Chapter 11
Fluorochrome-Based Methods for Fungal Sample Examination

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Contents

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11.1 Introduction

Fungi are eukaryotic, usually filamentous, spore-producing organisms and can be obligate parasites, nonobligate parasites, or biotrophs, developing several interactions with plants, animals, or the environment and can be used to produce food and enzymes for industrial processes [27]. Most fungi species have microscopic structures and studies on this organism group depend on various microscopy techniques types.

Stokes related the fluorescence phenomenon in 1852, describing a photon molecular absorption generating the emission of another photon with greater wavelength, the principle from which it was possible to develop techniques of fluorescence microscopy [20]. Therefore, natural or induced fluorescence characteristics have been explored for organisms and macromolecules localization explaining several types of biological phenomena, mainly by techniques of epi-fluorescence or laser confocal microscopy.

These techniques enable many morphological and physiological analyzes in cells and tissues, locating cellular components, interaction with plants, nuclear dynamics, reactive oxygen species accumulation, and cellular death.

Fluorescence microscopy studies may include analyzes of fluorescence or autofluorescent samples. Some fungi like Basidiomycota are autofluorescent, and

others such as *Cercospora* spp. produce fluorescent phytotoxins. Other studies types are conducted by inducing fluorescence in the samples. This may occur through the use of fluorochromes, immunofluorescence techniques, nucleic acids hybridization, and molecular markers. Fluorochromes are molecules capable of specifically binding to cellular components by inducing their fluorescence under the excitation of certain wavelengths. These components can be the fungal cell wall, nuclei, chromosomes, mitochondria, and others. Other fluorochromes may indicate physiological aspects, such as cell death, accumulation of reactive oxygen species, or evidence of defense reactions in plant tissues colonized by fungi. Immunofluorescence, also called immunostaining, is a technique where fluorescent molecules are attached to antibodies corresponding to antigens to which they will be located. Nucleic acid hybridization, called FISH (fluorescent in situ hybridization), allows the use of nucleic acid probes attached to fluorescent molecules. These probes are complementary to target sequences, which can identify specific regions on chromosomes, the expression of certain genes by mRNAs, or specific groups of organisms using regions of phylogenetically important DNA. One of the most commonly used molecular markers is fluorescent proteins such as GFP (green fluorescent protein). By genetic transformation, the fluorescent markers genes are associated with the genes whose products will be localized. Thus, the expressed proteins are localized through the fluorescent protein anchored.

In this chapter, protocols for fluorescence microscopy will be discussed in studies on fungi using only fluorochromes or autofluorescence of structures for localization techniques.

All following procedures were done at the Electron Microscopy and Ultrastructural Analysis Lab at Federal University of Lavras, using an inverted Epi-Fluorescence Zeiss Axio Z.1 and an inverted Laser Confocal Zeiss LSM780 Observer Z.1 and Zen 2012 software.

11.2 Sample Preparation to Observe Fungi Nuclei and Chromosome

Ascomycota fungi have the characteristic of maintaining monocariotic state but may present some dicariotic cells throughout the intermediary processes of their reproduction, such as plasmogamy and karyogamy. In the case of Basidiomycota, they may have different phases throughout their life cycle, monocariotic or even dicariotic (Fig. 11.1e). Basidiomycota may present multinucleate cells, as basidia before nuclei migration to the basidiospores, and the in certain *Rhizoctonia* hyphae (Fig. 11.1f). These nuclear dynamics processes can be studied using fluorescence markers, such as DAPI, one of the most used. Often, DAPI is used associated with immunofluorescence and hybridization techniques, for proteins or DNA sequences specific localization in nuclei. Other fluorescent nuclei dyes can be used, such as SyBrGreen and Propidium Iodide (in the case of living cells).

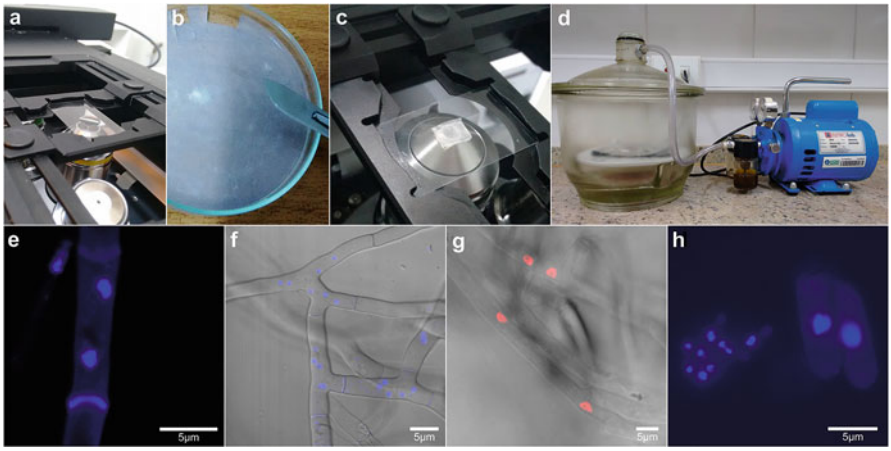

Fig. 11.1 Fungi nuclei and chromosome observations by fluorochrome-based methods. (**a**) Positioning of DAPI drops above coverslip coupled in an inverted microscope. (**b**) Sampling of *Rhizoctonia solani* colony growth in SNA media. (**c**) Transferring of colony fragment to the dye. (**d**) Vacuum pump linked to a hermetically sealed recipient. Laser scanning confocal micrographs of *Rhizoctonia solani* from binucleated (**e**) and multinucleated lineages (**f**) marked with DAPI; *Rhizoctonia* sp. nuclei marked with Propidium Iodide (**g**); and *Colletotrichum* chromosome at metaphase observation by germ tube burst method using DAPI (**h**)

11.3 DAPI Methods

Materials to prepare fungi for EFM or LSM observation. The necessary materials can vary depending on the protocol used.

