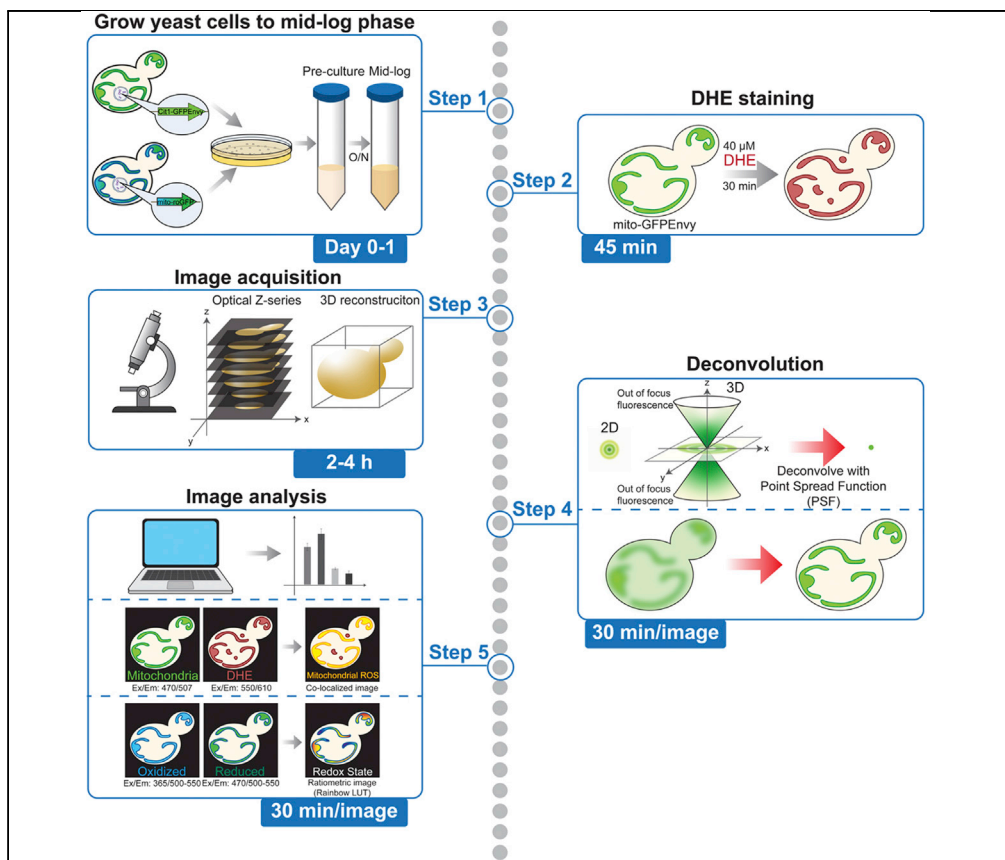


Protocol

Live-Cell Imaging of Mitochondrial Redox State in Yeast Cells



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HIGHLIGHTS
Protocols for analysis of mitochondrial redox state and ROS in *S. cerevisiae*

Analysis is performed in living cells at the resolution of individual organelles

Methods for analysis using commercial and open-source software are provided

These protocols can be adapted for use in other cell types

The redox state of mitochondria is one indicator of the functional state of the organelles. Mitochondria are also the primary endogenous source of reactive oxygen species (ROS). Therefore, the redox state of the organelles also reflects their function in ROS production. Here, we provide step-by-step protocols for live-cell imaging and quantification of mitochondrial redox state using the genetically encoded fluorescent biosensor, mitochondria-targeted redox sensing GFP (mito-roGFP), and mitochondrial ROS using the membrane-permeant small molecule dihydroethidium (DHE) in budding yeast cells.

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Protocol

Live-Cell Imaging of Mitochondrial Redox State in Yeast Cells

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SUMMARY

The redox state of mitochondria is one indicator of the functional state of the organelles. Mitochondria are also the primary endogenous source of reactive oxygen species (ROS). Therefore, the redox state of the organelles also reflects their function in ROS production. Here, we provide step-by-step protocols for live-cell imaging and quantification of mitochondrial redox state using the genetically encoded fluorescent biosensor, mitochondria-targeted redox sensing GFP (mito-roGFP), and mitochondrial ROS using the membrane-permeant small molecule dihydroethidium (DHE) in budding yeast cells.

For complete details on the use and execution of this protocol, please refer to Liao et al. (2020c).

BEFORE YOU BEGIN

This protocol is for live-cell imaging and quantification of mitochondrial superoxides and redox state using DHE and mito-roGFP, respectively, in yeast cells.

DHE reacts with superoxides to form 2-hydroxyethidium, and has been extensively used to examine superoxide levels in different model organisms (Benov et al., 1998; Zhao et al., 2003). DHE staining coupled with flow cytometry has been applied to quickly sort large number of cells according to ROS levels (Lam et al., 2011). However, this method does not provide subcellular resolution of mitochondrial ROS. For live-imaging and analysis of mitochondrial ROS, yeast cells that express mitochondria-targeted GFPenvy are stained with DHE. A z-series of DHE and GFP fluorescence of mid-log phase living yeast cells is obtained using wide-field imaging, and out-of-focus fluorescence in the acquired images is removed or reassigned by digital deconvolution. Mitochondrial ROS is then measured as the fluorescence intensity of DHE-stained superoxide that co-localizes with mitochondria. Here, we describe methods for yeast propagation, staining with DHE, image acquisition and deconvolution, and quantitative analysis of deconvolved images using commercially available and open-source software.

Redox-sensitive GFP (roGFP) (Dooley et al., 2004; Hanson et al., 2004) is a GFP variant which contains surface-exposed cysteines that undergo oxidation or reduction depending on the local environment. roGFP has two widely spaced excitation peaks at ~400 nm and ~480 nm, and one emission peak at ~510 nm. Oxidation of the cysteines within roGFP results in an increase in excitation at ~400 nm; reduction of these cysteines increases excitation at ~480 nm. Thus, the ratio of the fluorescent signals produced by roGFP upon excitation at 480 and 400 nm reveals the relative amounts of reduced and oxidized roGFP, which reflects the redox state of the environment.



Two versions of roGFP are widely used: roGFP1 and roGFP2. roGFP1 is based on wild-type GFP, which has higher relative amplitude of excitation maxima at ~400 nm compared to that at ~480 nm. roGFP2 is based on S65T GFP, which has higher relative amplitude of excitation maxima at ~480 nm compared to that at ~400 nm. roGFP1 and roGFP2 contain the same cysteine insertions and respond to changes in redox state with similar kinetics. However, roGFP1 is less pH sensitive than roGFP2 and its dynamic range extends further into the reduced range (Dooley et al., 2004; Hanson et al., 2004; Schwarzlander et al., 2008). Therefore, roGFP1 may be more useful for monitoring more reducing compartments such as mitochondria or the cytosol, and compartments with variable pH, such as endosomes.

To detect the redox state in the mitochondrial matrix in living yeast cells, we fused the mitochondrial targeting sequence of ATP9 to roGFP1, and inserted this construct into the yeast genome at the HO locus (Liao et al., 2020a). These experiments can be performed using plasmid-borne mito-roGFP (Vevea et al., 2013). However, although mito-roGFP is a ratiometric biosensor, which provides a read-out of mitochondrial redox state that is not affected by biosensor levels, there is less cell-to-cell variation in the levels of expression of integrated compared to plasmid-borne mito-roGFP. As a result, a single image acquisition and deconvolution pipeline can be used for analysis of all cells imaged using integrated mito-roGFP.

Here, we describe methods for propagation of yeast that express mito-roGFP, imaging the biosensor, image deconvolution, and quantitative analysis of the ratio of reduced to oxidized mito-roGFP. These protocols can be adapted for use of roGFP variants that detect specific redox active agents (e.g., glutathione or hydrogen peroxide), are targeted to different subcellular compartments or are expressed in other cell types (Schwarzlander et al., 2016).

Preparation of Cells in Mid-Log Phase

⌚ Timing: 30 min/step

1. At least 4 days before the beginning of the experiment, streak out the strains of interest on rich glucose-based media (Yeast Extract Peptone Dextrose, YPD) plates and incubate for 3 days at 30°C. After colonies have grown, plates can be sealed with parafilm and stored at 4°C for up to 4 weeks.
2. On the morning before the day of imaging, prepare pre-cultures of cells of interest. Inoculate cells from a single colony on solid media into 5 mL of synthetic complete glucose-based (SC) liquid media in a 50-mL conical tube and grow to mid-log phase (optical density at 600 nm (OD_{600}) = 0.1–0.3) at 30°C with shaking at 200 rpm.

Note: Cells are plated on YPD solid media because the growth rate on YPD is higher than that on SC. However, since YPD is autofluorescent, cells used for imaging are propagated using SC media. Investigators may choose other media (e.g., synthetic drop-out media) as needed for their application.

