Chapter 20

Methods to Detect Apoptotic-Like Cell Death in Filamentous Fungi

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Abstract

Fungi are capable of undergoing apoptotic-like cell death, and display many of the characteristic features of apoptosis observed in multicellular organisms. These features include nuclear condensation, DNA fragmentation, translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the plasma membrane, and increased levels of reactive oxygen species (ROS). Several assays can be used to detect apoptotic cells, and here we describe adaptations of assays such as TUNEL, Annexin V, and Evan's Blue for the investigation of apoptotic-like cell death in fungal hyphae. We also present approaches for monitoring nuclear condensation and production of ROS.

Key words: Apoptosis, Filamentous fungus, Nuclear staining, TUNEL, Annexin V, Evans Blue, Reactive oxygen species

1. Introduction

Apoptosis is a form of programmed cell death critical for the development and homeostasis in multicellular organisms (1). The process of apoptosis depends on numerous extra- and intracellular pro- and antiapoptotic signals that are integrated to activate apoptotic effectors only when necessary. Two separate, but overlapping, mechanisms of inducing apoptosis have been identified (2). For the first mechanism, intracellular death messengers lead to disruption of mitochondrial membrane potential. The subsequent release of cytochrome *c* from mitochondria activates the apoptotic executors; cysteine proteases that are known as caspases. Alternatively, signals from membrane-bound death receptors can lead to mitochondria-independent activation of caspases.

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Morphological and biochemical changes follow activation of either apoptotic pathway. These changes include nuclear and cytoplasmic condensation, DNA fragmentation, plasma membrane alterations including the translocation of phosphatidylserine (PS) from the cytoplasmic to the extracellular side of the membrane, increased levels of reactive oxygen species (ROS) indicative of cellular oxidative stress, and partitioning of cytoplasm and nuclei into membrane bound-vesicles (apoptotic bodies). The latter structures are known to contain ribosomes, morphologically intact mitochondria, and nuclear material. In vivo, apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (1).

Apoptotic-like cell death has been reported in several fungi, with many of its underlying features resembling apoptosis of multicellular organisms, including DNA condensation and fragmentation, exposure of PS and high levels of ROS. For example, we previously reported that the isoprenoid compound farnesol induces apoptotic-like cell death both in *Aspergillus nidulans* (3) and *Fusarium graminearum* (4). Several assays have been developed for the detection of mammalian cells undergoing apoptosis. In this chapter, we describe assays that were adapted to detect apoptotic-like cell death in fungal hyphae.

One of the first morphological changes associated with apoptosis is chromatin condensation, similar to that observed in mitosis. Nuclear condensation can be easily detected by different fluorochromes that label nuclear material, followed by visual analysis with fluorescence microscope. We prefer using Hoechst 33258 for nuclear staining since this fluorochrome stains chromatin on the nuclear DNA, whereas DAPI, for example, stains both mitochondrial and nuclear DNA. The next assay described, TUNEL (TdT-mediated dUTP nick end labeling), is the standard method for identification and quantification of apoptotic cells. This method detects DNA fragmentation by using terminal deoxynucleotidyl transferase (TdT) to incorporate dUTP tagged with biotin, DIG, or fluorescein into the blunt ends of double-stranded DNA breaks (5, 6). In order to allow the TdT and dUTP to enter fixed hyphae, their cell wall and plasma membrane have to be permeabilized prior to the enzymatic reaction. The assay described here uses FITC-conjugated dUTP, which can be directly visualized by fluorescence microscopy, thereby avoiding the second incubation step with streptavidin or anti-DIG antibody necessary to detect dUTP tagged with biotin or DIG respectively.

