

Video Article

A Fluorescence Microscopy Assay for Monitoring Mitophagy in the Yeast *Saccharomyces cerevisiae*

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Abstract

Autophagy is important for turnover of cellular components under a range of different conditions. It serves an essential homeostatic function as well as a quality control mechanism that can target and selectively degrade cellular material including organelles¹⁻⁴. For example, damaged or redundant mitochondria (**Fig. 1**), not disposed of by autophagy, can represent a threat to cellular homeostasis and cell survival. In the yeast, *Saccharomyces cerevisiae*, nutrient deprivation (e.g., nitrogen starvation) or damage can promote selective turnover of mitochondria by autophagy in a process termed mitophagy⁵⁻⁹.

We describe a simple fluorescence microscopy approach to assess autophagy. For clarity we restrict our description here to show how the approach can be used to monitor mitophagy in yeast cells. The assay makes use of a fluorescent reporter, Rosella, which is a dual-emission biosensor comprising a relatively pH-stable red fluorescent protein linked to a pH-sensitive green fluorescent protein. The operation of this reporter relies on differences in pH between the vacuole (pH ~ 5.0-5.5) and mitochondria (pH ~ 8.2) in living cells. Under growing conditions, wild type cells exhibit both red and green fluorescence distributed in a manner characteristic of the mitochondria. Fluorescence emission is not associated with the vacuole. When subjected to nitrogen starvation, a condition which induces mitophagy, in addition to red and green fluorescence labeling the mitochondria, cells exhibit the accumulation of red, but not green fluorescence, in the acidic vacuolar lumen representing the delivery of mitochondria to the vacuole. Scoring cells with red, but not green fluorescent vacuoles can be used as a measure of mitophagic activity in cells^{5,10-12}.

Protocol

Selecting suitable control yeast strains

The assay relies on the experimenter visually evaluating the accumulation of red fluorescence in the yeast vacuole. As such the assay is subjective and relies on the selection of suitable control strains and growth conditions. A wild type control is used to indicate the levels of autophagy normally expected. As a negative control a strain null for expression of one of the core autophagy genes (e.g., *ATG3*) is suitable; such strains cannot deliver material (e.g., mitochondria, nucleus) to the vacuole

1. Transformation of yeast cells with plasmid DNA

Yeast cells can be easily made competent for transformation by treating with LiAcetate. Commercial kits may be purchased (e.g., EasyComp, Invitrogen) and published protocols are available¹³.

In this protocol we use wild type strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), and some members of a comprehensive library of deletion mutant strains (e.g., *Δatg3*) derived from it, each lacking expression of a different non-essential gene. The deletion strain library represents a valuable tool for investigating autophagy using Rosella-based probes. The reporter employed in this protocol is mt-Rosella for mitochondria (**Fig. 2A**), encoded by pAS1NB:mt-Rosella¹².

The following steps are based on the use of cells made competent using the *EasyComp* Transformation Kit and stored frozen at -80°C for convenient use.

1. Equilibrate Solution III (EasyComp Transformation Kit) to room temperature 30 minutes before adding to yeast cells.
2. Add 2-2.5 μl (~100 ng/μl) of plasmid DNA encoding the reporter (pAS1NB:mt-Rosella) to a sterile 1.7 ml snap-cap plastic microcentrifuge tube.
3. Add 5 μl of freshly thawed suspension of competent yeast cells and mix by gentle pipetting.
4. Add 50 μl of Solution III (EasyComp Transformation Kit) and vortex to mix. 1.5) Incubate the transformation reactions for 1 hour at 28-30°C with vigorous mixing every 15 minutes using a vortex mixer or manually by flicking the tube with the finger.
5. Carefully transfer the transformation mixture to a single YMM agar growth plate supplemented with histidine, methionine and uracil, and gently distribute the cells around the surface of the agar with a sterile glass spreader. Yeast cells are fragile at this stage and gentle handling can increase the yield of transformants.
6. Incubate growth plates for 2 to 4 days at 28-30°C or until colonies appear about 2-3 mm in diameter.