11.3.1 Materials

1. Nucleus marker DAPI (4′6-Diamidine-2′-phenylindole dihydrochloride) (VectaShield® H-1200 Vector®) mounting medium.
2. Calcofluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) 0.01% (p/v) prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
3. Fungi colony growth within a clear media, such as Synthetic Nutrient-poor Agar (SNA) [4], with living mycelia, or fixed fungi fragments.
4. Kolle handle with a needle
5. Scalpel.
6. Tweezer.
7. Bunsen burner.
8. Alcohol.
9. Automatic pipette.
10. Slides and regular coverslips (e.g. 20 × 20 mm).

11. Large coverslips (e.g. 24 × 40 mm).
12. Nail polish or other coverslip sealants.
13. Vacuum pump linked to a hermetically sealed container.
14. Stereomicroscope.
15. EFM or LSM, vertical or inverted.
16. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49).
17. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around fourfold zoom. The bright-field image was acquired using the TPM-T detector.

11.3.2 Methods

The methods presented below are for marking filamentous fungi nuclei, and variations will be presented and discussed.

11.3.3 Culture Block on Inverted Microscope

Firstly, we will show a quick preparation to observe nuclei in fungi (Fig. 11.1e, f) using an inverted microscope (EFM or LSM) as made by Melo et al. [17].

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Put 10 μL of DAPI-VectaShield mounting media above the coverslip (Fig. 11.1a). In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed. Avoid the coverslip corners, where the objective lens does not reach. Develop it in a room with diffuse light, or as dark as possible.
3. Take colony fragments with around 0.5 × 0.5 cm with a scalpel (Fig. 11.1b), choosing the region of interest using a stereomicroscope, if needed. The use of clear media like SNA allows better light transmission. Besides, it favors the fungi sporulation and reduces the aerial mycelia growth rate. The colony fragments may be fresh or fixed.
4. Transfer the upside-down colony fragment above the DAPI drop. The mycelia should be in contact with the dye (Fig. 11.1c).

5. Leave it in the dark for 5–20 min. The incubation time will depend on the fungus species. The thinner cell wall species as well as the younger mycelia usually need less time to mark the nuclei. Some thicker cell wall species demand incubation with vacuum for dye infiltration. This can be done using a vacuum pump linked to a hermetically sealed container (Fig. 11.1d). In these cases, it is recommended to use younger cultures and colony border regions.
6. Observe in inverted EFM or LSM.

If the LSM is equipped with a detector like T-PMT, the bright-field images acquired overlaid with fluorescent nuclei may dispense the need of other fluorochromes for fungi cell walls marking, such as Calcofluor White. Sometimes, DAPI colors well the cell walls also. Once the LSM is not equipped for bright field images, the Calcofluor White use is recommended. For this, the DAPI needs to be applied before and directly in the culture fragment. After this, 10 μL of the 0.01% (p/v) Calcofluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2 are positioned above coverslip, as described in step 2. Follow, the colony fragment DAPI-treated should be positioned upside-down above the Calcofluor drop and incubate for around 20 min in darkness before observation. The excitation/emission parameters are the same used for DAPI in EFM or LSM.

11.3.4 Slide and Coverslip Preparation

Below is a procedure to observe fungi nuclei using slide and coverslip (vertical or inverted microscope, EFM or LSM):

1. Put 10 μL of DAPI- VectaShield mounting media above a previously cleaned slide.
2. Sample fungi structures from fresh or fixed cultures using a needle previously cleaned with alcohol and flamed. Choose the region of interest using a stereoscopic microscope, if needed. In this case, the DAPI fluorescence signal in slides prepared with fixed structures may persist for weeks, if slides kept in the refrigerator, in darkness.
3. Transfer the specimens to DAPI drop.
4. Cover the drop with specimen with coverslip and seal with nail polish or other coverslip sealants.
5. Leave it in the dark for 5–20 min. The time may vary, as discussed above. A vacuum step may be required.
6. Observe in a vertical or inverted microscope, EFM or LSM.

11.4 Culture Block on Inverted Microscope and Propidium Iodide in Nuclei Marking

Propidium Iodide can be used as a fungi nuclei marker in the case of living cells (Fig. 11.1g). After the cell death, the fluorochrome passes through the nuclear envelope and occupies the cytosol, marking the entire cell. Thus, this marker is important in cellular death studies, as will be discussed later.

11.4.1 Materials

1. Propidium Iodide (Sigma-Aldrich, CAS-11348639001) work solution 1.0 μg. mL^{-1} prepared in 0.01 M PBS pH 7.2.
2. Fungi colony growth within a clear media, such as SNA, with living mycelia.
3. Scalpel.
4. Tweezer.
5. Automatic pipette.
6. Large coverslips (ex. 24 × 40 mm).
7. Stereomicroscope.
8. Inverted EFM or LSM.
9. For EFM, a filter cube that works near 500 ηm excitation and 630 ηm emission (e.g. Filter cube Chroma TxRed#39004).
10. For LSM, excitation with Argon 514 ηm laser line and emission filter with 620–660 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around fourfold zoom. The bright-field image was acquired using the TPM-T detector.

11.4.2 Method

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Put 10 μL of Propidium Iodide work solution (PIWS) above the coverslip. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed. Avoid the coverslip corners, where the objective lens does not reach. Develop it in a room with diffuse light, or as dark as possible. It is recommended to test PIWS dilutions, as it is possible that concentrations as 0.1 and 0.01 $\mu g.mL^{-1}$ may provide good results depending on the case.

