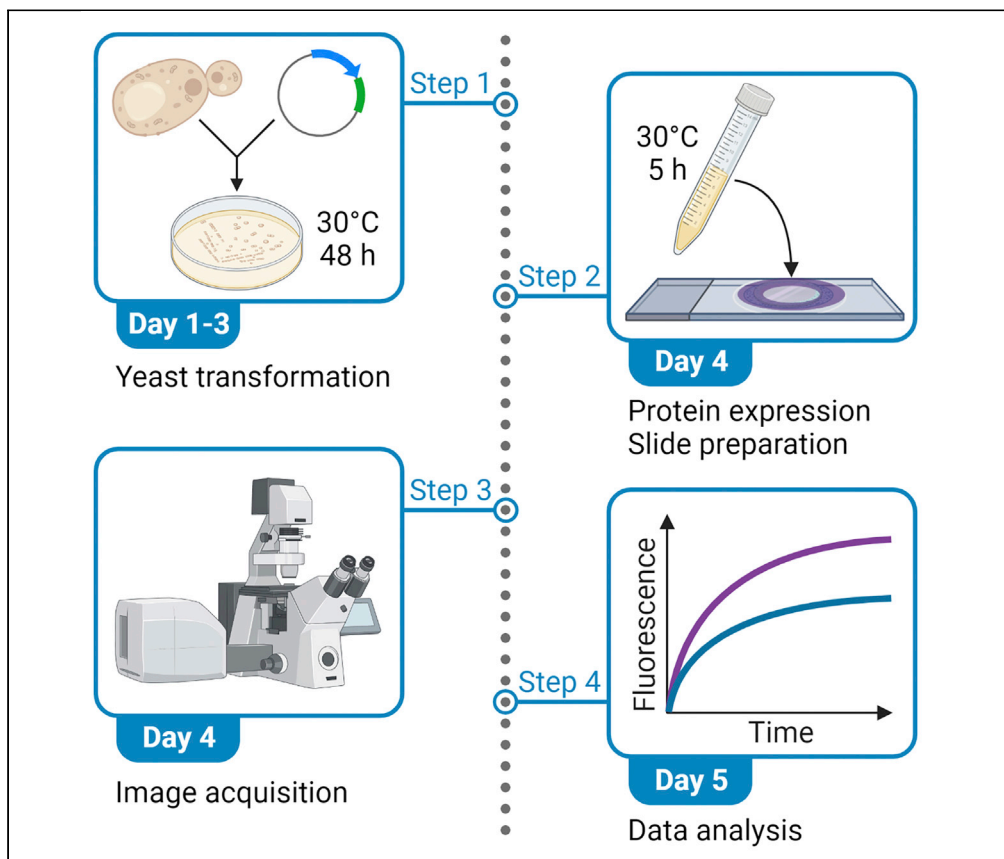# STAR Protocols

Protocol

Monitoring condensate dynamics in *S. cerevisiae* using fluorescence recovery after photobleaching

This protocol describes the use of fluorescence recovery after photobleaching (FRAP) to investigate the dynamics of Matrin-3 (MATR3) condensates in live budding yeast. We detail how to generate yeast strains containing MATR3 with an enhanced green fluorescent protein (eGFP) tag and induce MATR3-eGFP expression. We provide steps to prepare slides of immobilized yeast cells and perform FRAP imaging and data analysis. This protocol can be broadly applied to study condensate dynamics of a range of proteins in different model systems.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Transforming and expressing protein-of-interest in yeast cells

Preparing slides of immobilized live yeast cells for confocal imaging

Optimized FRAP experiments to study condensate dynamics in live yeast cells

Qualitative and quantitative analysis of FRAP experiments to assess phase separation

STAR Protocols

Protocol

Monitoring condensate dynamics in *S. cerevisiae* using fluorescence recovery after photobleaching

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SUMMARY

This protocol describes the use of fluorescence recovery after photobleaching (FRAP) to investigate the dynamics of Matrin-3 (MATR3) condensates in live budding yeast. We detail how to generate yeast strains containing MATR3 with an enhanced green fluorescent protein (eGFP) tag and induce MATR3-eGFP expression. We provide steps to prepare slides of immobilized yeast cells and perform FRAP imaging and data analysis. This protocol can be broadly applied to study condensate dynamics of a range of proteins in different model systems. For complete details on the use and execution of this protocol, please refer to Sprunger et al. (2022).

BEFORE YOU BEGIN

This protocol describes the steps to analyze condensates of MATR3-eGFP in live *S. cerevisiae* cells by FRAP (Sprunger et al., 2022). We have also used this protocol for analysis of FUS-CHOP-GFP in yeast (Ryan et al., 2019). This protocol could be used for studying any fluorescently tagged protein expressed in yeast.

Plasmid preparation

⏱ Timing: Variable

1. Obtain pAG423GAL-MATR3-eGFP (see key resources table) or another expression vector encoding the gene-of-interest with a fluorescent protein tag from a suitable source.
2. Prepare plasmid DNA using a QIAGEN Miniprep Kit to yield a concentration of 50–300 ng/μL (see Manufacturer Protocol).

Yeast transformation

⏱ Timing: 4 days

Note: This protocol is adapted from a standard protocol using polyethylene glycol (PEG) and lithium acetate (Gietz and Schiestl, 2007).

3. Grow cultures of W303aΔ*hsp104*, or an alternative strain if desired, in YPD (Yeast Extract-Peptone-Dextrose) media for 16–20 h. Start enough yeast culture such that sufficient yeast will be available for the subsequent steps (the volume varies by number of strains to be generated).

Note: Prepare commercially available YPD media by dissolving the manufacturer's specified mass of YPD media in 1 L of Milli-Q water, autoclaving at 121°C for 20 min, cooling, and storing at 20°C–25°C for up to one year.

4. After 16–20 h of growth, dilute cultures to an OD_{600nm} of 0.3 in YPD in a baffled flask. Total volume should include 10 mL of media per plasmid to be transformed and an additional 20 mL of media for controls. Grow at 30°C with shaking at 250 RPM for approximately 4 h.
5. Once the culture reaches early-log phase growth (OD_{600nm} 0.5–0.8) harvest and wash the cells.
   a. Pellet cells in a 50 mL falcon tube by centrifuging for 5 min at 3,200 × g (4000 RPM).
   b. Decant the supernatant by pouring off the water and resuspend the cell pellet in 50 mL of sterile water (autoclaved Milli-Q water).
   c. Repeat steps 5a and 5b until the cells have been washed three times in sterile water.
   d. After the final centrifugation, pour off the bulk of the supernatant and remove remaining supernatant by pipet.
6. Prepare the transformation master mix. *N* = the number of plasmids to transform plus 2. Vortex to mix after adding each component.
   a. Add *N* × 70 μL of sterile water to the cell pellet.
   b. Add *N* × 240 μL of sterile 50% (w/v) polyethylene glycol (PEG)-3350 to the cell mixture.
   c. Add *N* × 36 μL of sterile 0.1 M lithium acetate to the cell mixture.
   d. Add *N* × 10 μL of sterile boiled and cooled sonicated salmon sperm DNA to the cell mixture.
7. Label a 1.5 mL tube for each plasmid to be transformed, as well as a no DNA negative control. Add 2 μL of 50–300 ng/μL plasmid to each tube, or nothing to the negative control.
8. Vortex transformation mixture a final time and aliquot 360 μL of transformation mixture into each tube. Firmly close each tube and vortex to mix.
9. Incubate the transformation tubes in a 42°C water bath for 20 min.

