Video Article

Analysis of Autophagy in *Penicillium chrysogenum* by Using Starvation Pads in Combination With Fluorescence Microscopy

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URL: https://www.jove.com/video/52577
DOI: doi:10.3791/52577

Keywords: Microbiology, Issue 96, starvation, degradation, autophagy, microscopy, microscope slide, cavity, fungi, *Penicillium chrysogenum*

Date Published: 2/1/2015

Citation: Scheckhuber, C.Q. Analysis of Autophagy in *Penicillium chrysogenum* by Using Starvation Pads in Combination With Fluorescence Microscopy. *J. Vis. Exp.* (96), e52577, doi:10.3791/52577 (2015).

Abstract

The study of cellular quality control systems has emerged as a highly dynamic and relevant field of contemporary research. It has become clear that cells possess several lines of defense against damage to biologically relevant molecules like nucleic acids, lipids and proteins. In addition to organelle dynamics (fusion/fission/motility/inheritance) and tightly controlled protease activity, the degradation of surplus, damaged or compromised organelles by autophagy (cellular 'self-eating') has received much attention from the scientific community. The regulation of autophagy is quite complex and depends on genetic and environmental factors, many of which have so far not been elucidated. Here a novel method is presented that allows the convenient study of autophagy in the filamentous fungus *Penicillium chrysogenum*. It is based on growth of the fungus on so-called 'starvation pads' for stimulation of autophagy in a reproducible manner. Samples are directly assayed by microscopy and evaluated for autophagy induction / progress. The protocol presented here is not limited for use with *P. chrysogenum* and can be easily adapted for use in other filamentous fungi.

Video Link

The video component of this article can be found at https://www.jove.com/video/52577/

Introduction

Filamentous fungi are excellent model systems for the study of developmental processes. They offer several experimental benefits like cheap cultivation, high numbers of progeny and genetic accessibility. The last point is of particular relevance for the construction of transformants that allow investigating the importance of so-far uncharacterized genes for various cellular mechanisms. Filamentous fungi have been instrumental for the elucidation of several elements and mechanisms of cellular quality control pathways like protease activity for the degradation of aberrant proteins, mitochondrial dynamics for maintaining mitochondrial integrity and autophagy for the removal of surplus and/or dysfunctional cell components and for maintaining cell viability in times of starvation[1,2,3].

There are several experimental techniques available for the study of autophagy in filamentous fungi [2]: (i) investigation of vacuoles if they contain dense autophagic bodies when proteases are inhibited by transmission electron microscopy [4], (ii) visualization of autophagosomes by monitoring GFP-Atg8 foci via fluorescence microscopy [5,6] and (iii) detection of acidified autophagosomal structures by using the fluorescent dye monodansyl cadaverine [7].

Here, a novel method of growing *Penicillium chrysogenum* for autophagy studies is presented. The main element is the 'starvation pad' which simply consists of 1 % agarose dissolved in sterilized tap water. Additional compounds (*e. g.,* stressors, scavengers, autophagy modulators) can be added to the pad as long as they don't display auto-fluorescence. The pad is located in microscope slides that contain a shallow central cavity. This pad in inoculated either with a spore suspension or with small mycelium fragments. The latter is advisable if the strain of interest fails to sporulate efficiently (*e.g.,* Δ*atg1* strains [8]). The slides are positioned in wet chambers (these can be easily constructed by using empty pipette tip boxes) to prevent desiccation of the sample and incubated at room temperature. *P. chrysogenum* is able to grow for a few days under these conditions. Autophagy can be observed microscopically by vacuolar enlargement which is a positive marker for fungal autophagy. In this contribution, a *P. chrysogenum* strain (Wisconsin 54-1255) is used that forms green fluorescent protein that is targeted to peroxisomes by its C-terminal 'SKL' sequence [9]. Therefore it is possible to monitor the degradation of peroxisomes. It is feasible to label also other compartments of the cell (*e.g.,* mitochondria) by using appropriate localization signals and to analyze their degradation. Although data from *P. chrysogenum* Ws54-1255 (GFP-SKL) is presented here, it is certainly possible to use the 'starvation pad' method also for other filamentous fungi (*e.g., Neurospora crassa*, *Sordaria macrospora*, Aspergillus species, *etc.*).