3. On the evening before the day of imaging, use the appropriate volume of each pre-culture to inoculate 5 mL of SC in a 50-mL conical tube and grow cells overnight (16 h) at 30°C with shaking at 200 rpm to mid-log phase (about $0.5-1 \times 10^7$ cells/mL; $OD_{600} = 0.1-0.3$). The volume (V) of pre-culture that is used for inoculation is calculated using the following formula:

$$\text{Final } OD_{600} \times 5000 \mu\text{L} = \text{Preculture } OD_{600} \times 2^{\text{growth time/doubling time}} \times V$$

Note: The doubling time of yeast cells that are propagated in SC liquid media is typically 2 h. Therefore, to obtain cells in mid-log phase ($OD_{600} = 0.2$) after overnight (16 h) growth, add 13 μL of a pre-culture ($OD_{600} = 0.3$) to 5 mL SC. Different strains or mutants may have different doubling time. Calculate the required volume using the formula above.

Note: Our imaging conditions are optimized to visualize mid-log phase cells grown in SC. Stationary phase yeast or cells propagated using different carbon sources or media may have different metabolic states compared to mid-log phase yeast grown on SC and may therefore require different imaging conditions.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---------------------------|---|
| Chemicals, Peptides, and Recombinant Proteins | | |
| Paraquat | Sigma-Aldrich | 856177 |
| Dihydroethidium (DHE) | Invitrogen | D11347 |
| 30% Hydrogen peroxide (H_2O_2) | Sigma-Aldrich | 31642 |
| 1,4-Dithiothreitol (DTT) | Sigma-Aldrich | D9779 |
| Bacto-yeast nitrogen base (YNB) containing ammonium sulfate and no amino acids | BD Difco | 291920 |
| Adenine | Sigma-Aldrich | A9126 |
| Uracil | Sigma-Aldrich | U1128 |
| L-arginine | Sigma-Aldrich | A8094 |
| L-histidine | Sigma-Aldrich | H5659 |
| L-leucine | Sigma-Aldrich | L8912 |
| L-lysine | Sigma-Aldrich | L8662 |
| L-methionine | Sigma-Aldrich | M9625 |
| L-phenylalanine | Sigma-Aldrich | P5482 |
| L-tryptophan | Sigma-Aldrich | T8941 |
| L-tyrosine | Sigma-Aldrich | T8566 |
| Yeast extract | BD Difco | 212750 |
| Bacto-peptone | BD Difco | 211677 |
| D-(+)-Glucose | Sigma-Aldrich | G8270 |
| Experimental Models: Organisms/Strains | | |
| EJG168 (<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CIT1-GFPEnvy-HIS3</i>) | (Liao et al., 2020a) | N/A |
| JYY134 (<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ho::GPDp-mito-roGFP::KanMX4</i>) | (Liao et al., 2020a) | N/A |
| Recombinant DNA | | |
| HO-GPDpro-mito-roGFP1::KanMX4-HO | (Liao et al., 2020a) | N/A |
| Software and Algorithms | | |
| Volocity 5.5 | Quorum Technologies | https://www.quorumtechnologies.com/volocity/volocity-downloads/legacy |
| Fiji | (Schindelin et al., 2012) | https://fiji.sc/ |
| GraphPad Prism7 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--------------------------------------|--------------------------|--------------------------|
| Other | | |
| Spectrophotometer | Beckman | BU530 |
| Microscope | Zeiss | Axioskop 2 |
| Objective lens | Zeiss | 100×/1.4 Plan-Apochromat |
| CCD camera | Hamamatsu | Orca-ER |
| LED illumination system | CoolLED | pE-4000 |
| Excitation filter (GFP) | Chroma | ET470/40× |
| Excitation filter (mCherry) | Chroma | ET572/35× |
| Emission filter (eGFP/mCherry) | Chroma | 59222 |
| Emission filter (eGFP) | Chroma | 49002 |
| Falcon 50-mL conical centrifuge tube | Corning | 352098 |
| Microscope slides | ThermoScientific | 3050 |
| Microscope coverslips | ThermoScientific | 3406 |
| Incubator Shaker | New Brunswick Scientific | E24 |

MATERIALS AND EQUIPMENT

Yeast Extract Peptone Dextrose Yeast Media (YPD)

| Reagent | Stock Concentration | Final Concentration | Amount |
|---------------|---------------------|---------------------|--------|
| Yeast extract | N/A | 6.7% (w/v) | 10 g |
| Bacto-Peptone | N/A | 2 % | 20 g |
| Glucose | N/A | 2 % | 20 g |

Dissolve reagents in 1 L of ddH₂O. Autoclave at 121°C and 0.5 bar for 20 min

Synthetic Complete Glucose-Based Yeast Media (SC)

| Reagent | Stock Concentration | Final Concentration | Amount |
|--|------------------------------------|---------------------|--------|
| Yeast nitrogen base (YNB) containing ammonium sulfate and no amino acids | N/A | 6.7% (w/v) | 6.7 g |
| Glucose | N/A | 20 g/L | 20 g |
| Adenine | 2 mg/mL in 0.05 M HCl | 20 mg/L | 10 mL |
| Uracil | 2 mg/mL in 0.5% NaHCO ₃ | 20 mg/L | 10 mL |
| L-arginine | 1 mg/mL | 10 mg/L | 10 mL |
| L-histidine | 1 mg/mL | 10 mg/L | 10 mL |
| L-leucine | 10 mg/mL | 100 mg/L | 10 mL |
| L-lysine | 3 mg/mL | 30 mg/L | 10 mL |
| L-methionine | 2 mg/mL | 20 mg/L | 10 mL |
| L-phenylalanine | 5 mg/mL | 50 mg/L | 10 mL |
| L-tryptophan | 2 mg/mL | 20 mg/L | 10 mL |
| L-tyrosine | N/A | 30 mg/L | 30 mg |

Adjust pH to 5.5 with NaHCO₃ or HCl, and add ddH₂O to 1 L

Alternatives: We recommend use of an epifluorescence microscope that is equipped with a high numerical aperture (e.g., 1.4) 100× oil-immersion objective lens, a high-quantum efficiency, and low-noise cooled CCD or sCMOS detector, a piezo-electric focus stepper on

the objective lens or stage, and an LED illuminator with excitation/emission filters for imaging DHE and GFP.

STEP-BY-STEP METHOD DETAILS

DHE Staining and Image Acquisition

⌚ Timing: 2–4 h

Cells that express mitochondria-targeted GFP_{Envy} are stained with DHE and imaged by fluorescence microscopy to visualize superoxides and mitochondria.

1. Transfer 1 mL of mid-log culture grown in SC liquid medium to a 1.5-mL microcentrifuge tube and add DHE to a final concentration of 40 μM (4 μL of a 10 mM stock is added to 1,000 μL of cell suspension). Return the remaining cells in the 50 mL conical tube to the incubator and propagate at 30°C with shaking at 200 rpm. These cells can be stained and used to prepare fresh slides for imaging, as needed.

Note: YPD media (rich, glucose-based media containing yeast extract, peptone and dextrose) is autofluorescent and should not be used for fluorescence imaging.

2. Incubate for 30 min at 30°C in the dark.
3. Concentrate cells by centrifugation at 6,000 $\times g$ for 30 s, remove the supernatant, and resuspend the cell pellet in 1 mL SC.
4. Repeat step 3 once.

Note: Steps 3 and 4 are wash steps to remove unincorporated DHE. If high background is observed in the DHE channel, increase the number of washes.

5. Concentrate cells by centrifugation at 6,000 $\times g$ for 30 s, and remove supernatant, leaving 10–20 μL supernatant in the tube (Figure 1).
6. Resuspend the pellet in the residual medium, transfer 1.7 μL of the cell suspension to a microscope slide, and cover with a coverslip.

⚠ **CRITICAL:** Each slide should be imaged for 10 min or less (see Limitations for alternative mounting strategies for longer imaging). If additional slides are needed, prepare them using the remaining culture in step 1 and repeat steps 2–6.

⚠ **CRITICAL:** For optimal imaging, cells must be resuspended in the appropriate volume of supernatant. Typical volumes for cell suspensions for imaging are shown in Figure 1.

7. To image DHE and mitochondria-targeted GFP, locate a field of cells and acquire a z-series using the following settings and excitation/emission filters:
 - GFP_{Envy}: excitation/emission 470/507 nm, 20% power, typical exposure time: 200 ms (Slubowski et al., 2015)
 - DHE: excitation/emission 550/610 nm, 80% power, typical exposure time: 300 ms (Benov et al., 1998; Zhao et al., 2003)
 - 1 \times 1 binning (if camera chip has sufficient pixels, 2 \times 2 binning may be used; final pixel dimension should be < 0.1 μm).
 - 21 z sections with 0.3 μm spacing (total depth 6 μm , covering most yeast cells).
 - Acquire GFP_{Envy} and DHE z-stacks sequentially for greater speed and stability

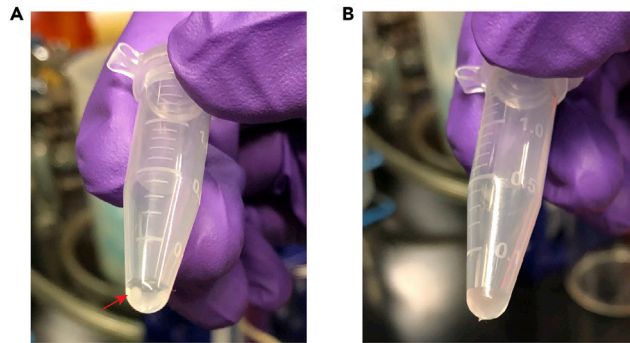


Figure 1. Adequate Volume of Cell Suspension for Live Imaging

(A) Representative image shows the volume of media that is retained with the cell pellet (red arrow).
(B) Representative image of the appropriate volume and turbidity of resuspended yeast for live-cell imaging.

