Visualization of Fungi During Wood Colonization and Decomposition by Microscopy: From Light to Electron Microscopy

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Chapter 17

Visualization of Fungi During Wood Colonization and Decomposition by Microscopy: From Light to Electron Microscopy

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Abstract

Fungi are the principal decomposers of wood together with xylophage insects and, as such, have a central role in nutrient cycling of forest ecosystems. These fungi are also envisaged as promising tools for converting wood and waste of wood industries into chemicals, as alternative to fossil chemicals. At the same time, wood decomposers pose a threat to wooden building materials and are intensively fought. As a consequence, intense researches have been conducted over the past 50 years to identify the fungi responsible for wood decomposition, the mechanisms by which they do so, the wood properties involved in resistance or sensitivity to attacks and ways to preserve woods. Many tools are now available to study fungal colonization of wood, including: "omics" techniques, enzymatic assays, spectrometry, etc. However, all these approaches provide bulk information and the data obtained by these methods contain no information on the localization of fungi, the stage of decomposition of the wood and the potential interactions between microorganisms. In these regards, microscopy approaches provide complementary information that can strengthen conclusions. The present chapter describes a diverse range of microscopy approaches, from simple bench light microscopy to confocal and electron microscopies, to shed light on the way fungi colonize wood tissues.

Key words Wood, Fungi, Reflected light microscopy, Confocal imaging, Electron microscopy, Correlative microscopy, Wood colonization, Wood decomposition

1 Introduction

Deadwood is key component in forest ecosystems because of its central position in organic and inorganic nutrient cycling, and because it provides habitat together with nutrient sources for a diverse range of organisms. If deadwood volume is typically low in managed forests where most wood is harvested, it can represent a carbon stock of a comparable or even greater size than that of standing tree biomass in natural forests [1]. Because of its physico-chemical properties, wood is resistant to the rapid colonization by microorganisms and represents a nutrient pool with a relatively

Francis Martin and Stephane Uroz (eds.), *Microbial Environmental Genomics (MEG)*,
Methods in Molecular Biology, vol. 2605, https://doi.org/10.1007/978-1-0716-2871-3_17,

slow turnover [2]. In the same time, wood is an ecological-used building material with growing interest whose mechanical properties must endure over time and should not be altered by microorganisms and insects. However, natural durability of wood against microorganisms and insects is highly variable as described in European standard [3]. Recently, wood has been considered as a very promising source of biomass for production of biofuels and biocommodities. Indeed, use of microorganisms to convert wood and wastes from the wood industry into chemicals as fossil alternative is the focus of intense and growing research efforts [4, 5]. These different reasons have prompted intense researches to identify (micro)organisms responsible for wood degradation, the mechanisms by which they do so and the wood properties that explain the different degrees of resistance to biological attacks over the past 50 years.

Wood is mainly made of cellulose and hemicelluloses embedded in lignin, with lignin protecting the polysaccharides from microbial decomposition [6, 7]. In addition, low-nitrogen (N) concentrations, low pH, accumulation of defensive toxic compounds (extractives), and occurrence of anatomical barriers such tylosis or torus prevent the attack of wood by most (micro)organisms [8, 9]. Apart from xylophage insects (i.e., termites and others) that decompose wood thanks to endosymbionts, fungi are the main microorganisms responsible for wood decomposition. Wood decomposing fungi are traditionally categorized in three groups: white rots, brown rots, and soft rots [9–12]. Even though this historical classification do not entirely reflect the complexity of the processes and the plasticity of fungal abilities, it holds true for major characteristics, in particular, the fact that if most of these fungi are able to depolymerize lignin, then only white rot fully convert it to CO_2 and H_2O [13, 14]. Yet, the relative rates of decomposition of lignin and cellulose vary greatly according to the white rot species of fungi and the conditions within the wood [15, 16]. Conversely, brown rot only fully decomposes hemicellulose and cellulose, and yields lignin residues with longer forest floor residence times and unique biogeochemical attributes that can be of interest for industries. Only 6% of all described wood decay fungi are known to cause a brown rot and most belong to the *Polyporaceae* [12]. They are predominantly associated with gymnosperms, whereas white rot fungi are associated with angiosperm trees. Soft rots appellation comes from the spongy texture of the wood surface they attack but these fungi, belonging to Ascomycetes and Deuteromycetes, are chemically more similar to brown rots than white rots since they decompose cellulose and hemicellulose while lignin is only modified slightly. However, the cavitation or erosion mechanisms and the corresponding enzyme repertoire used by these fungi to mine carbohydrates from the cell wall is less described [17].

Typical white rot degradation employs lignocellulolytic enzymes including cellulases, laccases, and manganese peroxidases [18–20]. By contrast, brown rot fungi employ a two chelated-mediated Fenton system that permits non-enzymatic deconstruction of cell walls based on oxidative reactions [21].

The number of fungal species capable of decomposing wood is not known exactly; however, reports indicate that there are ca. 1600 to 1700 species of wood-degrading fungi in the North America [22]. Wood decomposition in natural environments typically takes tens of years to complete and is characterized by the successive development of fungal communities with an initial dominance of decomposers, but also the support of ectomycorrhizal (ECM) fungi during late decay [23–25]. Early colonizers of wood are often ruderal opportunists arriving as spores, or endophytes latently present in functional sapwood and are dominated by Basidiomycetes while later colonizers, dominated by Ascomycetes, arrive as spores or via the soil as mycelium. Yet, the identity of primary colonizers together with interspecific interactions of fungi largely determines the establishment of later arriving species, a phenomenon referred to as the priority effect. The axial alignment of tracheids, vessels, and fibres and the radial arrangement of the xylem ray parenchyma facilitate access into the wood and allow widespread distribution of hyphae within the xylem [12]. Access to adjacent cells occurs via pit apertures, or direct penetration may take place directly through the cell wall. As the result of fungal decomposition, deadwood changes sequentially as following: (i) density decreases along with increasing water content, (ii) the concentration of lignin relative to wood dry mass increases while lignin concentration in relation to the deadwood volume decreases, and (iii) the concentration of nutrients like N, K, Ca, and Mg relative to wood mass often increases.

Many tools are available to study fungal colonization of wood either *in situ* or in controlled environments. Amplicon metabarcoding and cultivable approaches give access to the diversity and identity of wood colonizers. Metagenomics, (meta)transcriptomics, and enzymatic assays inform on the activities which are deployed by the fungi during wood colonization and degradation. However, all these approaches provide bulk information, and the data obtained by these methods contain no information on the localization of fungi and the stage of degradation of the wood. Spatial heterogeneity is high, even at scales smaller than a few centimetres for wood degrading fungi [26, 27]. Furthermore, it does not capture interactions between microorganisms. Finally, a spatially resolved experimental set-up can allow assessment of temporal patterns, tracking the sequence as fungi progressively unlock carbohydrates from lignocellulose. In these regards, microscopy approaches provide complementary information that can help interpretations. Depending on the goals, simple light microscopy