2. Confirming expression of Rosella in yeast cells

Before embarking on detailed experiments the correct localization and efficient expression of Rosella should be confirmed.

1. Using sterile toothpicks select for each strain 5 single colonies from the transformation plate and resuspend cells in 50 μ l of sterile water in separate sterile 1.7 ml snap-cap plastic microcentrifuge tube.
2. Place 10 μ l of each cell suspension onto separate labeled glass microscope slides (76 x 26 mm) and cover the cell suspension with a square coverslip (22 x 22 mm).
3. Immediately examine the cells for fluorescence emission using a fluorescence microscope (e.g., Olympus Fluoview FV500). Look for strong green and red fluorescence and labeling typical of mitochondrial localization (**Fig. 2B** and **2C**).
4. The remainder of each colony examined for fluorescence in this way should be inoculated onto a fresh YMM growth plate.
5. Incubate plates at 28-30°C for 2 days until colony are approximately 2-3 mm in diameter.

3. Cell growth and induction of autophagy

Autophagy can be induced using a number of different experimental conditions, including entry into stationary phase, change in carbon source, administration of rapamycin, or nitrogen starvation. We routinely use nitrogen starvation as the method to induce mitophagy.

1. Inoculate a single colony into 10 ml of growth medium containing ethanol and the appropriate auxotrophic supplements.
2. Incubate yeast cultures at 28-30°C with orbital shaking (200 rpm) for approximately 48 hours. The cells should be in mid-log growth phase.
3. Pellet cells from duplicate 1ml samples of culture in sterile 1.7 ml snap-cap plastic centrifuge tubes by centrifugation at 2,000 x g for 2 minutes at room temperature. Carefully remove and discard the supernatant without disturbing the cell pellet.
4. Wash the cells three times with 1 ml sterile distilled water by resuspension followed by centrifugation to remove residual medium.
5. After the third wash resuspend the cells in 100 ml of growth medium or nitrogen-starvation medium.
6. Re-inoculate the resuspended cells into 10 ml of fresh growth medium or 10 ml nitrogen-starvation medium. Incubate with orbital shaking for 6-12 hours to allow induction of mitophagy. After starvation prepare cells for viewing by fluorescence microscopy.

Note: Labeling of the vacuole with CMAC-Arg

When first establishing the assay it is useful to confirm under the fluorescence microscope delivery of Rosella to the vacuole. Although the yeast vacuole is a large organelle whose location can often easily be determined by reference to transmitted light images, in some strains of yeast and under some growth conditions the vacuole can be fragmented and difficult to locate.

The vacuole can be readily labeled using a coumarin-based vacuole dye such as CMAC-Arg (7-amino-4-chloromethylcoumarin, L-arginine amide). The action of vacuolar proteases on the dye results in bright blue fluorescence labeling of the vacuole. The blue emission can be readily distinguished from the red and green emissions of Rosella¹¹. Labeling with CMAC-Arg does not need to be performed routinely, but it is recommended for those who are not familiar with the location and appearance of the vacuole in yeast.

4. Labeling of the vacuole with CMAC-Arg

1. Add the CMAC-Arg (100 mM final concentration) to growing or nitrogen-starved cells prior to imaging. The CMAC-Arg solution can be prepared in advance, but as the solution is light sensitive it needs to be protected from exposure to light.
2. Incubate at 28-30°C with orbital shaking (200 rpm) for 30 minutes.
3. Pellet cells by centrifugation at 2,000 x g for 2 minutes at room temperature.
4. Discard the supernatant.
5. Wash the cells three times with sterile distilled water by resuspension followed by centrifugation to remove residual medium and excess CMAC-Arg dye.
6. Mount the cells for imaging as described below.