Protocol

1. Preparation of *P. chrysogenum* for the Starvation Experiments

1. If the *P. chrysogenum* strain of interest is kept on rice ('green rice'), place 2-3 rice grains covered with sporulating mycelium into a 1.5 ml microcentrifuge tube. Fill it with 500 µl YGG (10 g/l KCl, 20 g/l glucose, 10 g/l yeast nitrogen base, 5 g/l K_2HPO_4, 20 g/l yeast extract).
    1. Vortex the tube for 30 sec so that spores can detach from the rice efficiently.
    2. Incubate the tube for 1 day at room temperature (*e.g.*, between 20 and 25 °C).
    3. Prepare a 1/50 dilution of this spore suspension in sterile tap water, mix well.
2. If the *P. chrysogenum* strain of interest is kept on an agar plate (*e.g.*, YGG agar plate), transfer small pieces of the mycelium into a 1.5 ml microcentrifuge tube containing 400 µl sterile tap water and approximately 100 mg glass beads by using a sterile tooth pick or pipette tip.
    1. Vortex the tube for 2 min so that the mycelium becomes fragmented. Use the contents of the tube immediately.

2. Preparatory Steps for Cultivation of *P. chrysogenum* on Starvation Pads

1. Switch on a heating plate and set it to approximately 60 °C.
2. Dissolve 2 g of agarose in 100 ml tap water by cooking the solution in a microwave oven. Take care that this solution is completely clear after heating. If it is not, cook it again until the agarose is dissolved completely. Let the agarose solution cool down to approximately 60 °C before use.
3. Fill 1.5 ml microcentrifuge tubes (2-4, depending on the number of samples) with 400 µl sterile tap water each (no additional compounds added) or 400-x µl (addition of x µl of compound solution in step 2.5) and place them onto the heating plate for at least 1 min so that the water inside gets warm.
4. Place microscope slides that contain a central cavity onto the heating plate for at least 1 min.
5. Add 400 µl 2 % agarose to the solution in the 1.5 ml microcentrifuge tubes and vortex briefly. After this step if desired, add additional compounds (*i.e.,* 1 µM rapamycin). If this is done, vortex briefly after each additional substance is pipetted to the tube. Put the tubes back onto the heating table to prevent premature solidification.
   NOTE: The total volume in the microcentrifuge tube should be 800 µl.

3. Preparation of Microscope Slides Containing Starvation Pads

1. Pipette 140 µl of the agarose solution into the cavity of the microscope slide (which is still located on the heating plate). Try to avoid making bubbles if possible.
2. Immediately place a cover slip onto the agarose solution.
   NOTE: This will result in a flat surface.
3. Put the microscope slide onto the lab bench for approximately 4 min to allow the agarose pad to solidify.
4. Carefully remove the coverslip from the agarose pad by sliding it off with the help of a thumb or index finger (wear gloves to avoid contamination!).
5. Place the microscope slide with the pad into a wet chamber to prevent desiccation. Recommendation: Use an empty tip box that still has the insert for holding the pipette tips. Add sterile water to the box so that the bottom is covered. Place the microscope slides onto the insert and close the box.

4. Inoculating the Starvation Pads with *P. chrysogenum* and Incubation

1. Pipette 5 µl of the 1/50 spore dilution (if the cultures were grown on rice) or 5 µl of the undiluted solution containing mycelium fragments (if the cultures were grown on an agar plate) onto the centre of the starvation pad.
2. Close the wet chamber and wrap it in a plastic bag (this serves as additional protection against desiccation of the starvation pads). Be careful not to move the box too much because otherwise the inoculation drops will be disturbed.
3. Store the wrapped box at RT for at least 20 h before analyzing the samples.

5. Microscopic Analysis of the Samples

1. After 20 hr of incubation the samples are ready for microscopic analysis; put the microscope slide on dry tissue paper (the bottom tends to become wet).
2. Pipette a small volume of water (ca. 50 µl) onto the starvation pad. Put a coverslip onto the pad and squeeze out surplus water. Remove the surplus water with a tissue paper.
3. Put the microscope slide onto the observation table of the fluorescence microscope. To get an overview of mycelium growth, use a 20X objective. Use 63X or 100X objectives for studying the localization of GFP in the hyphae. In order to prevent gradual desiccation of the sample do not analyze the samples for longer than 15 min before putting them back into the wet chamber.
4. Repeat 5.3 at regular intervals (*e.g.*, after 40 hr and 60 hr of growth).
   NOTE: The sample can be put back into the wet chamber. It is not necessary to remove the coverslip as it does not affect mycelium growth.

