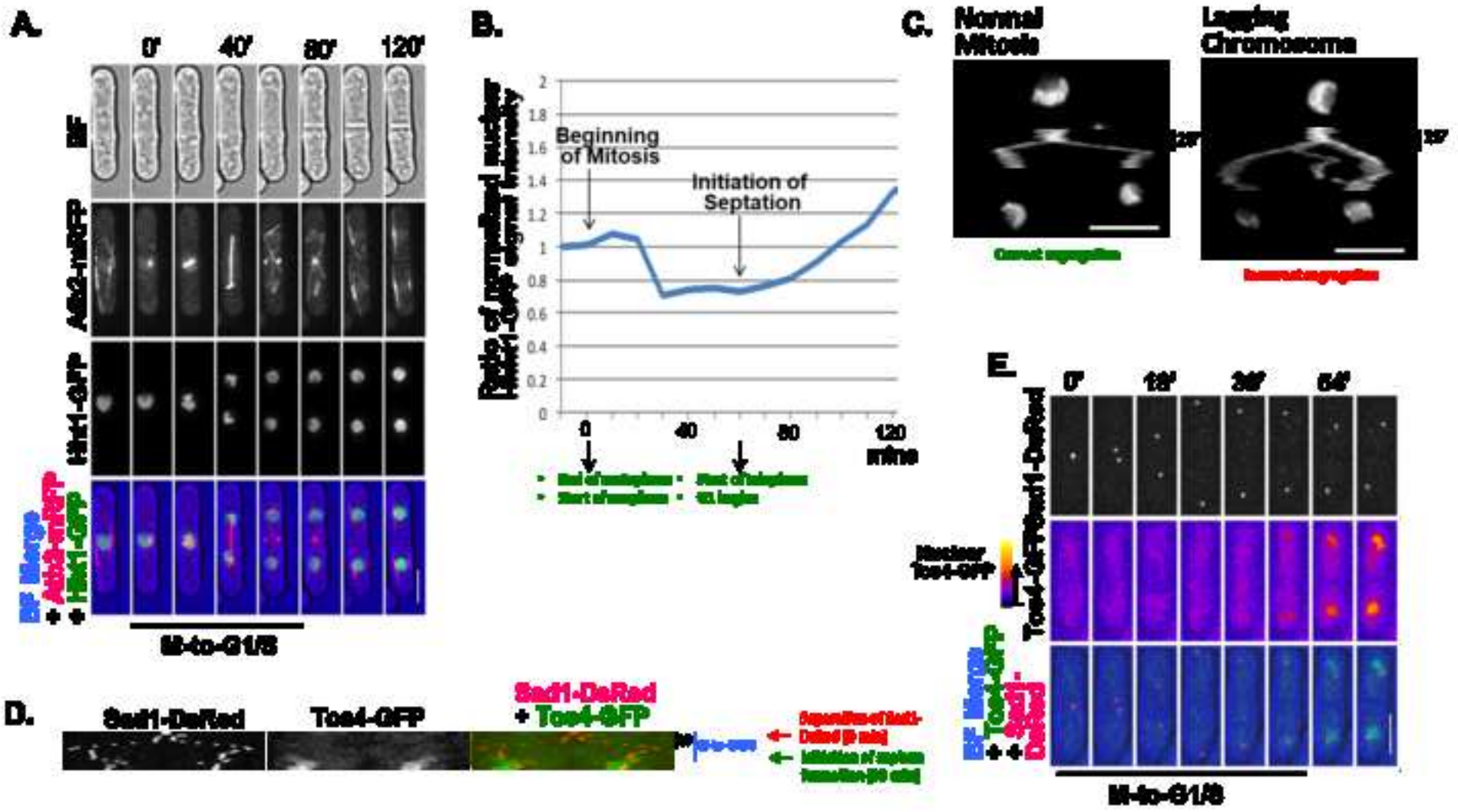

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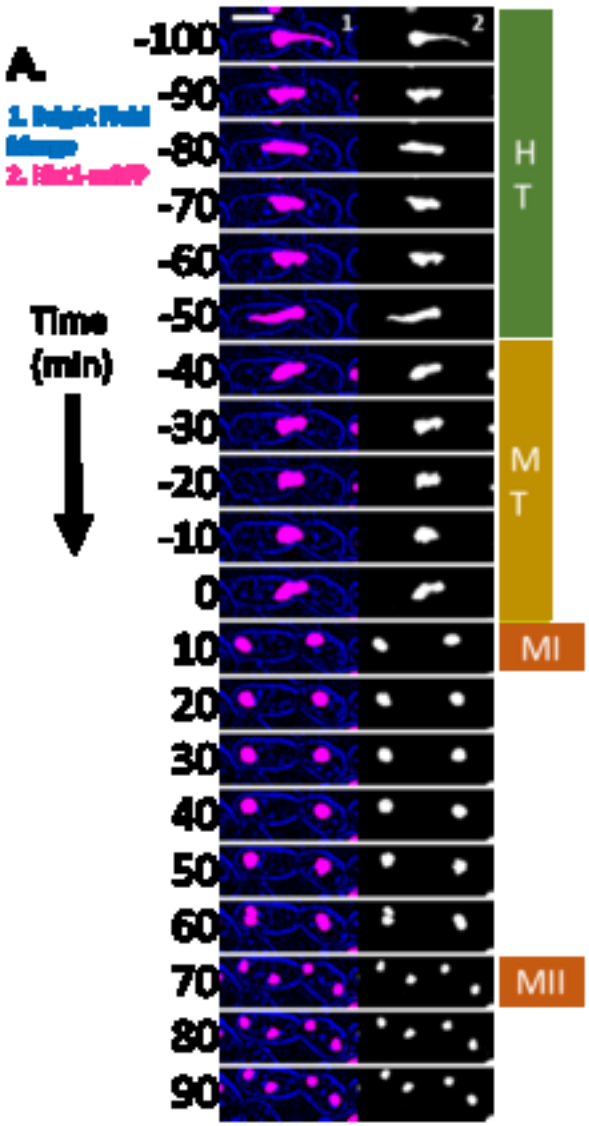