3. Take colony fragments with around 0.5 × 0.5 cm with a scalpel, choosing the region of interest using a stereoscopic microscope, if needed. The colony fragments should be fresh.
4. Transfer the upside-down colony fragment above the PIWS drop. The mycelia should be in contact with the dye.
5. Leave it in the dark for 15–20 min.
6. Observe in inverted EFM or LSM.

11.5 Sample Preparation to Observe Fungi Chromosome

Karyotyping involves characterize the number, morphology, and size of chromosomes of a species, and the main methods used for fungi are pulsed-field gel electrophoresis (PFGE) and germ tube burst method (GTBM) [28]. The techniques are important to determine the polymorphisms in the size and number of chromosomes between strains from the same species, or even extra chromosome presence. The study of these chromosomes is very important, since they may contain pathogenicity-essential genes of fungi to cause plant diseases [9]. Following, the GTBM method for *Colletotrichum* chromosome observation (Fig. 11.1h).

11.5.1 Materials

1. Petri dishes with PDA culture media.
2. PD liquid culture media.
3. Sterilized 40 μm miracloth filter.
4. Autoclaved 1.5 mL microtubes.
5. Centrifuge for microtubes.
6. Neubauer chamber.
7. Bunsen burner.
8. Tweezers.
9. Mili-Q water.
10. Sterilized distilled water.
11. Autoclaved slides.
12. Coverslips.
13. Poly-L-lysine.
14. Rubber glue.
15. Automatic pipette.
16. Wet chamber.
17. Incubator adjusted for 22 °C.
18. Bright field microscope.
19. Thiabendazole (TBZ) solution 50 $\mu g.mL^{-1}$.
20. Methanol.

21. Glacial acetic acid.
22. DAPI 1 μm.mL^{-1} and VectaShield mounting medium.
23. Propidium Iodide 1 μm.mL^{-1}.
24. Nail polish.
25. EFM or LSM.
26. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (e.g. Filter cube Zeiss #49). (A 100× objective was used on an inverted EFM).
27. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range.

11.5.2 Method

The following method was refined by Gonçalves [11], adapted from Taga et al. [24], to observe chromosomes from *Colletotrichum* species.

1. Growth of fungi in PDA at 22 °C in darkness. After sporulation, the conidia suspension is acquired with mili-Q water and scraping and collected with an automatic pipette.
2. The suspension is filtered with miracloth 40 μm filter and centrifuged within microtubes at 3000–3500 *g* for 5 min.
3. After centrifugation, the supernatant should be discarded and the pellet washed with sterile water twice. Resuspend the pellet in a nutrient medium and adjust the concentration to 2 to 3 × 10^6 conidia.mL^{-1} using a Neubauer chamber.
4. The previously autoclaved slides are treated with poly-L-lysine solution and marking a rectangle on the slide with the rubber glue.
5. Pipetting 450–600 μL conidia solution into the rectangle marked with the rubber glue on the face of the poly-L-lysine slide.
6. Place the slides in a humid camera and incubate them in the dark at 22 °C. Monitor the germination after 6 h of incubation using a light microscope, prolong the incubation time if necessary.
7. Remove the liquid excess from the slide surface without drying out completely.
8. Add 400–600 μL of the nutrient medium containing thiabendazole (TBZ) at the final concentration of 50 μg.mL^{-1} to stop mitosis during metaphase.
9. Incubate the slide at 22 °C in the dark for 2–3 h, then remove the TBZ solution using an automatic pipette and remove the rubber glue using tweezers.
10. Slowly immerse the slide in mili-Q water to wash off excess TBZ that may still be present on the blade and remove excess water using a filter paper, but still leaving the slide moist.
11. Dry the slide by passing it over a flame quickly, without letting it dry completely or overheating the slide.
12. Add 20–25 μL of the propidium iodide 1 μg.mL^{-1} on the slide surface and wait 15 min. After, immerse the slide in sterile water to remove the dye excess.

13. Add 15–20 μL of the VectaShield mounting medium with DAPI and incubate in the dark for 10–15 min and then seal the slide with coverslip and nail polish.
14. Observe in EFM or LSM.

The DAPI-VectaShield mounting media may be used instead of the DAPI and VectaShield mounting media separated.

11.6 Sample Preparation to Observe Fungi Cell-Wall

The fungi cell wall staining with fluorochromes is very important in many studies, such as during infection processes in plant tissues, delimitating the septa during the nuclei dynamic studies, and associated with other methods, such as immunolabeling or hybridization. Calcofluor White is a commonly used dye for glucans, as the fungi chitin and the plant cellulose. On the other hand, AlexaFluor 488® WGA Conjugate (Alexa488-WGA) marks exclusively fungi. Thus, fungi-plant interaction can be studied with both used together, Alexa488-WGA to dye fungi and Calcofluor marking plant tissues, as will be discussed later.

11.7 Calcofluor White Staining

This fluorochrome dyes the fungi cell wall (Fig. 11.2a) usually with a short time incubation, around 10–30 min, depending on species and cell type. The procedure below was performed with a 7-days-old *Pyricularia oryzae* colony.

11.7.1 Materials

The necessary materials can vary depending on the protocol used. The samples can be mounted in slide-coverslip with fungal structures in 0.01 mg.mL^{-1} CalcoFluor in a vertical microscope or with culture block facing-dawn above 10 μL of 0.01 mg. mL^{-1} CalcoFluor in large coverslip on an inverted microscope, as discussed in DAPI methods. The excitation and emission conditions are also similar.