to more complex instrumentation like confocal or electron microscopy can be used to measure the level of colonization, the stage of degradation or analyze mechanisms of attacks and of interactions [12, 28–30]. Traditional light microscopy has the advantages of being fast, cheap, and of requiring a minimal set of equipment that is found in most laboratories [12]. It permits fast check of the level colonization of wood and it allows the qualitative monitoring of the colonization process up to the cell level. However, the level of resolution can be dampened by out of focus signals, leading to blurry images. This problem can be solved by using confocal microscopy (CLSM), which permits to acquire 3D views of samples. Sample preparation for confocal microscopy is rather minimal, as it only requires fungal staining. Additional information on decomposition processes can be obtained by coupling fungal staining with specific dyes or antibodies that target cell wall components [31–34]. Investigation of fungal wood colonization by fluorescence microscopy techniques can also take advantage of the changes in autofluorescence of the wood compounds by using spectral or sequential modes on confocal microscope [35]. Last, scanning electron microscopy (SEM) allows for reaching additional levels of comprehension of the colonization process by giving access to the nanometre scale and revealing complementary features that cannot be visualized by optical microscopy (e.g., bacteria, spores, oxalate crystals, surface alteration of cell walls, visualization of hyphae through bordered pits, etc.). In addition, SEM equipped with field emission gun (FEG) can be used to obtain high-resolution pictures (e.g., fungal bacterial interactions, visualization of changes in cell wall porosity due to fungal decaying activity, etc.). Moreover, the coupling between SEM imaging and X-ray microanalysis provides information on certain physiological processes. Yet, identification of structures on SEM images can be tricky since no dye can be used to highlight specific compounds or tissues. Thus, it may require a good knowledge of wood and fungal structures. Correlative confocal/SEM microscopy (CLEM) can be valuable to solve this problem by combining on the same image information obtain with CLSM and SEM. CLEM is a well-established and widely used technique in several research fields in biology which is emerging in wood science (Loussert Fonta and Humbel [36] and references inside). Since wood is a fibrous and porous material, imaging the same area of a sample with both SEM and CLSM methods and overlaying the images in live offers a valuable approach to distinguish between tissues and dissect biological events at sub micrometre scale.

In this chapter, we describe a set of methodologies to track fungal colonization of wood from samples taken in the field or produced in more controlled environment and we provide

guidance on how to use and adapt the methods. A summary of the methods described, usage, and advantages is given in Table 1 and the general workflow in Fig. 1. The experimental methodologies given here are generic and may need to be adapted to specific conditions depending on the microorganisms studied, the type of wood, and the imaging methods chosen.

2 Materials

Biological Material

1. Wood sticks (*in situ* studies).
2. Wood chips/wafers/sections (*in vitro* studies).
3. Fungal cultures (*in vitro* studies).

Media

1. Malt agar (*in vitro* studies).

Reagents

- *All techniques except dark field and SEM images*

  Phosphate Buffer Saline 10× pH 7.4 (PBS 10×, 1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4): NaCl 15.2 g, Na2HPO4 2 g, NaH2PO4, 0.94 g, add 200 mL ultrapure water qsp 200 mL. Adjust pH 7.4

  PBS 1×. Dilute PBS 10× in ultrapure water (1/10th)

- *Wide field light microscopy*

  Trypan Blue (Merk, catalog number: T6146).

  Safranin O (Merk, catalog number: S2255).

  KOH 10%.

  30% H_2O_2.

  10% HCl.

  Lactoglycerol: Lactic acid:glycerol:distilled water (1:2:1).

  Glycerol.

  Distilled water.

- *Fluorescence microscopy*

  Wheat Germ Agglutin-Alexa Fluor 488 (Thermo-Fisher catalog number: W11261)

  Tween 20 (Merk, catalog number: P1379)

  20% glycerol solution or anti-fading mounting solution

  Nail polish

Table 1
List of methods described in the chapter, purpose of use, limitations, and references of the machines used for illustrations

Method	Type of samples	How does it work?	For which purpose?	Foreseen limitations	Notes	Specifications of the equipment required	Equipments used to generate figures
Dark field episcopy	Wood block (1 cm^3), wood chips, thin sections	Reflected light, couples live multi-focus image computation	Allows for searching for fungal hyphae in wood structure without preparation	Rough imaging	Best results are obtained on dry samples because of the light diffraction caused by wood free water is suppressed	Dark field reflector	Leica DM 2700 M microscope equipped with an ICC50 camera
Wide field transmitted light microscope	Thin sections	Use color stains that reveal the fungi	Fast and cheap to observe the level of colonization	Rough imaging	Wood thin sections have to be bleached and hyphae must be stained	NA	Olympus CX31 microscope equipped with an Olympus Color view III CCD camera
Epifluorescence microscopy	Thin sections, wood particles, chips or surface of small blocks	Combine fluorescent dye to reveal fungal cells and wood autofluorescence	To observe the level of colonization with higher definition than transmitted light	Limited resolution in 3D	Autofluorescence can be problematic for imaging	Filter cubes and lasers for WGA Alexa fluor 488 (exc: em) and wood auto-fluorescence (DAPI/UV exc 335–425 nm; em long path >420 nm or RFP exc 620, em long path >660 nm)	Leica DM 2700M microscope equipped with a DFC7000T CCD camera and a XYZ scanning stage, filter cubes L5 (EX = 480/40, LP505 dichroic, BP527/30) or I3 (EX = 450–490,LP510 dichroic, LP515) for Alexa Fluor 488; Y5 filter (EX = 620/60,LP660 dichroic, BP700/75) or DAPI (EX = 335–425, LP400 dichroic, LP470) for wood-auto-fluorescence (polyphenol compounds)
Laser scanning confocal microscopy (CLSM)	Thin sections, wood particles, chips or surface of small blocks	Combine fluorescent dye to reveal fungal cells and wood autofluorescence	Imaging in 3D of fungal colonization, possibility to make quantitative measurements	Spatial resolution can be limiting, little information on structural and morphological context of the hyphae, inorganic elements cannot be visualized, fine phenotypic characterization of the fungal response to a	Spectral deconvolution can be very handy to obtain high-quality images and reduce the background noise due to the auto-fluorescence of wood tissues and instead take	Standard imaging: confocal equipped with 405 nm or 561 nm and 488 nm laser beam lines. Deconvolution mode : confocal equipped with white-light laser technology or with	Zeiss LSM 780 equiped with highly sensitive spectral detector GaAsp of 32 channels, laser lines : 405 nm, 488 nm, 561 nm, 633 nm

				particular environment (wall thickness, spore ornamentation,...) may be difficult	advantage of this auto-fluorescence.	a highly sensitive spectral detector GaAsp of 32 channels, laser lines : 405 nm, 488 nm, 561 nm, 633 nm.	
Scanning electron microscopy (SEM)	Thin sections, wood particles, chips or surface of small blocks	Visualize local differences in electron density.	Ideal technique to describe cellular morphology and environment	It might be difficult to distinguish directly specific types of molecules or even between hyphae and cellulose fibrils	The main benefits of this technique are its high depth of field and the high-spatial resolution (no limitation by optical constraints)	Basic imaging: standard tabletop SEM equipped for backscattered electronic detection. Resolutive imaging: electron detectors for imaging in controlled pressure mode (backscattered electrons and secondary electrons detectors), and in high vacuum mode (backscattered electrons, secondary electrons in chamber, In-lens secondary electron detectors)	Tabletop Microscope TM3000 (Hitachi) equipped with secondary electron detector (basic tabletop SEM) and SIGMA HD-VP Zeiss (Resolutive imaging SEM)
Field Emission Gun (FEG) Scanning Electron Microscope	Thin sections, wood particles, chips or surface of small blocks	Electron beam with a higher brightness and a smaller diameter	To perform very accurate observations of sample with low impact energy and thus to perform microanalysis and observations at high resolution (10 nm)		This is particularly relevant to observe sample with low current beam and voltage (protection of sensible samples)	In-lens secondary electron detectors in high vacuum mode for high resolution and high magnification observations, Cryo-Sas and cryo-stage (−160°;+60 °C) to insert and maintain cryo-fixed samples in cryo-mode at high vacuum	SIGMA HD-VP Zeiss
Correlative Imaging CLSM/SEM (CLEM)	Small dry samples (2–5 mm^2)	Imaging the same area with both SEM and CLSM	Combine the levels of information of CLSM and SEM	Difficulties to set up, requires expensive equipments that are not frequent in labs		Indexed sample holder to manage correlative microscopy with CLSM equipment	SIGMA HD-VP Zeiss/LSM 780 Zeiss