Representative Results

To demonstrate the utility of the protocol detailed above peroxisome degradation in the *P. chrysogenum* strain Ws54-1255 (GFP-SKL) was analyzed. In this strain GFP-SKL is usually imported into peroxisomes [9]. This results in the appearance of multiple spherical shapes when the sample is analyzed via fluorescence microscopy. If autophagy occurs, vacuoles enlarge. GFP-SKL becomes incorporated into vacuoles by autophagy (pexophagy). Due to the fact that GFP is resistant to degradation by vacuole proteases it labels these organelles [10]. Therefore the two parameters for observing autophagy (pexophagy) in this strain, vacuole enlargement and localization of GFP-SKL to vacuoles, are conveniently monitored by fluorescence microscopy.

At 20 hr of incubation, GFP is never observed in vacuoles (**Figure 1**). Also, vacuole enlargement is not taking place. However, at 40 hr of growth GFP-labelled vacuoles become visible in some of the hyphae, indicating autophagy (pexophagy) to become active (**Figure 1**). At 60 hr of growth the amount of GFP-labelled vacuoles has increased further. As a negative control a Δ*atg1* mutant of *P. chrysogenum* was utilized (Δ*atg1* (GFP-SKL)) (**Figure 2**). Neither enlarged nor GFP-labelled vacuoles can be observed in this strain. As a positive control, the autophagy-stimulating drug rapamycin is used (**Figure 3**). After 40 hr of growth GFP-labelled vacuoles become visible. At 60 hr, the hyphae are completely filled with huge GFP-containing vacuoles (**Figure 3**). This finding indicates that, as expected, massive autophagy takes place here. Collectively, these results demonstrate the validity of the experimental set-up.

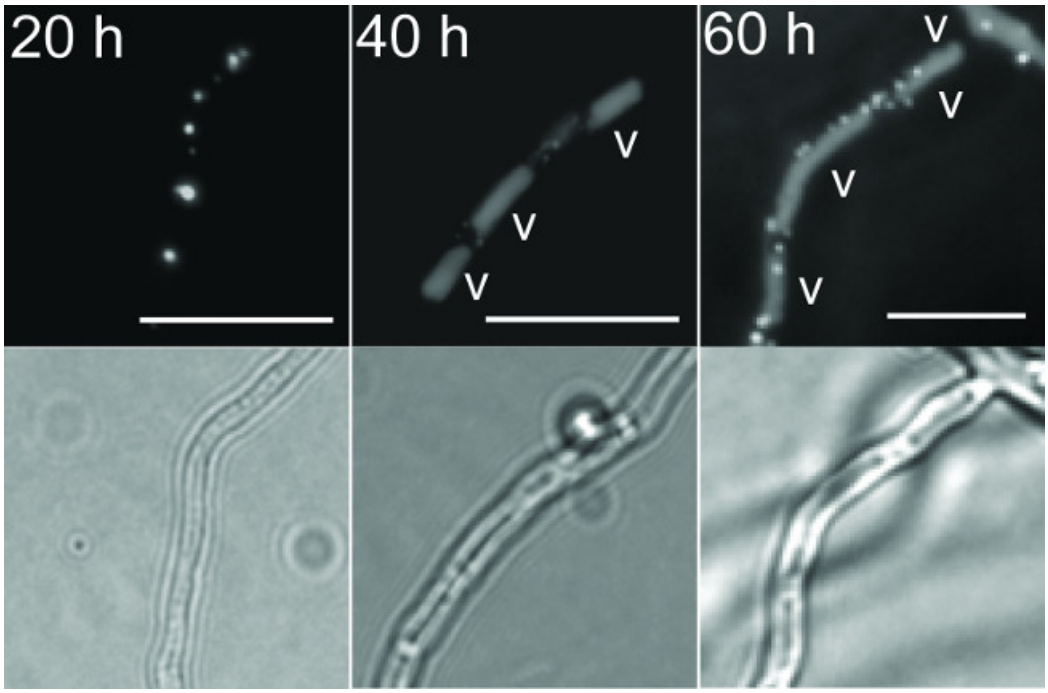

Figure 1: Localization of GFP-SKL in Ws54-1255 (GFP-SKL) during cultivation on starvation pads. At the indicated times, cultures grown on starvation pads were analyzed using fluorescence microscopy. Representative images are shown for each time point. Corresponding bright field areas are shown below each fluorescence channel image. White “v”: GFP-SKL localized to vacuoles indicating peroxisome degradation. Scale bars: 10 µm.