11.8 AlexaFluor 488® WGA Conjugate Staining

Alexa488-WGA labels the fungi cell wall (Fig. 11.2b) and the time incubation may vary depending on species and cell type. Another very important point is that some fungi with thick conidia cell walls such as *Pyricularia* and *Alternaria* require a

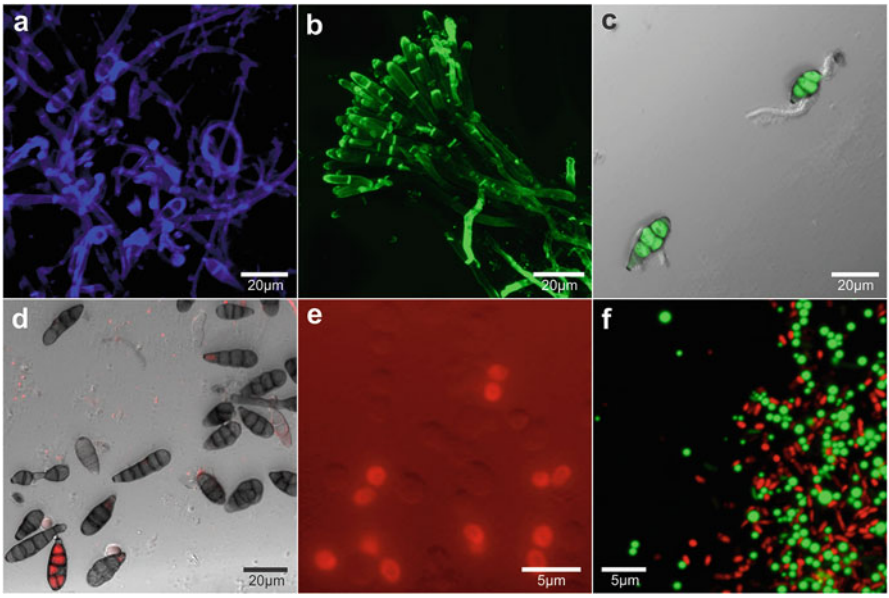

Fig. 11.2 Fungi structures were fluorochrome-stained. (**a**–**b**) Fungi cell wall dyed with CalcoFluor White (**a**) and AlexaFluor488–WGA (**b**). (**c**) Reactive oxygen species labeled with DCF-DA. (**d**–**e**) Cellular-death observation using Iodide Propidium for conidia of filamentous fungi (**d**) and yeasts (**e**). (**f**) Live-dead test for yeasts and bacteria using Iodide Propidium for dead (red) and Syto9 for living cells (green)

vacuum-infiltration step for 20 min to efficient dyeing [13]. Fungus with thin cell walls such as *Fusarium* can be marked without vacuum step. The method shown was made with common bean leaves infected with fungus.

11.8.1 Materials

1. Wheat Germ Agglutinin (WGA) AlexaFluor 488® Conjugate (Alexa488-WGA) (ThermoFischer, CAT-W11261) 10 µg.mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
2. 0.01 M PBS pH 7.2.
3. Scalpel, tweezers, and scissors.
4. Sterilized 96-well ELISA plates.
5. Vacuum pump linked to a hermetically sealed container.
6. Clean coverslips.
7. Glass piece (2 × 2 × 1 cm).
8. Aluminum paper.
9. Automatic pipette.
10. Clean tips.

11. Large coverslips (e.g. 24 × 40 mm).
12. Inverted EFM or LSM.
13. For EFM, a filter cube that works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE).
14. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range, for Alexa 488. A Plan-Apochromat 63×/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution.

11.8.2 Method

1. Sampling of common bean leaves infected with fungus and cutting in 4 × 4 mm pieces.
2. After, the leaves fragments are place within Elisa plates with 10 μg.mL^{-1} Alexa488-WGA.
3. Wrap the Elisa plate with aluminum foil and keep it at vacuum (Fig. 11.1d) for 1 h.
4. Wash with PBS.
5. Put a leaf fragment above the previously cleaned large coverslip on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. Put the leaf fragment above the coverslip with the region of interest facing down.
7. Place a glass piece above the sample (to minimize the irregular topography of the sample).
8. Observe in inverted EFM or LSM.

11.9 Reactive Oxygen Species (ROS) in Fungi

Reactive oxygen species (ROS) are ubiquitous in fungi living cells, with high damaging potential but are also essential for gene expression signaling and development in several biological processes [14] such as the programmed cellular death [10]. One way to study the ROS dynamics is using the 2′,7′-Dichlorofluorescein diacetate (DCF-DA) fluorochrome. DCF-DA dyes several ROS, as hydrogen peroxide (H_2O_2), peroxyl (ROO•), hydroxyl (HO•), peroxynitrite anion (ONOO-) and nitric oxide (•NO), and it's useful for assessing various stress types in plants, such as osmotic, thermal, and by pathogen infection [2, 13, 19, 29]. Another fluorochrome variant, H_2DCFDA, was important to understanding the ROS role during rice blast pathogenesis [6]. The strobilurin resistance in *Alternaria alternata* is caused by a non-synonymous mutation that changes the cytochrome b product, replacing a glycine by an alanine at the condon 143 (G143A mutation) [5]. Resistant strains remain with low DCF-DA signal when cultivated in PDA media plus high concentration fungicide (Fig. 11.2e), while the wild type presents a high signal (not shown).

11.9.1 Materials

1. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) (Sigma, CAS-4091-99-0) 10 μM in filtered dimethyl sulfoxide (DMSO).
2. Conidia suspension without fixation.
3. Water-Agar 1.5% in thin layer on a Petri dish.
4. Kolle handle with a needle.
5. Scalpel.
6. Tweezer.
7. Automatic pipette.
8. Slides and regular coverslips (e.g. 20 × 20 mm).
9. Large coverslips (e.g. 24 × 40 mm).
10. Nail polish or other coverslip sealant.
11. EFM or LSM, vertical or inverted.
12. For EFM, a filter cube that works near 488 ηm excitation and 525 ηm emission (ex. Filter cube Zeiss #38HE).
13. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution. The bright-field image was acquired using the TPM-T detector.