   ⚠ CRITICAL: Check that temperature of the water bath is exactly 42°C. Higher temperatures may kill the yeast and lower temperatures will decrease transformation efficiency.

10. Pellet the cells in the transformation mixture by centrifugation at 2,000 × g (4600 RPM) for 1 min. Remove the supernatant.
11. Resuspend the pelleted cells in 250 μL of sterile water and plate on solid synthetic yeast media with dextrose and appropriate drop-out (SD-His, see Recipe, when using pAG423GAL-MATR3-eGFP). Different expression vectors may require different drop-out conditions.
12. Incubate the plates at 30°C for 2–3 days until colonies are evident.
13. Scrape up all colonies on each plate to prepare 30% (w/v) glycerol stocks for storage at −80°C and/or inoculate liquid raffinose synthetic yeast media with histidine drop-out (SRaff-His, see Recipe).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
XL10-Gold Ultracompetent cells	Agilent	Cat# 200315
Chemicals, peptides, and recombinant proteins		
BD Difco™ Yeast Nitrogen Base without Amino Acids	Fisher	Cat# DF0919-07-3
Complete Synthetic Mixture-His	Sunrise Science	Cat# 1006-100
Dextrose (D-Glucose)	Fisher	Cat# D16-10
Galactose	Fisher	Cat# BP656-500
Glycerol	Fisher	Cat# BP229-4
Lithium Acetate	Sigma-Aldrich	Cat# 517992-100G

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Poly(ethylene glycol)-3350	Sigma-Aldrich	Cat# P4338-1KG
Raffinose	MP Biomedicals	Cat# 114010022
Sonicated Salmon Sperm DNA	Agilent	Cat# 201190
UltraPure™ LMP Agarose	Invitrogen	Cat# 16520-050
YPD media	Sigma-Aldrich	Cat# Y1375-1KG
Experimental models: Organisms/strains		
S. cerevisiae: W303aΔhsp104 (MATa, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1, hsp104::KanMX)	Schirmer et al. (2004)	N/A
Recombinant DNA		
pAG423GAL-MATR3:WT-eGFP	Sprunger et al. (2022)	N/A
Software and algorithms		
Excel	Microsoft	https://www.office.com/
GraphPad Prism 9	GraphPad	https://www.graphpad.com/
Fiji/Image J	Schneider et al. (2012)	https://imagej.nih.gov/ij/
ZEN Black	ZEISS	https://www.zeiss.com/
Other		
1.5 mL Safe-Lock Tubes	Eppendorf	Cat# 022363204
15 mL Conical Centrifuge Tubes	Fisher	Cat# 07-200-886
50 mL Conical Centrifuge Tubes	Fisher	Cat# 06-443-19
Bemis™ Parafilm™ M Laboratory Wrapping Film	Fisher	Cat# 11772644
Baffled Erlenmeyer Flasks	Fisher	Cat# 11353454
Cel-Gro Tissue Culture Rotators	Thermo Scientific	Cat# 88882015
Corning™ LSE™ Digital Water Bath, 6 L	Fisher	Cat# 15671312
Coverslips 12 mm Circular Type 1.5	Fisher	Cat# 12-545-81
Drum for Cel-Gro Tissue Culture Rotators	Thermo Scientific	Cat# 88882155
Forceps, General Application	Fisher	Cat# 15644926
GENESYS™ 10S UV-Vis Spectrophotometer	Thermo Scientific	N/A
Immersion Oil Type F30CC	Fisher	Cat# NC1244982
Microscope slides	Fisher	Cat# 12-550-343
NanoDrop™ One	Thermo Scientific	Cat# ND-ONE-W
Eppendorf™ Excella™ E24 Incubator Shaker	Fisher	Cat# 11320065
QIAprep Spin Miniprep Kit	QIAGEN	Cat# 27106
Petri Dish, Sterile 100 mm × 15 mm Polystyrene	Fisher	Cat# FB0875712
Precision Low Temperature Refrigerated Incubator	Thermo Scientific	Cat# PR205745R
Sally Hansen Dries Instantly Top Coat Nail Polish	Various	Cat# 45114
Scientific Industries SI™ Vortex-Genie™ 2	Fisher	Cat# 15547335
Self Sticking Labeling Tape	Fisher	Cat# 159015R
Semi-Micro Cuvettes, 1.5 mL	Fisher	Cat# 14955127
Thermometer	Fisher	Cat# 11551993
Thermomixer® C	Eppendorf	Cat# 5382000023
Zeiss LSM 880 Confocal with Airyscan	ZEISS	N/A

MATERIALS AND EQUIPMENT

Synthetic yeast media		
Reagent	Final concentration	Amount
Dextrose, raffinose, or galactose	2% (w/v)	20 g
Yeast Nitrogen Base	0.67% (w/v)	6.7 g
Yeast Complete Supplement Mixture with appropriate dropout (- Histidine)	Variable, indicated on container	× g
Agar (for preparing plates)	2% (w/v)	20 g
ddH$_2$O	N/A	To 1 L
Total	N/A	1 L

Media for preparing plates can be autoclaved at 121°C for 20 min with a stir bar, cooled with stirring for 45–60 min, then poured into petri dishes. Allow to stand undisturbed for 24 h, plates can then be bagged and stored at 4°C for up to one year. Liquid media should be sterile filtered and stored at 20°C–25°C for up to one year.

Alternatives: We utilized the Zeiss LSM 880 Confocal with Airyscan Fast imaging system which was equipped for Airyscan deconvolution. A confocal microscope with an equivalent detector system and other necessary features could also be used. Different yeast species and different proteins with a fluorescent tag could also be used with this protocol.

STEP-BY-STEP METHOD DETAILS
Yeast induction and slide preparation

⊘ Timing: 24 h

This protocol yields yeast cells that are expressing the protein of interest. Here, yeast cells are grown for 16–20 h in raffinose media to prime the cells for induction and then transferred to galactose media to induce expression of MATR3-eGFP. After induction, cells are harvested and immobilized on an agarose pad for imaging.