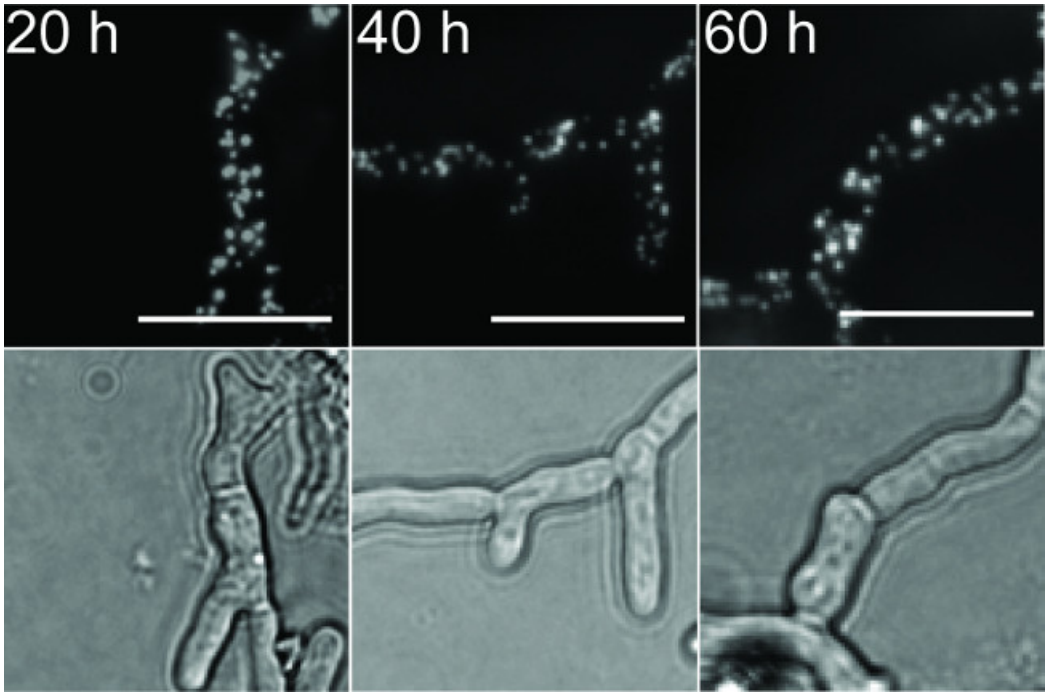

Figure 2: Localization of GFP-SKL in Δ*atg1* (GFP-SKL) during cultivation on starvation pads. At the indicated times, cultures grown on starvation pads were analyzed using fluorescence microscopy. Representative images are shown for each time point. Corresponding bright field areas are shown below each fluorescence channel image. Scale bars: 10 µm.