11.9.2 Methods

11.9.2.1 Conidia Suspension

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 10 μL of conidia suspension above the WA block.
3. Wait around 10 min for decanting.
4. Apply 10 μL of DCF-DA 10 μM above the WA block, and leave for 5 min in darkness.
5. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
7. Take the WA block with a dyed sample using a scalpel and transfer the upside-down block above the coverslip.
8. Observe in inverted EFM or LSM.

This procedure may be done for observation in a vertical microscope, using slide-coverslip preparing, mixing 5 μL sample suspension and 5 μL DCF-DA. In this case,

the fluorochrome concentration will be diluted, and maybe a higher concentration may be necessary.

11.10 Fungi Cellular Death Studies with Propidium Iodide

Experiments with cellular death marking of fungi can help to characterize phenotypes in many contexts, such as confirming the fungicide activity of a control product, instead fungistaticity. Another possible subject is the the programmed cell death (PCD), which may occur during the sexual and asexual reproduction, in some infection processes for phytopathogenic fungi, and in the non-self-recognition mechanism heterokaryon incompatibility (HI) [10]. The strobilurin resistance of *Alternaria alternata* is caused by the G143A change in cytochrome b [5], and low mortality of conidia when cultivated in PDA media plus high fungicide concentration (Fig. 11.2d). Other assay was for yeast mortality observation during a fermentation process (Fig. 11.2f). The cell-death marker used was Propidium iodide [8].

11.10.1 Materials

1. Propidium iodide (Sigma, CAS-25535-16-4) 1.0 μg.mL^{-1} prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
2. Fungi conidia suspension without fixation.
3. Yeast suspension from a fermentation process.
4. Water-Agar 1.5% in a thin layer on a Petri dish.
5. Kolle handle with a needle.
6. Scalpel.
7. Tweezer.
8. Automatic pipette.
9. Slides and regular coverslips (e.g. 20 × 20 mm).
10. Large coverslips (e.g. 24 × 40 mm).
11. Nail polish or other coverslip sealants.
12. EFM or LSM, vertical or inverted.
13. For EFM, a filter cube that works near 543 ηm excitation and 645 ηm emission (ex. Filter cube Zeiss #45). A 100× objective was used on an inverted EFM – yeast suspension.
14. For LSM, excitation with HeNe 543 ηm laser line and emission filter with 625–665 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution – *Alternaria alternata* conidia suspension. The bright-field image was acquired using the TPM-T detector.

11.10.2 Methods

The methods presented below are for filamentous fungi or yeast cell-death marking, and variations will be considered.

11.10.3 Conidia or Yeast Suspension

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 10 μL of conidia or yeast suspension above the WA block.
3. Wait around 10 min for decanting.
4. Apply 10 μL of Propidium iodide 1.0 $\mu g.mL^{-1}$ above the WA block, and leave for 5 min in darkness.
5. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
7. Take the WA block with dyed sample using a scalpel and transfer the upside-down block above the coverslip.
8. Observe in inverted EFM or LSM.

This procedure may be done for observation in vertical microscope, using slide-coverslip preparing, mixing 5 μL sample suspension and 5 μL Propidium iodide. In this case, the fluorochrome concentration will be diluted, and maybe higher concentration may be necessary.

11.11 Live-Dead Test for Fungi Cells

The methods presented below are for yeasts and bacteria, and variations will be considered. In Fig. 11.2f, observation of yeasts and bacteria from a probiotic product was analyzed. The following protocol was developed based on Stiefel et al. [23] and Batista et al. [1].

11.11.1 Materials

1. Propidium iodide (Sigma, CAS-25535-16-4) 1.0 $\mu g.mL^{-1}$ in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
2. Syto9 (Thermo Fischer-S34854) 20 μM in filtered DMSO.

3. Probiotic yeasts and bacteria suspension.
4. Water-Agar 1.5% in a thin layer on a Petri dish.
5. Kolle handle with a needle.
6. Scalpel.
7. Tweezer.
8. Automatic pipette.
9. Slides and regular coverslips (e.g. 20 × 20 mm).
10. Large coverslips (e.g. 24 × 40 mm).
11. Nail polish or other coverslip sealants.
12. EFM or LSM, vertical or inverted.
13. For EFM, a filter cube that works near 543 ηm excitation and 645 ηm emission (ex. Filter cube Zeiss #45) in Propidium iodide localization; filter cube to works near 488 ηm excitation and 535 emission (ex. Filter cube Zeiss #38HE) in Syto9 working.
14. For LSM, excitation with HeNe 543 ηm laser line and emission filter with 625–665 ηm range in Propidium iodide localization; excitation with Argon 488 ηm laser line and emission filter with 520–550 ηm range in Syto9 observation. (Plan-Apochromat 63×/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around twofold zoom).

11.11.2 Method

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 5 μL of yeast suspension above the WA block.
3. Wait around 10 min for decanting.
4. Above the same surface apply 5 μL of Syto9 20 μM and leave for 40 min in darkness.
5. After, apply 10 μL of Propidium iodide 1.0 $\mu g.mL^{-1}$ above the WA block, and leave for 5 min in darkness.
6. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
7. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
8. Take the WA block with a dyed sample using a scalpel and transfer the upside-down block above the coverslip.
9. Observe in inverted EFM or LSM.

This method may be adapted also for filamentous fungi and other organisms, adjusting the concentration and the time incubation. The Syto9 fluorochrome dye all cells and the Propidium iodide the dead cells only. Thus, the merged green and red channels will show the living cells green and the dead cells in red color

(Fig. 11.2f). If the Syto9 is not available, it may be done using only the Propidium iodide, merging the red channel with the bright field image (Fig. 11.2d).