1. Inducing yeast.
   a. Prepare a 5 mL culture of MATR3-eGFP yeast in liquid raffinose synthetic yeast media with histidine drop-out (SRaff-His, see Recipe). This can be inoculated from newly transformed plates of yeast or from glycerol stocks of yeast (see yeast transformation step 13). Incubate this culture in a rotating drum or in a shaker at 30°C for 16–20 h.
   b. Preparing yeast for induction.
      i. Measure the OD_{600nm} of the SRaff-His yeast cultures.
      ii. Calculate the volume of culture needed to prepare 5 mL of culture at OD_{600nm} 0.5 and transfer this volume to a new tube.
      iii. Pellet the transferred cells at 3,200 × g (4,000 RPM) for 5 min and decant the supernatant.
      iv. Resuspend the cell pellet in 5 mL of liquid galactose synthetic yeast media with histidine drop-out (SGal-His, see Recipe).
      v. Incubate the yeast cultures in a rotating drum at 30°C for 5 h.

Note: You may need to induce for more or less time depending on the expression level of your protein of interest.

2. Agarose pad slide preparation.
   a. Preparing agarose pads.
      i. Add 40 mg of low melting point agarose and 1 mL of liquid SGal-His media to a 1.5 mL tube with safe-lock.
      ii. Heat the solution to 100°C in a thermomixer. Vortex and flick the tube to dissolve the agarose into the media. Once melted, flick the tube to dislodge bubbles.

Note: The agarose/galactose solution can be used repeatedly once dissolved. Store at 20°C–25°C after use and reheat for next use to melt the agarose. This solution can be stored up to 6 months.

      iii. Apply a single layer of lab tape to about a half-dozen microscope slides. Ensure that the tape lies completely flat, with no bubbles, and does not wrap around to the back of the slide. These slides are simply used for spacing to achieve an appropriately thick agarose pad and will not be used for imaging.
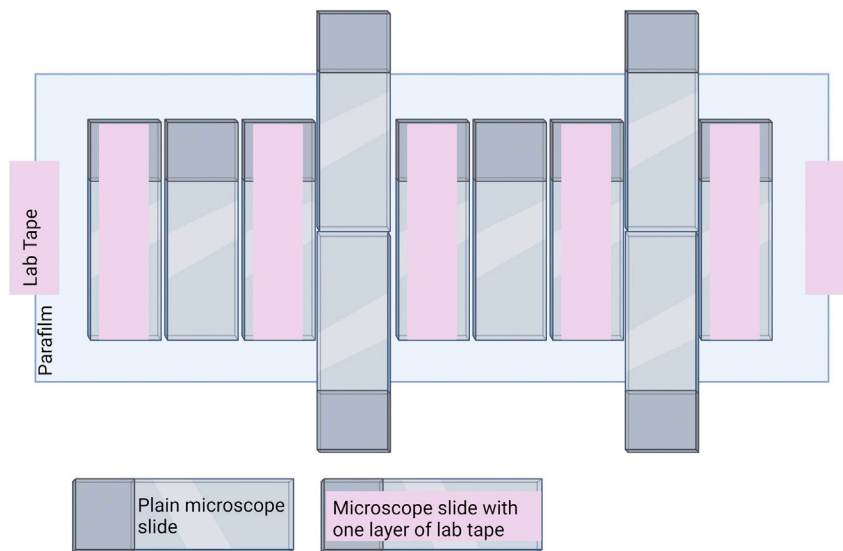
Figure 1. Diagram of slide layout alternating plain and taped microscope slides on a piece of parafilm
Taped slides are used to achieve equally thick agarose pads on all slides. Created with BioRender.com.

b. Forming agarose pads.
   i. Tape a clean piece of parafilm down to the lab bench. Lay out alternating clean microscope slides and taped microscope slides (see Figure 1). Be sure to keep the slides flat and even with each other.
   ii. Carefully pipet 23 µL of melted agarose/galactose solution to the center of a microscope slide that does not have tape applied.
   iii. After a brief pause (~5 s) to allow the solution to cool slightly, lay another clean microscope slide perpendicular across the agarose drop to set the thickness of the agarose pad (see Figure 2 and problem 5).
   iv. Allow the agarose pads and slides to set for approximately five minutes to ensure that the agarose has solidified.
   v. Carefully separate the paired slides by lifting off the top slide and laying it down with the agarose pad face up (see Figure 3A). Try not to let the agarose pad fold over on itself. Use whichever slide the agarose pad sticks to.
c. Harvesting and applying yeast to agarose pads.
   i. Harvest the yeast from the expression cultures by pelleting the cells at 3,200 × g (4000 RPM) for 5 min. Decant the supernatant.
   ii. Resuspend the cell pellet in 200 µL of galactose media and transfer the cells to a 1.5 mL tube.

Optional: At this stage, the yeast cells could be incubated with Hoechst stain for nuclear imaging if desired.

   iii. Apply 5 µL of resuspended yeast directly to the center of the agarose pad (see Figure 3B).
   iv. Lay a 12 mm circular Type 1.5 glass coverslip on top of the yeast and agarose pad (see Figure 3C).
   v. Stabilize the coverslip with a pair of forceps while gently applying clear nail polish around the edges of the coverslip (see Figure 3D). The nail polish should contact the full perimeter of the cover slip and should also contact the microscope slide in a full perimeter beyond the agarose to be sealed.
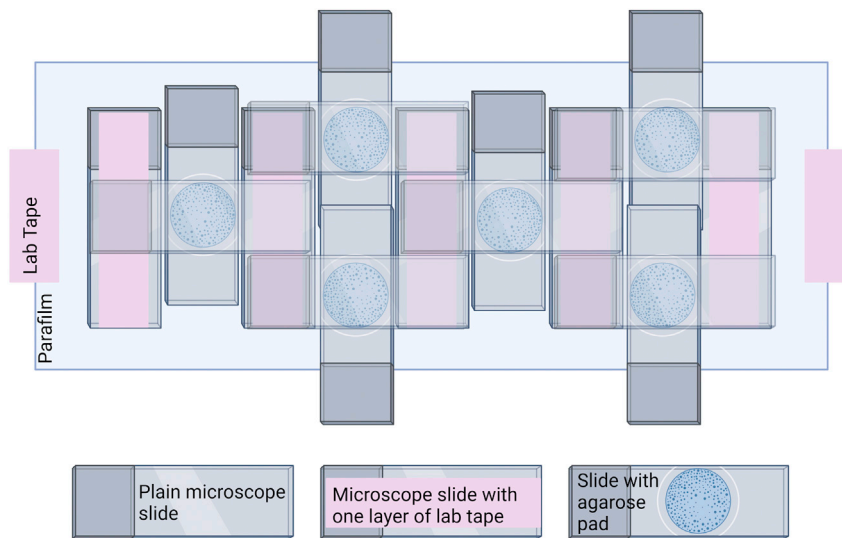
Figure 2. Diagram of layout of slides for formation of agarose pads of uniform thickness
Created with BioRender.com.

vi. Allow the nail polish to dry for several minutes, then apply a second layer of nail polish to ensure the agarose pad is fully sealed (see Figure 3E).

vii. Store and transport the slides horizontally to the microscope for imaging. Slides are suitable for imaging up to several hours after preparation.