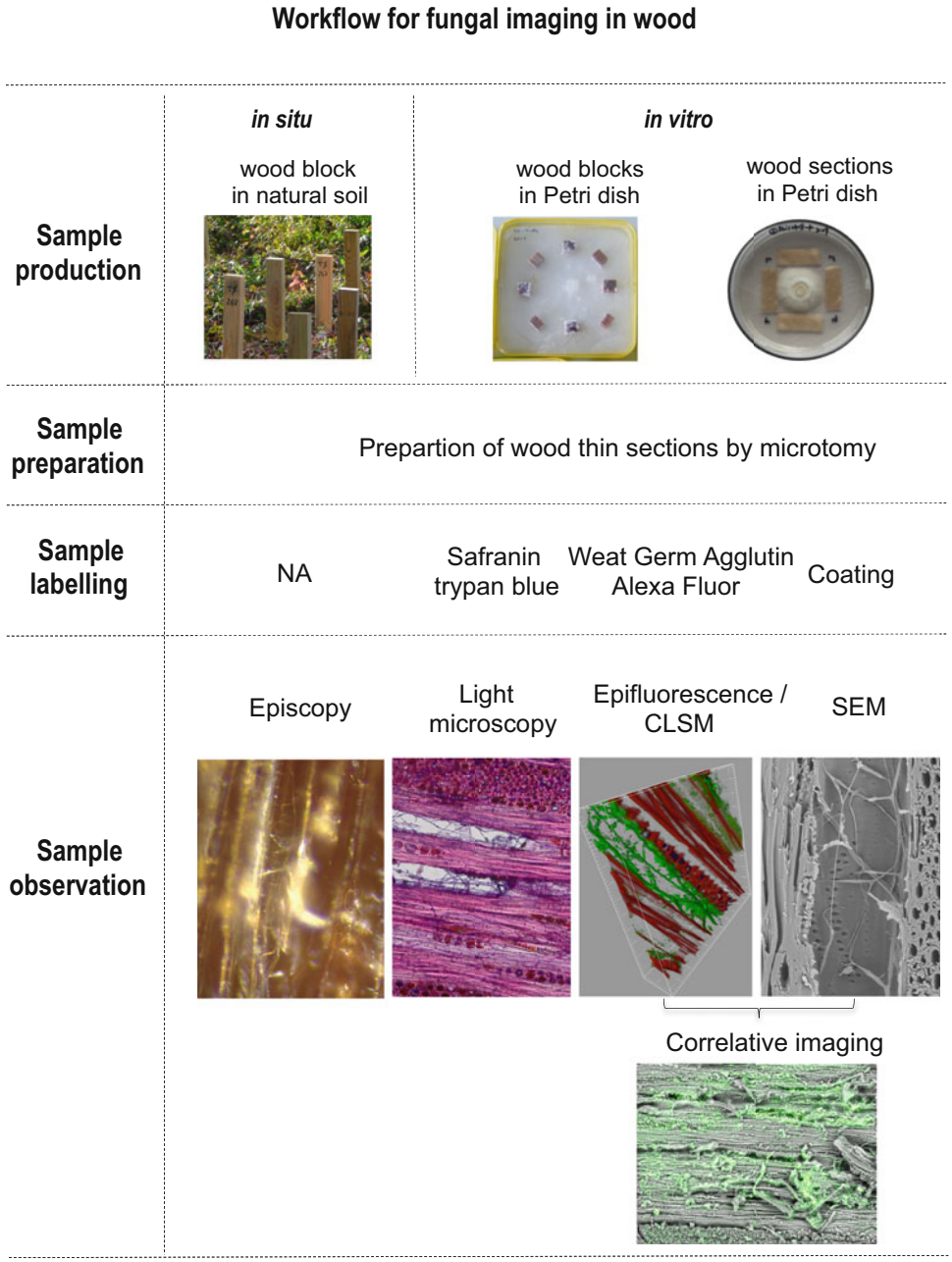

Fig. 1 Workflow from sample production to observation

Consumables

- *In vitro assay*

  O-ring RS PRO, Ø int. 9.6 mm, Ø ext.14.4 mm, thickness 2.4 mm, in Rubber Nitrile

  90 mm diameter Petri dishes

1 L Schott bottle or Erlenmeyer

Scalpel

- *Wide field light microscopy*

  Tweezers

  Pipet tips 1000 µL and 200 µL

  2 mL Eppendorf tubes

  Microscopy slides and cover slips

- *Fluorescence microscopy*

  Tweezers

  Pipet tips 200 µl

  2 mL Eppendorf tubes

  3 cm diameter Petri dish

  Microscopy slides and cover slips

- *SEM & SEM—FEG*

  SEM holders (stub)

  Coating target gold/palladium and/or platinum-tungsten

Equipment

- *For in vitro assays*

  Microbiology hood or gas burner device to work under aseptic conditions

  Steam sterilization device/autoclave

  Cultivation chamber with regulated temperature and humidity (optional)

- *For sample preparation & imaging*

  Sliding microtome (e.g., Leica SM200 R)

  Coater (e.g., Leica ACE 600 system, for SEM)

  Cryo-shuttle (e.g., Leica VCT 100 shuttle, for SEM-FEG)

Light/episcopy/epifluorescence/CLSM/SEM/FEG-SEM microscopes—Details of the different microscope specifications recommended are given in Table 1. Optical microscopes should be equipped with a combination of at least 10× and 40× magnification objectives, but 20×, 50× long distance and 63× immersion objectives can be very useful.

3 Methods

3.1 Production of Material

3.1.1 In Situ *Assays*

1. Place the samples (i.e., wood material) on soil or bury them depending on the goals of the experiment (*see* **Note 1**)
2. Incubate for at least two to three months in field condition (*see* **Note 1**)

3.1.2 In Vitro *Assay*

Different types of *in vitro* assays can be set up depending of the goals of the experiments using wood sections, wood block or wafers. We propose here a protocol derived from European Norm Standards for wood colonization by fungi that is easy to implement in any laboratory (*see* **Note 2**)

1. Prepare 2% (w/v) Malt agar medium in a Schott bottle or Erlenmeyer and autoclave it.
2. Pour around 20 mL of medium in 9 cm Petri dishes.
3. When the culture medium used is solid, cut a plug of the pre-culture of fungi of interest with a scalpel and transfer the plug at the centre of the box in axenic conditions. Flip the plug upside down so that hyphae are in direct contact with the new culture medium.
4. Incubate for 1 week at the optimal temperature and humidity (e.g., 22 °C/60% RH for *Trametes versicolor*)
5. In axenic conditions, place steam-sterilized wood samples (e.g., beech wood samples of 40*15*5 mm) on steam-sterilized O-rings (*see* **Note 3**)
6. Incubate for desired time in optimal conditions (e.g., 8 weeks at 22 °C/60% RH in the dark for *Trametes versicolor* on beech wood samples of 40*15*5 mm) (*see* **Note 4**)

3.2 Sample Preparation Before Imaging

1. Prepare 30 μm thin longitudinal wood section with a sliding microtome (*see* **Notes 5**, **6**).
2. Harvest wood sections in water or 1× PBS buffer and proceed to staining of section according the imaging technique used (see next sections) (*see* **Note 7**).
3. Keep sections in sterile distilled water or PBS buffer at 4 °C before further processing.

3.3 Dark Field Epimicroscopy Imaging

1. Put the wood specimen (block, chip, and slice) on the microscope stage (76 × 50 mm; mechanical resolution = 1 μm; Leica).
2. Directly investigate the sample for hyphal occurrence. Example of picture that can be expected are shown in Fig. 2. Picture was obtained with Leica N Plan Epi dark field (BD) objectives (10×/0.25 BD, 20×/0.40 or 50×/0.75).

3.4 Wide Field Transmitted Light Microscope Imaging

1. Bleach thin sections by boiling it in a KOH 10% solution at 90 °C for 1 h. Wood pigments should be destroyed.
2. Wash thoroughly in distilled water to remove excess of KOH.
3. If the sections are thicker or they are not enough bleached (i.e., the sample is still yellow in colour), repeat **steps 1** and **2**.