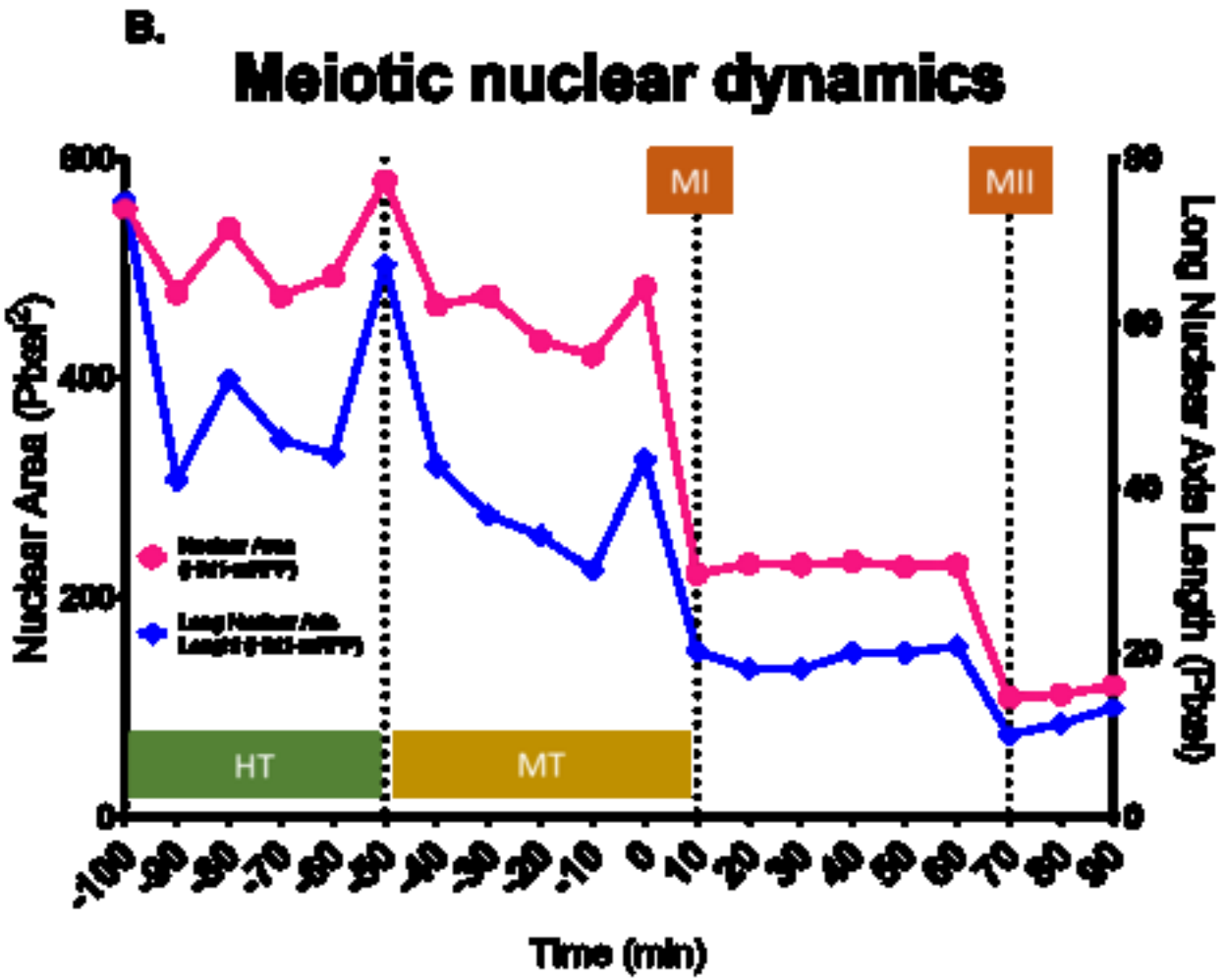

Figure 5

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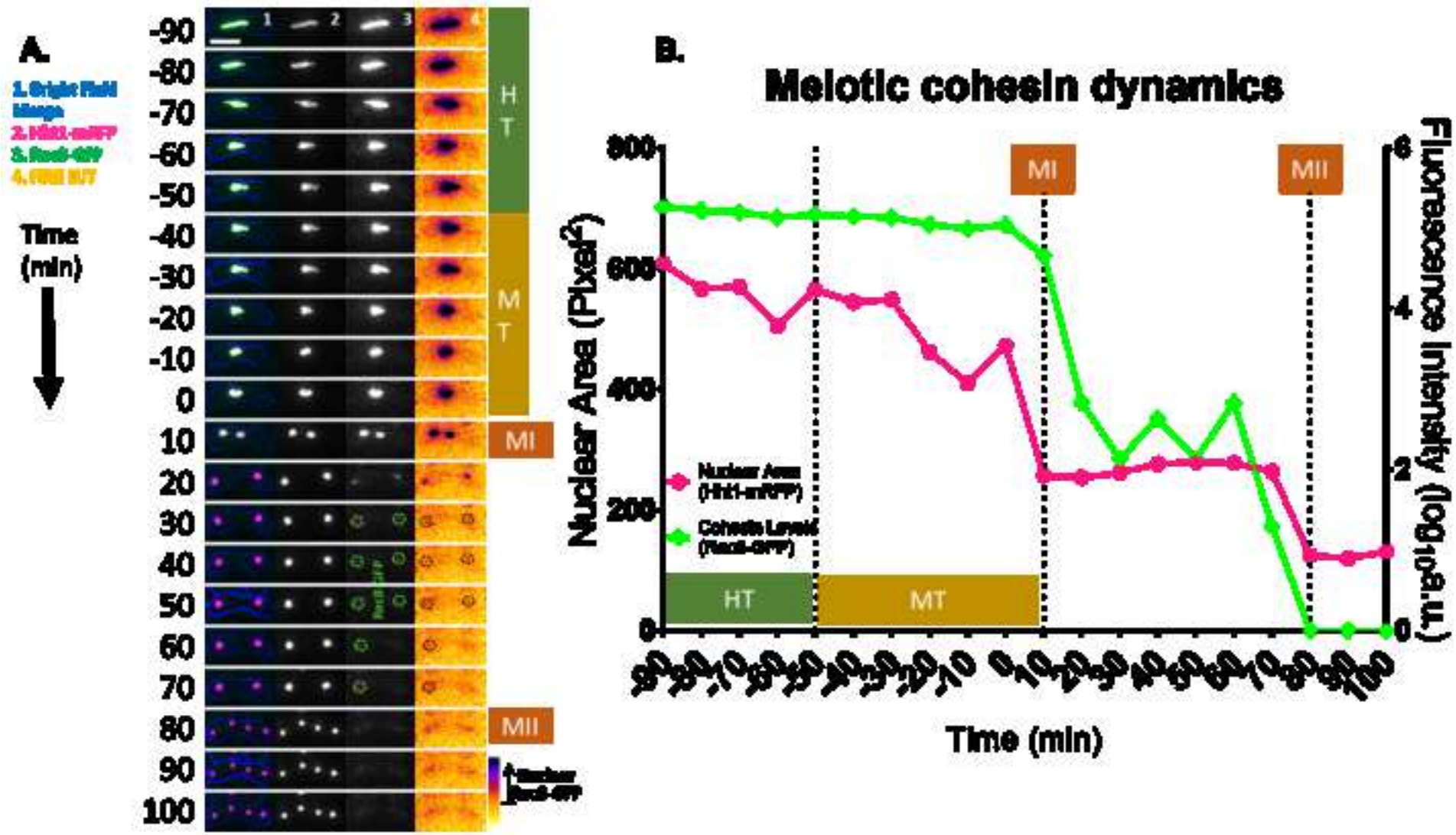

Video1 for readers to use

Video2 for readers to use

## Table I. Markers of nuclear and cytosolic dynamics

| NUCLEUS | | | | |
|---|---|---|---|---|
| **Protein** | **Function** | **Comments** | **Tag** | **References** |
| **Hht1** | Histone H3 involved in DNA packaging | Used to examine chromosome dynamics | Hht1-mRFP | **5,14** |
| **Tos4** | Tos4 function occurs during G1 phase | Employed to study G1 timing | Tos4-GFP | **22,23** |
| **Rad21/ Rec8** | Cohesin subunits involved in keeping sister chromatids together following replication | Used to analyze cohesion stability during mitotic (Rad21) and meiotic segregation (Rec8) | Rad21-GFP/Rec8-GFP | **6,24** |
| **Rad11** | RPA activity occurs during mitotic DNA repair, meiotic recombination, and ends in metaphase | Employed to study DNA repair in mitosis and recombination dynamics in meiosis | Rad11-YFP | **5,13** |
| **Rad52** | Rad52 acivity takes place during mitotic DNA repair and meiotic recombination | Employed to study DNA repair in mitosis and recombination dynamics in meiosis | Rad52-CFP | **5,13** |
| **Cnp1** | Histone H3 CENP-A localizes specifically at the centromere | Used to follow chromosome segregation dynamics in mitosis and meiosis | Cnp1-mCherry | **13** |
| **Swi6** | HP1 homologue protein involved in heterochromatin formation | Employed to study heterochromatin mobilization at the centromere and telomeres | Swi6-GFP | **13** |