5. Mounting live yeast cells for confocal laser scanning microscopy (CLSM)

1. Melt 2 mg of low melting point agarose in separate 1 ml aliquots of growth medium and nitrogen-starvation medium by incubating at 70°C. The molten agarose is maintained at 70°C.
2. Gently pellet the cells in 1 ml aliquots of each cell culture by centrifugation at 2,000 x g for 2 minutes at room temperature and discard the supernatants.
3. Wash the cells three times with 1 ml sterile distilled water by resuspension and centrifugation as in step 5.2.
4. Resuspend the washed cells in 100 μ l of sterile distilled water.
5. To mount the cells spot 20 μ l of molten agarose onto a labelled glass microscope slide (76 x 26 mm).
6. Immediately spot 10 μ l of the washed cell suspension onto the agarose bed.
7. Cover the agarose bed with a coverslip (22 x 22 mm). The cells will spread across the agarose surface beneath the coverslip.
8. To prevent dehydration and movement of the coverslip during imaging carefully seal the edges of coverslip with a thin film of nail polish.
9. When the nail polish is completely dry (10 min) the slide is ready to view by fluorescence microscopy Beware: nail polish can cause damage to microscope objectives.
10. Cells mounted using this technique can be observed up to 1 hour after mounting.

6. Visualizing autophagy using CLSM

The level of autophagy in a population of cells is routinely assessed by determining the number of cells showing red vacuolar fluorescence before and after the induction of autophagy. Ideally the progression of autophagy is monitored at selected time points following induction of autophagy (**Fig. 3**). This is best performed by visualizing through the microscope binocular. It is important that the correct controls are employed (e.g., $\Delta atg3$ mutant) and that the microscope settings remain unchanged between observing different samples (e.g., use of neutral density filters, filter selection).

Yeast cells are relatively small requiring a good quality fluorescence microscope (e.g., Olympus Fluoview FV500) equipped with excitation/emission filters suitable for the separate visualization of GFP and/or pHluorin (green fluorescence emission) (FITC filter) and DsRed.T3 (red fluorescence emission) (TRITC filter).

- Using a 60x water objective view non-starved wild type cells using alternately the FITC and TRITC filters. Wild type cells should exhibit a typical cellular distribution of mitochondria at the periphery of the cells that show both red and green fluorescence. Note that this distribution of mitochondria is dependent upon the pinhole setting of the confocal microscope. We generally use a lower pinhole value of 125 μm that only allows the mitochondria to be observed as a series of dots localized at the periphery of the cell (**Fig. 2C**) so that the vacuole is not obscured¹². If a high pinhole value of (200 μm) is used this allows the typical filamentous mitochondrial network distributed throughout the cell (and the changes it undergoes) to be observed¹¹. No other structures in the cell should be fluorescently labeled.
- Sequentially view cells for each of the time points following transfer to starvation medium. Visualize alternately using the green and red emission filters. At the 6 h starvation time point red fluorescence, but no corresponding green emission should be discernable in the vacuole. Vacuolar localization can be separately confirmed using CMAC-Arg (**Fig. 2C**). View ~200 cells and count the number that show only red fluorescence in the vacuole (i.e. vacuolar uptake of mitochondria).
- Count vacuolar accumulation for all time points observing >200 cells and then plot percentage of cells showing vacuolar accumulation.
- Examine autophagy-negative control cells expressing mt-Rosella before starvation and following 6 h starvation.

7. Representative results:

Here, we show typical results obtained using mt-Rosella, expressed in wild type and Δatg3 mutant cells. Under growing conditions with ethanol as carbon source, both wild type and Δatg3 mutant cells exhibit a cellular distribution of fluorescence (red and green) typical of mitochondria in yeast cells. Red and green fluorescence emission is not detected in the vacuole (**Fig. 2B** and **2C**). When subjected to nitrogen starvation for 6 h and beyond then, in addition to red and green fluorescence corresponding to mitochondria, wild type cells also exhibit the accumulation of red, but not green fluorescence in the vacuolar lumen (**Fig. 2B**; **Fig. 3**). However, in Δatg3 mutant cells neither red nor green fluorescence accumulates in the vacuolar lumen (**Fig. 2C**; **Fig. 3**).