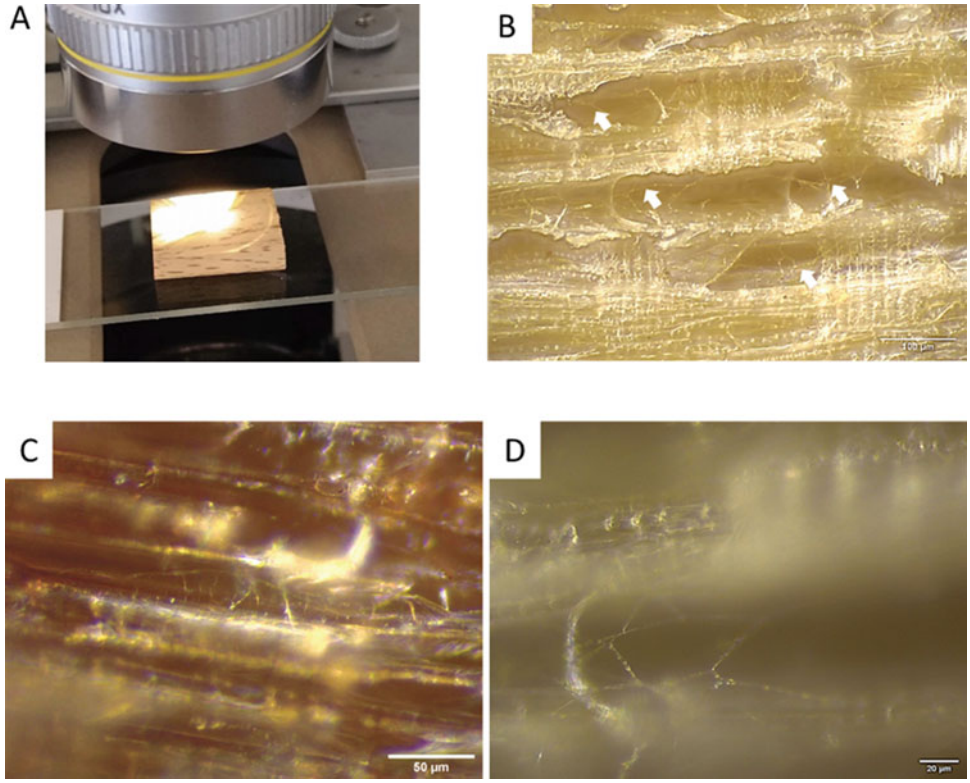

Fig. 2 Dark field reflected light microscopy observations. (**a**) Experimental setup for observation. Beech wood sample dimensions are 20*15*5 mm. (**b**) Picture of the beech wood sample showing numerous fungal hyphae (white arrows) in wood vessels. (**c**, **d**) Observations of Scot pine wood blocks, hyphae ar clearly visible. All observations were performed with a Leica N Plan Epi dark field (BD) objectives (10×/0.25 NA, 20×/0.40NA or 50×/0.75 NA) in (**b**), (**c**) and (**d**), respectively. The LIB-Z module of the LAS software was used to obtain a real-time multi-focus imaging

4. Wash the samples in 30% H_2O_2 at room temperature for 30–60 min (depending of the slice thickness), until you get a complete discoloration of tissues.
5. Wash the samples in distilled water (3–4 times) to remove all the H_2O_2.
6. Soak slices in 0.64N (10%) HCl for 40 min.
7. Transfer wood sections in a 0.05% trypan blue in lactoglycerol (*see* **Note 8**).
8. Incubate for 1 h at 90 °C in a dry or water bath in the staining solution.
9. Transfer wood sections in lactoglycerol solution and wash with distilled water until total removal of the stain in excess (the section does not release blue stain anymore).

   Option: counterstain section with 0.1% (w:v) Safranin O in distilled water for 1 min (*see* **Note 8**).

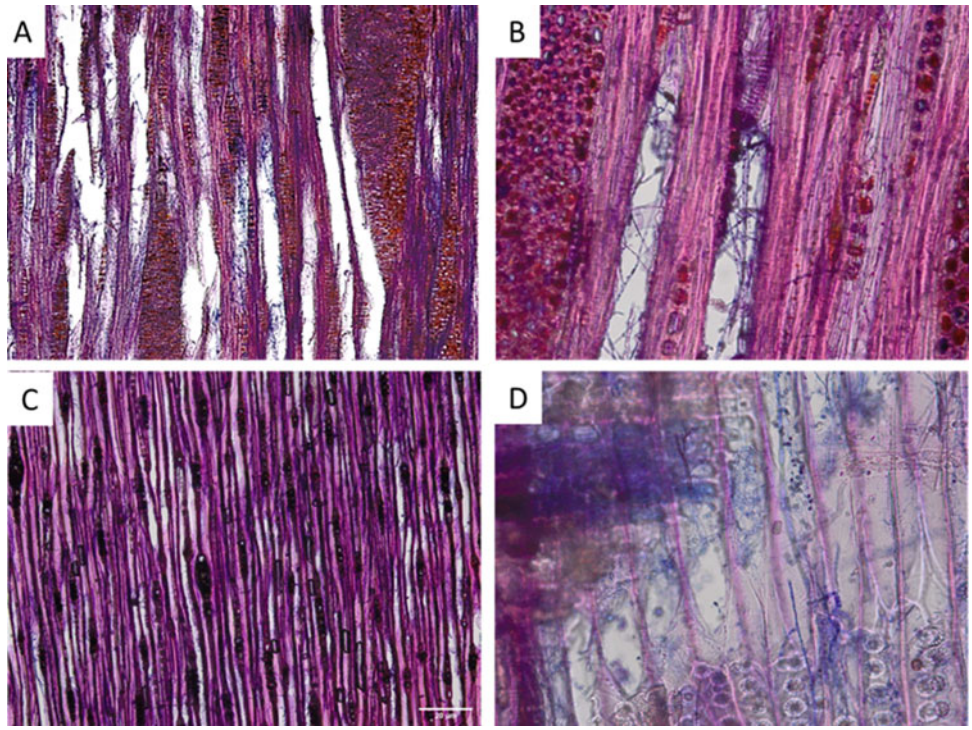

Fig. 3 Wide field light microscopy observations. Representative examples of trypan blue staining of fungal hyphae in tangential sections of Beech wood (**a**, **b**) and in radial sections of Scot Pine (**c**, **d**). Images were acquired using 10×/0.25 NA (**a**, **c**) or 40×/0.65 NA (**b**, **d**) Plan C N objectives (Olympus).

10. Observe under light microscope. Wood tissues should appear in pink and fungal structures in blue (Fig. 3). Images were acquired using 10×/0.25 NA and 40×/0.65 NA PlanC N objectives (Olympus).

3.5 Epifluorescence and Laser Scanning Confocal Imaging

3.5.1 Labelling of Fungi Prior Imaging

1. Incubate the sample in PBS-WGA-AF488 (10 μg/mL in 1× PBS pH 7.4) (*see* **Notes 9** and **10**) for at least 30 min in the dark in a 2 mL Eppendorf tube (*see* **Note 11**). Adapt the volume of labelling solution so that the sample is fully immersed.
2. Rinse three times with 1× PBS (5 min each) in a 3 cm diameter Petri dish under gentle agitation to remove the excess of dye from wood tissues.
3. Mount the sample on a glass slide with anti-fading mounting solution or 20% glycerol solution and add a cover slip.
4. Seal the cover slip to the glass slide with nail polish to avoid dehydration during imaging. Slides can be kept in the dark at 4 °C for several weeks.