| **Sad1** | Sad1 is involved in localizing the spindle pole body to the nuclear envelope | Employed to analyze chromosome segregation in mitosis and meiosis | Sad1-mCherry | **10** |
| **CYTOSOL & MEMBRANE** | | | | |
| **Atb2** | Tubulin alpha 2 involved in microtubule cytoskeleton organization | Used to examine chromosome segregation in mitosis and meiosis | Atb2-mRFP | **7,14** |
| **Ync13** | Exocytosis and endocytosis regulator involved in vesicle-mediated transport | Employed to study cell-wall integrity during cytokinesis | Ync13-mECitrine | **25** |
| **Rlc1** | Myosin II subunit involved in contractile ring contraction | Used to explore the dynamics of septum maturation and contraction | Rlc1-mCherry | **25** |
| **Ccr1** | NADPH-cytochrome p450 reductase that is part of the ER, plasma, and mitochrondrial outer membrane | Employed to examine membrane-associated dynamics such as endocytosis, exocytosis, and cytokinesis | Ccr1N-GFP | **5** |
| **Gef1** | Rho guanyl-nucleotide exchange factor involved in the establishment and maintenance of cell polarity | Used to examine global, microtubule-dependent cell polarity dynamics | Gef1-mCherry-GBP | **26** |
| **Fus1** | Formin involved in actin fusion focus assembly during cytogamy | Used to study fusion dynamics associated with fission yeast mating | Fus1-sfGFP | **27** |
| **Mnn9** | Mannolsyltransferase subunit involved in | Employed to study cargo maturation in the Golgi as it | Mnn9-mCherry | **28** |