The Annexin V assay detects translocation of PS from the inner side of the plasma membrane to the outer layer (7). This test allows the detection of cells at early stages of apoptosis, because changes in PS asymmetry become apparent before the other morphological changes associated with apoptosis, including DNA fragmentation. Annexin V is a member of the annexin

family of calcium-dependent phospholipid-binding proteins and preferentially binds to negatively charged phospholipids like PS. By conjugating FITC to Annexin V, it is possible to identify and quantify apoptotic cells by fluorescence microscopy. Annexin V does not bind cells with an intact plasma membrane, but it can falsely detect the inner membrane PS of lysed (necrotic) cells. Therefore, simultaneous staining with propidium iodide (red fluorescence), which will be excluded from intact cells, allows the discrimination of intact (FITC–, PI–), early apoptotic (FITC+, PI–) and late apoptotic or necrotic hyphae (FITC+, PI+). An extra step has to be added to the protocol in order to remove the cell wall from hyphae and allow the detection of exposed PS on the external surface of protoplasts. Late apoptotic hyphae can also be detected by Evans Blue staining. Normal hyphae with an intact plasma membrane exclude Evans blue and remain their natural color. Dying hyphae that have undergone plasma membrane lysis are unable to exclude the dye and stain deep blue. Finally, we describe the detection of increased ROS production by apoptotic cells using two staining methods. The first method uses 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) which is permeable to protoplasts and undergoes intracellular conversion by nonspecific esterases to nonfluorescent 2′,7′-dichlorofluorescein (DCFH). DCFH oxidizes in the presence of H_2O_2 and other ROS to form 2′,7′-dichlorofluorescein (DCF), which emits bright green fluorescence (8). The second method uses Nitro blue Tetrazolium (NBT), a pale yellow compound that is reduced by ROS to a blue-purple formazan precipitate (9, 10). Superoxide was reported to be the major oxidant species responsible for reducing NBT to formazan (11).

2. Materials

2.1. Detection of Nuclear Condensation

1. Phosphate Buffered Saline (PBS) 10× stock solution: 1.37 M sodium chloride, 27 mM potassium chloride, 100 mM sodium dihydrogen phosphate (NaH_2PO_4), 18 mM potassium dihydrogen phosphate (KH_2PO_4). Adjust pH to 7.2 with HCl if necessary and autoclave before storing at room temperature (see Note 1).
2. Fixing Solution (see Note 2): 1× PBS, 5% dimethyl sulfoxide (DMSO), 3.7% formaldehyde. Better if prepared fresh but can be stored at room temperature for 1 week.
3. Nuclear Staining Solution: Prepare stock of 1 mg/mL Hoechst 33258 (Molecular Probes) and store at 4°C in the dark for several months. Prepare working solution fresh by diluting to 0.1–0.5 mg/mL (see Note 3).

4. Mount Solution: 1× PBS buffer, 50% glycerol, 0.1% *n*-propyl-gallate. Divide into aliquots and store at –20°C.
5. Sterile 22 mm × 22 mm square glass coverslips.
6. Staining jars for coverslips (see Note 4).
7. Precleaned microscopy slides (available from Fisher or VWR).
8. Fluorescence microscope.

2.2. TUNEL Assay

1. This method uses all materials described in the previous Subheading 2.1, however, a different fixing solution is routinely used. Substitute PBS with PEM; 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) pH 6.7, 25 mM ethylene glycol tetra-acetic acid (EGTA) pH 7.0, and 5 mM magnesium sulfate ($MgSO_4$).
2. Digestion Solution: Dissolve 50 mg/mL lysing enzymes (Sigma-Aldrich), 67.5 mg/mL beta-D glucanase (Interspex), and 22 mg/mL driselase (Sigma-Aldrich) in PEM and incubate on ice for 15 min. Centrifuge at 2,000 rpm (447 × *g*) for 5 min and transfer the supernatant to a new tube and add 1 mL of egg white (aliquot previously and store at –20°C) (see Note 5).
3. Extraction Solution: 100 mM PIPES pH 6.7, 25 mM EGTA pH 7.0, and 0.01% Igepal CA-630.
4. PEM/BSA: Dissolve 0.1% bovine serum albumin (BSA) in PEM.
5. DNase: Dilute RQ1-RNA free DNase (Promega, Cat. No. 610A, concentration 1 U/μL) to 50 U/mL of PBS. Use fresh.
6. TUNEL Reaction Solution (In Situ Cell Death Detection Kit, Fluorescein: Boehringer Mannheim, Cat. No. 1684795): Remove 100 μL Label Solution from vial two and reserve for two negative controls. Add total volume of vial one (50 μL) to the remaining 450 μL Label Solution in vial two to obtain 500 μL TUNEL Reaction Solution. Mix well to equilibrate components. The TUNEL Reaction Solution should be prepared immediately before use and should not be stored.