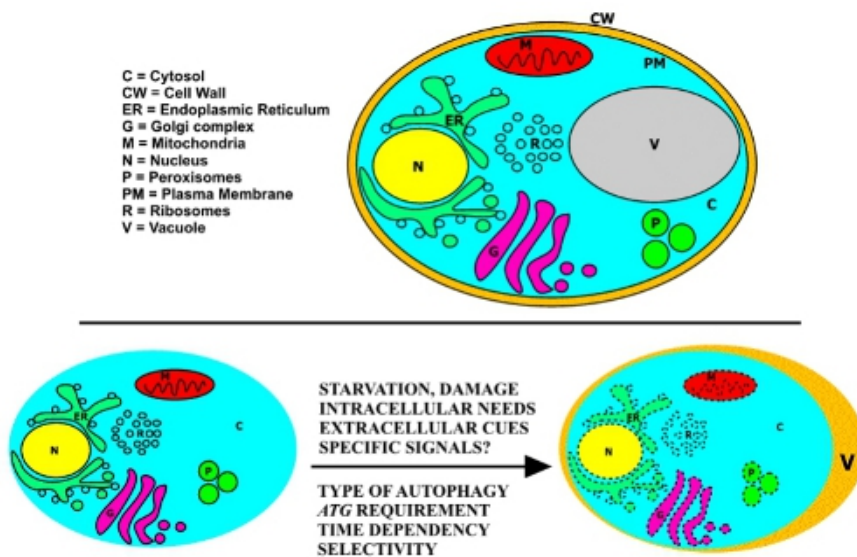
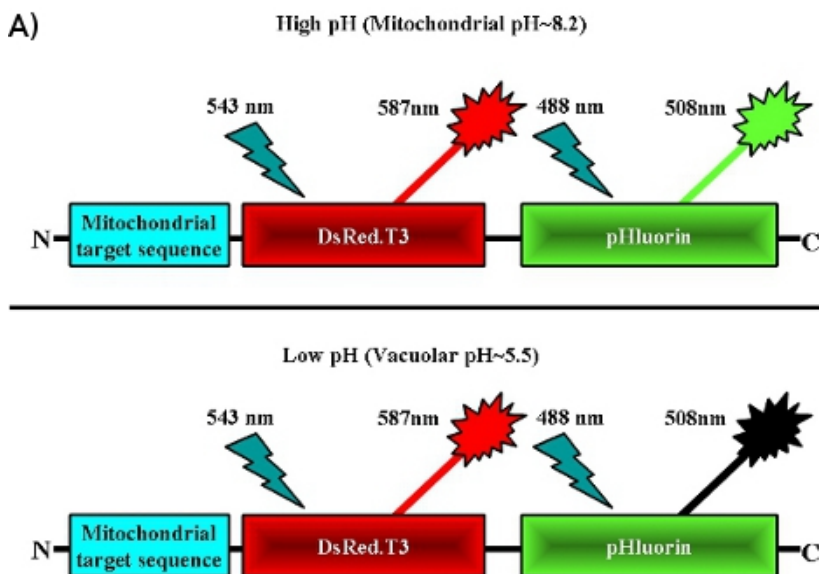


Figure 1. Top, scheme of organelles and compartments in a yeast cell. Bottom, a vacuole-centric view of a yeast cell is presented indicating autophagic degradation of different organelles and compartments. Some of the differences between the various organelle-specific types of autophagy (e.g., mitophagy, nucleophagy) include the specificity (cargo selection) of the engulfed contents, various intracellular needs and extracellular cues (e.g., starvation, damage), specific signals, ATG genes and time dependency.