Note: We typically prepare 2–3 slides per strain in case a slide is not properly sealed or if slides break during acquisition.

**FRAP imaging**

⏱ Timing: 3–4 h

A Zeiss LSM880 Confocal Microscope with Airyscan was employed to acquire FRAP data for MATR3-eGFP. This section details the parameters and steps for performing FRAP experiments on MATR3-eGFP with this imaging system (see Figure 4). Although our yeast strains are immobilized between the agarose pad and coverslip, we found that the MATR3 condensates move throughout the cell. To circumvent this limitation, we streamlined acquisition and opened the pinhole for a greater depth of view. Considerations for optimization of different protein systems are found in the troubleshooting section.

3. Microscope Setup.
   a. Turn on the microscope system and ZEN Black software with the 488 nm and 405 nm laser lines turned on. We used an alpha Plan-Apochromat 100× / 1.40 Oil DIC M27 objective.
   b. Just before imaging each slide, confirm that the nail polish seal is intact by checking that a yeast film is visible beneath the coverslip. Add a drop of 30C immersion oil to the objective and place the microscope slide onto the stage.

Note: We do not utilize a heated chamber for live yeast cell imaging.

Note: We recommend the use of 30C immersion oil due to the prolonged imaging of each slide. This oil will prevent overheating of the sample.
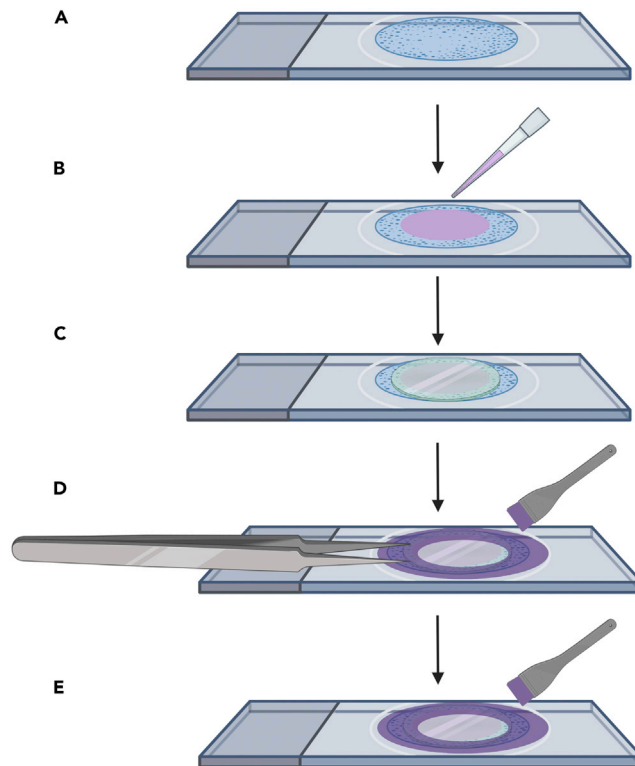
Figure 3. Process of preparing slides for imaging
(A) Separate slides and place slide with agarose pad facing up.
(B) Apply 5 μL of resuspended yeast (pink) directly to the center of the agarose pad.
(C) Add a 12 mm circular coverslip (green) on top of the yeast.
(D) Stabilize the coverslip with a pair of tweezers and apply clear nail polish (purple) around the coverslip.
(E) After a few minutes, apply a second coat of nail polish.
Created with BioRender.com.

    c. Under the Locate tab of ZEN, use GFP epifluorescence to locate and focus on the yeast cells (see Figure 5A).

    d. Switch to the Acquisition tab of ZEN to setup the parameters for FRAP imaging as follows (see Figure 5B):

       i. Check the "Time Series", "Bleaching", and "Regions" boxes.

Note: To acquire images with appropriately fast time frames, we do not utilize Z-stack imaging.

       ii. Under "Imaging Setup", setup GFP detection from the LSM Tab.

       iii. Under "Acquisition Mode" set the frame size to 520 × 520 pixels, speed to 2.02 μsec, line averaging of 2, bit depth of 8 bits, bidirectional scanning, and 4× zoom (see problem 3).

       iv. Under "Channels" setup a single track for EGFP with the 488 nm laser set to 1.5% and 824 V gain. Open the pinhole to 5.38 Airy units (AU) (see problem 4).

Note: The laser power represented in percentage is a relative number between systems. Furthermore, the absolute power compared to laser percentage can vary on the same system depending on the age of the laser source. As such, one should always optimize laser power when starting a new set of experiments. See problem 1 for more information about
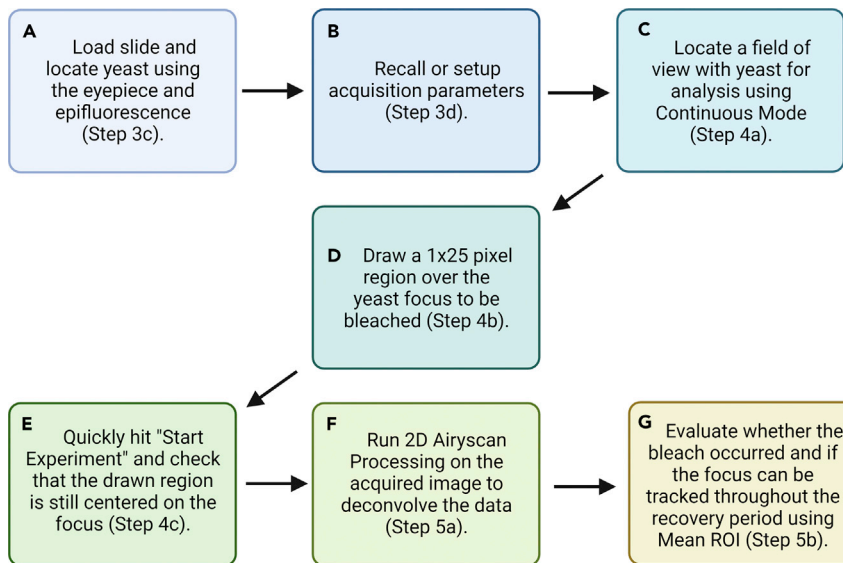
Figure 4. Schematic detailing the steps to setup, acquire, and process a FRAP experiment
Created with BioRender.com.

optimizing laser power during bleaching, or problem 4 for more information about optimizing laser power to minimize general photobleaching.