2.3. Annexin V Assay

1. Citric acid: 0.5 M citric acid, adjust to pH 6.0 with NaOH. Autoclave before storing at 4°C.
2. Solution 1: 0.8 M ammonium sulfate ($(NH_4)_2SO_4$), 100 mM citric acid. Autoclave before storing at 4°C.
3. Solution 2: 1% yeast extract, 2% sucrose, and 20 mM $MgSO_4$. Autoclave and store at 4°C.
4. Protoplasting Solution: In a 250 mL sterile flask, add 10 mL of Solution 1 and 20 mL of Solution 2. Dissolve 200 mg of Driselase, 300 mg of Glucanex, and 50 mg of Lysing enzymes in 5 mL of Solution 1 in a falcon tube and place on ice for 15 min. Because the enzymes dissolve poorly, they should be gently mixed every minute. In order to clarify, centrifuge at 2,000 rpm (447 × *g*) for

5 min and then filter-sterilize through a 0.2 μm syringe filter directly into the same flask in which the other solutions were added (see Note 6). Dissolve 500 mg of BSA in 5 mL of Solution 1 and filter sterilize into the same flask (see Note 7).

5. Sucrose Solution: 1 M sucrose in autoclaved distilled water.
6. 1× Binding Buffer (Annexin V-FITC Apoptosis Detection Kit II, Calbiochem, Cat. No. CBA059): Dilute the 4× Binding Buffer from the kit to 1× in 1 M sucrose.
7. FITC-tagged Annexin V (Annexin V-FITC Apoptosis Detection Kit II, Calbiochem, Cat. No. CBA059).
8. Propidium iodide (PI) (Annexin V-FITC Apoptosis Detection Kit II, Calbiochem, Cat. No. CBA059) (see Note 8).
9. Items 4–8 from Subheading 2.1. Because protoplasts that have failed to adhere to slides can interfere with microscopy, we recommend the use of Gelatin Coated Adhesive or Colorfrost/Plus Microscope Slides (Fisher).

2.4. Evans Blue Staining

1. 1× PBS.
2. Evans Blue Solution: Dilute 1% Evans Blue (Sigma) in PBS.
3. Bright-field microscope.
4. Items 5–7 from Subheading 2.1.

2.5. ROS Detection

1. Solutions 1–6 from Subheading 2.3.

2.5.1. 2′,7′-Dichlorofluorescein Diacetate Staining

2. DCF Solution: Prepare 1 mM stock solution of 2′,7′-dichlorofluorescein diacetate (DCF, Molecular Probes) in DMSO. Divide into aliquots and store at −20°C. Prepare working solution fresh by diluting to 50 μM in water (see Note 9).
3. Items described in item 9 of Subheading 2.3.

2.5.2. Nitro Blue Tetrazolium Staining

1. MOPS: Prepare 5 mM solution of 3-(*N*-Morpholino) propanesulfonic acid (MOPS) in water. Adjust to pH 7.6 with NaOH.
2. NBT Solution: Make 2.5 mM solution of Nitro Blue Tetrazolium (NBT) in MOPS and store for up to 2 weeks at room temperature and protect from light.
3. Bright-field microscope.
4. Items 5–7 from Subheading 2.1.