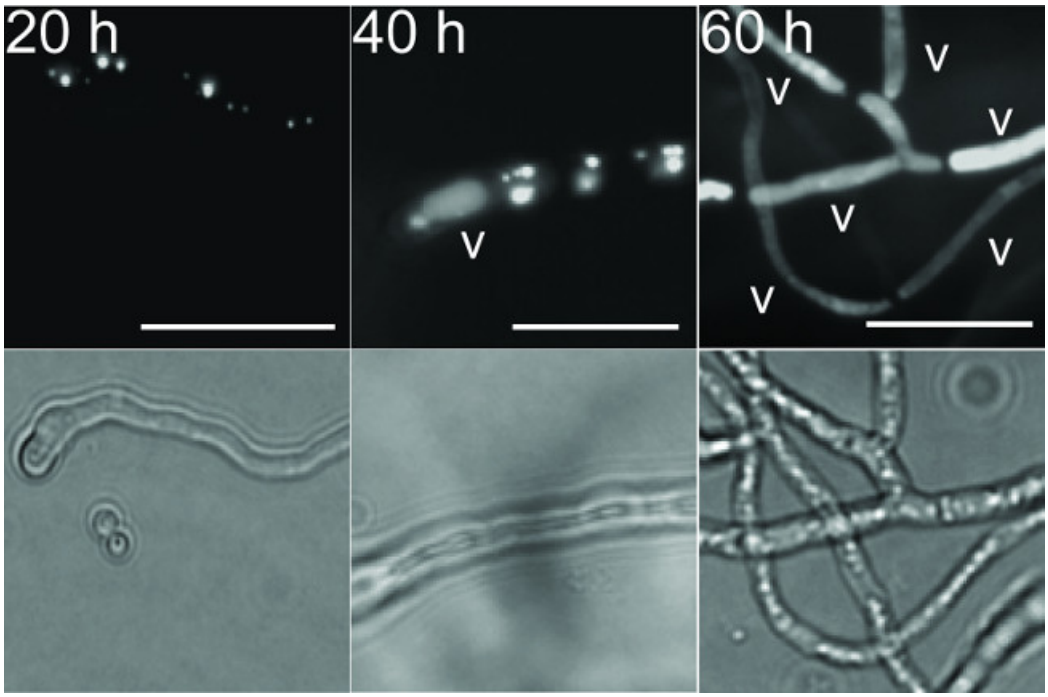

Figure 3: Localization of GFP-SKL in Ws54-1255 (GFP-SKL) treated with rapamycin for the induction of autophagy. At the indicated times cultures grown on starvation pads supplemented with 1 µM rapamycin were analyzed using fluorescence microscopy. Representative images are shown for each time point. Corresponding bright field areas are shown below each fluorescence channel image. White "v": GFP-SKL localized to vacuoles indicating peroxisome degradation. Scale bars: 10 µm.

Discussion

The method presented here allows the convenient and reproducible study of autophagy in *P. chrysogenum*. For example, it can be used for screening the efficacy of various compounds whether they are capable of modulating the autophagy response of this fungus or not. The results

with rapamycin demonstrate that inhibition of TOR signaling leads to a pronounced induction of autophagy in *P. chrysogenum* which has also been demonstrated for other organisms [11].

It is possible to use mycelia grown on starvation pads for analysis in more sophisticated microscopes (*e. g.*, confocal laser scanning microscopes). It does not matter whether the sample is put below the objective of above the objective (inverted microscope). Various fluorescent proteins targeted to certain compartments can be used to monitor the fate of different organelles during autophagy (*e.g.,* mitochondria -> mitophagy; endoplasmic reticulum -> ER-phagy, *etc.*). In summary, the technique described here extends the spectrum of methods for microscopically studying fungi [12].

The protocol is generally straightforward and easy to use. However, it is very important that the starvation pads never desiccate as this will result in artefacts and cell death. Therefore it is strongly suggested that (i) the microscope slides containing the still liquid agarose solution are removed immediately from the heating plate (step 3.3), (ii) that the cover slip for making a flat surface is removed from the starvation pad after a maximum of 5 min (steps 3.3 and 3.4) , (iii) that the microscope slide is always kept in the wet chamber when not analyzed (steps 3.5 and 5.4) and (iv) that microscopic analysis does not take more than 15 min (step 5.3). Another important aspect is that the starting amount of spores or mycelial fragments pipetted onto the starvation pad should be not too high or too low. This can be easily controlled by dilution of the stock by using sterile tap water. The 1/50 dilution described in 1.1.3 works well for *P. chrysogenum* spore suspensions. It is the recommended dilution for use with our fungi but might require some adjustments.

A limitation of the method is that it is not advisable for time-lapse imaging experiments for more than 15 min. This is due to the gradual desiccation of the starvation pad once it is removed from the wet chamber. If longer observation times are needed it is possible to add small amounts of sterile tap water to the fringe of the starvation pad although this does not prevent some desiccation in central regions of the pad. Another limitation is that the method is not strictly quantitative. In unicellular organisms it is possible to score events per cell (*e.g.*, amount of cells containing GFP in the vacuole). In contrast, *P. chrysogenum* as a filamentous fungus is characterized by a multicellular architecture. Cells are divided by septae which contain a central pore that allows the passage of cytoplasm and organelles. Therefore samples can be only analyzed 'semi quantitatively' (*e.g.*, number of hyphae containing GFP localized to vacuoles). To achieve significance it is very important that the number of hyphae analyzed is high enough (at least 80 per time point and condition tested but more is definitively preferable).

The utilization of starvation pads for studying autophagy can be easily adapted for use in other filamentous fungi. Therefore the protocol presented here is not only limited to *P. chrysogenum*. It should also be possible to adapt it for with single-celled fungi, *i.e.* yeasts. Transfer of the protocol to higher organisms (*e.g.*, nematodes) probably requires more specialized adaptations. It will be interesting to see whether the method described in this work also finds its use in other fields.

Disclosures

The author has nothing to disclose.

Acknowledgements

CQS receives a fellowship from the LOEWE Excellence Cluster for Integrative Fungal Research (IPF). The author would like to thank Ida J. van der Klei for the *P. chrysogenum* strains used in this work and Andreas S. Reichert for the gift of rapamycin.

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