

## Video Article

# A New Method for Qualitative Multi-scale Analysis of Bacterial Biofilms on Filamentous Fungal Colonies Using Confocal and Electron Microscopy

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## Abstract

Bacterial biofilms frequently form on fungal surfaces and can be involved in numerous bacterial-fungal interaction processes, such as metabolic cooperation, competition, or predation. The study of biofilms is important in many biological fields, including environmental science, food production, and medicine. However, few studies have focused on such bacterial biofilms, partially due to the difficulty of investigating them. Most of the methods for qualitative and quantitative biofilm analyses described in the literature are only suitable for biofilms forming on abiotic surfaces or on homogeneous and thin biotic surfaces, such as a monolayer of epithelial cells.

While laser scanning confocal microscopy (LSCM) is often used to analyze *in situ* and *in vivo* biofilms, this technology becomes very challenging when applied to bacterial biofilms on fungal hyphae, due to the thickness and the three dimensions of the hyphal networks. To overcome this shortcoming, we developed a protocol combining microscopy with a method to limit the accumulation of hyphal layers in fungal colonies. Using this method, we were able to investigate the development of bacterial biofilms on fungal hyphae at multiple scales using both LSCM and scanning electron microscopy (SEM). This report describes the protocol, including microorganism cultures, bacterial biofilm formation conditions, biofilm staining, and LSCM and SEM visualizations.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/54771/>

## Introduction

Fungi and bacteria have many opportunities to interact with each other because they cohabit in most terrestrial environments. Due to their diversity and their ubiquity, these interactions are important in many biological fields, including biotechnology, agriculture, food processing, and medicine<sup>1,2</sup>. Molecular interactions require a certain degree of proximity to allow exchanges between the partners, and in some cases, a physical association of the partners is necessary for a functional interaction<sup>3</sup>. A common physical association between bacteria and fungi is the formation of bacterial biofilms on fungal surfaces<sup>4</sup>. This direct contact between bacterial cells and fungal hyphae permits intimate interactions that are involved in various biological processes. For example, in medicine, the study of biofilm formation of *Pseudomonas aeruginosa* on the opportunistic fungal pathogen *Candida albicans* could provide insights into the link between biofilm formation and virulence<sup>5</sup>. In agriculture, studies suggest that plant-growth-promoting rhizobacteria and biocontrol bacteria have an increased efficiency when associated with a fungus in a mixed biofilm. For example, *Bradyrhizobium elkanii* have enhanced N<sub>2</sub>-fixing activity when associated with *Pleurotus ostreatus* in a mixed biofilm<sup>6</sup>. Finally, in bioremediation, bacterial-fungal mixed biofilms have been used for the remediation of polluted sites<sup>7,8</sup>.

LSCM is particularly suitable to study biofilms since it allows for a three-dimensional observation of living hydrated biofilms with minimum pretreatments, thereby maintaining biofilm structure and organization. Thus, biofilm analysis by LSCM is very informative, especially to determine the time course of the biofilm formation and the detection of characteristic stages<sup>9,10</sup>, from the adhesion step to the development of a mature biofilm. It is also particularly adapted to visualize the biofilm structure and matrix<sup>11,12</sup> or to quantify the biofilm size<sup>13,14</sup>. Although this method is suitable to study biofilms on abiotic or thin biotic surfaces, studying bacterial biofilm on a fungal filamentous colony is still very challenging. Indeed, most filamentous fungi build thick, complex, tridimensional networks in culture. Even if thick objects can be imaged by confocal microscopy, the attenuation of the laser penetration and the fluorescence emission often decrease the quality of the final images over a depth of 50 μm<sup>15</sup>. Moreover, because fungal colonies are not rigid, it is difficult to handle the microorganisms without disturbing the biofilms. Due to the thickness of the samples, the few microscopic analyses of bacterial biofilms on fungal hyphae are usually only performed on a small part of the fungal colony, therefore containing only few hyphae<sup>16,17,18</sup>. All this limits our ability to describe biofilm distribution on the fungal colony and thus can bring biases into the analysis in case of the heterogenic distribution of the biofilm within the fungal colony.

To overcome such difficulties, we report a method for the growth and the analysis of bacterial biofilm on fungal hyphae. This method was applied to study the biofilm formation in *Pseudomonas fluorescens* BBc6 on the hyphae of the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N.

