Kinetic Analysis of Phagosomal ROS Generation

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Running Head: Phagosomal ROS

Abstract

Phagosomal ROS generation is critical for our immune defense against microbial infections. Quantitative assessment of phagosomal ROS production is required to understand the complex relationship between the phagocyte and the microbe, in particular for pathogens that resist phagosomal destruction. ROS detection is difficult due to the transient nature of the reactive species and their multiple interactions with the environment. Direct labelling of phagocytic prey with a ROS sensitive dye allows to target the dye into the phagosome and to follow the kinetics of phagosomal ROS production on a single phagosome base. Here we describe the basic labelling procedure, the quality assessment and the imaging technique to achieve this kinetic analysis.

iii. Key Words

NADPH oxidase, Phagosome, Microbial killing, Yeast, ROS sensitive dyes, Covalent labelling, DCF, $DCFH_2$-SE, Imaging, Flow cytometry,

1. Introduction

The primary role of the phagocyte NADPH oxidase, NOX2, is the production of superoxide anion, which is converted to other reactive oxygen species (ROS), inside phagosomes to kill ingested microbes (1, 2). To this end, the phagocyte assembles and activates the enzyme specifically at the phagosomal membrane immediately upon formation of the phagosome. The importance of its role for host defense in infection becomes evident when NOX2 is absent or non-functional as in Chronic Granulomatous Disease (3). Some pathogens either resist to ROS in the phagosome or inhibit NOX2 activation (4, 5). To investigate the mechanism of phagosomal ROS production and the associated pathologies, quantification of ROS inside the phagosome is required. From the wide choice of ROS detection methods only few are compatible with localized, time-resolved detection. The phagosome is particularly challenging because of the harsh intraphagosomal conditions in terms of changing pH, ROS, proteases and salt concentrations (6).

Covalent labelling of the phagocytic prey with a ROS sensitive dye assures that the dye is localized inside the phagosome. Dichlorodihydrofluoresceine ($DCFH_2$) is a fluorescein derivative that becomes fluorescent upon oxidation. Dichlorofluorescein (DCF) is the main oxidation product (7, 8). $DCFH_2$ is not specific and should be considered as a general indicator of ROS (*see* **Note 1 and 2**).We describe a general method to attach $DCFH_2$ to heat killed yeast as well as other particles containing free amine groups. The procedure involves preparation of the particles, the labelling reaction, and a second reaction to de-protect the dye. Since the dye is sensitive to chemical- and photo-oxidation all steps need to be performed at low oxygen tension and as little light exposure as possible. The quality of the preparation is best evaluated by flow cytometry. $DCFH_2$-labeled yeast can be opsonized and used for time-resolved fluorescence microscopy or flow cytometry (9–11). Double or triple labelling of the

particles with several dyes allows the detection of multiple parameters with the same particle (12, 13). We describe here the case of a reference dye that is rather stable in the phagosomal environment, which allows the independent quantification of phagocytosis. We will also provide a basic protocol for live cell imaging of phagosomal ROS production. The imaging conditions strongly depend on the features of the microscope. A number of controls are required to find the appropriate settings.

2. Materials

1. 100 mM sodium bicarbonate buffer, 0.84 g $NaHCO_3$ dissolved in 100 ml of water and then adjusted at pH 8.3 to suspend yeast during the reaction

2. Clean and dry DMSO

3.- 1.5 M d'hydroxylamine solution at pH 8.5 prepared the same day

4.- $DCFH_2$-SE (2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester, Molecular probes D-2935) 5 mg, re-suspended in 0.5 mL DMSO, sufficient for $2x10^9$ yeast (check appropriate number of particles when used with other microorganisms or beads) (*see* **Note 3**)

5.- Alexa405-SE (Molecular probes A30000) 1 mg, re-suspended in 0,1 ml DMSO, divide in 5 aliquots, evaporate DMSO in speedvac, storage at -20°C for approximately 6 months, resuspend each aliquot in 20 µL DMSO just before use.