Note: Power and exposure time can be adjusted according to signal intensity. We typically acquire 5–6 z-stacks per slide, and 2–3 slides per sample to image 100 cells using this setting.

Image Deconvolution Using Velocity

⌚ Timing: 10–30 min/image

Images acquired using a wide-field microscope are deconvolved to remove out-of-focus fluorescence.

8. Enter pixel size in X, Y, and Z as 0.0645, 0.0645, and 0.3 μm , respectively. in *Edit* → *Properties* (Figure 2A).
9. Go to *Actions* → *Create New* → *Calculated PSF* (Point Spread Function).
10. Set up PSF parameters by entering **microscope type**, **immersion medium refractive index**, **objective lens numerical aperture**, and **emission wavelength**. For this example, enter wide-field, 1.52, 1.4, and 507 nm and 610 nm for GFPEnvy and DHE, respectively (Figure 2B).
11. Go to *Tools* → *Iterative Restoration*. We use 60 iterations and a 100% confidence criterion for termination. Start the deconvolution (Figure 2C).

Note: Pixel size should be set based on the microscope and camera used and the actual spacing of the z-stacks. Iteration settings should be set based on the signal strength of fluorophores of interest. The deconvolved images may be distorted if the number of iterations is too high. Conversely, the out-of-focus signal will persist if too few iterations are performed.

Analysis of Superoxide Levels in Mitochondria Using Velocity

⌚ Timing: 0.5–1 h/image

Superoxide levels are represented as mean intensity of DHE fluorescence that co-localizes with GFPEnvy-labeled mitochondria.

12. Draw a region of interest (ROI) in an area without cells. Go to the *Measurement* tab, and export data including mean intensity to a spreadsheet for analysis as mean background intensity (Figure 3A).
13. Draw an outline of a cell using a freehand ROI tool (Figure 3B).
14. Create a protocol in the *Measurement* tab to obtain mitochondrial DHE: *Find Objects* → *Clip Objects to ROIs* → *Measure Objects*. Identify mitochondria in *Find Objects* by thresholding

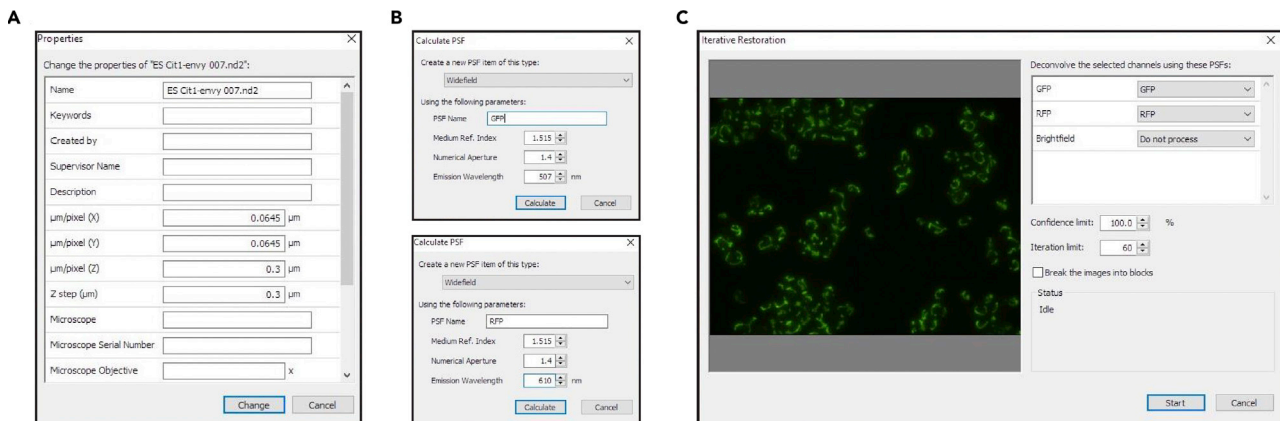


Figure 2. Screenshots of Key Steps in Image Deconvolution Using Velocity
Screenshots of step 8 (A), step 10 (B), and step 11(C) in image deconvolution using Velocity.

the intensity of GFPEnvy signals. Clip these mitochondrial objects to ROIs which represent outlines of mitochondria and measure mean intensity in the DHE channel (Figure 3B).

- Mitochondrial superoxide levels are the mean DHE intensity in mitochondria minus background intensity.

Analysis of Colocalization of Superoxide and Mitochondria Using Velocity

⌚ Timing: 0.5–1 h/image

Colocalization of superoxides with mitochondria is measured using Manders' overlap coefficient or Pearson's correlation coefficient between DHE and GFPEnvy signals.

- Draw a region of interest (ROI) in an area where there are no cells. Go to *Colocalization* tab, and *Set up Thresholds from ROI* (Figure 4A).
- Select Channel X and Channel Y as DHE channel and GFPEnvy channel, respectively (Figure 4B).
- Draw an outline of a cell using a freehand ROI tool. Pearson's Correlation and Overlap Coefficient are shown in the *Thresholded Statistics* panel (Figure 4B).

Image Deconvolution Using Fiji

⌚ Timing: 0.5–1 h/image

Since Velocity is obtained from a commercial source, we provide an alternative method using the open-source software, Fiji, to perform image deconvolution and analysis.

- Download and install Diffraction PSF 3D (https://imagej.net/Diffraction_PSF_3D) and Iterative Deconvolve 3D (https://imagej.net/Iterative_Deconvolve_3D) plugins from the Fiji website (<https://imagej.net/Fiji/Downloads>).
- Create a PSF using Diffraction PSF 3D: *Plugins* → *Diffraction PSF 3D*. Set up PSF parameters by entering immersion medium refractive index, objective lens numerical aperture, emission wavelength, image pixel size, image z step size, image width, image height, and image Z stack. Typical settings:
 - refractive index: 1.518
 - numerical aperture: 1.40
 - emission wavelengths: 507 nm or 610 nm for GFP Envoy and DHE, respectively
 - longitudinal spherical aberration at maximum aperture: 0

