Submission ID #: 68887
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/account/file-uploader?src=21010583

Title: Manipulation and Analysis of Cell Cycle-dependent Processes in Budding Yeast

Authors and Affiliations:
Michael G. Stewart[1,2,*], Talia C. Scheel[1,*], Ahmed A. Abouelghar[1,*], Sara E. Hoppe[1,*], Matthew P. Miller[1]

[1]Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, United States [2]Department of Molecular Biology and Genetics, Howard [2]Hughes Medical Institute, Johns Hopkins University School of Medicine

*These authors contributed equally

Corresponding Authors:

Michael G. Stewart mstewa64@jh.edu
Matthew P. Miller matthew.miller@biochem.utah.edu

Email Addresses for All Authors:
Michael G. Stewart mstewa64@jh.edu
Matthew P. Miller matthew.miller@biochem.utah.edu
Talia C. Scheel talia.cahoon@biochem.utah.edu
Ahmed A. Abouelghar ahmed.abouelghar@biochem.utah.edu
Sara E. Hoppe Sara.Hoppe@biochem.utah.edu

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 26
Number of Shots: 41

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Matthew Miller:** We study how dividing cells faithfully pass on their chromosomes during mitosis, focusing on the molecular machines and mechanisms that ensure accurate chromosome segregation.
   1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.4.1.*

What research gap are you addressing with your protocol?

1.2. **Sara Hoppe:** We synchronize cells to study molecular processes that change with the cell cycle; without these methods, key changes would be hidden in an unsynchronized cell population.
   1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

1.3. **Sara Hoppe:** Compared to other synchronization methods, alpha-factor arrest in bar1 mutants provides a cleaner, reversible G1 arrest, allowing us to track an entire yeast culture progressing synchronously through the cycle.
   1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.2*

How will your findings advance research in your field?

1.4. **Matthew Miller:** Our work reveals dynamic protein localization and activity changes through the cell cycle, shedding light on key mitotic processes like chromosome segregation and spindle maintenance.
   1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figure 2*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Yeast Culture and Cell Cycle Synchronization: α-Factor Arrest-Release

Demonstrator: Talia C. Scheel

2.1. To begin, inoculate yeast into 25 milliliters of YPAD *(Y-P-A-D)* media **[1]** and incubate overnight to reach an optical density at 600 nanometers between 0.5 and 2.0 **[2]**.

2.1.1. WIDE: Talent adding yeast inoculum to the YPAD culture.

2.1.2. Talent placing the flask with YPAD medium on a shaker. **TXT: If culture is OD_{600} > 2.0 in morning, dilute to OD_{600} = 0.2–0.4 and grow one more cell cycle.**

2.2. Dilute the yeast cells to an optical density at 600 nanometers of 0.5. **[1]**. Add alpha-factor to the culture to reach a final concentration of 1 microgram per milliliter **[2]**.

2.2.1. Talent diluting yeast culture to the specified optical density using fresh YPAD medium.

2.2.2. Talent pipetting alpha-factor stock solution into the flask to achieve the final concentration.

2.3. At 2.5 to 3.5 hours after alpha-factor addition, count the percentage of non-budded, shmooed cells under a microscope to assess the cell arrest **[1-TXT]**.

2.3.1. LAB MEDIA: AlphafactorArrestedCellsT0.tif: **TXT: Proceed to release when 90–95% cells are shmooed**

2.4. Then, spin down the culture in a centrifuge at 3,000 *g* for 3 to 5 minutes at 23 degrees Celsius **[1]**. Carefully pour off the supernatant to remove the alpha-factor **[2]**. Resuspend the cell pellet in 25 milliliters of YPAD containing 1 percent dimethyl sulfoxide to wash the cells **[3].** After the first wash, transfer the cells to a fresh tube to remove residual alpha factor **[4-TXT]**.

2.4.1. Talent placing the culture tube into the centrifuge and setting it to the required speed and time.

2.4.2. Talent pouring off the supernatant from the centrifuged culture.

2.4.3. Talent pipetting fresh YPAD with dimethyl sulfoxide into the centrifuge tube and resuspending the pellet.

2.4.4. Talent pouring off supernatant, resuspending in leftover volume, and transferring into a new centrifuge tube. **TXT: Repeat washing in the same tube**

for a total of three washes

2.5. Next, add YPAD to the washed cells to bring the final volume to 25 milliliters **[1]** and transfer the suspension into a new flask for further incubation or use **[2]**. Collect the zero-minute time point sample immediately after release and fix the sample **[3-TXT]**.

2.5.1. Talent adding the appropriate volume of YPAD to the cell pellet.

2.5.2. Talent pipetting cells into a clean flask.

2.5.3. Talent pipetting an aliquot of the cell culture into a labeled microcentrifuge tube. **TXT: Take samples every 15 min at 23 °C and fix each sample immediately upon collection**

2.6. At 60 minutes post-release, assess synchrony of the cell population under a light microscope **[1]**.

2.6.1. LAB MEDIA: AlphafactorArrestedCellsT60_arrows.png

2.7. If desired, add alpha-factor again at 60 minutes after release to a final concentration of 1 microgram per milliliter to block further cell cycle progression **[1]**.