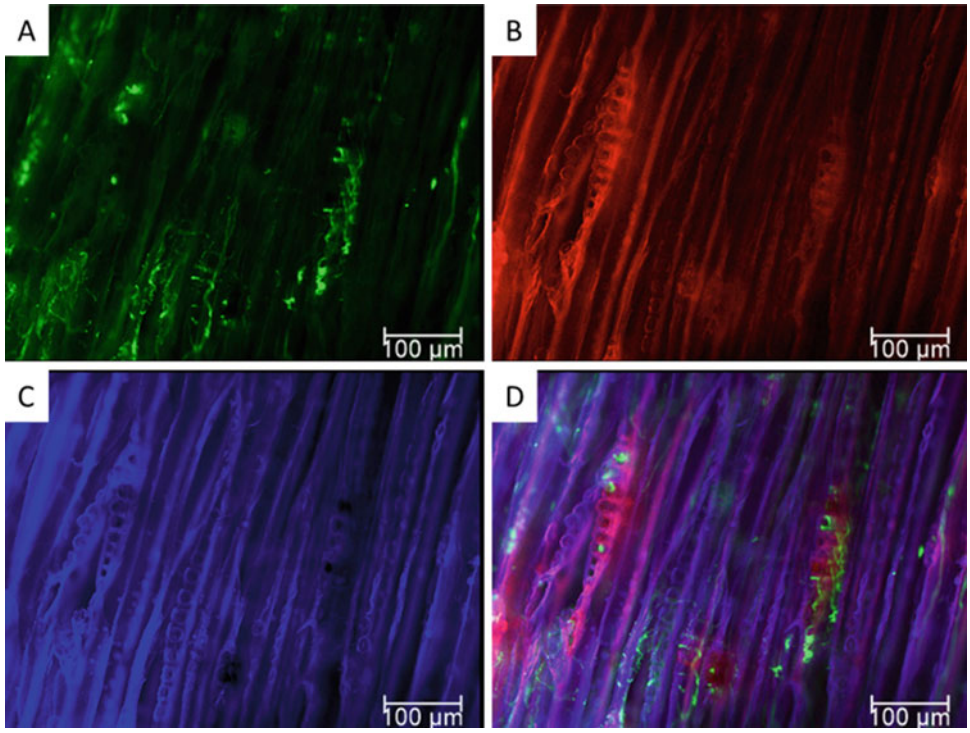

Fig. 4 Auto-fluorescence of wood tissue under variable excitation wavelengths visualized by epifluorescence. (**a**) hyphae and wood auto-fluorescence visualized with L5 filter channel (exc. 480/40, LP505 dichroic, BP527/30), (**b**) wood auto-fluorescence in the Y5 (red—exc.620/60, LP660 dichroic, BP700/75) and (**c**) DAPI (blue—UV excitation, exc. 335–425, LP400 dichroic, LP470) filter channels. (**d**) overlay of the three images. Note the weak non-specific signal on green channel and the perfect overlay of DAPI and Y5 channels (wood structures). Objective used was N Plan 20×/0.40 NA (Leica)

3.5.2 Epifluorescence Imaging

1. Make the focus on the sample by using transmitted light
2. Choose appropriate filter cubes to image WGA-AF 488 dye and wood auto-fluorescence (*see* **Notes 12** and **13**) (Fig. 4). Objective used was N Plan 20×/0.40 NA (Leica).
3. Examine first with ocular and then adjust the camera settings for picture acquisition.
4. Acquire images sequentially in grey levels with two different filters to make the difference between specific (hyphae) and non-specific (wood structures) labelling. Use Z-stack if possible to obtain more resolved images (Figs. 5 and 6). Objectives used were N Plan 40×/0.65 NA and N Plan 20×/ 0.40 NA (Leica).
5. Images can then be either post-processed with manufacturer's or free available software such as FIJI [37].

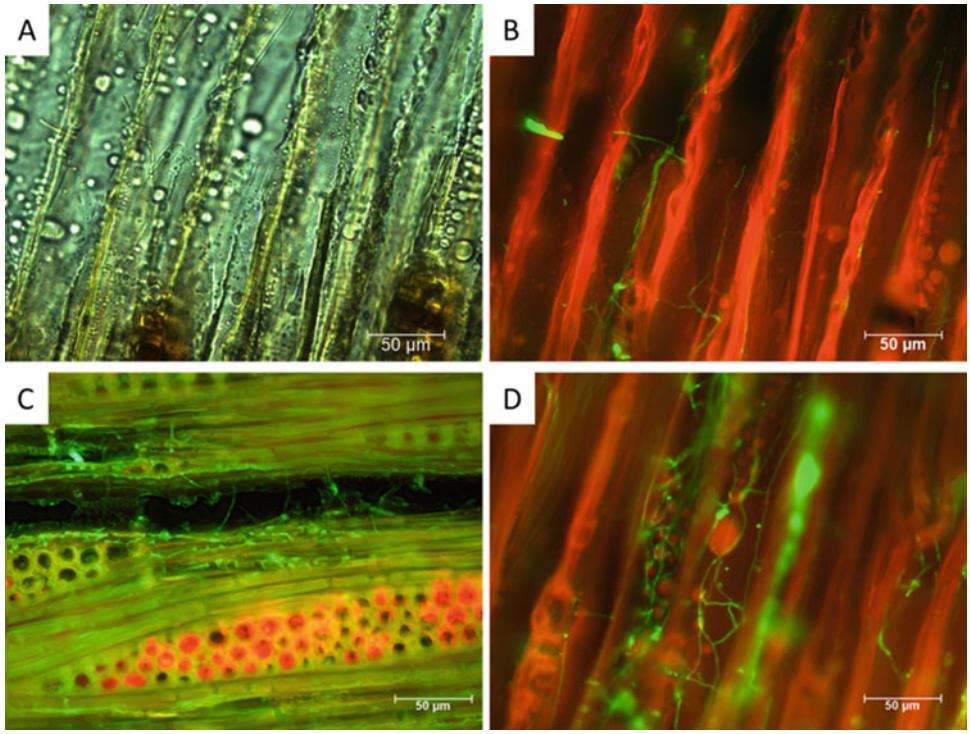

Fig. 5 Epifluorescence microscopy observations. (**a**) Wide field transmitted light of radial section of *Pinus sylvestris* wood sample colonized by fungi. (**b**) Same observation field but in fluorescence mode, after staining by WGA-AF 488 conjugated probe illustrating the benefits of fluorescent labeling of fungal structures. Observations of hyphae in beech wood (**c**) or in Scot pine wood (**d**) with the of L5/Y5 combination filters. Fungal hyphae appear in green and wood auto-fluorescence in red. Acquisitions were performed in gray levels and false colors LUT were applied. Objective used was N Plan 40×/0.65 NA (Leica)

3.5.3 Confocal Imaging

Regular Imaging

1. Use 488 nm laser excitation together with 500–550 nm emission wavelengths for WGA-Alexa fluor. Parameters for wood imaging depend on the type of wood but usually good autofluorescence can be obtained with 405 and 561 nm lasers. Specific stains that bind to cell walls such as safranin-O (exc. 488 nm, emission 520–600 nm) can be added to increase fluorescence of cell walls.
2. Perform imaging using parameters adapted to the type of data requested and to the time constrains (scanning time, magnification, and image size). Sequential rather than simultaneous acquisitions of the fungal and wood fluorescent signals should be preferred to obtain better signal to noise results and limit cross-talks between fluorescent signals.
3. Use a combination of tile scan and the Z-stack functions to obtain global 3D views of the fungal hyphae within the wood.
4. For 3D data visualization, use one of the many free or commercial software packages offering for 3D rendering (Fig. 7a).