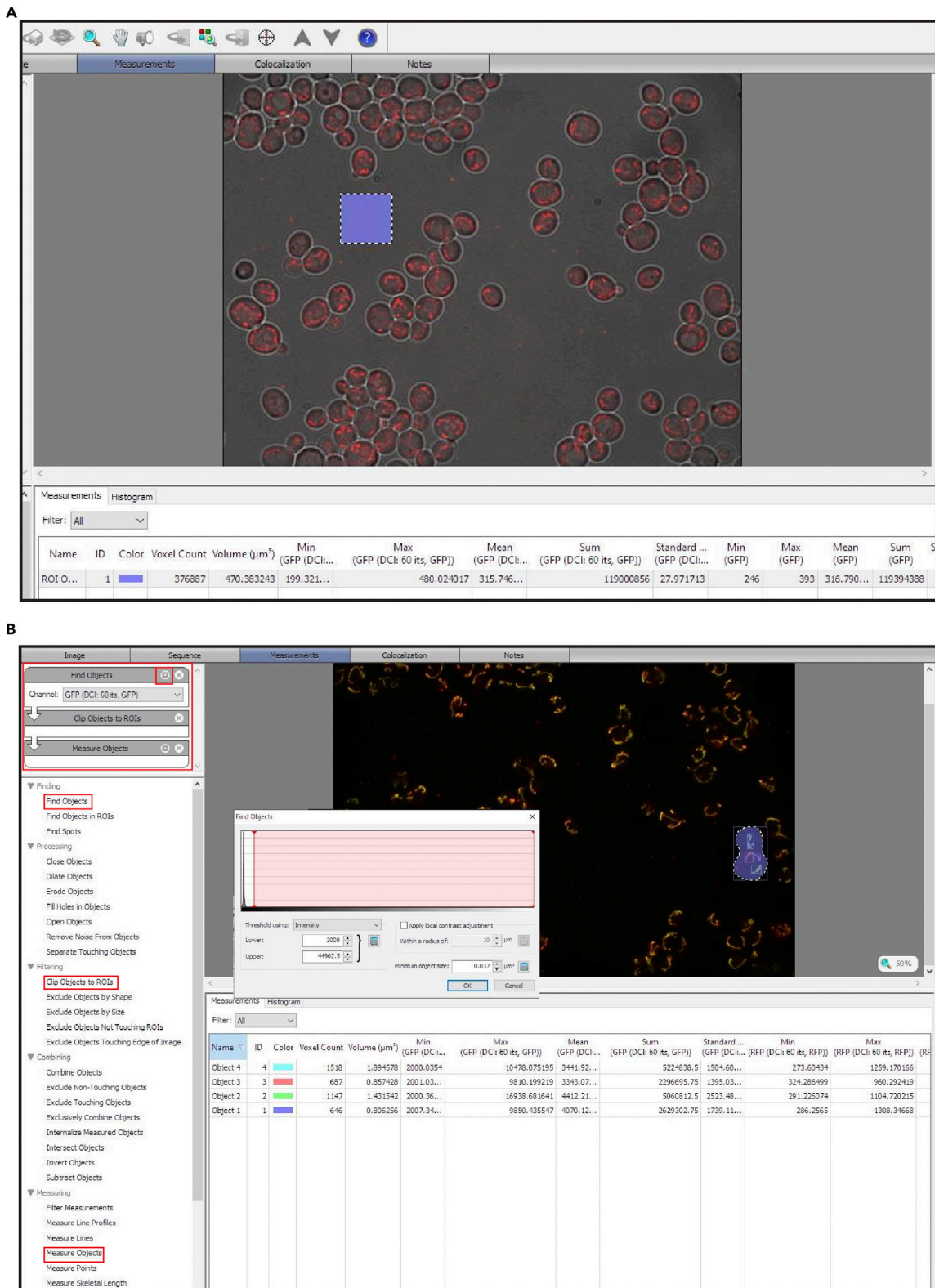


Figure 3. Screenshots of Key Steps in Analysis of Superoxide Levels in Mitochondria Using Velocity
Screenshots of step 12 (A) and step 14 (B) in analysis of superoxide levels in mitochondria using Velocity.

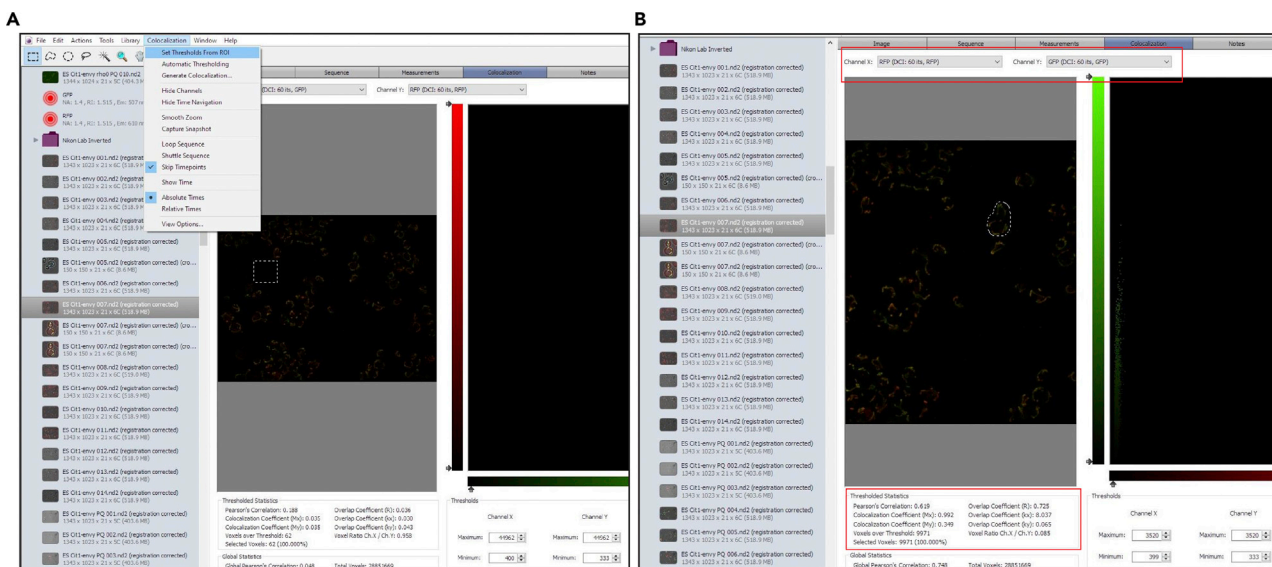


Figure 4. Screenshots of Key Steps in Analysis of Colocalization of Superoxide and Mitochondria Using Velocity. Screenshots of step 16 (A) and steps 17 and 18 (B) in analysis of colocalization of superoxide and mitochondria using Velocity.

- image pixel spacing: 64.50 (substitute your own spacing in nm if applicable)
- slice spacing: 300 (substitute your own spacing in nm if applicable)
- image width: 1,343 (substitute your own image size in pixels if applicable)
- image height: 1,023 (substitute your own image size in pixels if applicable)
- image depth: 21 (substitute your own z stack size if applicable)
- normalization: sum of pixel values = 1

Name your PSF with the corresponding wavelength for easy recognition (Figure 5A).

10. Deconvolve selected images: *Plugins* → *Iterative Deconvolve 3D*. Select the image and corresponding PSF, set Wiener filter gamma to 0, maximum iterations to 60, and delta for termination to 0. The deconvolution will be terminated automatically if iterations do not produce better resolution (Figure 5B).
11. Save the deconvolved file.

Analysis of Superoxide Levels in Mitochondria Using Fiji

⌚ Timing: 0.5–1 h/image

12. Open deconvolved images in Fiji using the Bio-Formats importer: *Plugins* → *Bio-Formats* → *Bio-Formats Importer*. Select “split channels.” Keep the brightfield channel, deconvolved DHE channel and GFPEnvy channel open, and close unused channels (Figure 6A).
13. Subtract the background intensity in the DHE channel:
 - a. Generate a maximum projection of DHE channel: *Image* → *Stacks* → *Z project*. Set the start and stop slice to include all slices. Select “Average Intensity” as the projection type (Figure 6B).
 - b. Go to *Analyze* → *Set measurements*, and check “Mean gray value.”
 - c. Use the *Rectangle selection tool* to select a background region where there are no cells and measure the mean intensity in this region (Figure 6C).

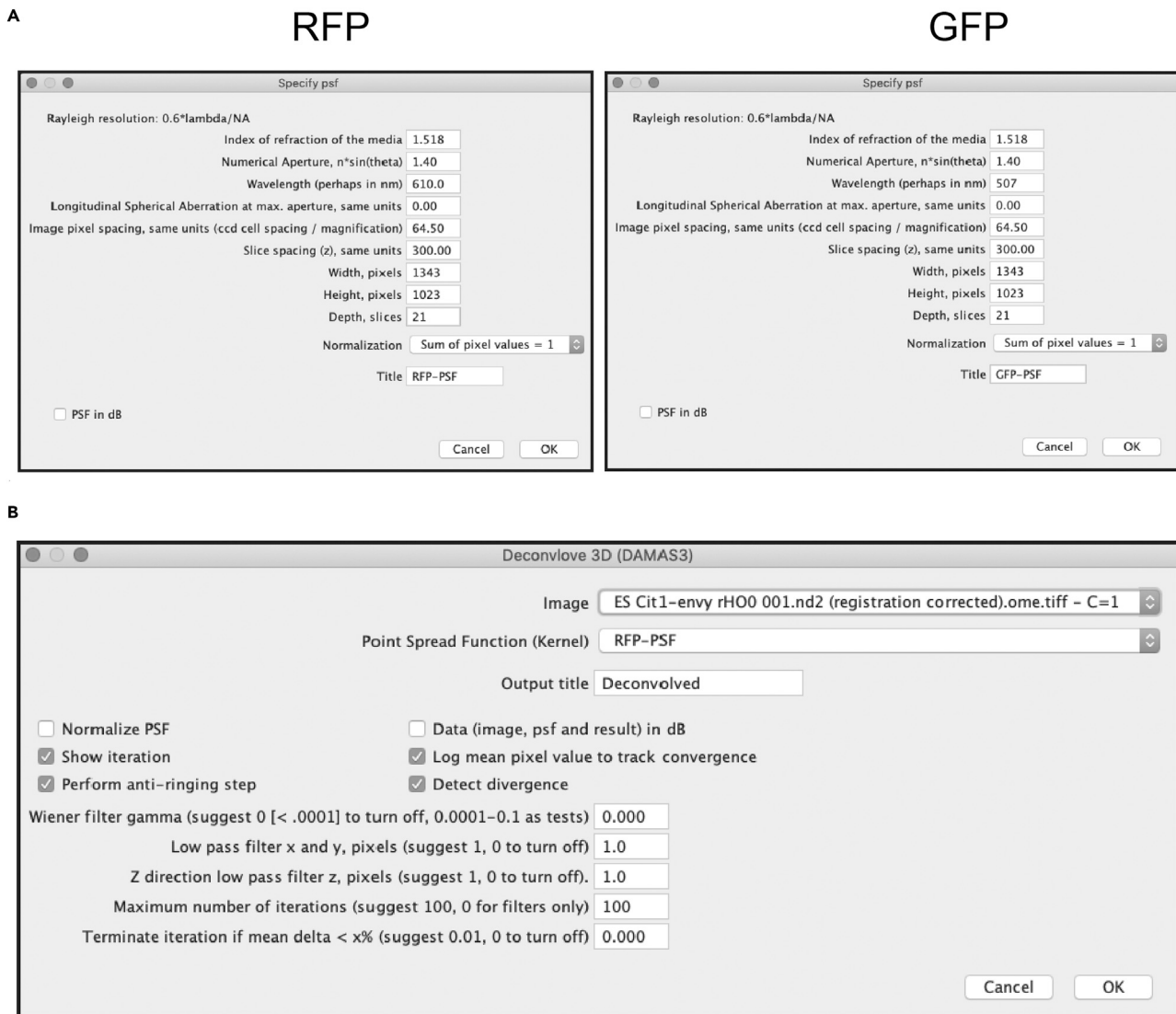


Figure 5. Screenshots of Key Steps in Image Deconvolution Using Fiji

Screenshots of step 9 (A) and step 10 (B) in image deconvolution using Fiji.