| | | | | |
|---|---|---|---|---|
| | protein N-linked glycosylation in the Golgi apparatus | progresses from *cis* -Golgi to *trans* -Golgi | | |
| **Num1** | Cortical anchoring factor for dynein involved in horse-tailing | Used to examine mitochondria-dependent dynein anchoring during nuclear oscillation in meiotic prophase I | Num1-yEGFP | **29** |

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 60x Plan Apochromat objective lens | Olympus | AMEP4694 |
| Adenine | Sigma | A-8751 |
| Agar | 7558B | IB4917-10 kg |
| Agarose | Sigma | A9539-500g |
| Belly Dancer rotator | Stovall | US Patent #4.702.610 |
| Biotin | Sigma | B-4501 |
| Boric acid | Sigma | B-6768-5kg |
| CaCl2·2H20, | Sigma | C3306-500G |
| Citric acid | Sigma | C-0759 |
| Convolve 3D software plug-in | OptiNav, Inc. | n/a |
| CoolSnap HQ CCD camera | Roper | n/a |
| Cover slip | VWR | 16004-302 |
| CuSO4·5H20, | END | CX-2185-1 |
| DeltaVision deconvolution fluorescence microscope | GE Healthcare/Applied Precision | n/a |
| Diffraction PSF 3D software plug-in | OptiNav, Inc. | n/a |
| Edinburgh minimal medium (EMM) Notrogen | Sunrise | 2023;1kg |
| FeCl2·6H2O | END | FX9259-04 |
| Glucose | Sigma | G-7021 |
| Heat plate | Barnstead | Thermo Lyne |
| Histidine | Sigma | H8125-100g |
| Huygens deconvolution software | SVI | n/a |
| Imaris deconvolution software | Bitplane | n/a |
| Incubator | Shell lab | #3015 |
| Inositol | Sigma | I-5125 |
| Iterative Deconvolve 3D software plug-in | OptiNav, Inc. | n/a |
| KCl | Mallinckvadt | 6858-04 |
| Lanolin | Sigma | L7387-1kg |
| Leucine | Sigma | L8912-100g |
| Lysine | Sigma | L5626-500g |
| Malt extract (ME) | MP | 4103-032 |
| MgCl2·6H20 | AMRESCO | 0288-500G |
| MetaMorph | Molecular Devices | n/a |
| Microscope slides | VWR | 16004-422 |
| MnSO4, | Mallinckvadt | 6192-02 |
| Molybdic acid | Sigma | M-0878 |
| Na2S04 | Mallinckvadt | 8024-03 |
| Nicotinic acid, | Sigma | N-4126 |
| Pantothenic acid | Sigma | P-5161 |
| Paraffin wax | Fisher | s80119WX |
| Pombe glutamate medium (PMG) | Sunrise | 2060-250 |

| | | |
|---|---|---|
| softWorx v3.3 image processing software | GE Healthcare | n/a |
| Sporulation Medium powder | Sunrise | 1821-500 |
| Temperature controlled centrifuge | Beckman | Allwgra 6KR xentrifuge |
| Uracil | AMRESCO | 0847-500g |
| Vaseline | Equaline | F79658 |
| yeast extract | EMD | 1.03753.0500 |
| Yeast extract plus supplements (YES) | Sunrise | 2011-1kg |
| ZnSO4·7H2O | J.T.Baker | 4382-04 |

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tel. 617.945.9051
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| | |
|---|---|
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| Author(s): | Wilber Escorcia, Kuo-Fang Shen, Ji-Ping Yuan, and Susan L. Forsburg |

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**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Susan L Forsburg |
| Department: | Molecular & Computational Biology |
| Institution: | University of Southern California |
| Title: | Professor |
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# Authors comments:

We would like to express our gratitude for the insightful comments made by the reviewers of our manuscript. Their attention to detail and generous suggestions made revising this work a less difficult endeavor. We would especially like to thank reviewer #1 for his/her extensive input. His/her contribution to the review of this work will certainly enhance the quality of the published paper and video.

Below each editor's or author's comment, we have placed our response to the suggested change or edit.

# Editorial comments:

## *Changes to be made by the Author(s):*

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- **We have checked and revised the manuscript for this.**

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

- **Both abstract and summary have been revised.**

3. Please ensure the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

- **We believe the current introduction covers these criteria.**

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly.

- **We made sure to use the active voice and imperative sentences throughout the manuscript.**

5. The Protocol should contain only action items that direct the reader to do something. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

- **We feel we have provided enough direction in each protocol step to answer the "how" question.**

7. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

- **We made sure to write easy-to-follow directions in the Fiji software section.**

8. 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.