Note: By partially opening the pinhole, the detectors receive more signal from a larger Z slice. Since we are unable to use multiple Z-slices in our experimental setup, this helps us collect more Z information. Additionally, a deeper field of view will help minimize the impact of Z drift of our focus-of-interest. However, opening the pinhole further could be detrimental by allowing more than one focus within the Z plane.

   v. Under "Time Series" use 100 cycles with an interval of 0 for a total of 2 min of acquisition. With these acquisition settings, our frame interval is about 1.3 s per frame.
   vi. Under "Bleaching" set to acquire five scans before bleaching. Bleach for 50 iterations. Use a different scan speed for bleaching at 16 μsec/pixel. Activate the 405 nm laser at 1% and the 488 nm laser at 70% for bleaching (see Note above, and see problem 1). A decrease in GFP signal of at least 50% should be achieved consistently with these parameters.

Note: On ZEN Black, all of these acquisition parameters can be saved under the experimental manager to be recalled for future imaging sessions.

4. Acquiring a FRAP Experiment.
   a. For a new experiment, first use Continuous mode to locate a field of view with multiple yeast cells and focus on the condensate of interest.

   ⚠ CRITICAL: To prevent pre-photobleaching of the sample, be sure to complete this process quickly and turn off Continuous mode before proceeding to the next step.

Note: Continuous mode uses the laser power, detector gain, pixel dwell time, and scan speed settings which were set in Acquisition Parameters (see step 3d).

Note: In selecting a focus for bleaching, try to find one which is rather stable in its position (i.e., not moving around the cell or drifting away slowly). Furthermore, a focus must >500 nm in diameter for bleaching.
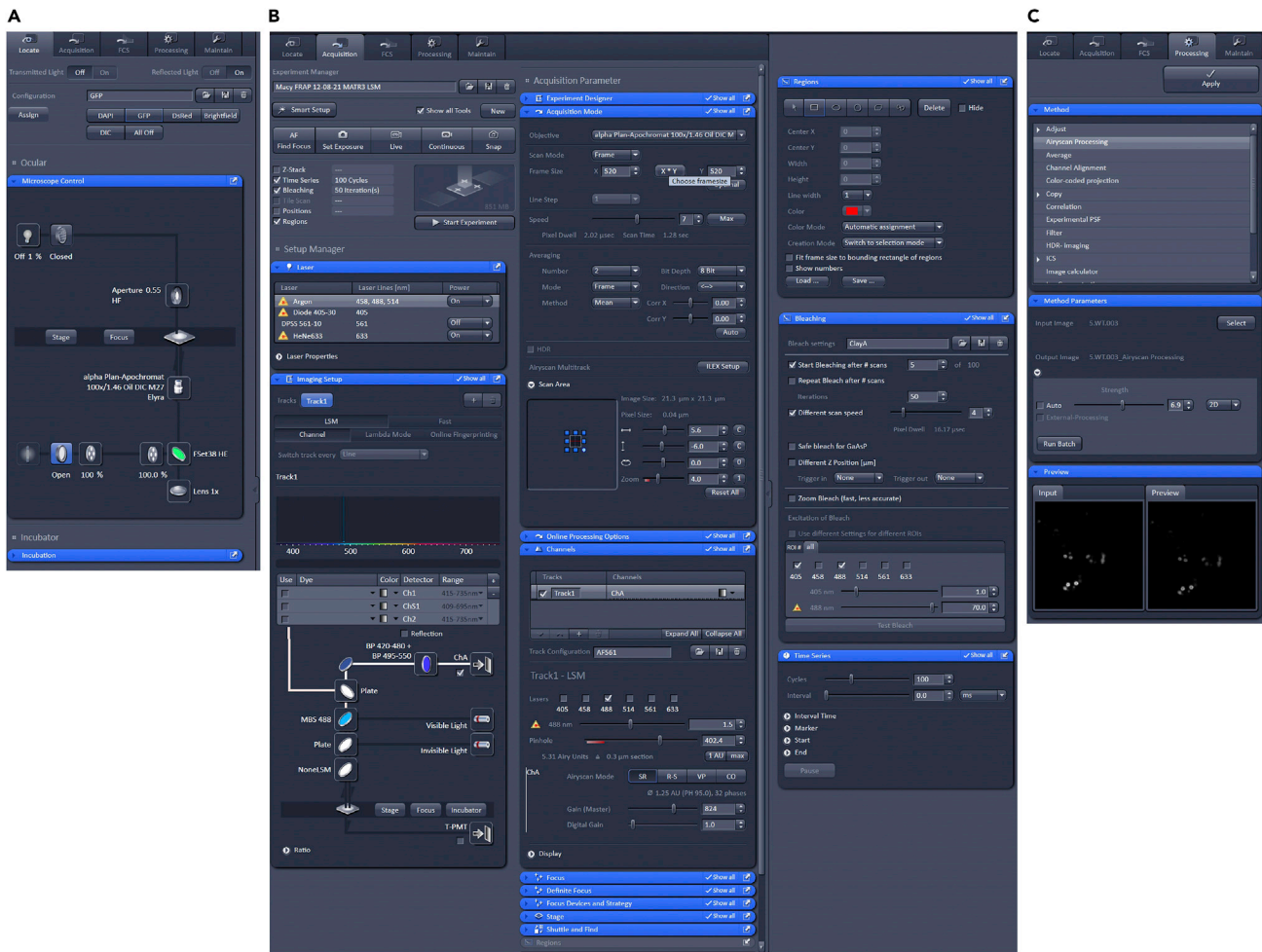
Figure 5. Setting up ZEN Black software and adjusting key parameters
(A) Details of the "Locate" tab including the light path. Corresponds to step 3c.
(B) Details of the "Acquisition" tab including experimental setup, light paths, and acquisition parameters. Corresponds to step 3d.
(C) Details of the "Processing" tab including parameters for Airyscan Processing. Corresponds to step 5a.

    b. Under "Regions" draw a horizontal rectangular region of interest (ROI) across the widest point of the condensate of interest.

       i. Adjust this rectangle to a width of 25 pixels and a height of 1 pixel.

       ii. Recenter on the condensate if necessary (see problem 2).

    c. Without delay, start experiment to acquire the data.

Note: Upon initiation, check that the drawn ROI is still aligned with the condensate of interest, which may have shifted during setup. If the ROI is no longer aligned, or if the condensate disappears from view during acquisition, the experiment should be aborted as it will not be a useful trial.