3. Methods

Before performing the techniques described below, apoptosis should be induced. The conditions for induction of apoptosis, including dosage, exposure time, culture phase, temperature,

media, etc., should be tested for each apoptotic inducer and fungal species. Ideally, the optimized conditions should induce apoptosis in the majority of the hyphae and in a dose-dependent manner. A control using the same volume of the solvent used to dissolve the apoptotic inducer should always be run in parallel with each experiment. Also, when performing the assays for the first time, it is advisable to use a known stimulator of apoptosis as a positive control (e.g., (3)).

3.1. Detection of Nuclear Condensation

1. Using sterile procedures, place up to four coverslips in the bottom of a 10 cm plastic Petri plate.
2. Inoculate 10^6 spores in 10 mL of appropriate liquid medium and gently pour into the plate containing the coverslips (see Note 10).
3. Incubate at temperature and time appropriate for each experiment (see Note 11).
4. Treat hyphae with apoptotic inducer diluted in fresh medium pre-incubated at the growth temperature. Include a parallel control sample that is not treated.
5. Transfer coverslips with forceps to a staining jar containing 10 mL of Fixing Solution. It is important to keep track of the side of the coverslip containing the growing hyphae. One suggestion to avoid confusion is to always keep the hyphal side facing you as you transfer the coverslips. Fix for 15 min at room temperature.
6. Transfer the coverslips to a new staining jar containing 10 mL of PBS and incubate for 5 min. Repeat this step two times (total of three washes) adding new buffer to the jar for each wash.
7. Transfer the coverslips to a new staining jar containing 10 mL of Nuclear Staining Solution, cover with aluminum foil to protect from light, and incubate for 5 min.
8. Rinse coverslips briefly in a beaker containing water. Dry the excess of water by carefully blotting the edge of the coverslip with a Kimwipe. Alternatively, the coverslips may be propped against a box resting on a Kimwipe to briefly drain. Make sure that the coverslips do not dry out (a few min should be enough). Always keep track of the side of the coverslip containing the hyphal growth.
9. Pipette 10 μL of Mounting Solution onto a microscope slide. Gently lay the coverslip on the Mounting Solution, with the hyphae facing down, avoiding the formation of bubbles.
10. Remove excess Mounting Solution that spread out from under the coverslip by carefully blotting with a Kimwipe (see Note 12).

11. Seal the edges of the coverslip with transparent nail polish.
12. Visualize samples using a fluorescence microscope, using a 60× objective and a UV filter set (see Note 13). Slides can be stored at 4°C in the dark for several months.
13. The proportion of hyphae with condensed nuclei should be determined and compared to the untreated control. Condensed nuclei are typically much smaller in size and lack the nucleolar shadow (i.e., small intranuclear region that does not stain) that is usually visible in normal nuclei. At least 200 hyphae should be counted in each sample.

3.2. TUNEL Assay

1. Follow steps 1–5 from Subheading 3.1 for growth and fixation of hyphae, but use the Fixing Solution described for the TUNEL Assay.
2. Wash coverslips three times with PEM for 5 min to remove Fixing Solution.
3. Cut a piece of parafilm and stretch it uniformly over an acrylic sheet or other smooth surface; the blunt side of a clean razor blade can be used to smooth out the parafilm and eliminate air bubbles. Pipette 200 μL of Digesting Solution such that it forms a drop on the parafilm. Gently lay the coverslip on the drop with hyphae facing down. Incubate at 28°C for 15–120 min (see Note 14). At each digestion time point (i.e., 15, 30 min, etc.), transfer one coverslip to a staining jar containing PEM and leave there until the final time point.
4. Wash coverslips three times with PEM for 5 min to remove the digestive enzymes.
5. Transfer the coverslips to a new staining jar containing 10 mL of Extraction Solution and incubate for 5 min at room temperature to permeabilize hyphal membranes.
6. Wash twice in PEM and once in PEM/BSA for 5 min.
7. Using parafilm as described in step 3, incubate the positive control with DNase at 37°C for 1 h. Meanwhile, keep the other samples in PEM/BSA.
8. Cover the bottom of a shallow plastic box with parafilm. Divide the TUNEL Reaction Solution into enough drops for each sample, including the positive control (use at least 50 μL for each one). Pipette 50 μL of Label Solution reserved for the negative controls (see Note 15). Lay coverslips on drops with hyphae facing down, keeping track of each different sample. Place 2 moistened cotton balls in the edges of the box and cover it with aluminum foil. Incubate at 37°C for 1 h. All following steps should be protected from light.
9. Wash coverslips three times with PEM/BSA for 5 min.