- **The protocol sections we highlighted provide sufficient information required to understand the objective of this protocol.**

9. 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.

- **We have removed all commercial language and limit it to the materials table.**

For example: VALAP, GE Healthcare/Applied Precision, Issaquah, WA, DeltaVision restoration microscope, Olympus 60x NA 1.4 PlanApo objective, softWorRx (v3.3 or higher), 12-bit Roper CoolSnap HQ, etc.

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

- **We feel our discussion addresses these criteria well.**

11. For all the figures with microscope, please include a scale bar.

- **All microscope images have a scale bar.**

12. Please alphabetically sort the materials table.

- **The table is alphabetized.**

13. Please obtain explicit copyright permission to reuse any figures if from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- **All the work in this protocol is original.**

# Reviewers' comments:

## *Reviewer #1:*

## *Manuscript Summary:*

In this manuscript the authors have provided a thorough explanation of the method of live imaging of fission yeast. The authors have provided a complete workflow for the entire process. Details on cell culture and sample prep are provided. Details on data acquisition including imaging parameters and optical component selection are shown. Some common tools for image analysis specifically for nuclear processes including the use of Fiji are provided. Finally, interpretation of the imaging data including specific plots and analyses are provided, as well as a list of useful proteins to tag for visualization.

## *Major Concerns:*

The authors have focused on the analysis of nuclear processes in this method, but I feel that the authors would provide a greater benefit to the scientific community if they **expanded to include other cellular processes other than the nucleus**. This change would not take much effort for the authors since all parts of the method can be generalized for any cellular process. The only place the method would need to be expanded is to require a larger list of proteins to tag for other processes and a brief explanation of how to interpret the data using montages, kymographs and intensity quantifications, etc. I'm not suggesting this be a comprehensive list of all cellular process but including details on proteins which tag other organelles would be extremely useful to the community.

- **We have included a note that refers readers to Table I, which contains select markers for dynamics occurring in the nucleus as well as the cytosol. This suggestion was very helpful.**

My only real concern with the method the way it is written are the details on image thresholding and ROI selection for measurements. The authors have shown proteins for the method that are bright and easily segmented. The specific use of the default thresholding method in Fiji works well for these

particular proteins, but for greater applicability to the community a discussion of other segmentation methods for markers that may be dim and/or diffuse would be useful. Careful selection of the thresholding algorithm is an important part of the image processing workflow and the selection of the wrong method will lead to erroneous results.

- **We make mention of this caveat and refer readers to the appropriate sources for learning how to distinguish between thresholding methods based on their experimental requirements.**

Finally Figure 5A is confusing the way it is constructed now. I can appreciate the fact that the figure is trying to relay a lot of information in a small format, but its current construction makes it difficult to figure out what is going on. I would propose removing the inverted LUT column and separating the transmitted light and the fluorescence channels into their own columns. I further understand that some of the proteins appear/disappear over time and therefore will have blank panels down the montage of when they are not on. Leaving the blank panels will help clarify the timing of the proteins with respect to the cell cycle and make the figure much clearer.

- **We have decided to leave this figure without any modifications for the following reasons:**
    a. **We added an interpretation box that along with the manuscript description will help readers better understand the panels in this figure.**
    b. **Because the BF image is made from a meiosis movie, which goes for 8 hours, the transmitted light is too dim to offer any cell details other than the cell outline. This is done so as to not to photo-bleach our sample prematurely. Thus, the bright field merge helps use to use color inversion to make the pombe outline easier to see.**
    c. **The LUT panel is helpful to consider when dealing with diminishing signal, for it allows readers to detect small changes in fluorescence. Thus, we wish to feature it when possible.**
    d. **Including more blank images at the end of MII is difficult because sporulation introduces cell-shifting, which moves the focal plane.**
    e. **Overall, we feel that the changes we made thanks to the recommendations of all the reviewers makes this figure more understandable.**

# *Minor Concerns:*

-Section 3.1.1 I appreciate the use of the tip box for the slide making apparatus, but it's unclear to me what purpose this serves. Wouldn't the lab bench work just as well?

- **The lab bench works just fine. We recommend using a makeshift slide-maker for consistency. Thus, we added a note to that section of the protocol that encourages the use of a slide-maker but leaves it up to the reader to decide.**

-Section 3.1.2 the figure reference here should be 1C.

- **We have corrected this.**

-Section 3.1.2 line 180. "The size of the agarose pad is typically inversely proportional to the length of imaging time." This should read "The size of the agarose pad is typically proportional to the length of imaging time." A larger agarose pad for long timelapses and vice versa.

- **We have corrected this.**

-Section 3.1.3 Nail polish typically doesn't quench fluorescence and is not usually autofluorescent. However, sealing large gaps is typically hard to accomplish with nail polish and it may kill the cells.

- **This is a great point. We have modified this protocol step to match the reviewer's suggestion.**

-Section 3.2.4 17949 g is a very specific setting. Is this necessary?

- **It was a typo that we have corrected.**

-Section 3.2.5 As this section now reads, one should place 20uL of cell suspension onto the pad and then invert the pad and touch it to a lint-free paper towel. Is this the correct order? If removing excess liquid from the pad, shouldn't that be done before placing the cells? If removing excess liquid from the 20uL of cell suspension, wouldn't allowing a couple minutes for the excess liquid to absorb into the agarose take care of the problem? Line 213 Should read "Figure 1D" but now reads "Figure D". Line 214 should read "Figure 1F" but now reads "Figure 1E".