5. Data Processing and Analysis.

Note: This section details how to determine if an experiment meets the criteria to be counted as a trial for qualitative reporting. It is helpful to perform this analysis during an imaging session to keep track of how many additional trials are needed per strain.
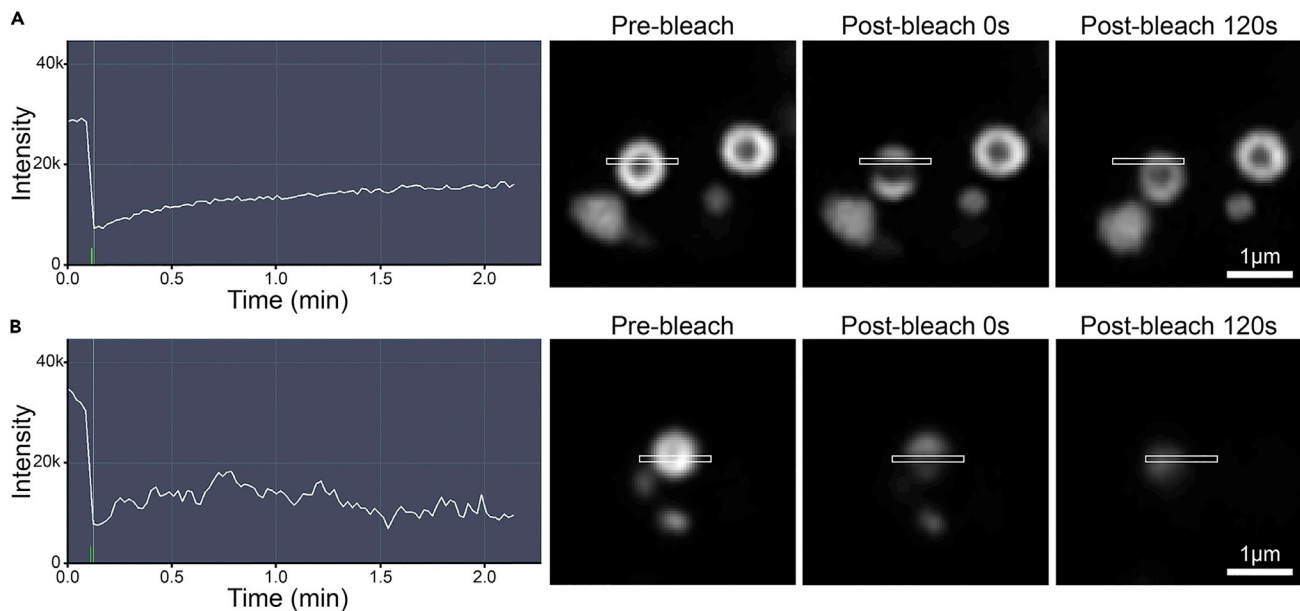
Figure 6. ZEN Black software screen shots of "Mean ROI" automatically generated recovery curves and corresponding micrographs

Generated recovery curves plot the signal intensity within the drawn ROI against time. In the case of bleached focus drift in the X, Y, or Z directions, the generated curve does not represent recovery within the bleached region.

(A and B) Data presented of an experiment which does meet the criteria of a trial (6A) and an experiment which cannot be counted as a trial (6B). White box indicates bleached region. Note that due to drift, the data shown in experiment A cannot be quantified, but does demonstrate recovery. Scale bar, 1 μm.

a. Under the "Processing" tab of ZEN Black, select Airyscan Processing and run 2D processing on your newly acquired image (see Figure 5C). For all MATR3-eGFP data, we processed images with a 6.9 Airyscan parameter.

Note: All images in a data set need to be processed using the same Airyscan Parameter. For a new data set, run 2D Airyscan processing on a variety of images using the automatic function and note their resulting Airyscan parameter. Choose a representative or mean Airyscan parameter and reprocess all images in the data set using this parameter.

b. Evaluate the deconvolved image under the "Mean ROI" tab. This tab will automatically generate a curve of GFP signal measured within the drawn ROI versus time (see Figure 6). Additionally, it shows the deconvolved micrographs adjacent to the generated recovery curve.

Note: Although a recovery curve is automatically generated in the "Mean ROI" display, this curve may not be an accurate depiction of recovery observed in the experiment. In our experiments, the bleached focus nearly always drifted in the X, Y, and sometimes Z direction. Since the drawn ROI does not drift with the bleached focus, the ROI does not remain aligned with the bleached region if the focus drifts. The automatically generated curve plots the signal detected within the ROI against time, which does not represent recovery within the bleached region in the case of focus drift. The main use of the generated recovery curve is to observe the percent decrease in signal from pre-bleach to post-bleach images.

c. Evaluate if the experiment can be counted as a trial by ensuring that each experiment meets each of the following criteria:
   i. The focus was bleached.

    ii.   The bleached region is in the center of the focus, not on the edge.

    iii.   The GFP signal decreased at least 50% from pre-bleach to post-bleach (evaluate with automatically generated curve).

    iv.   The focus remains within the field-of-view over the two-minute recovery period such that recovery or lack-thereof is evident.

Note: In Figure 6A, the experiment meets all of the above criteria to be counted as a trial. Clear recovery of the focus can be observed by two minutes post-bleach, although the focus has drifted in the XY plane. Because of this movement of the focus, recovery cannot be accurately quantified and analysis should instead be done qualitatively. In Figure 6B, the experiment does not meet the requirements to be counted as a trial. The bleached region is on the edge of focus rather than in the center, which makes recovery more difficult to observe. Additionally, the focus drifts in the Z direction in addition to some XY drift, which means the focus is not clearly within the field-of-view. As such, we cannot observe whether the focus recovered from the bleaching or not. Note that the recovery curve for Figure 6B is very jagged due to movement of the focus, indicating that it cannot be used to accurately assess if the focus recovered or not.

d. Save a copy of the table in "Mean ROI" to have the real time stamps of each frame.

e. Save all images with both raw and processed files for your records.

f. Obtain at least ten trials per strain for qualitative publication of the results.

EXPECTED OUTCOMES

Phase separation of biomolecules such as proteins and nucleic acids is an essential physiological process. Upon aberrant phase transitions, condensates can convert from a dynamic liquid-like state to a gel- or solid-like state. Monitoring condensate properties can therefore indicate if phase separation has been perturbed (Sprunger and Jackrel, 2021). FRAP is a useful technique to investigate the dynamics of condensates in living cells. FRAP experiments on living yeast cells are challenging due to the small size of yeast cells, the small size and dynamics of their internal structures, and the mobility of live yeast cells which grow in suspension.

Following the details outlined in this protocol, we performed FRAP experiments on MATR3 wild-type and disease-associated MATR3 mutants to investigate internal dynamics of foci comprised of these proteins (see Figure 7). After bleaching the foci across a line, return of fluorescence to that line was monitored over two minutes. Fluorescence of MATR3^{WT} and MATR3^{F115C} shell-like condensates rapidly recovered, indicating that these structures have liquid-like composition. In contrast, the misshapen foci of MATR3^{S85C}, MATR3^{P154S}, and MATR3^{T622A} did not display any recovery over the two-minute observation period, indicating more gel- or solid-like properties. Movement of these foci within the nucleus precluded this data from quantitative analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

⏱ Timing: 1–2 days

In the case of MATR3 FRAP experiments, the foci were moving too much within the cells to allow quantification. We were unable to drift correct our data due to the lack of a suitable reference point. As such, we only reported qualitative results (see Figure 7). If the bleached foci remain stable throughout the observation period, the data can be quantitatively analyzed to produce FRAP curves which are fitted to calculate several parameters including time-to-half-recovery and immobile fraction. Below are the steps to follow for quantitative analysis, which can be performed in Fiji/Image J.