6.- 1 mL syringes, 23G syringe needles

7. Inert gas (N_2 or Argon) from bottle or wall outlet

8.- Dark room with dim red light if possible

9. Centrifuge for yeast (1100 x g)

10. Slowly rotating wheel or shaker for tubes during reaction

11. Human serum (H4522,Sigma Aldrich)

12. Chamber for live cell microscopy

13. Flow cytometer with 488 nm laser

14. Wide-field inverted fluorescence microscope with appropriate filters for GFP or FITC detection (e.g; excitation at 480/15 nm, 495-nm beam splitter, and emission at 525/50 nm) and a fast, sensitive camera driven by a software for time-lapse acquisition (*see* **Note 4**)

3. Methods

3.1 Particle labelling with $DCFH_2$

The following protocol as outlined in Fig 1, has been developed for heat-killed yeast (*Saccharomyces cerevisiae*). We have also used this protocol for zymosan and *Candida glabrata*. In the literature, a similar labelling has been used for living microorganisms and for inert polystyrene beads (14, 15, 12). (*see* **Note 5**)

1. Yeasts, *S. cerevisiae*, are grown in YPD-medium at 30°C at 220 rotations per minute until they reach an exponential growth rate (absorbance at 600 nm between 0.2 and 0.4). They are spun down at 1100 x g and washed twice with PBS. They are resuspended at approximately 10^9 cells/ml and heat killed by boiling at >95°C for 60 min. Clumps are removed by filtration through filter paper of coarse porosity. Heat-killed yeast are washed in H_2O, adjusted to 10^9 particles/ml and stored at -20°C. This preparation is stable for several years. (*see* **Note 6**)

2. For labelling, wash yeasts twice in bicarbonate buffer, centrifuge at 1100 x g.

3. Degas 10 mL bicarbonate buffer with nitrogen gas in a closed 15 mL Erlenmeyer flask for 60 min. The flask is closed with a rubber gasket pierced with 2 syringe needles. One of the

needles is connected to the gas outlet and reaches into the buffer solution, the other does not reach into the buffer and serves for the escape of the gas from the flask.

4. Wash yeast once with degassed bicarbonate buffer.

5. Resuspend yeast in degassed bicarbonate buffer at 10^9/mL and transfer into black Eppendorf vial sealed with a rubber gasket.

6. Degas Eppendorf vial with syringe needles through gasket

7. Add $DCFH_2$-SE to yeast (250 µL dye solution for 10^9 yeast) by injecting through the gasket with a small (1 mL) syringe (*see* **Note 7**).

8. Incubate for 1 h at room temperature with constant gentle agitation and protect from light at all times (*see* **Note 8**).

9. Prepare the solution of hydroxylamine 1.5 M during the incubation time

Prepare 25 mL at 210 mg/mL of hydroxylamine in distilled water and adjust pH to 8.5 with a 5 M NaOH solution while stirring the solution. The solution will be at 3 M hydroxylamine and shall be diluted 1:2 to obtain the final solution at 1.5 M (*see* **Note 9**).

10. Add hydroxylamine solution directly to the yeast suspension to obtain 100 mM final concentration. Incubate for 45 min at room temperature. The hydroxylamine solution deprotects the ester groups of $DCFH_2$ (Fig. 2) and neutralizes dye molecules that are not bound to the particles.

11. The yeast need to be washed intensively to remove unbound dye. Centrifuge for 5 min at 1100 x g and wash 3 times with degassed PBS. Recover the first supernatant for later testing (*see* **Note 10**).

12. Incubate in degassed PBS overnight at room temperature with gentle agitation.

13. Add 3 more washes with PBS and recover the last supernatant for testing.

14. Store aliquots in light-protected vials under inert gas if possible at -20°C. Practically, aliquots of 20 µL with 10^9 yeasts/mL in eppendorff tubes are prepared and then briefly flushed with N_2 and then closed.

15. Preparation for phagocytosis requires opsonization (in most cases). The labeled yeast are diluted 10 times and then opsonized with human serum. An equal volume of yeast and serum should be mixed (for example 50 µL of yeast at 10^9 yeasts/mL and 50 µL of human serum). The sample is then incubated for 1 h at 37°C, washed twice with PBS and then resuspended in a suitable buffer for phagocytosis (see 3.3). The label does not seem to affect the outcome in terms of phagocytosis. Opsonized yeast are stored at -80°C. Even at that temperature, the opsonization is not stable and opsonized particles should not be stored for more than one month.

3.2 Assessment of labelling quality

The quality of the labelling is best assessed by flow cytometry. All particles should be labeled and the fluorescence intensity of the population should be homogenous (Fig. 3). However, a factor of 10 in fluorescence intensity between the weakest and the strongest particles is common. Treatment of the particles with H_2O_2 (50 mM) and HRP (50 U/mL) for 24 h at 22°C shifts the whole population to higher fluorescence (7). For freshly labeled yeast, DCF fluorescence shifts by a factor of ≈ 10 from untreated to H_2O_2 / HRP-treated particles. (*see* **Note 11**).