- **The paper drying step is more time-efficient than gravity absorption. We made a note of this because meiosis movies are time sensitive during the stage where karyogamy and nuclear fusion are happening.**
- **The figure mislabeling was also corrected.**

-Section 3.2.7 line 226 should be Figure 1H

- **This has been corrected.**

-Section 4.1.3 Depending on the experiment and what is to be learned, I think this objective requirement is slightly too high. I would recommend at minimum a 40x objective with NA > 1.2.

- **We agree with the reviewer and modified the manuscript to reflect his/her recommendation.**

-Section 4.1.4 Please reference the ImageJ deconvolution package by name, cite their paper (if applicable) and link to the online reference material.

- **We refer the readers to the proper software websites and citations.**

-Line 273 "and 13 5-um sections" This is a confusing way to reference the z-stack settings. Do the authors mean 13 sections covering 5 microns total distance? Or do the authors mean 13 sections with 5 micron spacing? The former is appropriate but is dependent on the objective employed while the latter would miss the cells entirely. If this is the only place the z stack settings are mentioned, the authors should talk about proper z sampling dependent on the objective used and formalize the z stack settings in the manner "13 sections with 0.38 micron spacing covering a total depth of 5um".

- **We agree with the reviewer and have implemented her suggested change.**

-Section 4.1.9 "using 9-13 serial z-sections with 5um spacing is enough to resolve the thickness of a fission yeast cell". Please clarify as discussed in the previous comment.

- **We have made this correction as per the reviewer's previous suggestion.**

-Section 5.1.1 The authors referenced ImageJ above and Fiji here. For people not familiar with either, this will be confusing. If the authors meant Fiji throughout, please change all references.

- **We have changed ImageJ for Fiji throughout the manuscript for more clarity.**

-Section 5.1.3 is mislabeled. It should be 5.1.6. Additionally, it's not necessary to go through all ROIs by hand and select measure each time. In the ROI manager, under the more button, there is a button labeled multi measure. In the subsequent pop-up box, make sure "measure all slices" is selected, deselect the "one row per slice" option and then click OK. This will measure all the ROIs iteratively.

- **We have added a note which stipulates that if cell-shifting isn't an issue, automatic iteration is the prefer option.**

-Section 5.1.4 line 350 should be 5.1.7

- **This has been corrected.**

-Line 358 histograms should be profiles

- **This has been corrected.**

-Section 5.1.5 line 364 should be 5.1.8

- **We have modified this.**

-Section 5.1.6 line 372 should be 5.1.9

- **We have corrected this.**

-Section 5.1.7 line 380 should be 5.1.10. It's important to note also for the montage instructions that not necessarily every frame needs to be included, so adding a line about the increment setting would be useful.

- **We implemented the reviewer's suggestion.**

-Section 5.1.8 line 394 should be 5.1.11. In using the time stamper tool, the end point is not a setting, but it is necessary to input the appropriate time between frames.

- **We agree with the reviewer and have modified this step.**

The transmitted light images in Figure 2 are noisy due to too little light intensity from the transmitted light bulb. It would be beneficial to other readers to have brighter images to better illustrate the texture and septa for starved and dividing cells, respectively. Additionally, it would be convenient for readers to have the transmitted light and fluorescence images be from the same field of view. This would help readers to correlate the appearance of the different channels in deciding whether their cells are healthy.

- **These images have been changed for better quality ones.**

-Line 436,439 states Hht1-mRFP but Figure 2A shows Hht1-GFP. Please clarify.

- **We have corrected this mistake in the manuscript.**

-Figure 3D would be much clearer if the kymograph was extended in the vertical direction and if septation were marked in time on the side of the kymograph. As it stands now, I can't correlate the change in GFP expression with the division and whether that happens before septation.

- **This has been changed to reflect the reviewer's preference.**

-Figure 3E would benefit from having the transmitted light included as a separate channel.

- **We prefer to have a merge channel in this figure.**

-Figure 4A Please label the second column. Is it segmented Hht1-mRFP?

- **We have placed numbers atop each panel that identifies it with its markers.**

# *Reviewer #2:*

# *Manuscript Summary:*

The authors describe the practical application of live-cell microscopy in studies of events associated with mitosis and meiosis in fission yeast, a favorite model in the fields of cell and molecular biology. The provided protocols describe steps from media preparation, through cell cultivation, to microscopy of living cells, and the subsequent quantitative image analysis with the popular imageJ/fiji package. The manuscript is informative and reads very well, the procedures are well-described and complemented with illustrative representative results. Critical steps/parameters of the workflow are properly highlighted, and limitations of the method are discussed. The image analysis part of the workflow is of interest also to researchers outside the fission yeast community. Overall, the manuscript provides very useful information and deserves to be published. I have only some minor comments and suggestions to further improve the paper, as detailed below.

Minor Concerns:

- it would be great to include sample raw image data (eg. the files used to produce Figs. 2-5) to help users learn to use fiji (perhaps by making the data available through Figshare).

- **We will submit an avi movie with the final manuscript.**

- the authors might want to state more explicitly that fiji (not just plain imageJ) is needed for the described analyses

- **We have corrected this throughout the manuscript.**

- line 180: please explain why "the size of the agarose pad is typically inversely proportional to the length of imaging time". This was not clear to me.