2.7.1. Talent pipetting alpha-factor into the culture flask to reach the final concentration. **TXT: Confirm release: check uniform small-budded cells before addition**

2.8. Continue collecting time point samples every 15 minutes up to 180 minutes or for as long as needed **[1-TXT]**.

2.8.1. Talent collecting successive samples at defined intervals into labeled tubes. **TXT: 23 °C: Metaphase ~45–60 min; Anaphase ~60–90 min; G1 by ~120 min post-release**

3. Yeast Fixation and Slide Preparation for Imaging

Demonstrator: Ahmed Abouelghar

3.1. To fix the yeast cells, centrifuge 1 milliliter of culture for 1 minute at maximum speed **[1]**. Aspirate the supernatant completely **[2]** and resuspend the pellet in 500 microliters of fixative solution **[3-TXT]**.

3.1.1. Talent placing microcentrifuge tubes containing culture into a benchtop centrifuge and initiating a spin at maximum speed.

3.1.2. Talent using a pipette to remove all supernatant without disturbing the pellet.

3.1.3. Talent pipetting the fixative solution into the tube and gently resuspending the pellet. **TXT: Incubate at 23 °C for 2-15 min**

3.2. After centrifuging the fixed cells, aspirate the supernatant and resuspend the pellet in 500 microliters of 0.1 molar potassium phosphate buffer at pH 6.4 **[1-TXT]**.

3.2.1. Talent removing the supernatant and resuspending the pellet in fresh potassium phosphate buffer. **TXT: Store cells at 4 °C until ready to image**

3.3. Before imaging, centrifuge the fixed cells at 23 degrees Celsius for 1 minute at maximum speed **[1]**. Once the supernatant is aspirated **[2],** resuspend the pellet in 10 to 100 microliters of Triton, DAPI, and sorbitol solution **[3]**.

3.3.1. Talent spinning the fixed cells in a benchtop centrifuge.

3.3.2. Talent using a pipette to remove all supernatant without disturbing the pellet.

3.3.3. Talent pipetting the Triton/DAPI/sorbitol staining solution into the tube and gently mixing the contents.

3.4. Pipette approximately 0.8 microliters of the stained cell suspension directly onto the center of a clean coverslip **[1]**. Use a pipette tip to gently spread the droplet into a circular area approximately 1 square centimeter in size **[2]**.

3.4.1. Talent dispensing a small droplet of stained cells onto a clean coverslip.

3.4.2. Talent using a pipette tip to gently spread the droplet into an even circle.

3.5. Place the coverslip onto a microscopy slide **[1]**. Using a Kimwipe, gently press around the edges of the coverslip to evenly distribute the sample **[2]**. Then, seal the edges of the coverslip with nail polish to secure the sample **[3-TXT]**.

3.5.1. Talent placing the coverslip onto the slide.

3.5.2. Talent gently dabbing around the coverslip edges with a Kimwipe to settle the cells.

3.5.3. Talent applying nail polish along the edges of the coverslip using a fine brush.

4. Imaging Yeast Cells to Analyse Cell Cycle-Dependent Protein Localization

Demonstrator: Sara Hoppe

4.1. Take the prepared slide to a microscope equipped with a 60 times magnification, 1.42 numerical aperture oil-immersion objective and a Red, Green, Blue, and Far-Red laser and filter set **[1]**. Focus the microscope on the yeast cells adhered to the coverslip **[1]**.

4.1.1. Talent placing the slide onto the microscope stage and securing it with clips.

4.1.2. SCREEN: 68887_screenshot_1.mp4: 00:02-00:10

4.2. Once focused, tune the exposure settings for each fluorescence channel to achieve a signal-to-noise ratio of at least 3 to 1 **[1-TXT]**. Adjust the acquisition settings to collect 14 to 20 Z-stack images, with each slice spaced 0.2 micrometers apart, covering a total Z-depth of 2 to 4 micrometers **[2]**.

4.2.1. SCREEN: 68887_screenshot_2.mp4: 00:08-00:22 **TXT: Adjust other channel exposures similarly avoiding overexposure of fluorescent puncta**

4.2.2. SCREEN: 68887_screenshot_2.mp4: 00:24-00:36

4.3. On microscopes equipped with post-processing modules, select **Deconvolution** and **Quick Projection** options for each Z-stack image **[1]**. Acquire Z-stacks of the sample, capturing enough images to record approximately 100 to 200 yeast cells for each experimental condition or time point **[2]**.

4.3.1. SCREEN: 68887_screenshot_3.mp4: 00:03-00:10.

4.3.2. SCREEN: 68887_screenshot_3.mp4: 00:10-00:20

4.4. For analysis, open the projected images in ImageJ software. Adjust the brightness and contrast of each fluorescence channel to enhance visibility **[1]**.