- d. Subtract the mean background mean intensity: **Process** → **Math** → **Subtract**. Enter the mean background intensity from the last step. Process all slices (Figure 6D).

Note: If background intensity is still visible after subtraction, adjust the value provided to **Process** → **Math** → **Subtract** to remove most of the background. If the background intensity is low, rolling ball background subtraction can be used as an alternative method.

14. Outline a cell in the brightfield channel using the freehand selection tool and add it to the ROI Manager (Figure 7A).
15. Use ROI Manager to recall the ROI on both GFPEnvy and DHE channels, and then duplicate the cell image in ROI: **Image** → **Duplicate**. Check the "Duplicate stack" box and set the range to include all slices. Remove the cell outline ROI once the duplication is done (Figure 7B).
16. Threshold the GFPEnvy channel to create masks of mitochondria: select the GFPEnvy channel, **Image** → **Adjust** → **Threshold**. Adjust the threshold to segment mitochondria in the GFPEnvy

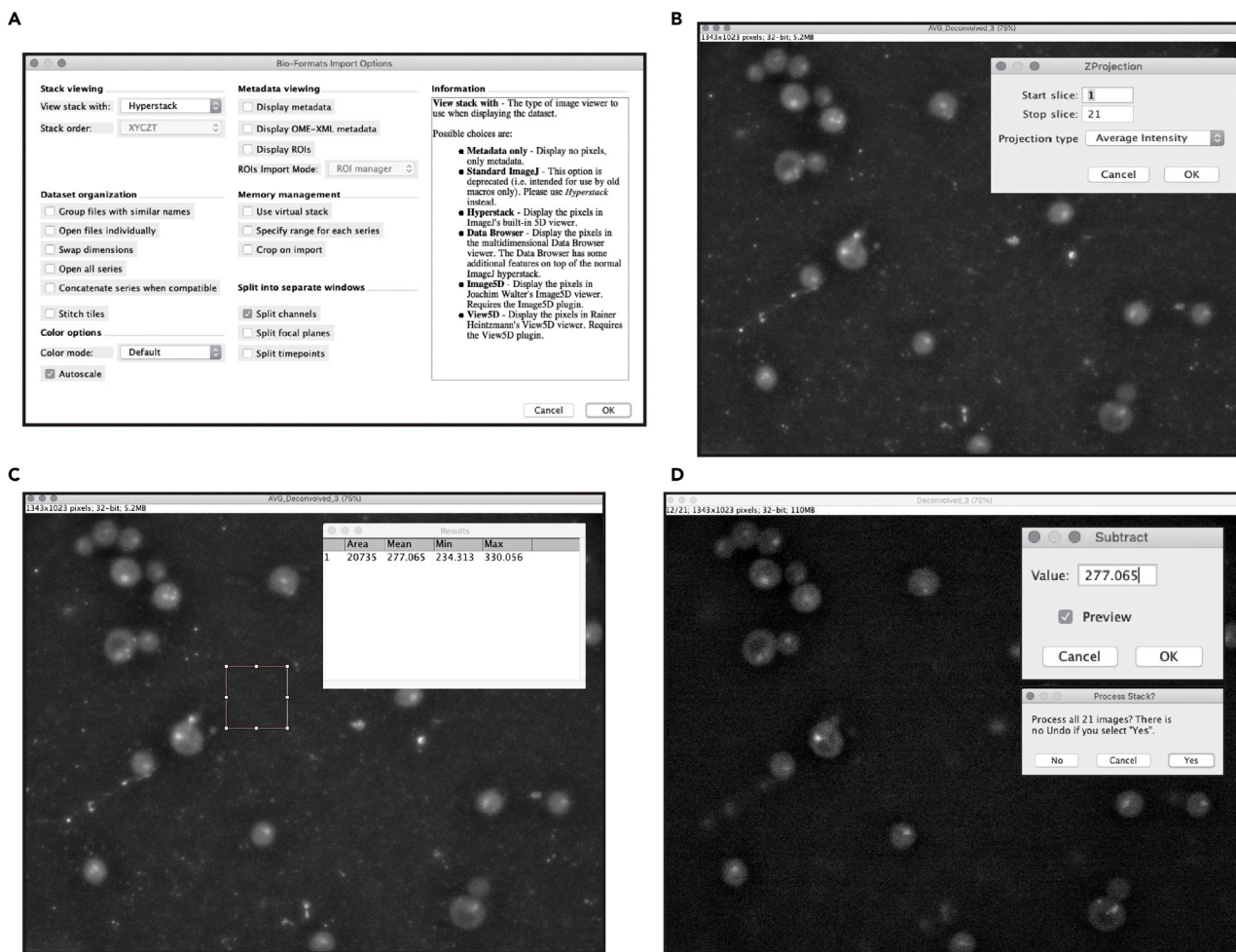


Figure 6. Screenshots of Key Steps in Image Import and Background Subtraction Using Fiji

Image import and background subtraction are used in step 12 (A) and step 13 (B–D) in analysis of superoxide levels in mitochondria using Fiji, and step 22 (A) and step 23 (B–D) in analysis of colocalization of superoxide and mitochondria using Fiji.

channel, and then click **Apply**. When the pop-up “Convert Stack to Binary” comes up, uncheck “calculate threshold for each image” and check “Black background (of binary masks).” Apply to all slices in the stack (Figure 7C).

Note: Automatic threshold “IsoData” can be used to recognize mitochondria in the GFPEnvy channel.

17. Add all the masks to the ROI Manager: Select the stack containing the binary masks of mitochondria. **Analyze** → **Analyze Particles**. Keep Size and Circularity to default values to include all mitochondria. Select “Add to Manager” and “Exclude on edges.” Process all slices in the stack (Figure 7D).
18. Go to **Analyze** → **Set measurements**, check “Area” and “Integrated density.” Clear the results before proceeding to the next step (Figure 7E).
19. Measure the DHE signal using the mitochondria mask in ROI Manager: Select the window containing the DHE channel. Go to **ROI Manager** and click **Measure**. Export data to a spreadsheet for analysis.