1. Open deconvolved FRAP images in ImageJ with ROIs displayed.
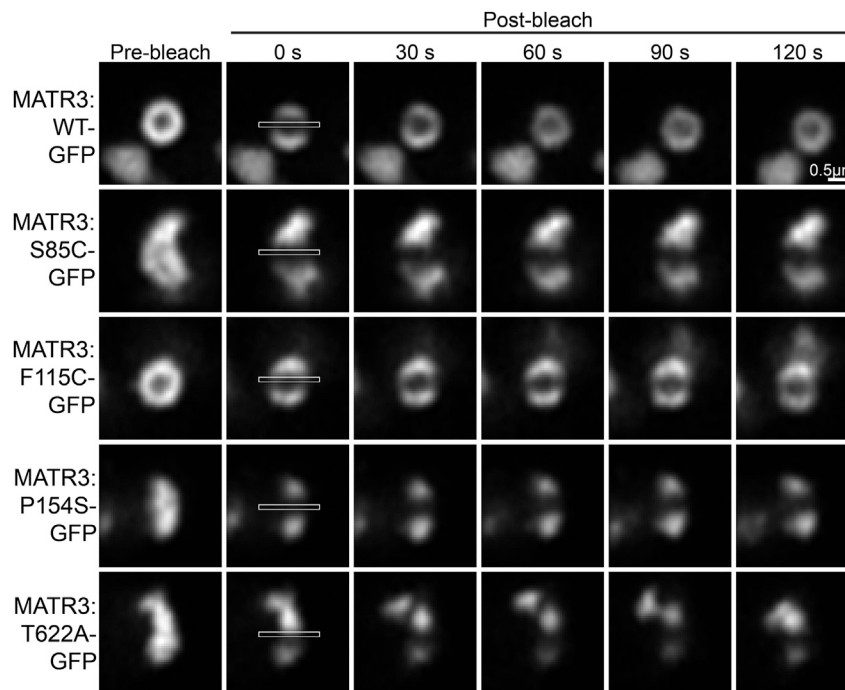
Figure 7. FRAP of MATR3-eGFP and mutants

MATR3-GFP foci were photobleached in a 1 × 25 pixel line, and recovery was monitored over 2 min. MATR3^{WT} displays full recovery within 2 min. MATR3^{F115C} showed partial recovery by 2 min while MATR3^{S85C}, MATR3^{P154S}, and MATR3^{T622A} do not recover. Images from one trial are shown, which are representative of at least ten trials per strain. White box indicates bleached region. Scale bar, 0.5 μm. Data is reproduced from Sprunger et al., *iScience*, 2022.

2. Copy the bleach ROI and place on a background region where there are no cells.
3. Use the multi-measure tool to calculate the integrated GFP density for each ROI. Copy and paste the resulting values into Excel for calculations.
4. Subtract the background signal from the bleached ROI signal.
5. Normalize the average pre-bleach signal to one and the minimum post-bleach value to zero.
6. Find the true time values from the saved table (see Saving Time Table).
7. Using GraphPad Prism, plot your background corrected and normalized values against time to generate a FRAP curve.
8. Fit the curve in GraphPad Prism with a nonlinear regression exponential curve (see Figure 8). The calculated value corresponding to the signal plateau represents the mobile fraction. The calculated value corresponding to half-life represents the time-to-half-recovery.

LIMITATIONS

The ability to perform FRAP experiments in live yeast is largely limited by the size and mobility of the cells and/or foci. Due to resolution limitations of this microscope system, the FRAP protocols we have detailed should not be performed on foci which are smaller than 500 nm. While our agarose pad slide preparation effectively immobilizes yeast cells, condensates still drift and tumble within the cells. We have tried to minimize the impact of this drifting by opening the pinhole and imaging less mobile condensates. However, condensate drift can limit the ability to quantify results and may in some cases prevent stable observation of recovery. Further, choosing the more stable condensates for analysis can lead to bias in the dataset. Other limitations include the requirement for a fluorescent tag appended to MATR3, which could alter its stability and phase separation properties. Further, the use of a 2 micron plasmid, which is a high copy number plasmid, can lead to different expression levels of MATR3-eGFP in different cells which may skew the resulting dataset.
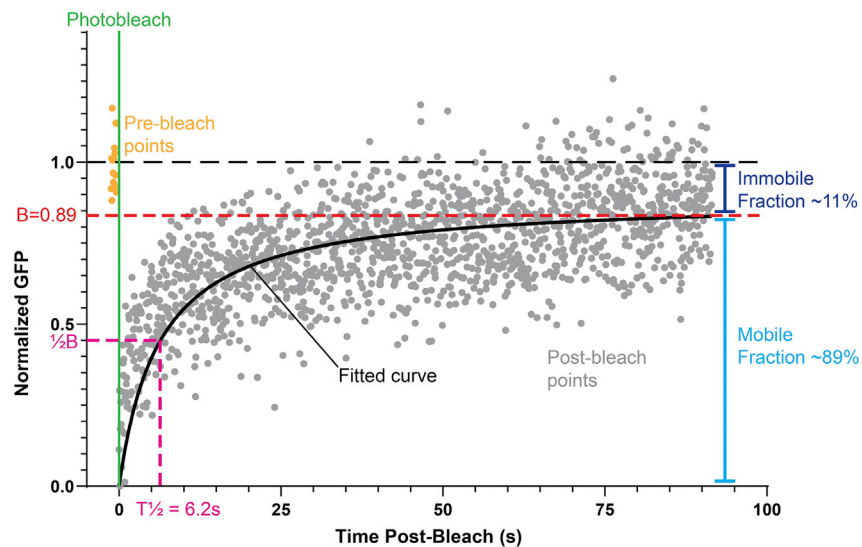
Figure 8. Sample FRAP curve of Normalized GFP versus Time Post-Bleach (s)
Data points with negative time values are pre-bleach points which were normalized to 1.0 (orange dots). Photobleaching occurred at time zero, denoted by a green line. The post-bleach minimum signal was normalized to zero. Data from post-bleach time points are shown as gray dots. The nonlinear regression curve was fitted to the data and is shown in black. The fitted curve plateaus at a B value of 0.89 GFP signal (red dashed line). The time from half recovery is extrapolated from half of B, and corresponds to a time of 6.2 s (pink dashed line). The mobile fraction is equal to B at 89% (represented by light blue line). The immobile fraction is 1-B, which equals 11% (represented by dark blue line).

TROUBLESHOOTING

Problem 1
Bleaching intensity.

Bleaching parameters are not effective at producing 50% decrease in signal (see FRAP Imaging Step 3d).