After double labelling, the fluorescence for both dyes detected by flow cytometry should be presented on a 2-parameter dot-plot. The points should form a cloud around a diagonal from left bottom to upper right (6) indicating that particles that are weakly labeled with one dye tend to be also weakly labeled with the other dye. Strongly labelled particles are highly fluorescent with both dyes.

3.3 Time-lapse imaging of phagocytosis of $DCFH_2$-labeled particles

Opsonized labeled yeasts are readily internalized by primary phagocytic cells and by neutrophil-like or macrophage-like cell lines. We have mostly used neutrophil-like, differentiated PLB985 cells. The actual imaging conditions depend strongly on the microscope setup. We will first describe a standard protocol and then list appropriate control experiments.

1. Use an inverted fluorescent microscope equipped with filters for GFP or FITC detection (e.g; excitation at 480/15 nm, 495-nm beam splitter, and emission at 525/50 nm) and a sensitive rapid camera for fluorescence monitoring. (*see* **Note 12**)

2. Freshly prepare phagocytic cells (10^6 cells/ml) in a physiological buffer (e.g. 10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl) containing divalent ions (1 mM Mg^{2+}, 2 mM Ca^{2+}), 1 mM glucose and a low concentration of protein (e.g. 1% human serum). Keep cells on ice.

3. Put cells on 25 mm coverslips fixed in a heated chamber (30 - 37°C, below 30°C no phagocytosis occurs) in an inverted microscope in 100 µL buffer and let them settle on the coverslip. Add 300 µl of buffer in order to avoid dehydration.

4. Add 10 µL $DCFH_2$-labeled yeast (at 10^8yeast/mL) to the coverslip and let them settle on the phagocytes.

5. Chose a field with phagocytes surrounded by the yeasts. (*see* **Note 13**)

6. Start time-lapse acquisition using an established protocol. Typically take images with 10 ms exposure at an interval of 10 s for a period of 30 min.

7. As the phagocyte itself and the phagosome within the phagocyte move in every direction, readjustment of the z-focus is often required. Therefore, the images have to be monitored during acquisition and the microscope focus adjusted accordingly.

8. End the recording if i) the phagosome is lost from focus or from the field of view, ii) significant bleaching occurs, iii) photooxidation of $DCFH_2$ renders all yeast particles fluorescent, even those that are not internalized.

9. Analyze data by choosing an ROI for each phagosome, adjusting this ROI to the x-y-movement throughout the recording and measure mean grey value of the ROI for each image. Automated analysis is possible with the Multi-Dimensional Motion Analysis module from MetaMorph software (Molecular Devices) or similar analysis modules.

3.4 Control steps for imaging protocol

A number of controls are required to optimize this protocol for each microscopy setup. The appropriate exposure time for each image depends on the intensity of the excitation light, the quality of the optical path and the sensitivity of the camera. DCF is sensitive to photobleaching. $DCFH_2$ is also sensitive to photooxidation that renders a fluorescent product.

1. To optimize between these two opposing constraints, it is useful to start with $DCFH_2$-labeled yeast alone to see how much light they support before photooxidation occurs.

2. Then, DCF-yeasts should be prepared by deliberate oxidation (50 mM H_2O_2 and 50 U/mL HRP for 24 h) (7). These oxidized yeasts will indicate the exposure time to obtain a signal with a satisfactory signal to noise ratio under the microscope.

3. Repeated exposure of these DCF-yeasts will also indicate whether photobleaching occurs under the chosen conditions. Photooxidation is not a linear phenomenon, for example, 10 x 300 ms exposure does not have the same effect as 5 x 600 ms exposure.

4. Control experiments either with cells that do not have a functional NADPH oxidase (PLB gp91^{phox} knock-out cells) or in the presence of an inhibitor of the NADPH oxidase will confirm that any increase in DCF fluorescence during the course of phagocytosis is indeed due to phagosomal ROS-production.