11.12 Sample Preparation to Observe Fungi-Plant Interactions

Below, a protocol for staining plant tissues with Calcofluor White and fungi with Alexa488 WGA. Before the staining steps, a clarification protocol was develop based on Warner et al. [26] and Minker et al. [18]. Common bean leaves infected with phytopathogenic fungus were used (Fig. 11.3).

11.12.1 Materials

1. CalcoFluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) 0.01 mg. mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
2. Wheat Germ Agglutinin (WGA) AlexaFluor 488® Conjugate (Alexa488-WGA) (Thermo Fischer, CAT-W11261) 10 µg.mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
3. Clarifying solution (6 M Urea, 30% Glicerol, 0.01% Tween 20).
4. 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
5. Potassium hydroxide (KOH) solution 10% (p/v).
6. Scalpel, tweezers and scissor.
7. Sterilized 96-well ELISA plates.
8. Vacuum pump linked to a hermetically sealed container.
9. Clean coverslips.
10. Glass piece (2 × 2 × 1 cm).
11. Aluminum paper.
12. Automatic pipette and clean tips.
13. Large coverslips (e.g. 24 × 40 mm).
14. Incubator.
15. Inverted fluorescence microscope.
16. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49) to CalcoFluor, and filter cube to works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE) to Alexa 488.
17. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range, for CalcoFluor; excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range, for Alexa 488. A Plan-Apochromat 63x/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution.

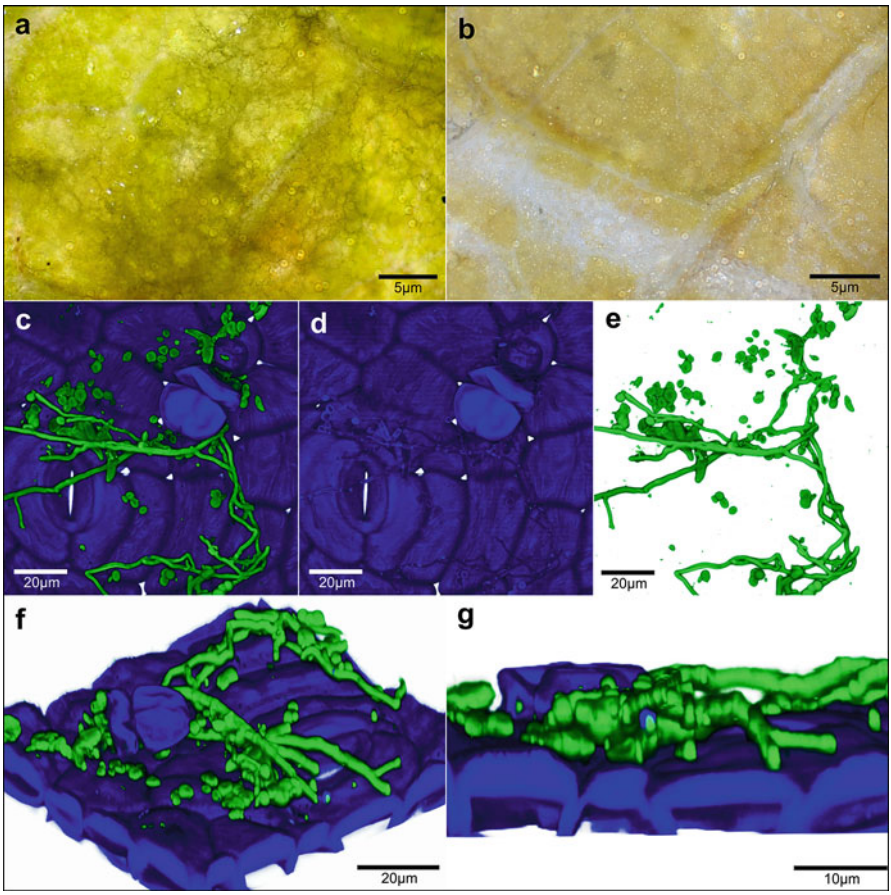

Fig. 11.3 Fungi-Plant interactions. Common bean leaf surface before (**a**) and after (**b**) clarifying treatment. (**c**–**g**) Common bean colonized superficially by fungi. Plant tissue dyed with CalcoFluor and fungi marked with AlexaFluor 488 – WGA. (**c**) 2D overlay of blue and green channels from 98 focal plans within 30 μm range in Z-axis using the maximum intensity projection method. (**d**–**e**) 2D of separated channels. (**f**–**g**) 3D software reconstruction from focal plans acquired

11.12.2 Methods

1. Sampling of common bean leaves infected with phytopathogenic fungus and cutting in 4 × 4 mm pieces.
2. After, the leaves fragments are placing within Elisa plates well containing KOH 10%, following incubation at 10 °C for 4 days in darkness.
3. Wash with PBS and replace for a new KOH 10% solution.
4. Other incubation steps at the same conditions.
5. PBS wash and transfer fragments to Elisa wells with clarifying mix [Urea 6 M, Glycerol 30% (v/v) and Tween 20 0.05% (v/v)].

6. After 4 days in clarifying mix at 10 °C and darkness, wash with PBS, and incubate in a new clarifying mix for 4 days.
7. Wash with PBS, and add 100 μL Alexa488–WGA 10 $\mu g.mL^{-1}$.
8. Wrap the Elisa plate with aluminum foil and keep it at vacuum (Fig. 11.1d) for 1 h.
9. Add 80 μL of CalcoFluor 0.01 $mg.mL^{-1}$ and incubate for 10 min at the darkness.
10. Put a previously cleaned large coverslip on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
11. Put the leaf fragment above the coverslip with the region of interesting facing down.
12. Place a glass piece above the sample (to minimize the irregular topography of the sample).
13. Observe in inverted LSM.
14. In this assay, the z-stack method was used to capture 98 focal plans within 30 μm ranges in Z-axis, to obtain the 2D with the maximum intensity method and the 3D reconstruction, using the Zen software (Carl Zeiss).