10. Follow steps 7–12 from Subheading 3.1.
11. The proportion of hyphae presenting TUNEL positive nuclei (green fluorescent nuclei) should be determined and compared to the untreated control (see Note 16). At least 200 hyphae should be counted in each sample. No TUNEL positive nuclei should be observed in negative controls and the majority of nuclei should be TUNEL positive in the positive controls (see Note 17).

3.3. Annexin V Assay

3.3.1. Generation of Protoplasts (See Note 18)

1. Inoculate 10^9 fresh spores in 50 mL of appropriate liquid medium in a 250 mL Erlenmeyer flask. Incubate with appropriate agitation, temperature, and time.
2. Treat hyphae with apoptotic inducer. Be sure to include a parallel control sample that is not treated.
3. Collect hyphae from each condition using centrifugation at 3,000 rpm ($1006 \times g$) for 3 min and discard supernatant.
4. Resuspend in 50 mL of Protoplast Solution and incubate at 30°C and 100 rpm. After about 3 h, monitor protoplast formation under the microscope. Protoplasts should appear slightly translucent when compared to undigested spores and hyphal fragments.
5. Filter the protoplast solution through sterile Miracloth into 50 mL falcon tubes. Centrifuge at 4,000 rpm ($1789 \times g$) for 10 min and discard supernatant.
6. Transfer flasks to ice (see Note 19) and wash protoplasts with 10 mL of cold Sucrose Solution. Gently re-suspend the pellet by pipetting up and down. Centrifuge at 4,000 rpm ($1789 \times g$) for 5 min and repeat the wash (see Note 20).
7. Resuspend protoplasts in 1 mL of cold Sucrose Solution and transfer to an Eppendorf tube (see Note 21).

3.3.2. Annexin V

1. Make different dilutions (1:5, 1:10 and 1:50) of resuspended protoplasts into 1× Binding Buffer and transfer 38 μL of each dilution to new Eppendorf tubes (see Note 22).
2. Add 2 μL of Annexin V and 2 μL of PI solutions to each tube.
3. Incubate at room temperature for 20 min in the dark.
4. Pipette 5 μL of Mounting Solution into a microscope slide. Add 10 μL of stained protoplasts to slides and place a coverslip on top.
5. Remove excessive Mounting Solution and seal the edges of the coverslip with transparent nail polish.
6. Analyze samples immediately using the fluorescein and rhodamine filters of a fluorescence microscope.

7. Count the number of Annexin V-positive (green fluorescent, especially at plasma membrane), PI-negative protoplasts (undetectable red fluorescence), which are scored as apoptotic.

3.4. Evans Blue Staining

1. Follow steps 1–4 from Subheading 3.1.
2. Transfer coverslips to a new staining jar containing Evans Blue Solution. Stain for 5 min at room temperature.
3. Wash coverslips three times with PBS for 5 min.
4. Mount slides as described in step 9 of Subheading 3.1 and analyze samples immediately using a bright field microscope.
5. Count the number of living hyphae (natural color) and dead hyphae (stained blue).