Potential solution
The strength of bleaching will need to be optimized for each microscope system and cellular structure. The parameters to be optimized include laser intensity during bleaching, scan speed during bleaching, and the number of bleaching iterations. A higher laser intensity can be used for a shorter duration of time (faster scan speed and fewer iterations), but this may kill the cell if too intense. Alternatively, a gentler bleach using less intense laser power for longer scan speeds and more iterations could be used, but if the focus is moving this could prohibit precise bleaching. We found that optimum bleaching was achieved in our system by using low intensity from the 405 nm laser line in addition to the high intensity 488 nm laser. Aim to achieve at least a 50% decrease from starting signal when optimizing bleaching parameters.
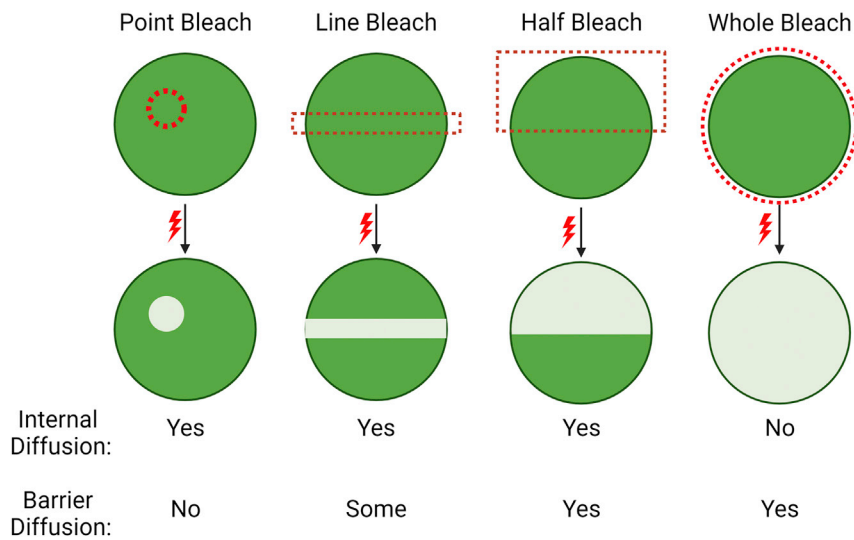
Problem 2
Bleaching mode.

The mode or shape of bleaching does not provide the desired information or reproducible results (see FRAP Imaging Step 3d).

Potential solution
Another useful aspect to consider when optimizing bleaching parameters is the type of photobleaching you are performing. There are two types of diffusion that one can observe using FRAP.

Figure 9. Diagram of different methods of photobleaching in a FRAP experiment including point, line, half, and whole bleach of the focus
Whether diffusion of molecules within the focus (internal diffusion) or across the external barrier of the focus (barrier diffusion) is being measured depends on the mode of bleaching utilized. Created with BioRender.com.

One is the diffusion of molecules inside the focus-of-interest (internal diffusion) and the other is diffusion of molecules into and out of the focus-of-interest (barrier diffusion). Different modes of bleaching can measure internal and barrier diffusion differently (see Figure 9). For example, point bleaching only measures internal diffusion while whole bleach only measures barrier diffusion. You can try different modes of bleaching while optimizing your experiments, but we recommend using a consistent ROI size. Bleaching can occur beyond the drawn ROI due to the diameter of the laser and diffraction as it passes through the sample. Sometimes, this can be circumvented by changing the mode of bleaching.

In our experience, it is best to spend one or two imaging sessions optimizing the bleaching parameters and mode for a new system. During this process, both gentle bleach conditions (lower laser intensity, longer scan speeds, more bleach iterations) and harsh bleach conditions (higher laser intensity, shorter scan speeds, and fewer bleach iterations) should be tested in each bleach mode of interest (whole, half, line, or point) until desirable and reproducible bleaches are obtained for the specific system being studied.

Problem 3
Speed vs. image quality.

Acquisition speed or image quality is not sufficient (see FRAP Imaging Step 3d).

Potential solution
Different protein systems will have different rates of diffusion as well as different ratios of mobile fraction to immobile fraction. These factors impact the time-to-half-recovery, which will influence the speed at which you should acquire images. For example, if a condensate has a time-to-half-recovery of 10 min, you would be able to acquire an image every 30 s or 1 min, and each image would have a much higher quality than could be achieved with rapid imaging. Our parameters are optimized for the recovery rate of MATR3-GFP condensates, where full recovery takes less than 1 min, and so we acquire an image every 1.3 s.

In a previous study, we performed FRAP experiments on FUS-CHOP-GFP which had a faster recovery rate than MATR3-GFP (Ryan et al., 2019). For those experiments, we acquired an image every 0.054 s which required sacrificing image quality. In this study, we utilized the Airyscan Fast detector, used an acquisition speed of 0.664 μsec/pixel, performed no line averaging, and acquired 1700 total images to reach 90 s of recovery time.

Problem 4
General photobleaching.

The entire field-of-view becomes dim by the end of the experiment (see FRAP Imaging Step 3d).

Potential solution
If you observe the entire field-of-view becoming dim by the end of your acquisitions, this is due to general photobleaching from repeated imaging. This can become especially problematic when acquiring hundreds of images quickly. To prevent general photobleaching, try using a lower laser intensity with a higher laser gain. Alternatively, you can correct for general photobleaching during quantitative analysis. To do so, measure the integrated GFP density of non-bleached cells in your experiment, plot this measurement against time, fit this data with a line, use the fit line to calculate the percentage of signal loss, and apply this to the bleach ROI signal.

Problem 5
Agarose pad size.

The agarose pad size is smaller than or much larger than the coverslip after solidifying (see Slide Prepation Step 2b).

Potential solution
The agarose pad should be at least as large as the coverslip, or slightly larger. We do not suggest using an agarose pad that is smaller than the coverslip as this would leave air bubbles trapped within the sealed part of the slide. If the agarose spreads across too large of an area of the slide after setting, the solution was too hot at the time of adding the top slide and the resulting agarose pad could be too thin. If the agarose does not spread to at least the size of your coverslip after setting, the agarose solution was too cold when adding the top slide and the resulting slide may be too thick. We optimized the volume of 23 μL of melted agarose solution for a round coverslip 12 mm in diameter. A different size of round coverslip would require optimization of the melted agarose volume used to make the pad. A square or rectangular coverslip may be used if the agarose pad exceeds the corners of the coverslip after setting. After placing the yeast cells and square coverslip on the agarose pad, cut away the extra agarose with a razor blade before sealing the slide with nail polish.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Meredith Jackrel (mjackrel@wustl.edu).

Materials availability
Plasmids newly generated in this study will be made readily available to the scientific community. Material transfers will be made with no more restrictive terms than in a Materials Transfer Agreement.

Data and code availability
The published article includes all data generated or analyzed during this study.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, M.L.S. and M.E.J.; Investigation, validation, and resources, M.L.S. and M.E.J.; Writing, M.L.S. and M.E.J.; Supervision and project administration, M.E.J.; Funding Acquisition, M.L.S. and M.E.J.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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