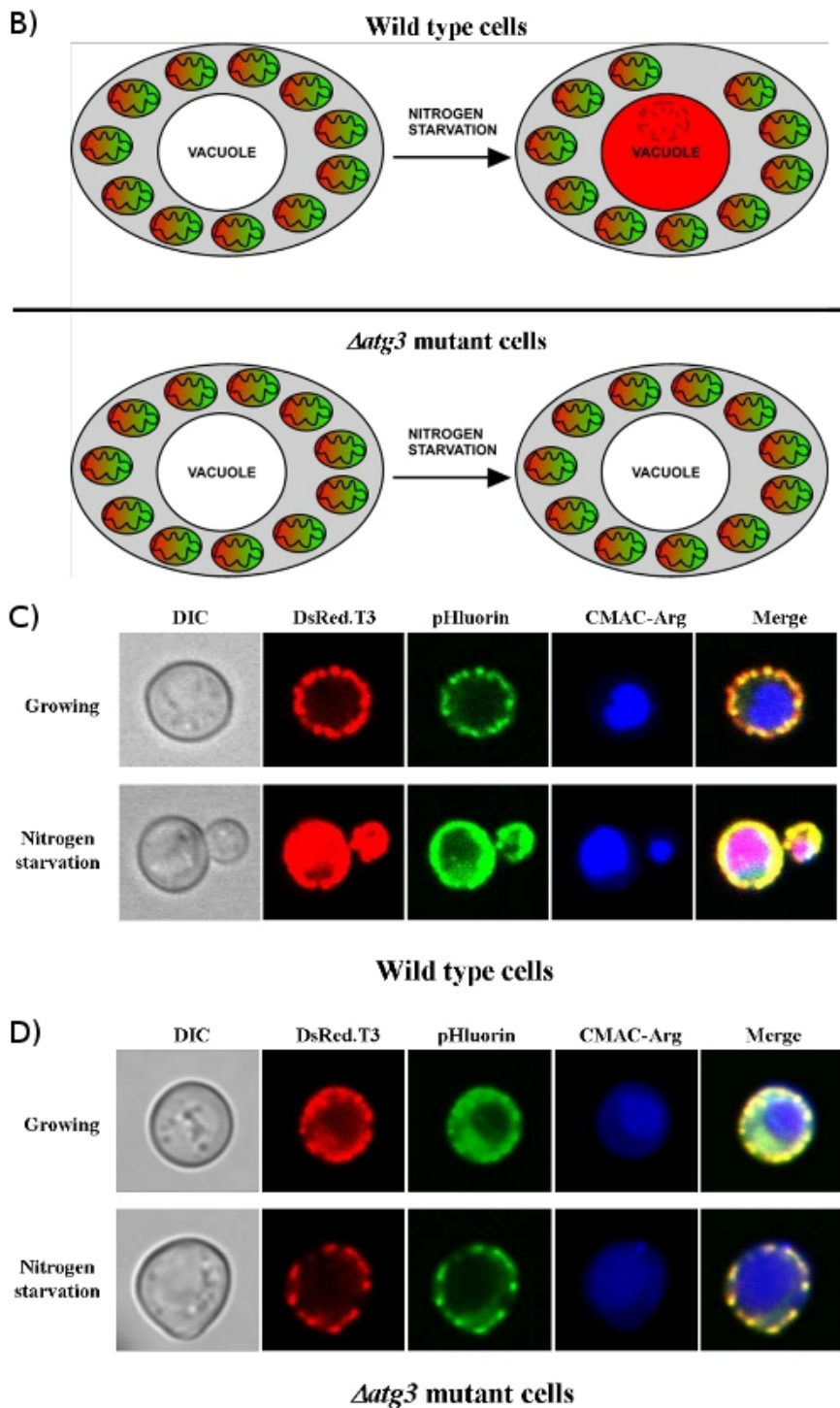


Figure 2. (A) Schematic representation of mt-Rosella. The mitochondrial leader sequence (in this case of citrate synthase) is used to target the Rosella biosensor to the mitochondrial matrix. Excitation and emission maxima for both red fluorescent protein (DsRed.T3) and green fluorescent protein (pHluorin) are indicated. At high pH (mitochondrial pH~8.2) the biosensor fluoresces both red and green, however, at low pH (vacuolar pH~5.5) it fluoresces only red. **(B)** Schematic representation of expected results in wild type and $\Delta atg3$ mutant cells expressing mt-Rosella. **(C)** Actual results obtained by CLSM for wild type cells under growing and nitrogen starvation conditions. From left to right: DIC (difference in contrast), RFP (DsRed.T3) fluorescence emission, GFP (pHluorin) fluorescence emission, CMAC-Arg fluorescence and merge image. **(D)** Actual results obtained by fluorescence microscopy for $\Delta atg3$ mutant cells under growing and nitrogen starvation conditions. From left to right: DIC (difference in contrast), RFP (DsRed.T3) fluorescence emission, GFP (pHluorin) fluorescence emission, CMAC-Arg fluorescence and merge image.

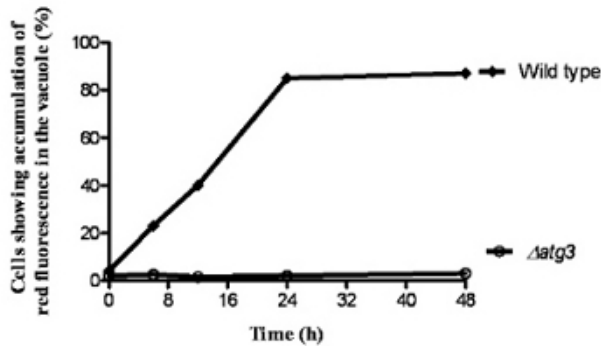


Figure 3. The percentage of wild type and $\Delta atg3$ mutant cells, expressing mt-Rosella and grown under nitrogen starvation, showing red fluorescence in the vacuole over a time course of 48 hours. The percentage of cells showing accumulation of red fluorescence in the vacuole was recorded at 0, 6, 12, 24 and 48 hours after commencing nitrogen starvation.

Discussion

In the representative images presented in **Fig. 2B** and **2C** it can be clearly seen that the use of organelle-specific fluorescent reporters and fluorescence microscopy provides evidence of mitochondrial localization/distribution in both growing and nitrogen-starved cells, and allows mitophagy to be visualized.