These two forest-soil microorganisms were previously described to form mixed biofilm-like structures<sup>19,20</sup>. This method can easily be further adapted to other filamentous fungal/bacterial systems. The method presented here is based on the combination of a fungal culture method, allowing for the growth of very thin fungal colonies, with LSCM and SEM imaging. This permitted us to obtain micro- ( $\mu\text{m}$  range) and meso- (mm range) scale views of the interaction between the two microorganisms, allowing for the qualitative characterization of the biofilm. We also showed that the samples can be observed with SEM, permitting structural analysis of the biofilm at the nano-scale level (nm range).

## Protocol

### 1. Preparation of Bacterial and Fungal Cultures

#### 1. Preparation of fungal cultures

1. Prepare cellophane membranes with the same diameter as the Petri dishes and boil them in EDTA (1 g/L in deionized water) for 30 min. Rinse the sheets with deionized water and autoclave them twice.
2. Prepare a fungal pre-culture by inoculating fungal agar plugs on the appropriate agar medium covered with an EDTA pre-treated cellophane membrane. Incubate it at the optimal growth temperature to obtain colonies approximately 1 cm in diameter.
  1. For *L. bicolor* S238N, incubate at 25 °C for 5 days on Pachlewski P5 agar medium, made of 0.5 g of diNH<sub>4</sub> tartrate, 1 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g of Maltose D+, 20 g of Glucose D+, 1 ml of 10% Thiamine-HCl, 1 ml of 1/10 diluted micronutrient solution (6 g/L ferrous sulfate; 0.27 g/L molybdenum trioxide; 8.45 g/L boric acid; 5 g/L manganese sulfate; 5 g/L cupric sulfate; 2.27 g/L zinc sulfate) made up to 1 L with deionized water; and 20 g of agar; pH 5.5.
3. From the fungal pre-culture, inoculate a new agar plate covered with the EDTA pre-treated cellophane membrane containing a low-carbon agar medium to promote the radial expansion of the colonies .
  1. To inoculate the Petri dish, gently scratch the external area (where cells have the same physiological state) of a fungal colony from the pre-culture with a scalpel and transfer the hyphae to the new agar plate.
  2. Incubate at the optimal growth temperature until the new colonies are approximately 1 cm in diameter. For *L. bicolor* S238N, incubate them at 25 °C for 5 days on P20 agar medium (0.25 g of diNH<sub>4</sub> tartrate; 0.5 g of KH<sub>2</sub>PO<sub>4</sub>; 0.25 g of MgSO<sub>4</sub>·7H<sub>2</sub>O; 1 g of Glucose D+; 0.5 ml of 10% Thiamine-HCl; 0.5 ml of 1/10 diluted micronutrient solution (cf 1.1.2.1), made up to 1 L with deionized water; and 20 g of agar; pH 5.5).  
 Note: Due to the pre-culture on cellophane, the second culture will not contain agar media in the central plugs. The agar plugs should be removed for further analysis of the biofilms, since these plugs introduce agar and nutrients that can create biases in the analysis. This step concerns only fungal species that are kept and propagated on agar plates, and it is not necessary for fungi that are propagated from spores or frozen stocks.

#### 2. Preparation of bacterial cultures

1. Use a sterile loop to collect 2 to 3 individual bacterial colonies from a culture on the appropriate agar medium and inoculate 25 ml of liquid Luria-Bertani (LB) medium (or any other appropriate medium, depending on the strain used). For *P. fluorescens* BBc6, incubate an overnight culture at 28 °C with gentle shaking (~150 rpm).  
 Note: Using a strain tagged with fluorescent protein, such as GFP, is preferable, because this avoids performing a double-staining (fungus and bacteria) prior to microscopic observations. The timing, the stirring speed, and the temperature of the incubation depend on the strain used, the goal being to obtain a culture in late exponential growth.

### 2. Preparation of *N In Vitro* Biofilm of Bacteria on the Fungal Colony

1. Centrifuge the bacterial culture at 5,000 x g for 3 min and suspend the pellet in 25 ml of sterile 0.1 M potassium phosphate buffer (25 g/L KH<sub>2</sub>PO<sub>4</sub> and 2.78 g/L K<sub>2</sub>HPO<sub>4</sub>, pH 5.8). Repeat this step once and adjust the final bacterial concentration to 10<sup>9</sup> cells/ml with the same buffer.
2. Fill a 6-well microplate with 5 ml of the bacterial suspension (or sterile 0.1 M potassium phosphate buffer for the negative control).
3. Cut the cellophane membrane of the fungal culture with a sterile razor blade to obtain squares of cellophane with a single fungal colony on each membrane. Carefully remove the cellophane squares containing hyphae from the solid medium using forceps and transfer the square of cellophane containing hyphae to a well of the microplate containing the bacterial suspension.
4. Gently shake the microplate while the fungal colonies are still attached to the cellophane; then, remove the cellophane sheets, leaving the fungal colonies in the plate.
5. Incubate the microplate with gentle agitation (~60 rpm) at a temperature adapted to the studied microorganisms.  
 Note: The time of incubation depends on the speed of establishment of the biofilm and the stage to be analyzed. For *P. fluorescens* BBc6/*L. bicolor*, incubate at 20 °C for 30 min to get early-stage biofilms and up to 16 hr to get mature biofilms.