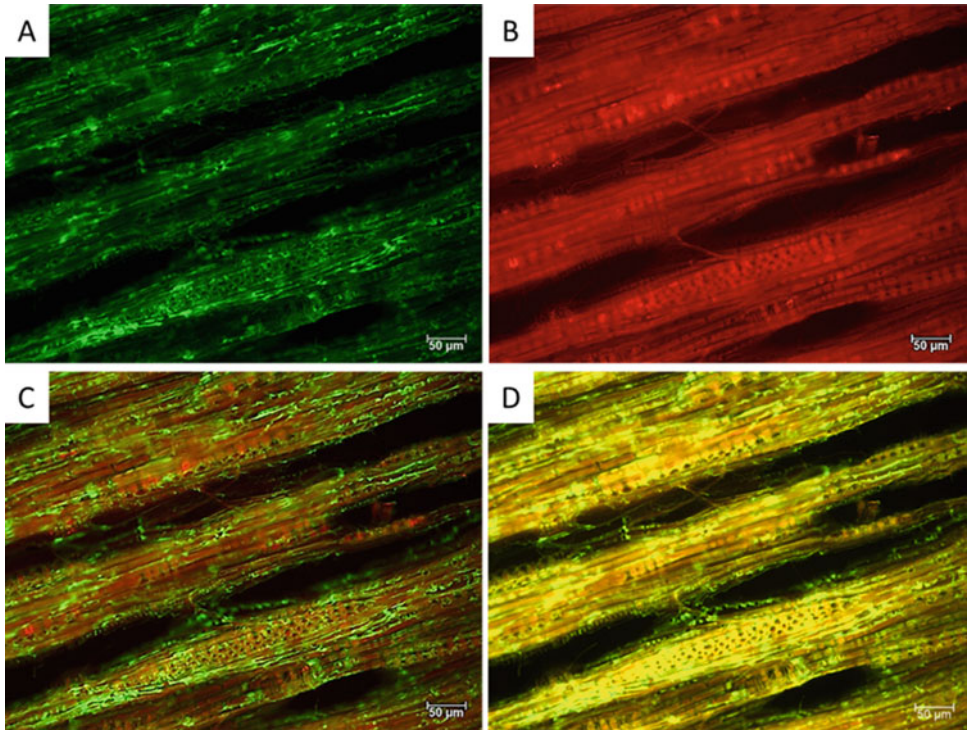

Fig. 6 Sequential vs. simultaneous acquisition in epifluorescence microscopy. Sequential acquisition with (**a**) L5 (green, narrow band filter cube, exc. 480/40, LP505 dichroic, BP527/30) and (**b**) Y5 (red, filter cube allowing only the detection of wood auto-fluorescence, exc. 620/60, LP660 dichroic, BP700/75) filters. Overlay of (**a**) and (**b**) is shown in (**c**) for comparison with the picture obtained with I3 filter (exc. 450–490, LP510 dichroic, LP515) allowing simultaneous excitation and detection of fungus and wood in (**d**). Objective used was N Plan 20×/0.40 NA (Leica)

Spectral Deconvolution

1. Prepare one slide with a fungal culture stained with WGA-AF488 (Subheading 3.5.1—slide 1), one slide with non-labelled wood sections (slide 2) and one slide with wood section colonized by fungi and labelled by WGA 488 (slide 3).
2. Acquire the emission spectrum of fungal cells labelled WGA-AF488 by exciting with laser 488 nm (slide 1) and the emission spectrum of wood excited with laser 405, 488, 561 and/or 633 nm depending on the best auto-fluorescence signal obtained (slide 2) (*see* **Note 14**)
3. Image wood sections colonized by fungi and labelled with WGA-AF488 (slide 3) with the deconvolution mode using the 2 spectra previously acquired (Fig. 7b).

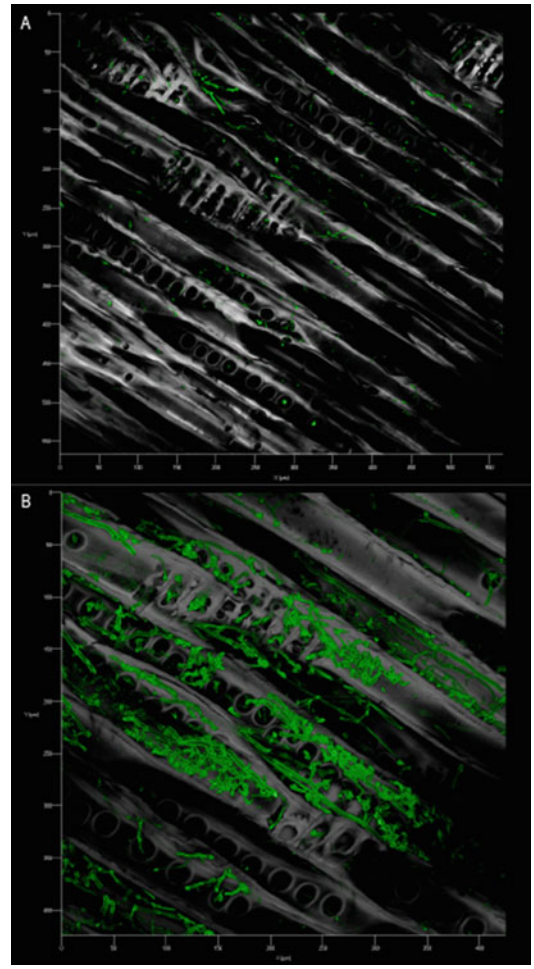

Fig. 7 Confocal Scanning laser microscopy observation of Pine wood colonized by fungi. Images were generated from a 30 μm section stained with WGA-AF 488 using the following parameters. (**a**) Sequential acquisition—channel 1 Wood autofluorescence (grey): exc. 561 nm, emission 562–695 nm GaAsp detector, channel 2—WGA AF488 fluorescence (green): exc. 488 nm, emission 490–545 nm—pixel dwell 3.15 μs, z-stack: 35 stacks over 32.39 μm. Numeric zoom 0.7×. (**b**) Spectral deconvolution imaging mode. Excitation lasers: 405 nm, 488 nm, 561 nm, emission 410–695, z-stack: 36 stacks over 31.48 μm GAasp detector—pixel dwell 1.58 μs. Numeric zoom 1×. Both the images were taken with Plan Apo 20×/0.8 NA objective. 3D reconstructions were generated with Zen 2012 SP2 software

3.6 Scanning Electron Microscopy Imaging

3.6.1 Coating (see Note 15)

Coating for Regular SEM Imaging

1. Put sample on a SEM stub adapted for the microscope used and place it in the coater at 45 mm away from the target (*see* **Note 16**).
2. Perform coating with air at vacuum chamber pressure of 10^{-4} mbar for 180 s and a thickness of the coating layer of 2–5 nm.
3. After 180 s, let chamber pressure returns to atmosphere and remove specimen.

Coating for High-Resolution Imaging with Field Emission Gun

1. Cryo-fix sample in liquid nitrogen and maintain it at −140 °C on the coater-stage by conduction of cold (i.e., liquid nitrogen using a metal braid) (*see* **Note 17**).
2. Use a cryo-shuttle (*see* **Note 18**) to maintain the sample at −140 °C during the transfer to the metallizer.
3. Perform coating at high vacuum (between 10^{-4} and 10^{-6} mbar) under Argon with the finest possible granulometry (2–3 nm) (*see* **Note 19**).

3.6.2 Regular SEM Imaging and High-Resolution Imaging with Field Emission Gun (FEG) Scanning Electron Microscope

1. Place the stub on the appropriate holder (regular SEM imaging) or use the cryo-shuttle to transfer the sample into SEM (high-resolution SEM-FEG imaging)
2. Adjust the SEM settings (working distance, voltage, current supply, and detection) and start observations.

Representative pictures obtained from both *in situ* or *in vitro* experiments with regular SEM and SEM-FEG are illustrated on Fig. 8.

3.7 Correlative Imaging

3.7.1 Sample Preparation

1. Harvest sample of interest.
2. Cut sample to appropriate dimension and orientation (*see* **Note 20**).
3. Label the fungal structure with WGA-AF488 as described above.
4. Wash in PBS buffer to remove the excess of dye.
5. Mount the sample on a stub.