The clarifying protocol showed was for leaf tissues. Adjustments are needed depending on the tissue type and the plant species, changing the incubation time. For example, the common bean pods can be clarified with 20 days in KOH 10% and 15 days in clarifying mix, changing the solutions every 5 days of incubation.

The importance of the clarification step is the high amount of autofluorescent compounds such as cell wall components, phenols, and alkaloids [21]. Thus, many protocols have been developing to minimize these fluorescence signals and optimize the specific fluorescent label [18, 25, 26]. The transparency increasing in plant tissues improves the three-dimensional structures imaging quality, especially valuable during the investigation of plant–fungi interactions, once it provides better fluorochrome infiltration, photon and or laser penetration, and so high quality of fluorescence signal [18]. Other clarification protocol with very satisfactory results was developed by Ursage et al. [25] which uses the *ClearSee* solution [xylitol 10% (w/v); sodium deoxycholate 15% (w/v); urea 25% (w/v)] in the clarification and for fluorochromes preparing. The *ClearSee* method also has the advantage of allowing the fluorescent proteins such as GFP and m-Cherry observation in plant tissues even after treatment. In cases where the sample preparation evolves alcohol gradients, for embedding and microtome cutting, clarification is not necessary. And, for sure, for non-autofluorescent samples.

Fungi-plant interactions fluorochrome-based studies can be made using other dyes combinations. Ha et al. [13] observed the wheat tissues colonization by *Pyricularia graminis-tritici* using Alexa488-WGA and *Fusarium graminearum* genetically transformed for GFP expression. In both cases, the fixed plant tissues were dyed with Propidium iodide. The plant cell wall marker, pontamine fast scarlet (S4B), is specific for cellulose and may be used combined with other fluorochromes, such as aniline blue, which label callose [7].

11.13 Important Plant Structures Defence Against Phytopathogenic Fungi

The plant resistance factors against phytopathogens are divided into biochemical and structural, preformed or postformed. Through histochemical analysis, some structural resistance components such as callose, lignin, and cuticle, and biochemical, as ROS, can be evaluated by microscopy techniques. Then, callose and lignin protocols are shown.

11.14 Callose Deposition

The callose polymer (β-1,3-glucan) is a plant cell wall component and may plant defense responses, and their deposition may increase in response to infections or resistance-inducing agents [7, 16]. Aniline blue is the fluorochrome used in callose deposition studies, such as in papillae observation [7]. Figure 11.4a shown callose deposition marked with aniline blue in tangerine leaf.

11.14.1 Materials

1. Aniline blue diammonium (Sigma, CAS-415049) 0.1 mg.mL^{-1} in PBS 0.01 M pH 7.2.
2. Tangerine leaves fragments with around 0.5 × 0.5 cm were previously fixed in 4% paraformaldehyde and clarified as seen before for common bean leaves.

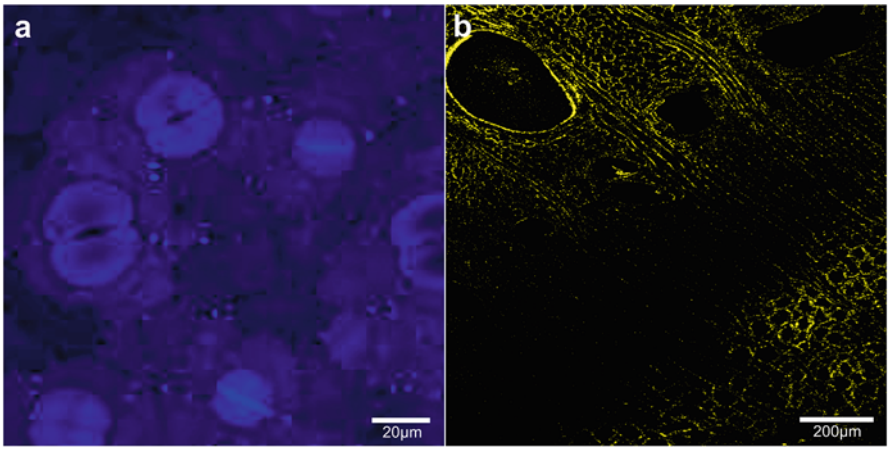

Fig. 11.4 (**a**) Callose deposition in tangerine leaf observed with Aniline blue fluorochrome. (**b**) Lignin localization in *Eucalyptus* cambial region using Auramine-O

3. Scalpel, tweezers, and scissors.
4. Sterilized 96-well ELISA plates.
5. Vacuum pump linked to a hermetically sealed container.
6. Clean coverslips.
7. Glass piece (2 × 2 × 1 cm).
8. Aluminum paper.
9. Automatic pipette and clean tips.
10. Large coverslips (e.g. 24 × 40 mm).
11. Inverted fluorescence microscope.
12. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49).
13. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range. The 40× objective was used with zoom resource.

11.14.2 Method

1. Take the clarified leaf fragment and incubate samples within Elisa plate wells in 80 µL Aniline blue 0.1 $mg.mL^{-1}$.
2. Wrap the Elisa plate with aluminum foil and keep it at vacuum for 1 h.
3. Incubate overnight at around 25 °C.
4. Wash with PBS.
5. Transfer the abaxial side to a large coverslip supported in the microscope stage.
6. Place the glass piece on a sample.
7. Observe with EFM or LSM the leaf surface (In the case of LSM, paradermic examination is possible).