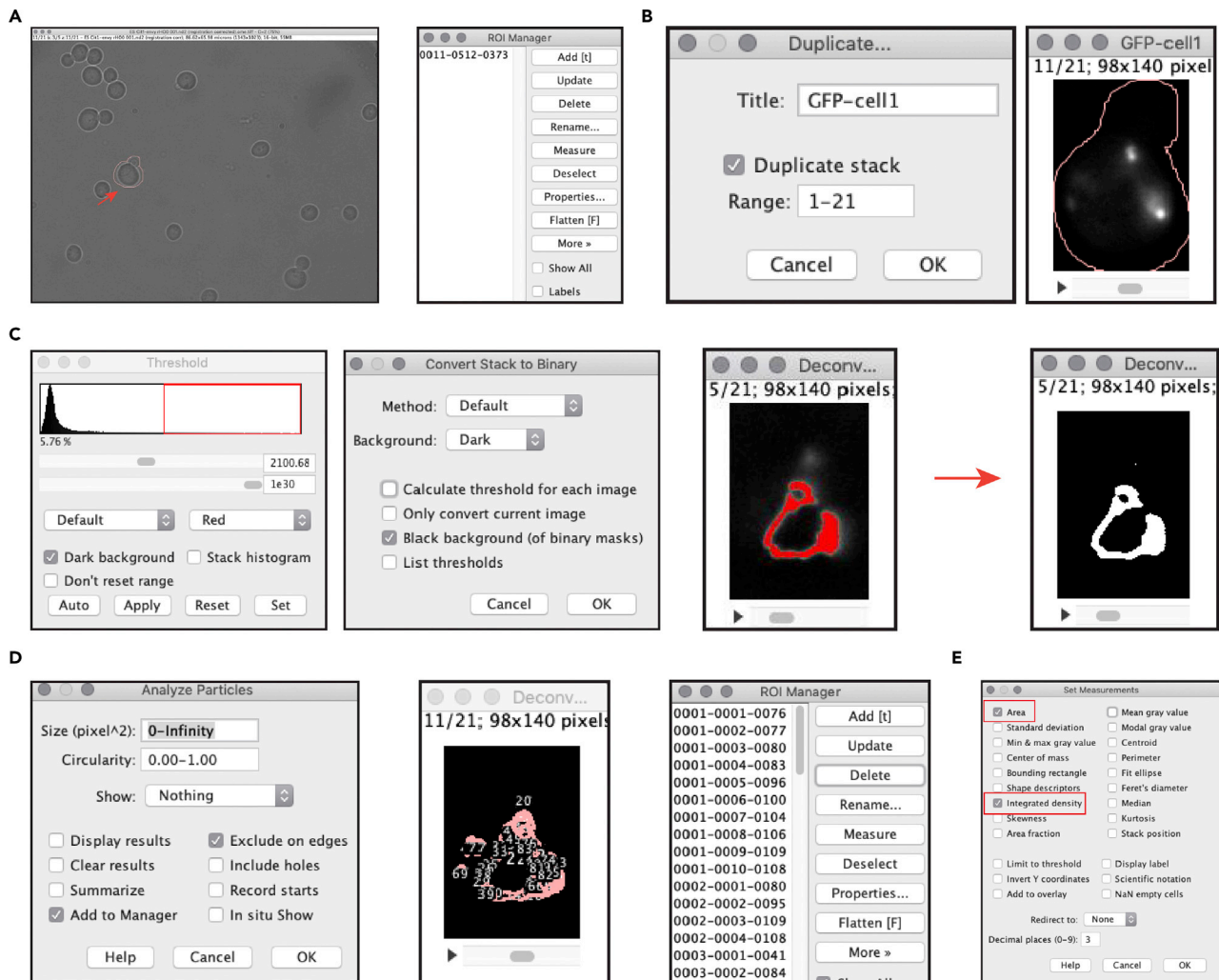


Figure 7. Screenshots of Key Steps in Analysis of Superoxide Levels in Mitochondria Using Fiji

Screenshots of step 14 (A), step 15 (B), step 16 (C), step 17 (D), and step 18 (E) in analysis of superoxide levels in mitochondria using Fiji.

20. In the spreadsheet, sum the area and integrated density (IntDen) in one cell. Then divide the summed integrated density by summed area to obtain the superoxide level in the mitochondria.

Analysis of Colocalization of Superoxide and Mitochondria Using Fiji

⌚ Timing: 0.5–1 h/image

21. If your Fiji does not include the Coloc2 plugin, download the Coloc2 plugin from the ImageJ website (https://imagej.net/Coloc_2) and install it, or activate the “Fiji” update site in *Help* → *Update*.
22. Open deconvolved DHE and GFPEnvy images in Fiji using the Bio-Formats importer: *Plugins* → *Bio-Formats* → *Bio-Formats Importer*. Select split channels. Keep the deconvolved DHE channel and GFPEnvy channel open, and close unused channels (Figure 6A).
23. Subtract the background intensity in the DHE and GFPEnvy channels:
 - a. Generate a maximum projection of DHE channel: *Image* → *Stacks* → *Z project*. Set the start and stop slice to include all slices. Select “Average Intensity” as the projection type (Figure 6B).
 - b. Go to *Analyze* → *Set measurements*, and check “Mean gray value.”

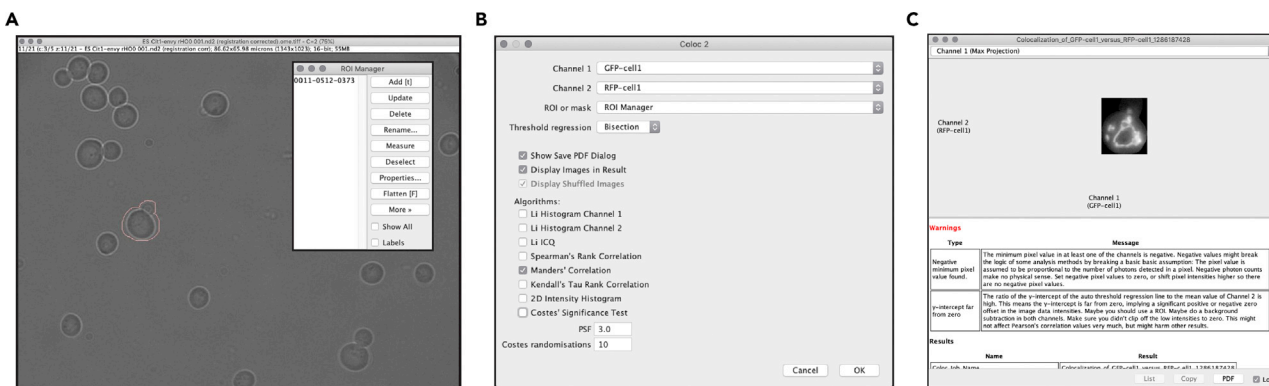


Figure 8. Screenshots of Key Steps in Analysis of Colocalization of Superoxide and Mitochondria Using Fiji
Screenshots of step 24 (A), step 25 (B), and an example of output (C) in analysis of colocalization of superoxide and mitochondria using Fiji.

- c. Use the **Rectangle selection tool** to select a background region where there are no cells and measure the mean intensity in this region (Figure 6C).
- d. Subtract the mean background mean intensity: **Process** → **Math** → **Subtract**. Enter the mean background intensity from the last step. Process all slices (Figure 6D).
24. Outline a cell in the brightfield channel using the freehand selection tool and add it to the ROI Manager (Figure 8A).
25. Perform Coloc2 analysis: **Analyze** → **Colocalization** → **Coloc2**. Select channel 1 and channel 2 as DHE and GFPEnvy channels, respectively. For ROI or mask setting, select "ROI Manager" to import the ROI selected from step 24. Use **Bisection** as threshold regression. For Algorithms, select "Manders' Correlation". Pearson's Correlation and Manders' Overlap Coefficient are shown in the pdf report. Output values are also shown in the Log window and may be saved as plain text (Figure 8B and 8C).

Mito-roGFP Image Acquisition

⌚ Timing: 2–4 h

roGFP1 is targeted to mitochondrial matrix using the targeting sequence of ATP9 (mito-roGFP). In the strain used for imaging, mito-roGFP is inserted into the yeast genome at the HO locus.

Note: The dynamic range of the oxidized and reduced states of mito-roGFP should be determined by imaging cells treated with 5 mM hydrogen peroxide (H₂O₂) or 1,4-dithiothreitol (DTT), respectively, for 30 min (Figure 12).

1. Transfer 1 mL of mid-log culture in SC to each of three 1.5-mL microcentrifuge tubes. Add H₂O₂ to 1 culture to a final concentration of 5 mM, and DTT to another culture to a final concentration of 5 mM. Incubate at 30°C for 30 min. These are used for detection of the dynamic range of the mito-roGFP1. Return the remaining cells in the 50 mL conical tube to the incubator and propagate at 30°C with shaking at 200 rpm. These cells can be used to prepare fresh slides for imaging, as needed.
2. Concentrate cells by centrifugation at 6,000 × g for 30 s, and remove supernatant, leaving 10–20 μL supernatant in the tube (Figure 1).
3. Resuspend the pellet in the residual medium, transfer 1.7 μL of cell suspension to a microscope slide, and cover with a coverslip.

△ **CRITICAL:** Each slide should be imaged for 10 min or less (see Limitations for alternative mounting strategies for longer imaging). If additional slides are needed, use the remaining culture from step 1 and repeat steps 2 and 3.

△ **CRITICAL:** For optimal imaging, cells must be resuspended in the appropriate volume of supernatant. Typical volumes for cell suspensions for imaging are shown in [Figure 1](#).

4. Locate a field of cells and acquire a z-series using the following settings and excitation/emission filters to image mito-roGFP1:
 - Oxidized mito-roGFP: excitation/emission 365/500–550 nm, 50% power, typical exposure time: 300 ms
 - Reduced mito-roGFP: excitation/emission 470/500–550 nm, 20% power, typical exposure time: 200 ms
 - 1 × 1 binning (if camera chip has sufficient pixels, 2 × 2 binning may be used; final pixel dimension should be <0.1 μm).
 - 21 z sections with 0.3 μm spacing
 - Set software to excite at 365 and 470 nm sequentially at each z step

Note: Power and exposure time can be adjusted according to signal intensity. We typically acquire 5–6 images per slide, and 2–3 slides per sample to obtain images of 100 cells.

Note: Excitation at 400 nm is optimal for visualizing the oxidized species of roGFP. However, it also excites reduced roGFP to a greater extent compared to excitation at 365 nm, which leads to reduced sensitivity in measuring mitochondrial redox state. Exposure of mito-roGFP1 to high-intensity illumination at 400 nm also leads to photoconversion of the protein ([Vevea et al., 2013](#)). Therefore, we recommend using 365 nm for excitation of oxidized mito-roGFP.

5. Deconvolve images using Volocity or Fiji software as described above.

Note: Iteration settings are dependent on signal. The deconvolved images may be distorted if the number of iterations is too high. Conversely, the out-of-focus signal will persist if too few iterations are performed.