3.7.2 Imaging (see Note 21)

1. Define the coordinates of the holder on the CLSM stage by using three physical reference points on the sample holder.
2. Optimize the acquisition settings on the CLSM as described in Subheading 3.5 (Fig. 9a)
3. Transfer the sample holder in the SEM.
4. Define the coordinates of the holder on the SEM stage by using the same reference points defined in **step 1**

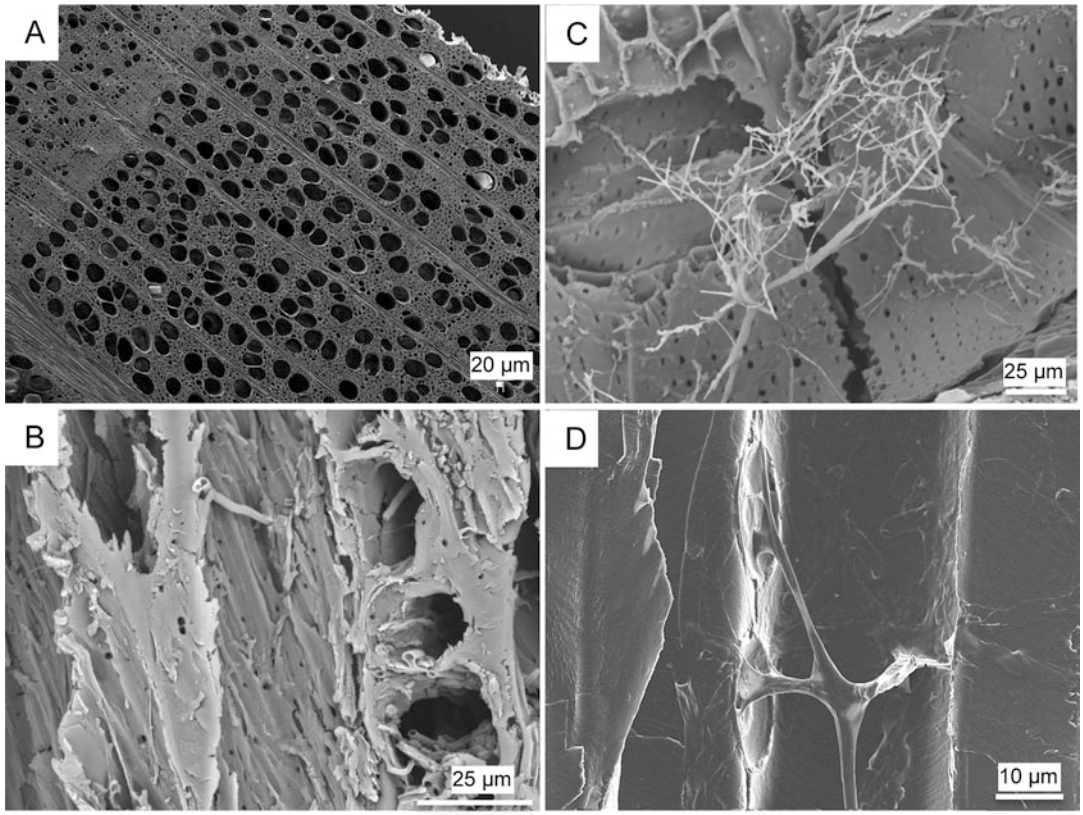

Fig. 8 Visualization of wood colonization by fungi by SEM (**a**–**c**) and FEG SEM (**d**). (**a**) Spruce wood infected *in vitro* with *Rhodonia placenta* (transverse section) (**b**) *Phanerochaete chrysosporium* in beech wood (transverse section). (**c**) *Rhodonia placenta* in oak vessel (radial section). (**d**) Hypha crossing bordered pits in Scot pine as observed using FEG-SEM with In-lens detector in high-resolution detection mode. Scale bars and magnification are indicated for each picture

5. Use automatic coordinate retrieval function if available in the software ("shuttle and find") or manually calculate the translation to achieve in the *Cartesian reference* to reach the region of interest
6. Acquire a SEM picture by using optimal settings (Fig. 9b).
7. Make the overlay of the CLSM and the SEM picture by aligning the reference points in the field of interest. An example of CLEM results is shown on Fig. 9c.

4 Notes

1. Samples for *in situ* experiments can be wood blocks, mini-stakes, or wafers. For colonization in environmental condition, samples can be buried in soil to maximize contact with wood-colonizing and saproxylic fungi. Depending of the wood

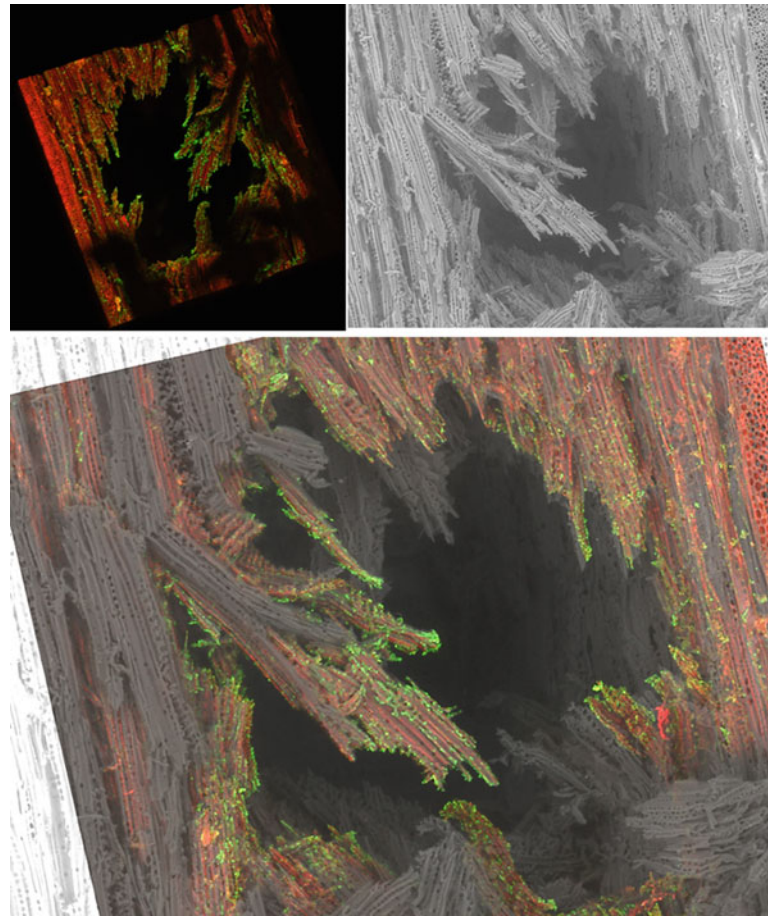

Fig. 9 CLEM observation of Beech wood particle colonized by *Trametes versicolor*. Images were generated from a 5*2 mm Beech wood particle. (**a**) Observation with CLSM. Image acquisition was performed in spectral deconvolution imaging mode. Excitation lasers: 405 nm, 488 nm, 561 nm, emission 410–695, emission 410–695, z-stack: 49 stacks over 320.73 μm, GAasp detector—pixel dwell 1.17 μs. Numeric zoom 0.6×. Both the images were taken with Plan Apo 10×/0.3 NA objective. Maximum intensity projection was generated with Zen black 2012 SP2 software. Image acquisition was performed in shuttle and find mode after dedicated sample holder calibration. (**b**) Observation with FEG SEM. Accelerating voltage used was 20 kV, backscattered electron detector (HDBSD) was used for acquisition. Image acquisition was performed in shuttle and find mode after dedicated sample holder calibration. (**c**) Overlay by CLEM. CLSM and FEG-SEM images have been overlaid by using the shuttle and find module of Zen blue 2012 software.

species tested and the aim of the experiment, samples should be let in contact with soil at least two to three months to detect a beginning of colonization. A great variability in the results could be expected with the same wood species used on a same experimental location as described in the literature

[26, 27, 38]. As a consequence, numerous replicates should be made. If mini-stakes are used, sections of different depth should be investigated [27, 28].