11.15 Lignin Localization in Plant-Fungi Interactions

Lignification has an important role in plant defense, in resistance to fungi mechanical penetration, and participating in gene expression signaling and metabolic pathways of the plant resistance [3]. The usual fluorescent markers in lignin labeling are Auramine-O [22], which dyes cutin also, and Basic fucsin [25], which dyes lignin only. In Fig. 11.4b, a cross section of *Eucalyptus* cambial region with lignin localization using the Auramine-O.

11.15.1 Materials

1. Auramine-O (Merck, CAS-2465-27-2) 1.0 mg.mL^{-1} work solution.
2. *Eucalyptus* cross sections of 7 μm thickness prepared in a microtome, in pith to bark direction.
3. Slides and coverslips.
4. Nail polish or other coverslip sealants.
5. Automatic pipette and tips.
6. EFM or LSM.
7. For EFM, a filter cube that works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE).
8. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range. An EC Plan-Neofluar 10x/0.30 M27 was used.

11.15.2 Method

1. Take the slides with sections and apply around 5 μL of Auramine-O 1.0 mg.mL^{-1} to cover all sample areas.
2. Incubate in darkness for 20–40 min.
3. Wash gentlly with PBS.
4. Cover with coverslip and seal.
5. Observe in an inverted or vertical microscope, EFM or LSM.

11.16 Sample Preparation to Observe Autofluorescent Fungi and Specific Structures

11.17 Autofluorescent Rust Fungi

Several Basidiomycota presents autofluorescence such as some mushroom producers and rust fungi [30, 31]. Its feature permits localization in the environment and plants. For *Hemileia vastatrix* and many other rust fungi, autofluorescence can be observed at the green or blue emission spectra. Here, we observed the autofluorescence of *H. vastatrix* pustules in coffee leaves (Fig. 11.5a).

11.17.1 Materials

1. Coffee leaf with rust fungi *Hemileia vastatrix* pustules.
2. Incubator at 20 °C.

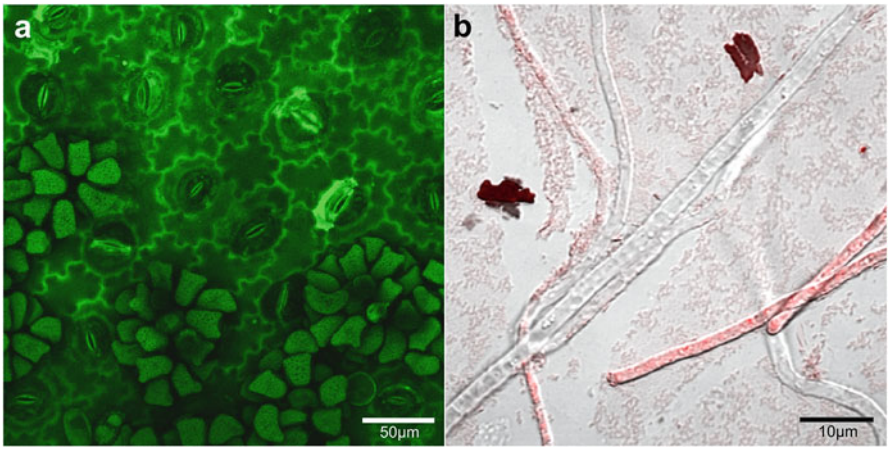

Fig. 11.5 (**a**) Autofluorescence of *Hemileia vastatrix* pustules in coffee leaves. (**b**) Autofluorescence of cercosporin crystals produced by *Cercoscopa coffeicola* in MEA

3. Dissecting scissor.
4. Tweezer.
5. Large coverslips (e.g. 24 × 40 mm).
6. Inverted EFM or LSM.
7. For EFM, a filter cube that works near 488 ηm excitation and 520 ηm emission (ex. Filter cube Zeiss #38 HE).
8. For LSM, excitation with 488 ηm laser line and emission filter with 490–560 ηm range. An LCI Plan-Neofluar 25×/0.8 Imm Korr DIC M27 objective was used, with 512 × 512 resolution.

11.17.2 Method

1. Put a previously cleaned large coverslip (ex. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Positioning a leaf fragment with pustules directed to an objective lens (upside-down). Small fragments, as 1 × 1 cm are desirable to minimize the irregular topography of the sample, as well as avoid the midrib.
3. Use a piece of glass as a weight to minimize the irregular topography of the sample.
4. Observe in inverted EFM or LSM.

11.18 Autofluorescent Cercosporin

The autofluorescence may be useful information to detect certain fungi metabolites and other endogenous fluorophores [15]. The cercosporin crystals produced by *Pseudocercosporella capsellae* are detectable at 561 nm excitation condition using a laser confocal microscope [12]. In the method described below will be shown the location of cercosporin crystals produced by *Cercoscopa coffeicola* in vitro (Fig. 11.5b).

11.18.1 Materials

1. *Cercoscopa coffeicola* colony growth in malt extract agar (MEA) 3 weeks at 20 °C.
2. Inverted EFM or LSM.
3. Scalpel.
4. Tweezer.
5. For EFM, a filter cube that works near 561 ηm excitation and 650 ηm emission (ex. Filter cube Zeiss #45).
6. For LSM, excitation with 543 ηm laser line and emission filter with 600–710 ηm range An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around three-fold zoom. The bright-field image was acquired using the TPM-T detector.

11.18.2 Method

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Take colony fragments with around 0.5 × 0.5 cm with a scalpel, choosing the region of interest using a stereoscopic microscope, if needed.
3. Transfer the upside-down colony fragment above the coverslip.
4. Observe in inverted EFM or LSM.

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