Analysis of Mitochondrial Redox State Using Volocity

⌚ **Timing:** 0.5–1 h/image

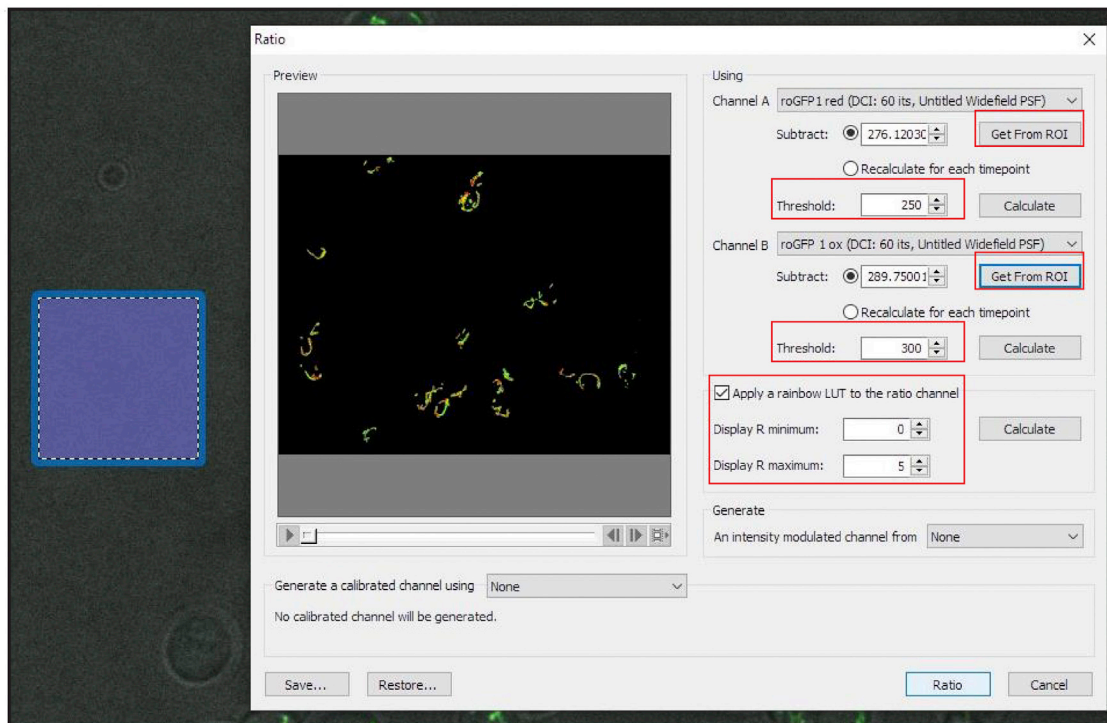
Mitochondrial matrix redox state is measured as the reduced to oxidized (R/O) ratio of mito-roGFP: the signal from excitation at 470 nm divided by that from excitation at 365 nm.

6. Import images into a Volocity library and create an image sequence with two channels.
7. Draw a region of interest (ROI) in an area where there are no cells.
8. Choose: **Tools** → **Ratio**. Calculate the background: **Get From ROI** ([Figure 9A](#)).
9. Adjust the threshold to include mitochondrial structures ([Figure 9A](#)).
10. Check the option to apply a rainbow LUT to the ratio channel ([Figure 9A](#)).

Note: Images in the ratio channel are used for presentation. Warmer colors represent more reducing environments and cooler colors represent more oxidizing environments. Images in the ratio channel should not be used for quantitative analysis.

11. Select the **Measurement** tab, select the ratio channel, and draw an ROI around the area of interest. Measure the ratio channel, excluding zero values. Multiple regions may be selected and measured at the same time. Export data to a spreadsheet for analysis ([Figure 9B](#)).

A



B

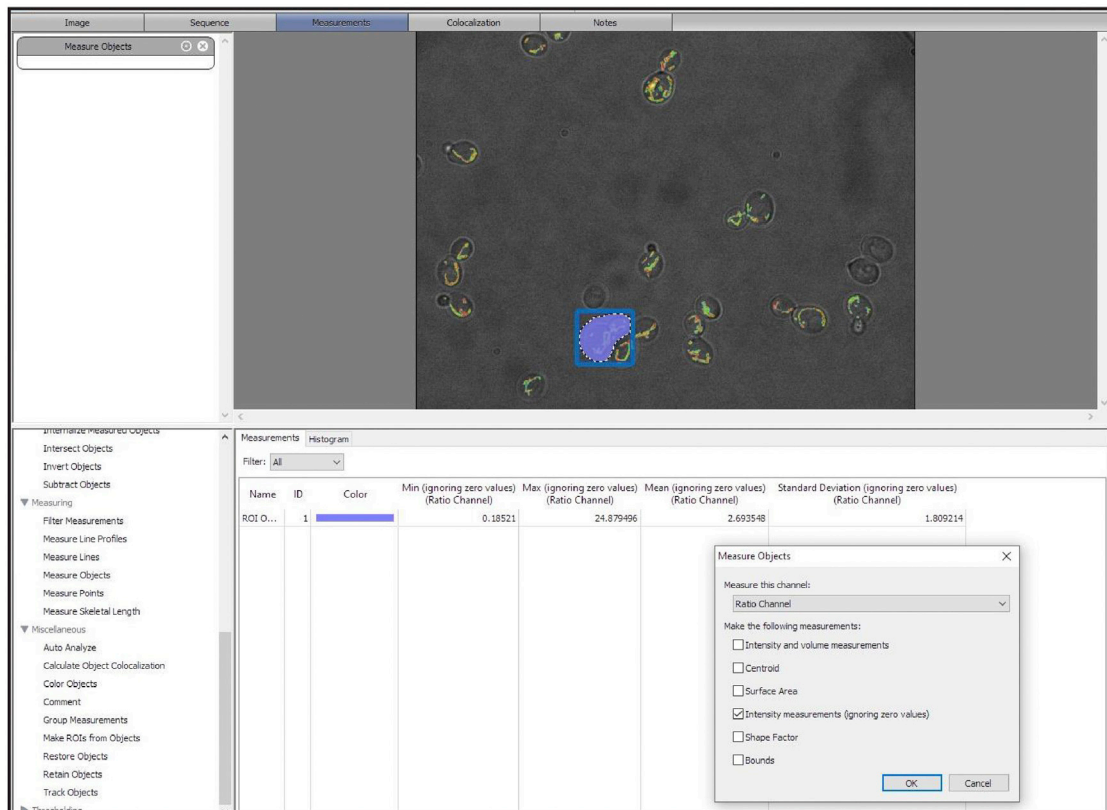


Figure 9. Screenshots of Key Steps in Analysis of Mitochondrial Redox State Using Velocity
Screenshots of steps 8–10 (A) and step 11 (B) in analysis of mitochondrial redox state using Velocity.

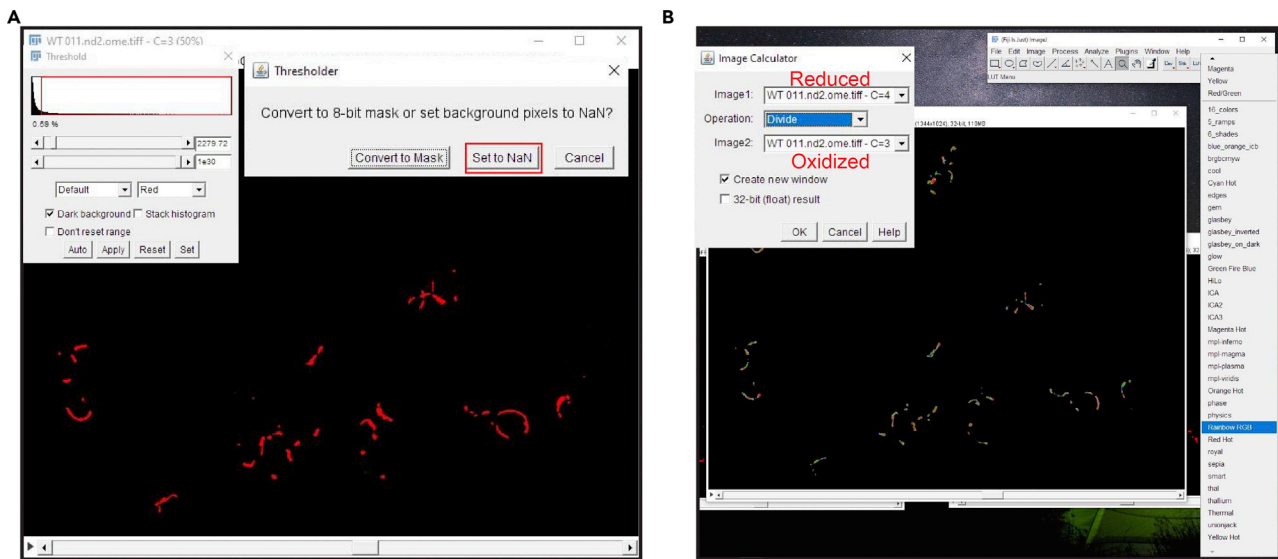


Figure 10. Screenshots of Key Steps in Analysis of Mitochondrial Redox State Using Fiji
Screenshots of step 10 (A) and step 11 (B) in analysis of mitochondrial redox state using Fiji.