4.4.1. SCREEN:68887_screenshot_4.mp4: 00:00-00:05, 00:19-00:29

4.5. To analyze cell cycle progression, count 100 cells for each time point and classify them as containing one or two nuclei **[1]**.

4.5.1. SCREEN: 68887_screenshot_5.mov

4.6. To calculate the percentage of cells showing Stu2-GFP *(Stu-two-G-F-P)* puncta, standardize the green channel brightness settings across all images **[1]**. Count cells showing Stu2-GFP puncta that co-localize with Spc110-mCherry *(S-P-C-One-ten-M-Cherry)* puncta as positive for kinetochore localization **[2].**

4.6.1. SCREEN: 68887_screenshot_7.mov: 00:04-00:16

4.6.2. SCREEN: 68887_screenshot_7.mov: 00:24-00:37

4.7. Next, to quantify the intensity of protein puncta, use the freehand selection tool to

outline the region of interest around the signal **[1]**. Add the selection to the Region of Interest Manager by pressing **T** or choosing the option from the **ROI Manager** menu **[2]**. In the **ROI Manager**, click **Measure** to calculate puncta intensity **[3]**. To visualize changes over time, plot the average intensity with 95 percent confidence intervals for each time point. Count approximately 100 cells per condition **[4]**.

4.7.1. SCREEN: 68887_screenshot_8.1-3.mov: 00:02-00:07, 00:15-00:21

4.7.2. SCREEN: 68887_screenshot_8.1-3.mov: 00:21-00:23

4.7.3. SCREEN: 68887_screenshot_8.1-3.mov: 00:23-00:33

4.7.4. SCREEN: 68887_screenshot_8.4.mov: 00:08-00:25

Results

5. Results

5.1. In G1-arrested cells, Stu2-GFP *(Stu-Two-G-F-P)* localized to a single spindle pole-proximal punctum, with additional dispersed signal along cytoplasmic microtubules **[1].** At 60 minutes after release, Stu2-GFP localized as two puncta adjacent to duplicated spindle poles marked by Spc110-mCherry *(S-P-C-One-Ten-M-Cherry)* **[2].**

5.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the green punctum near the center of the G1 cell at time 0 min*

5.1.2. LAB MEDIA: Figure 2A**.** *Video editor: Highlight the two green puncta and nearby red dots in the 60-minute cell image.*

5.2. By 90 minutes, during anaphase, Stu2-GFP appeared both near the spindle poles and along microtubules spanning the spindle axis **[1].** Following mitotic exit, at 120 minutes, Stu2-GFP reappeared on astral microtubules in the cytoplasm **[2].**

5.2.1. LAB MEDIA: Figure 2A. *Video editor: Highlight both green puncta in the 90-minute panel in Stu2-GFP panel*

5.2.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the 120-minute Stu2-GFP panel*

5.3. Quantification revealed that Stu2-GFP intensity near spindle poles increased during early mitosis, peaking before anaphase, and declined thereafter **[1].** The percentage of binucleate cells peaked at approximately 90 minutes, indicating synchronized entry into anaphase **[2].**

5.3.1. LAB MEDIA: Figure 2B (left graph). *Video editor: Highlight the rising curve that peaks around 60 minutes and falls after 90 minutes.*

5.3.2. LAB MEDIA: Figure 2B (right graph). *Video editor: Highlight the sharp peak in the curve near the 90-minute mark.*

1. **Inoculate**
   Pronunciation link: https://www.merriam-webster.com/dictionary/inoculate
   IPA: /ˈɪnəˌkjuleɪt/
   Phonetic spelling: IN-uh-kyoo-layt
2. **Optical (density)**
   Pronunciation link: https://www.merriam-webster.com/dictionary/optical
   IPA: /ˈɑptɪkəl/
   Phonetic spelling: OP-ti-kuhl
3. **Nanometer**
   Pronunciation link: https://www.merriam-webster.com/dictionary/nanometer
   IPA: /ˈnænəˌmitər/
   Phonetic spelling: NAN-oh-mee-ter
4. **Centrifuge**
   Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
   IPA: /ˈsɛntrəˌfjuʤ/
   Phonetic spelling: SEN-truh-fyooj
5. **Dimethyl sulfoxide**
   Pronunciation link: https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide
   IPA: /daɪˈmɛθəl ˌsʌlˈfɑːksaɪd/
   Phonetic spelling: dye-METH-uhl sul-FAWK-sahyd
6. **Resuspend**
   Pronunciation link: https://www.merriam-webster.com/dictionary/resuspend
   IPA: /ˌriːsəˈspɛnd/
   Phonetic spelling: ree-suh-SPEND
7. **Sorbitol**
   Pronunciation link: https://www.merriam-webster.com/dictionary/sorbitol
   IPA: /ˈsɔːrbɪtɒl/
   Phonetic spelling: SOR-bi-tol
8. **Synchrony**
   Pronunciation link: https://www.merriam-webster.com/dictionary/synchrony
   IPA: /ˈsɪŋkrəni/
   Phonetic spelling: SING-kruh-nee