- **That was wrongly stated, and we have corrected it in the manuscript**.

- references 18-20 are cited prior to refs. 15-17

- **This has been corrected.**

- line 78: MgCl2 quantity seems to be 10x higher than needed

- **That was indeed a typo and was promptly corrected.**

- lines 91, 119: uracil and adenine are not "amino acids"

- **We changed this to nutrients.**

- lines 104, 116, 128: agar will not dissolve without heating

- **We have corrected this in the manuscript. Now readers know that agar melts during autoclaving.**

- line 273, 285: optical slices of 5 um seem too thick (the whole fission yeast is thinner than that). Perhaps you meant 0.5 um?

- **This was incorrect and we changed it to the right magnitude: 0.5 um.**

- line 273: "1 GB of memory" - do you mean RAM or hard drive storage space? Please clarify.

- **We adopted the reviewer's suggestion.**

- line 384: I found the description of creating AVI movies a bit confusing. Users need to convert the hyperstack to AVI, not the montage image to AVI.

- **We changed it to say that a hyperstack is needed to generate an avi movie.**

- line 398: in my imageJ (1.52i) the Time Stamper tools is located under Image/Stacks, not in Plugins. Please double check.

- **We corrected this and adopted the reviewer's suggestion.**

# *Reviewer #3:*

# *Manuscript Summary:*

The manuscript outlines best practices for preparing and imaging fission yeast for live-cell studies of mitosis and meiosis. Methods for processing and analysis of the resulting images are also given. The authors show how fluorescent protein fusions can be used to follow structures important in mitosis and meiosis, and show how to recognize healthy and unhealthy cells. The advice on fusion proteins and image analysis will be useful for any S. pombe researchers whose work relates to the cell cycle or meiosis, and the tips for evaluating and maintaining cell health will be useful for anyone performing live imaging of fission yeast. The protocol has a useful level of detail, highlights potential pitfalls, and is clearly written.

# *Major Concerns:*

None.

# *Minor Concerns:*

-2.1.2: For the starter liquid culture, are there guidelines for the size of the tube (e.g. to guarantee sufficient aeration) or the shaking speed?

- **A note has been added to address this.**

-3.1.2: It would be helpful to say how thick the agarose pad should be for different time-lapse durations.

- **Pad thickness will depend on a researcher's reagents, agarose type, microscope temperature, etc. Thus, we added a note that addresses this by stating that optimization for each researcher's experiment needs is required.**

-3.2.4. "centrifuge at 17,949 x g" - this seems high. Is it a typo?

- **This type has been corrected.**

-4.1.4. Not all deconvolution algorithms are appropriate for quantitative analysis of intensity. Specific examples of ImageJ plugins, and deconvolution algorithms, should be given that allow images to be restored without distorting intensity data, and guidance should be given on avoiding artifacts. If the authors feel this exceeds the scope or space limitations of the article, they should give references that provide advice on deconvolution for quantitative analysis.

- **Readers are referred to the appropriate online guides and published literature on the subject.**

-4.1.8. "…as long as excitation and emission do not decrease …" This should probably read "…as long as emission does not decrease…."

- **We adopted the reviewer's suggested change.**

-5.1.3. "Use the Wand tool to highlight each ROI…" should probably be "Use the Wand tool to highlight each structure of interest..." As a side note, to measure a set of saved ROIs in current versions of Fiji, you can select all the ROIs in the ROI Manager and click Measure in the ROI Manager to obtain all the slice measurements in a single step.

- **We added a note stating that if cell-shifting is not an issue, the automated measurement option is preferred.**

-5.1.4. "Lack of overlap among signal histograms…." should be "Lack of overlap among profile plots…"

- **We corrected this to reflect the reviewer's suggestion.**

-Figure 5A. The legend does not explain the green circles in the lower half of the 3rd column, or the 4th column (shown in inverted Fire LUT).

- **We have labeled each of the image panels and have added a label that indicates the outlined foci are GFP signals.**

-Discussion: "…not exposing cells to high-frequency wavelengths…" should be "… not exposing cells to short wavelengths…"

- **This is correct. We changed it to reflect the reviewer's suggestion.**

Dear Vineeta Bajaj,

We appreciate all the constructive comments you made about our manuscript. Your insightful comments allowed us to modify this manuscript version into what we believe is a much clearer form. We would particularly like to thank you for the useful suggestions you provided for protocol steps that fit better in the discussion section. This made for a cleaner protocol segment. Also, we connected phenotypes to their correct figure panels in no small part thanks to your careful reading of this manuscript section. It is clear to us that your recommended changes have made this part a lot clearer than was the case in the previous version of our manuscript. All in all, we greatly appreciate your help to make this JOVE protocol more useful to all in the fission yeast community.

# Response to editor comments

1. Introduction

Please include a paragraph here describing how and what you are trying to do in your experiment. Introduce all the proteins used here.

*The goal of the manuscript is to present live cell imaging to study mitosis and meiosis in yeast. To do this xx protein is fluorescently tagged …. We studied the expression of xxx protein because….*

Bring out all the details here.

- We agree with Vineeta Bajaj on the need for more details in the introduction section. Thus, we added an additional paragraph (Lines 74-86) detailing the main sections of the protocol as well as the fluorescently tagged proteins featured in Figures 2-5.
- We also add a thesis sentence in this additional paragraph that voices the specific goal of this protocol.

2. Protocol

Please do not have paragraph of text in the protocol section.