5. The rapid and strong ROS production by most phagocytes may oxidize all available dye molecules on the particle. Thus the increase in DCF fluorescence may end due to dye saturation instead of the end of ROS production by the phagocyte (7, 6). (*see* **Note 14**)

4. Notes

1. $DCFH_2$ in its various forms is widely used since the 1960s (16). It is by no means a perfect dye. It has poor specificity, it is photoactivated and photobleached, and it has a moderate pH sensitivity. In the phagosome, $DCFH_2$ most likely detects H_2O_2 and HOCl (7). Today however, it is the only ROS sensitive dye commercially available as a succinimidyl ester. It would be extremely useful to have other dyes available for covalent labelling. Amplex Red would be a good candidate. Hydrocyanines are an interesting option because they exist in several colors and the oxidized variants are frequently used for protein labelling. Their succimidyl-esters are commercially available (Lumiprobe: https://www.lumiprobe.com; Biotium, https://biotium.com). It may be possible to label particles with cyanine dyes and then reduce them to obtain ROS sensitive particle (17). We are not aware that this has been performed to date.

2. Genetically encoded biosensors could be an alternative to chemical labelling (18, 19). Biosensors based on fluorescent proteins such as Hyper or roGFP are frequently used in redox

biology. For measuring ROS production in the phagosome, the sensor should be placed on or near the surface of the living microorganism. The synthesis of the bacterial or fungal cell wall is a complicated issue that is only partially understood. Targeting of a biosensor to the cell wall should be possible but has not been reported. The known biosensors are reversible and interact with the cellular antioxidant machinery. It is not clear whether they would be appropriate for ROS detection in the phagosome. The harsh conditions of the phagosome (pH, proteases, ROS, salt) may substantially alter the photophysical properties of these sensors.

3. The package size of $DCFH_2$-DA, SE (5 mg) is larger than required for most phagocytosis applications. Weighing the dye to prepare aliquots most likely causes some oxidation. Taking up the whole package into a solvent (DMSO), preparing aliquots and evaporating the solvent maybe an option. We have not assessed whether this is feasible without a major loss of dye or reactivity.

4. Particles such as yeasts are moving up and down in the Z direction along with the deformations of the phagocytes. In a wide field microscope, there is no spatial selection along the Z-axis and the fluorescence is integrated over a depth of several microns. On the contrary, with a confocal microscope, the axial sectioning leads to the detection of a single fluorescent plane. In confocal microscopy, the phagosome constantly moves in and out of the observation plane and renders any quantification difficult. If the reader wants to use a confocal microscope, an option is to record a Z-stack composed of a few planes at each time-point and to calculate the mean gray value of the yeast fluorescence for each plane. The highest value, corresponding approximately to the equatorial plane, could be taken for further quantification.

5. Labelling of live cells is possible, but faces 2 major problems. The actual labelling reaction in bicarbonate buffer at pH 8 – 9 is not toxic to many bacteria, yeast or even Leishmania (unpublished observation). The solvent DMSO may be toxic, but its concentration in the reaction medium can be reduced by using highly concentrated reagent solutions. On the contrary, the de-protection reaction with hydroxylamine is toxic to most microorganisms. Some references in the literature do not mention the second reaction. Avoiding the hydroxylamine step may be necessary to keep the cells alive, but the amount of "active" dye on the organisms may be much smaller. The second problem concerns the cost of the experiment. $DCFH_2$-DA, SE (D2935 Molecular Probes) is sold in packages of 5 mg, a quantity enough to label $>10^9$ cells. Preparing aliquots of the reactive dye is difficult as light and oxygen should be avoided as much as possible. For dead particles, it is best to use the entire package in one reaction and store the labeled particle. Living bacterial or fungal cells cannot be easily stored. They may either die or proliferate. We do not know, whether storage in 25% glycerol at -80°C is compatible with method. So the labelling reaction has to be performed immediately before the experiment, which costs an entire package of the reagent and several hours of work (*see also* **Note 2**).

6. Several techniques for "heat killing" of yeast exist. We have used the same technique for two types of yeast (*S. cerevisiae* and *C. glabrata*) (see 3.1.1) and observed similar labelling results. An advantage of this method is the large number of sites available for the labelling. The presence of a large number of attached dye molecules available for oxidation increases the amount of ROS detected before the saturation of the dye occurs and thus increases the detection window accessible for monitoring the labeled prey. Other heat killing protocols, namely lower temperature such as 70°C for 10 min (20), may change the number of available

amines for this reaction. The killing method also will change the surface of the yeast, which may affect the phagocytic process.