Analysis of Mitochondrial Redox State Using Fiji

⌚ Timing: 0.5–1 h/image

Mitochondrial matrix redox state is measured as the reduced to oxidized (R/O) ratio of mito-roGFP: the signal from excitation at 470 nm divided by that from excitation at 365 nm.

6. Convert the images to TIFF.
- △ **CRITICAL:** If the images are contrast enhanced in Volocity, the pixel values of the exported images may change. Using the histogram, verify that pixel values are not changed during the conversion. If the pixel values are changed, export the non-enhanced image from Volocity again.
7. Open images and change type to 32 bit: *Image* → *Type* → *32 bit*.
8. Draw a region of interest (ROI) in an area where there are no cells. Calculate the mean intensity in this ROI: *Analyze* → *Measure*.
9. Subtract the calculated mean background from the stack: *Process* → *Math* → *Subtract*.
10. Using the subtracted z stack, find the middle slice and threshold on mitochondria: *Image* → *Adjust* → *Threshold* and click *Apply* on the Threshold window. Apply to all slices in the stack. Check *Set background pixels to NaN* (Figure 10A).

Note: Image J occasionally crashes during the Threshold process. We find that the version 1.53e is not stable. Version 1.52 or 1.51 is more stable during the process.

11. Create the ratio z stack: *Process* → *Image Calculator*. Divide the reduced (470 nm excitation) stack by the oxidized (365 nm excitation) stack (Figure 10B).
12. Draw a ROI around the area of interest. Choose *Analyze* → *Tools* → *ROI Manager*, and click *Add* to store the ROI. Multiple regions may be stored in the manager. In ROI Manager, select all ROIs, then choose *More* → *Multi-Measure* to measure all stack slices. Export data to a spreadsheet for analysis.

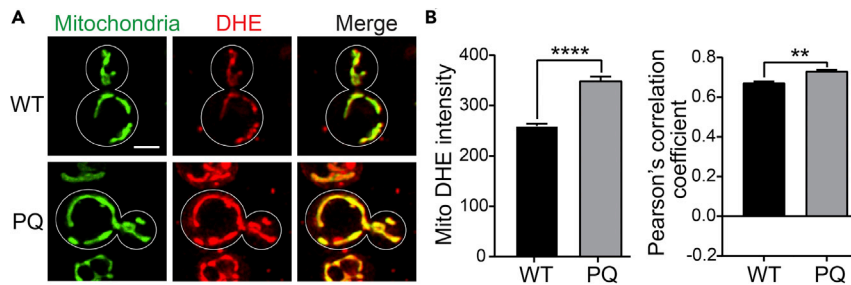


Figure 11. Measurement of Superoxide Levels by DHE Staining in Cells Treated with Paraquat

(A) Representative images of DHE staining to detect superoxide and targeted GFPEnvy to detect mitochondria in living yeast cells. Mitochondrial superoxide generation (red) was visualized using DHE in cells incubated \pm paraquat (PQ, 2.5 mM) for 8 h. Mitochondria (green) were visualized by tagging a mitochondrial marker protein (*CIT1*) at its chromosomal locus with GFPEnvy. Scale bar, 2 μ m.

(B) Quantification of mitochondrial superoxide levels by measuring mean DHE intensity that co-localizes with mitochondria (left). Colocalization of DHE-stained superoxides and mitochondria using Pearson's correlation coefficient (right). ($n > 250$ cells for each strain, two-tailed t-test, ** $p < 0.01$, **** $p < 0.0001$) (Liao et al., 2020a; Liao et al., 2020c).

- In the spreadsheet, multiply the mean intensity and area to obtain integrated intensity. Then divide the summed integrated intensity by summed area to obtain the mean ratio in the mitochondria.

EXPECTED OUTCOMES

DHE-stained superoxides are almost exclusively in mitochondria in yeast cells. We find that treatment with paraquat increases superoxide formation in mitochondria (Figure 11) (Liao et al., 2020a; Liao et al., 2020c).

Mitochondrial redox states measured using mito-roGFP are shown (Figure 12A). Treatment with H_2O_2 and DTT is used to determine the dynamic range of the probe, by generating fully oxidized and reduced states, respectively (Figure 12B). An advantage of the imaging-based approach is that it offers subcellular resolution of mitochondrial redox state. Mitochondria within individual yeast cells often have different redox states. For example, mitochondria in buds are typically more reduced compared to mitochondria in mother cells (Figure 12C) (Liao et al., 2020a; Liao et al., 2020c).

LIMITATIONS

The slide preparation method described above is used for short-term imaging. Since yeast cells are imaged in very small volumes of medium, the preparation will dry out or the cells will exhaust available nutrients within a short time (about 10 min). For long-term imaging in other studies, cells can be imaged using "pads" constructed from a slab cut from an agar growth plate. A protocol for long-term imaging is given in (Liao et al., 2020b).

Both DHE and mito-roGFP offer subcellular resolution, which can detect heterogeneity in the superoxide content and redox state of mitochondria within individual cells. However, they do not have the temporal resolution to detect bursts of ROS associated with ROS signaling. In addition, oxidized mito-roGFP is very sensitive to photobleaching and cannot be used for time-lapse imaging.

TROUBLESHOOTING

Problem

Low DHE intensity

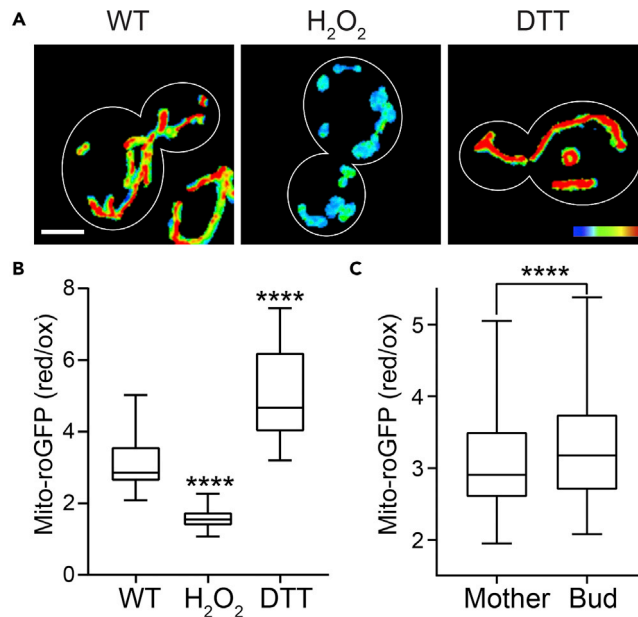


Figure 12. Measurement of Mitochondrial Redox State by mito-roGFP

(A) Representative images of mito-roGFP1 in wild-type (WT), and cells treated with 5 mM H₂O₂ or DTT. The heat map shows the ratio of the reduced to oxidized roGFP signals: warmer colors represent more reducing environments and cooler colors represent more oxidizing environments. Scale bar, 2 μm.

(B) Quantification of mitochondrial redox state shown in (A). H₂O₂ and DTT treatment reveals the dynamic range of mito-roGFP in cells ($n > 54$ cells for each strain, Kruskal-Wallis test with Dunn's multiple comparisons test, **** $p < 0.0001$).

(C) Quantification of mitochondrial redox state in WT mother cells and buds ($n = 154$, Wilcoxon matched-pairs signed rank test, **** $p < 0.0001$) (Liao et al., 2020a; Liao et al., 2020c).

Potential Solution

Increase DHE working concentration or incubation time (step 1).

Manipulate imaging settings. Increase LED or laser power; increase exposure time or gain (step 7).

Problem

High DHE background

Potential Solution

If high background is observed in the DHE channel, increase the number of washes (steps 3 and 4).

Problem

Cell-to-cell or replicate-to-replicate variability of mito-roGFP expression or ratios

Potential Solution

The same carbon source and media batch must be used for all biological replicates of a given experiment.

Use integrated mito-roGFP instead of plasmid-borne mito-roGFP.

Problem

Deconvolved images are distorted or the out-of-focus still persist

Potential Solution

Iteration settings should be set based on the signal strength of fluorophores of interest. The deconvolved images may be distorted if the number of iterations is too high. Conversely, the out-of-focus signal will persist if too few iterations are performed.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Liza A. Pon (lap5@cumc.columbia.edu).

Materials Availability

All strains or plasmids generated in this study are available from the Lead Contact.

Data and Code Availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.-C.L., E.J.Y., and L.A.P.; Validation, P.-C.L.; Formal Analysis, P.-C.L.; Investigation, P.-C.L.; Writing – Original Draft, P.-C.L. and E.J.Y.; Writing – Review & Editing, P.-C.L., E.J.Y., and L.A.P.; Supervision, L.A.P.; Project Administration, L.A.P.; Funding Acquisition, L.A.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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