2. Other norms are in use in the world and can be alternatively used. The US standard system is an interesting alternative, for instance: ASTM International (2007) ASTM Standard D1413-07e1. Standard Test Method for Wood Preservatives by Laboratory Soil-Block Cultures (ASTM International, West Conshohocken, PA). In addition, more refined protocols to mimic the colonization of wood by fungi from soil in the laboratory have been developed, see, for instance, Schilling et al. [29], Zhang et al. [39].
3. The use of O-rings will avoid excess of water and inappropriate fungal growth conditions in wood samples.
4. Depending of the experimental design (kinetic, end point, etc.) appropriate number of samples should be prepared and incubated in optimal conditions. Ideally, a minimum of 10 sample replicates by condition should be set up. *In vitro* experiments are carried out in laboratory, ideally in controlled temperature and humidity conditions. To evaluate mass loss of wood sample according to European standards, wood specimen dimensions are specified by the standard [40–42]. Adaptation of EN 113-2 protocol could be to use 40*15*5 mm wood sample and 9 cm diameter petri dishes
5. The best orientation of sections for visualizing hyphae in wood is longitudinal, either radial or tangential (parallel to the grain). Wood sections can be obtained in three plans (Fig. 10) and tangential or radial sections allow for a good visualization of hyphal colonization in the different wood tissues.
6. Thicker wood sections (60–100 μm), wafers (≥0.5 μm thick) or small block (2 mm^2 of section) could be used for scanning electron microscopy and imaging techniques based on episcopic light.

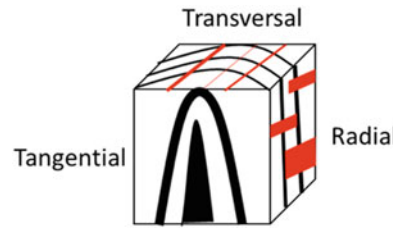

Fig. 10 Possible orientations for generation of wood sections with microtome. Growth rings are displayed in black and rays in red

7. To preserve physiological state for specific investigations, fixation of samples in 3% para-formaldehyde/ PBS solution can be used.
8. Trypan blue is a dye that will stain the chitin of fungal hyphae. In combination with a safranin O staining of wood tissues, it provides a good visualization of fungal structures occurring in a wood sample. Safranin-O binds to lignins and can also be used for fluorescent imaging (see below). Many other dyes can be used depending on the goals of the study, see Schwarze (2007) for an extensive list of available dyes for light microscopy.
9. Many fluorescent dyes are available to label fungal structure. Among them, the Wheat Germ Agglutinin lectin (WGA) conjugated to Alexa fluor dyes provides good results.
10. 0.1% Tween 20 can be added to the staining solution to improve stain penetration for recalcitrant samples. Staining solution can be kept at 4 °C for a week. Protect from light.
11. Penetration of the dye can be improved by incubating under 0.5 bar vacuum.
12. Different filters can be used for WGA 448 and wood autofluorescence; for instance, L5 (EX = 480/40, LP505 dichroic, EM = BP527/30) for WGA AF488 signal only or *I3* (EX = 450–490, LP510 dichroic, EM = LP515) for simultaneous WGA AF488 and wood autofluorescence. For wood autofluorescence (polyphenol compounds), Y5 filter (EX = 620/60, LP660 dichroic, EM = BP700/75) or DAPI (EX = 335–425, LP400 dichroic, EM = LP470) can be used. Figure 6 illustrates the wide spectrum of fluorescence emission from wood. Wood cell wall auto fluorescence is strong across a wide range of wavelengths due to polyphenols in cell wall [30, 43, 44]. Autofluorescence of the wood is detected after an excitation in the UV (filter D, Ex = 360/40 nm Fig. 6c) up to the red (filter Y5; Ex = 620/60 nm; Fig. 6b). To increase specificity of signal emission of AF488, a band pass filter (filter L5; Ex = 480/40 nm, Em = 527/30 nm) is required (Fig. 6a). Overlay of the red and blue channel shows identical patterns for wood structure and fluorescence for fungal structure which is found only in the green channel.
13. If the wood sample (in particular hardwood) is subjected to a basidiomycete causing white rot decay, autofluorescence of polyphenol compounds will be reduced due to lignin decomposition by the fungus.
14. Combination of several lasers can improve the quality and resolution of imaging by taking advantage the multiplicity autofluorescence compounds that are found in wood tissues. In the example shown (Fig. 7), combination of 405, 488, and 561 nm lasers was used to generate auto-fluorescence spectra

of Beech and Pine wood sections. Images of Fig. 7 were taken with Plan Apo 20×/0.8 NA objective.

15. Wood sample can be imaged without coating and preparation (dehydration) by using controlled pressure in SEM chamber. However, to improve image resolution and allow observations at higher magnification, coating of the sample is required. Indeed, wood is a non-conductive material. Thus, creating a very thin (few nanometers) conductive layer at the surface of the sample will avoid accumulation of electrons (charging) and improve the signal for topographic observations (secondary electron re-emission in the SEM). This step is achieved by coating the sample with gold/ palladium, platinum, or other heavy metals in ionized gas plasma (Argon) generated under vacuum. The choice of the coating techniques depends on the expected resolution and subsequent application (topography, elementary analysis, etc.).
16. The choice of the coating procedure is linked to the experimental aims (resolution of observations). If only the presence of structures related to the presence of fungi (hyphae, spores, oxalate crystals, etc.) are looked for, the gold/palladium coating is sufficient. In contrary, if SEM imaging will be coupled to elementary analysis by energy dispersive X-ray spectroscopy (EDS) or wavelength dispersive X-ray spectroscopy (WDS), it is better to use carbon sputtering (pure carbon "evaporation" with high-voltage current applied on carbon fiber or tip in vacuum).
17. Because wood is a hygroscopic material, dehydration of sample required for SEM observation will trigger deformations and observation of artifacts. To preserve sample integrity in their native state, a cryo-fixation in liquid nitrogen and lyophilization of the sample are often recommended. However, simple sample drying at 103 °C prior coating can give good results for preliminary structures inspection.
18. The use of a cryo-shuttle permits to do a sample transfer in the coater and the microscope under cryogenic conditions.
19. A fine carbon layer is transparent to primary electron beam but conductive and gives no interferences upon X-ray microanalysis.
20. Wood is a specific material to work with, since it is hydroscopic material and thus highly sensible to dimensional changes upon dehydration. For wood sample, the fastest and easiest way is to work with small dry samples (2–5 mm^2). The shape of the sample (wafer, section, or mini block) is not important since only the surface of the sample will be examined.
21. The basic principle of CLEM is to observe the same field with two different microscopy techniques. Thus, it is mandatory to

be able to determine, store, and transfer the coordinate of the region of interest on the sample from the confocal microscope to the SEM. Here, we took advantage of the shuttle and find solution developed by Zeiss but manually designed system can be used as well.

Acknowledgments

The authors would like to thank FCBA and particularly L Harvengt for providing an image of *in situ* the wood degradation experiment in forest soil (in Fig. 1). They thank SILVATECH for its participation to microscope observations and microanalysis (Silvatech, INRAE, 2018. Structural and functional analysis of tree and wood Facility, doi: 10.15454/1.5572400113627854E12 from UMR 1434 SILVA, 1136 IAM, 1138 BEF and 4370 EA LERMAB from the research center INRAE Grand-Est Nancy). SILVATECH facility, CR and AD are supported by the French National Research Agency through the Laboratory of Excellence ARBRE (ANR-11-LABX-0002-01).

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