### 3. Laser Scanning Confocal Microscopy Analysis of the Biofilm Formation

#### 1. Sample preparation

1. To remove planktonic bacteria and bacteria electrostatically-attached to the hyphae, rinse the fungus by transferring it to a new 6-well microplate filled with 5 ml of a strong salt solution (NaCl, 17 g/L)<sup>17</sup>; gently shake for 1 min.
2. Transfer the fungus to a new 6-well microplate containing 5 ml of sterile 0.1 M potassium phosphate buffer, gently shake for 2 min, and transfer the fungus to fresh potassium phosphate buffer.
3. Cut the part of the fungal colony to be imaged with a scalpel while keeping it in the potassium phosphate buffer, such that the obtained section contains about half of the fungal colony.

4. To stain the sample, transfer it to a Petri dish filled with sterile water containing an appropriate fluorescent dye (depending on the aim of the analysis), and incubate it in the dark.
    1. To visualize the fungal network, use, for example, Congo Red (0.1% final concentration, 5 min of incubation), wheat germ agglutinin (WGA) lectin conjugated to Alexa Fluor 633 (10 µg/ml final concentration, 10 min of incubation) or FUN1 (10 µM final concentration, 10 min of incubation).
    2. If using a non-fluorescent-tagged bacterium, counterstain the bacterial cells with a DNA-specific fluorescent probe among the numerous cell-permeant DNA dyes commercially available. For example, use DAPI (0.25 µg/ml final concentration, 10 min of incubation).
    3. To visualize the matrix proteins, use a protein stain<sup>21</sup>.
  5. After staining, rinse the sample by transferring it to a Petri dish lid containing 10 ml of sterile 0.1 M potassium phosphate and gently shake for 1 min.
  6. Half submerge a slide in the Petri dish lid and delicately bring the cut section to float above the slide. Then, slowly remove the slide from the buffer solution, allowing the sample to gently settle on the slide.  
Note: For this step, it is very important to proceed gently to avoid biofilm disturbance.
  7. Finally, add 10 µl of anti-fading mounting medium to the sample and cover it with a glass coverslip.  
Note: Once the slide is prepared, the microscopic analysis must be performed as soon as possible (within at most 30 min) to avoid modification in the biofilm structure.
2. **Confocal microscopic analysis**
1. Examine the slides under a confocal laser microscope with a 10X or 40X objective lens coupled to imaging software.  
Note: Here, a multi-beam scanning confocal microscope equipped with 405, 488, and 561 nm excitation lasers, a GaAsP PMT detector, and 10X 0.3 NA and 40X 1.2 NA objectives was used.
    1. Perform imaging using parameters adapted to the type of data requested and to the time constrains. Use laser excitation/emission wavelengths according to the stain used and to the manufacturer's recommendations. Use a combination of tile scan and the Z-stack functions to obtain global 3D views of the fungal colony and the biofilms.  
Note: Images on **Figure 3** were acquired using the following parameters: 8-bit/pixel, averaging = 2, pixel dwell = 1.58 µsec, tiles scan of 5x5 images with online stitching (10% coverage, threshold = 0.7); Z-step = 2 µm.
  2. For 3D data visualization, use one of the many free or commercial softwares (these offer many options for 3D rendering).
    1. For example, to display Z-stack data as 2D maximum intensity projections, subtract the bright-field channel, adjust the brightness and contrast, and merge channels to create a composite image. If they are present, eliminate slices without signal at the Z-stack extremities. Then, perform a 2D maximum-intensity projection and convert the result into RGB color. Use the "Z project" function in the ImageJ software<sup>22</sup> to obtain the images presented here by 2D maximum intensity projections.