7. Labelling with multiple dyes is possible. In particular, it is possible to label the yeast with Alexa 405–SE (Invitrogen A30000). Its fluorescence may serve as a reference in a ratiometric approach (6, 13). The fluorescence of Alexa 405 is quite stable with respect to pH or oxidation even within the phagosome. A solution of Alexa 405–SE (10 mg/mL in DMSO) can be added to the yeast together with $DCFH_2$-SE. Typically, 10 µL of Alexa 405–SE solution are added to 10^9 yeasts in 1 mL at the same time as $DCFH_2$-SE addition. Other succinimidyl ester dyes react under the same experimental conditions, butwhether they are stable inside the phagosome needs to be tested for each dye.

8. Protection of $DCFH_2$ against oxygen and light is tedious, but greatly improves the outcome. Any oxidation of the dye during the reactions, washing steps and storage will increase the background due to DCF being bound on the particle. (see paragraph on quality assessment).

9. Hydroxylamine is toxic, wear gloves and eye protection and dispose unused solution as chemical waste. The solution needs to be freshly prepared, at best during the first reaction period. The pH adjustment of this solution takes time. Concentrated, 5 mM NaOH needs to be added dropwise under stirring while monitoring pH with an electrode. If the solution is not properly stirred, pH may overshoot above 8.5 and then needs to be lowered by adding HCl.

10. At the end of the labelling procedure, yeasts are rose colored and the supernatant is light yellow due to unbound, oxidized dye. By oxidizing the dye (10 mM H_2O_2 + 10 U/ml HRP for 1 h) and measuring its absorbance (A), the concentration (c) can be determined.

$C = A / (\varepsilon \times l)$.

l is the optical path length of the measuring cuvette, usually 1 cm. The extinction coefficient (ε) of unbound DCF at pH 7.4 and 488 nm is 94,000 $M^{-1}cm^{-1}$ (7). The dye should be in excess to insure optimal labelling, thus at least some of the dye should remain unbound. Furthermore, at the end of the washing steps, no unbound dye should be detectable to make sure that the remaining dye is firmly attached to the particles.

11. With time, $DCFH_2$ oxidizes even under ideal storage conditions. It is worthwhile to test the basal and H_2O_2 / HRP-induced fluorescence by flow cytometer when older particles are used

12. DCF-particles are strongly fluorescent in the middle of the visual spectrum. Co-detection with fluorescent proteins is difficult since the latter tend to be weaker. Furthermore, excitation and emission spectra of DCF as well as fluorescent proteins are overlapping. Visualizing proteins tagged with mCherry in the same cell as fully oxidized DCF-particles has been possible in some but not all cases. Co-detection of DCF with the calcium indicator fura-2 is possible due to their clearly distinct excitation wavelengths (9). The red fluorescent pH indicator pHrodo is suitable for co-detection with DCF, even on the same particle (12).

13. Steps 5 and 7 require training. An experienced researcher will recognize cells that are likely to take-up a particle and he/she will lose less cells due to bad focus adjustment.

14. The amount of label on each particle depends on the number of accessible amine groups. Therefore, the type and size of the particle and its preparation method have a strong influence

on the maximal signal that can be obtained. Phagocytes are strong ROS producers and can rapidly saturate the entire label on the particles.

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Figures legends

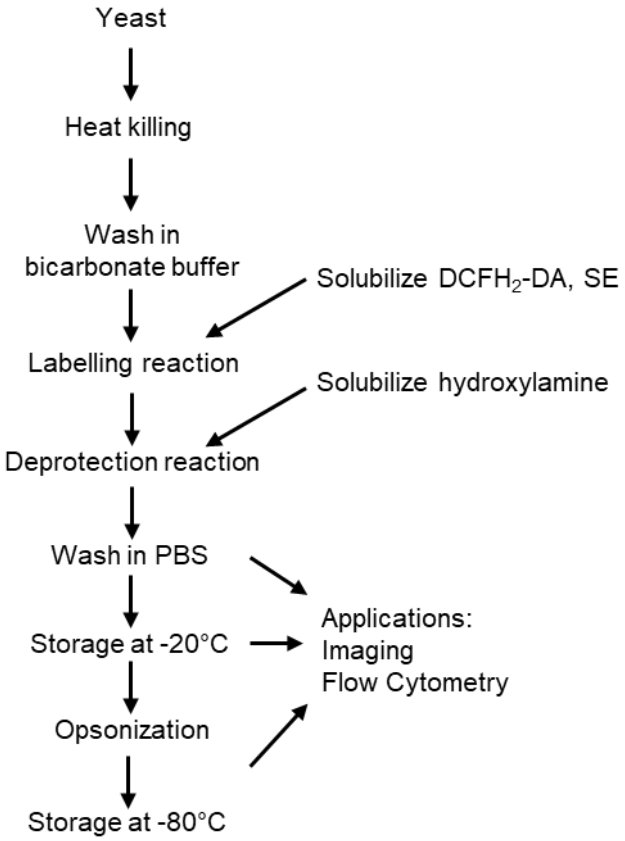

Figure 1: Workflow of the labelling procedure. Step by step representation of the labelling procedure.