3.5. Detection of ROS

3.5.1. 2′,7′-Dichlorofluorescein Diacetate Staining

1. Generate protoplasts as described in Subheading 3.3.1 (see Notes 23 and 24).
2. Transfer 20 μL of resuspended protoplasts to a new Eppendorf tube and add 20 μL of DCF Solution.
3. Mount slides as described in step 9 of Subheading 3.1 and analyze samples immediately by fluorescence microscopy using the fluorescein filter set.
4. Count the number of green fluorescent protoplasts, which reflects an increased ROS production.

3.5.2. Nitro Blue Tetrazolium Staining

1. Follow steps 1–4 from Subheading 3.1.
2. Place coverslips on parafilm (prepared as in step 3 of Subheading 3.2) with the hyphae facing up.
3. Immediately and carefully add 500 μL of NBT solution to a corner of each coverslip without disturbing the hyphae.
4. Incubate in the dark at room temperature for 30 min.
5. Drain the extra solution using a Kim wipe and mount on slide using growth media instead of mount solution.
6. Observe immediately using light microscopy and count hyphal cells that contain blue-purple NBT formazan deposits.

4. Notes

1. Unless indicated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ/cm and total organic content of less than five parts per billion.
2. Other chemical fixatives (i.e., glutaraldehyde, ethanol) could potentially be used as well.

3. The optimal concentration that results in good contrast between nuclear and cytoplasmic staining should be empirically determined for each stock solution.
4. Six-well microtiter plates can be used in place of staining jars. Transfer one coverslip to each well (hyphae facing up) and add 5 mL of solution.
5. Solution optimized for *A. nidulans*. Different fungi might require other enzymes that digest the components of their cell walls. Egg whites are obtained from fresh eggs that are cracked and filtered to remove yolks.
6. Even after the clarification the filter may clog. If that happens exchange the syringe filter.
7. Incubation at 37°C accelerates the process of dissolving the BSA.
8. PI is a potential carcinogen and must be handled with care.
9. The optimal concentration of DCF (25–50 μM) may vary between experiments and should be empirically determined.
10. Make sure that the coverslips are in contact with the plate to avoid hyphal growth on the underside of the coverslip. Sterilized forceps can be used to gently press the coverslips down.
11. The incubation period should be sufficiently long, enough for spores to break dormancy and form extending hyphae with at least eight nuclei. Note that this period should also include the incubation time with the apoptotic inducer.
12. Make sure all the excess of the Mounting Solution is removed, otherwise the nail polish will not stick well. In that case the coverslips may come off during microscopy or dry out from lack of sealing.
13. Although standard fluorescence microscopy is appropriate, the use of laser scanning confocal microscopy is preferable as it makes it possible to obtain images across multiple focal planes.
14. Because of variability between each batch of the Digestion Solution, it is advisable to set up several replicas for each condition and submit them to a range of digestion times (for example 15, 30, 60, and 90 min is recommended). The best digestion time will allow enough cell wall removal, so that TdT and FITC-dUTP penetrate the hyphae without destroying the fixed cells.
15. Reserve one untreated and one treated sample with the apoptotic inducer samples, digested for the longer incubation time, to be used as negative controls.
16. If all controls give the expected result and a low percentage of TUNEL positive nuclei are found in samples where apoptosis

was induced, it may be necessary to further optimize the conditions under which apoptosis is being induced (e.g., increase inducer concentration, increase exposure time).

17. If no TUNEL positive nuclei are found in the positive control, it is likely that the samples were not sufficiently digested or permeabilized.
18. The following Protoplast Generation protocol is optimized for *A. nidulans*.
19. From this step on, keep protoplasts on ice.
20. Be very careful manipulating the protoplasts since pipetting can disrupt the plasma membrane and increase the proportion of late apoptotic and necrotic cells.
21. Protoplasts can be kept on ice in a cold room overnight.
22. The purpose of using different dilutions is to have an adequate amount of protoplasts per field.
23. The same protoplasts can be used for the Annexin-V and ROS detection assays.
24. Cells treated with 1 mM of hydrogen peroxide, which induces oxidative stress, can be used as a positive control for the DCF staining.

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