## 4. Electron Microscopy Analysis

1. Prepare the samples as described for LSCM, except in step 3.1.2, transfer the biofilms to sterile water instead of potassium phosphate buffer after the rinse steps. This avoids the formation of salt crystals during the dehydration step, which would perturb SEM imaging.
2. Transfer the biofilms to a sample holder and remove excess water; only allow a small pellicle of water to cover the sample.
3. Transfer the sample to the chamber of a variable-pressure scanning electron microscope (VP-SEM); freeze it to -50 °C on a Peltier cooling stage; and allow it to slowly freeze-dry, directly in the SEM chamber, with 100 Pa of variable pressure set for 15 hr.
4. After completion of the freeze-drying, retrieve the sample and transfer it into a high-vacuum film deposition system. Coat the sample with 2 nm of platinum (quartz measurement) under argon plasma ( $2.5 \times 10^{-2}$  mbar, 35 mA).
5. Observe the coated sample with an SEM equipped with a field emission gun (FEG) using the high resolution "in lens" detector and an electronic high tension of 1 kV.

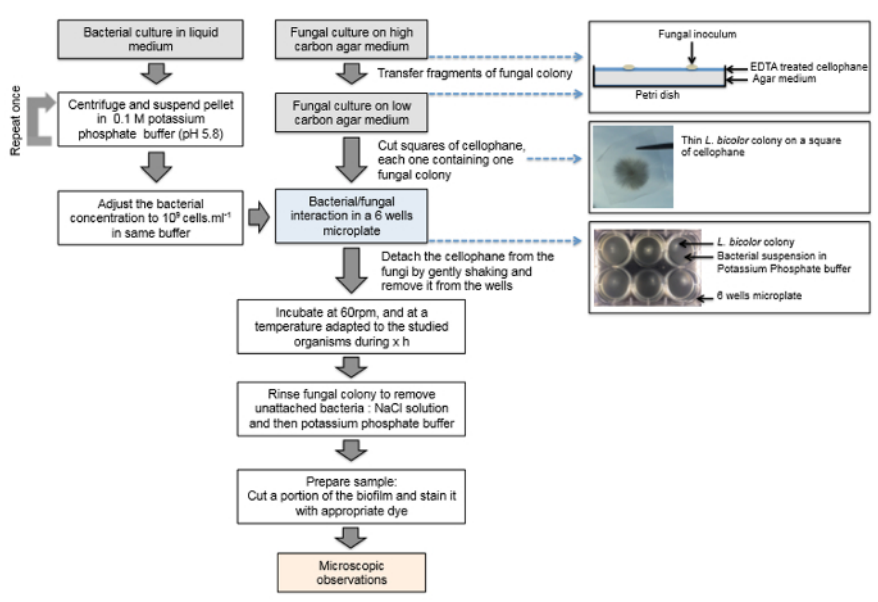
## Representative Results

The overall schematic procedure of fungal culture and biofilm preparation are given in **Figure 1**. The culture method allowed us to obtain fungal colonies 20 to 50 µm thick containing a few layers of hyphae, allowing micro- and meso-scale analyses of hydrated living biofilm using LSCM. The application of the method permitted the acquisition of high-quality images of *P. fluorescens* BBc6 biofilms on *L. bicolor* hyphae along the formation of the biofilm (**Figures 2-4**).