It is to be emphasized that this method is not restricted to following mitophagy. Autophagy of other cellular components or organelles (e.g., ribosomes, lipid droplets, peroxisomes, endoplasmic reticulum and nucleus) may be monitored with suitable labeling. Details of the Rosella biosensor construct, cell culture conditions and typical results for turnover of the nucleus (nucleophagy) have been reported^{11,12} illustrating the broader application of the method.

Use of organelle-specific fluorescent reporters for monitoring autophagy requires several considerations of construct design to be kept in mind. These include: (1) selection and fusion to the appropriate targeting signal to achieve organelle-specific targeting; (2) selection of a suitable fluorescent protein(s); (3) strong fluorescence emission; (4) correct organelle localization and/or distribution within the cell. Once the organelle-specific reporter has been successfully expressed in the chosen yeast host strain a second group of considerations become: (1) yeast growth conditions; (2) cell sample preparation; (3) imaging parameters as it is important that the parameters selected for CLSM (e.g., laser intensity, scan rate and mode, pinhole value, zoom value, objectives used or exposure for CCD-camera) are maintained throughout an experiment ensuring that comparisons can be made between control (growing cells) and starved samples; (3) autophagy induction and successful delivery of the reporter into the vacuole.

Overall, this fluorescent microscopy method is easy, relatively fast, and has potential application for high throughput screening for novel drugs that enhance or inhibit autophagy, and also for genes that regulate or modulate autophagy.

Disclosures

No conflicts of interest declared.

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