Please ensure that all the steps are as crisp as possible.

We cannot have paragraph of texts in the protocol section.

Also, the protocol section cannot be more than 10 pages and highlight cannot be more than 2.75 pages including headings and spacings. Please highlight complete sentences and not the part of a sentence.

Please ensure that the highlights form a cohesive story.

- We removed unnecessary sub-sections as indicated by Vineeta Bajaj.
- We removed unnecessary words.
- We replaced passive phrases, with imperative ones.

- We removed parts of protocol steps that contributed to paragraph blocks in the previous version of this manuscript.
- We modified highlights to fit 2 ¾ pages and to include complete sentences.
- The highlights now take you from yeast colony to live-cell images in a more sequential manner.
- We also eliminated unnecessary notes and moved others to the discussion section, where they fit better.

3. Live-cell imaging and processing (Lines 264-292)

Please use imperative tense throughout the steps in this section as if describing someone how to perform your experiment with all specific details.

Please ensure each step is showing a discrete action and describe how to do that step with all specific detail.

Please remove the redundancy from the steps and make it crisp with respect to your experiment and present discrete experimental steps only.

Discussion about the protocol can be moved to the discussion section.

The above step 3 ends with slides being equilibrated. E.g.,

4.1. step 4 should start with bringing and placing the slide on the microscope stage.

4.2. Then adjust the magnification to 40x or 60x to get a better view. Please also explain whether you adjust the objective and aperture manually or do you perform any button clicks in the associated software.

- We used more imperative language and modified steps to refrain from the passive voice.
- We changed some steps to add more clarity and better instructions for readers to follow.
- We eliminated redundant parts or merged them into more cohesive sentences for different steps.
- Step segments or notes that were superfluous in the protocol section were moved to the discussion section, where they fit better now.
- The last step of section 3 and the beginning portion of section 4 are now in the proper sequence of events.
- The move from 40x to 60x is mentioned and the functional details of microscope manipulation are addressed.

This is not a step, converted to a note instead (Line 273)

- We agree with the editor and retained that step as a note.

How do you use fiji to do so? Since this is marked for filming, please provide the steps in brief (Line 291).

- We removed the highlight from this step because it did not go well with the narrative we wish to have in the video. Readers, however, are provided with multiple citations that show them how to carry out that step.

How? Do you perform some knob turns in the microscope or button clicks in the software to adjust to the MES? (Line 304)

- We agree with the editor that this sentence was confusing. We rectified it by stating MESA is an approach and then had an additional sentence to explain its meaning.

This is not a note, hence converted to a step instead. 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 (Line 314).

- We agree with the editor and changed this step as suggested.

Please reword and make the step crisp.

- We carried out this throughout this section.

For the 8h time period do you move the cells back and forth to the incubator and to the microscope stage? Do you maintain the temperature on the stage itself with some sort of temperature controller? Please detail all specific details which will be helpful to reproduce the experiment.

- In section 3, we include a note where we made mention of the need for considering proper temperature control depending on their microscope systems.

How? (Line 321).

- We provide multiple citations so the reader can learn how to generate an appropriate experimental workflow.

How is this done? (Line 330)

- We also provide references for people to look up how to do pilot intensity analysis experiments.

This is not a step but just a discussion. Please consider moving this to the discussion section. (Line 333)

- We agreed with the editor and moved this to the discussion section.

How do you apply the same? (Line 340)

- This varies from software to software, so we made mention of that fact. Most software use for these purposes presents it as a choice when making image deconvolution and projection. We also provide references that talk about this part.

Please use imperative tense throughout the steps in this section.

Please ensure each step is shows a discrete action and describe how to do that step with all specific detail.

Also, we cannot have paragraph of text in the protocol section. Please ensure each step should not describe more than 2-3 action. Please consider making substeps.

- We modified our language and made it more imperative.
- We broke paragraph blocks into steps and sub-steps as suggested by the editor.
- Overall, we streamlined this section for easier understanding.

If 5.17 described how to do the step 5.16, please remove 5.16 and renumber accordingly (Line 451).

Please make substeps shown from step 5.1 -5.17. Each step should not have more than 2-3 action (Line 470).

- We renumbered steps to their correct sequence.
- We reduced the size of each step.
- We changed the language to be less passive and more imperative.

Please reword the result with respect to your experiment, you performed an experiment, how did it helped you to conclude what you wanted to and how is it in line with the title. Please do not generalize (Line 499).

Need more clarity for figure description.

Again, which panels show this transition. Please first explain which time point corresponds to what phase of mitosis to bring out clarity.

- We changed wording so the narrative of the results section is more coherent.
- We linked sentences describing nuclear dynamics to their correct figures and panels.
- We added sentences that explained what the results mean.
- We added specific times for each of the panels and figures mentioned in this section.

Why this is used? References if any? Include the reference (Line 546)

- Reference for this  marker is provided previously in the same paragraph.

Is this figure is cut? Please check. (Line 564)

- Figure 3D is not cut, but we could not show the rest of the kymograph because of cell-shifting, which cause the field of view to change in the last slices of that stack. We make mention of this effect in the section related to Fiji.

What is the time point here (which panel shows this)? (Line 598)

- We corrected this to show the panel and time when the specific phenotype is observed.

We cannot have interpretation panel in the figure itself. Please move the details to the representative result and refer to the time points as well to bring out clarity in understanding the figure (Line 606).

- We removed the interpretation box from figures 4 & 5.