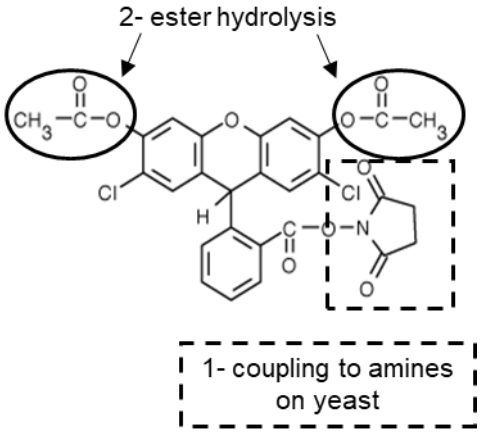

Figure 2: Formula of 2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green DCFH2-DA, SE) (adapted from Molecular Probes, Thermofisher)
The dashed box indicates the site of amine labelling in the first reaction. The full circles indicate the protective ester groups that need to be removed with hydroxylamine in the second reaction.

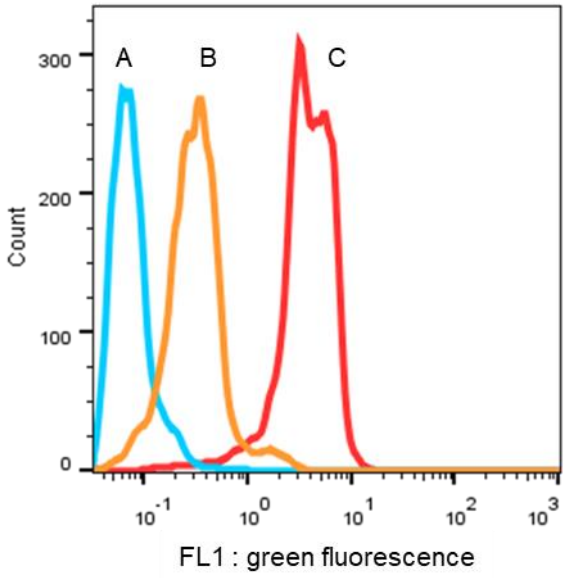

Figure 3: Analysis of labeled particles by flow cytometry. Comparison of fluorescence intensity (excitation 488 nm, emission 536 +/- 20 nm) of unlabeled yeast particles (A, blue), non-oxidized $DCFH_2$-labeled particles (B, orange) and $DCFH_2$-labeled particles oxidized by treatment with 50 mM H_2O_2, 50 U/mL HRP for 24 h (C, red).

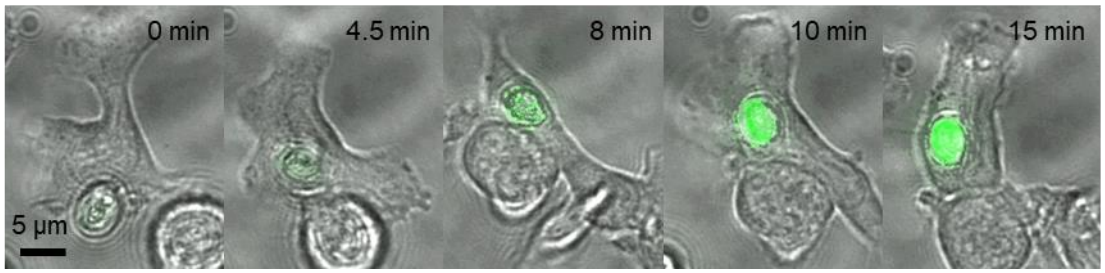

Figure 4: Images of DCF-labeled yeast in phagosomes. Superimposed images of transmitted light and green fluorescence showing the increase of phagosomal DCF fluorescence over time. Time 0 is near the closure of the phagosome. A neutrophil-like PLB985 cell has internalized a $DCFH_2$-labeled, opsonized yeast-particle.