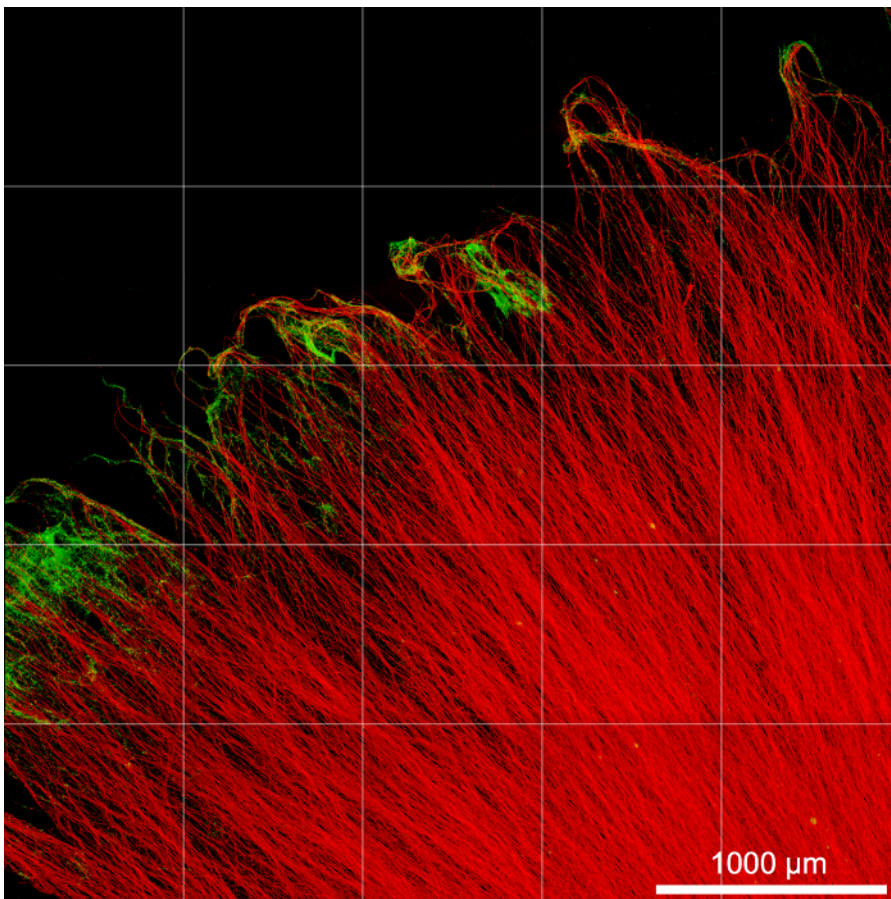
The meso-scale analysis of the biofilms demonstrated the heterogenic distribution of the biofilm of *P. fluorescens* BBc6 on the hyphae of *L. bicolor* S238N (**Figures 2 and 3**). Meso-scale analysis also allowed for the tracking of the biofilm formation over time (**Figure 3**) from the early steps, in which only some bacteria were attached to hyphae (**Figure 3a**), to the formation of a thick, mature biofilm (**Figure 3b**). The high resolution of the meso-scale images allowed us to perform micro-scale analysis on the same images in order to obtain the colony architectures (**Figure 3c-d**).

The micro-scale analysis combined with the specific labeling enabled us to go a step further in biofilm characterization. Here, SYPRO Ruby<sup>23</sup>, which labels most classes of proteins, was applied to the samples (**Figure 4**) and shows the presence of proteins in the matrix.

Finally, further details of the biofilm structure were obtained by SEM imaging of the same sample after dehydration and coating (**Figure 5**). SEM imaging provided access to the nanoscale level, thus giving access to the matrix structure.

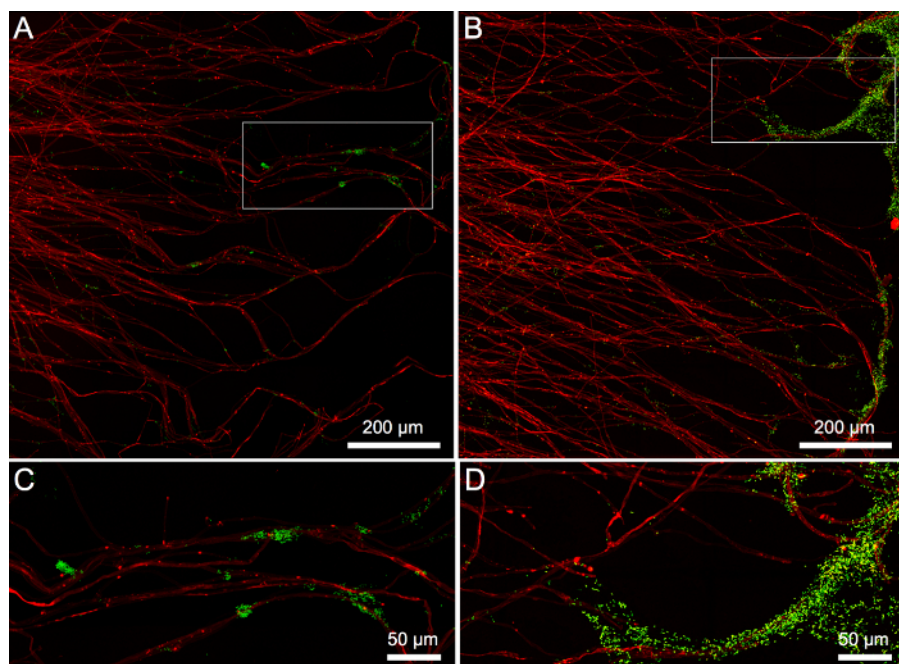


**Figure 1: Overall schematic procedure of fungal culture and biofilm preparation.** This figure describes the main steps of the method, from microorganism cultures to sample analyses. More details are given in the protocol. [Please click here to view a larger version of this figure.](#)

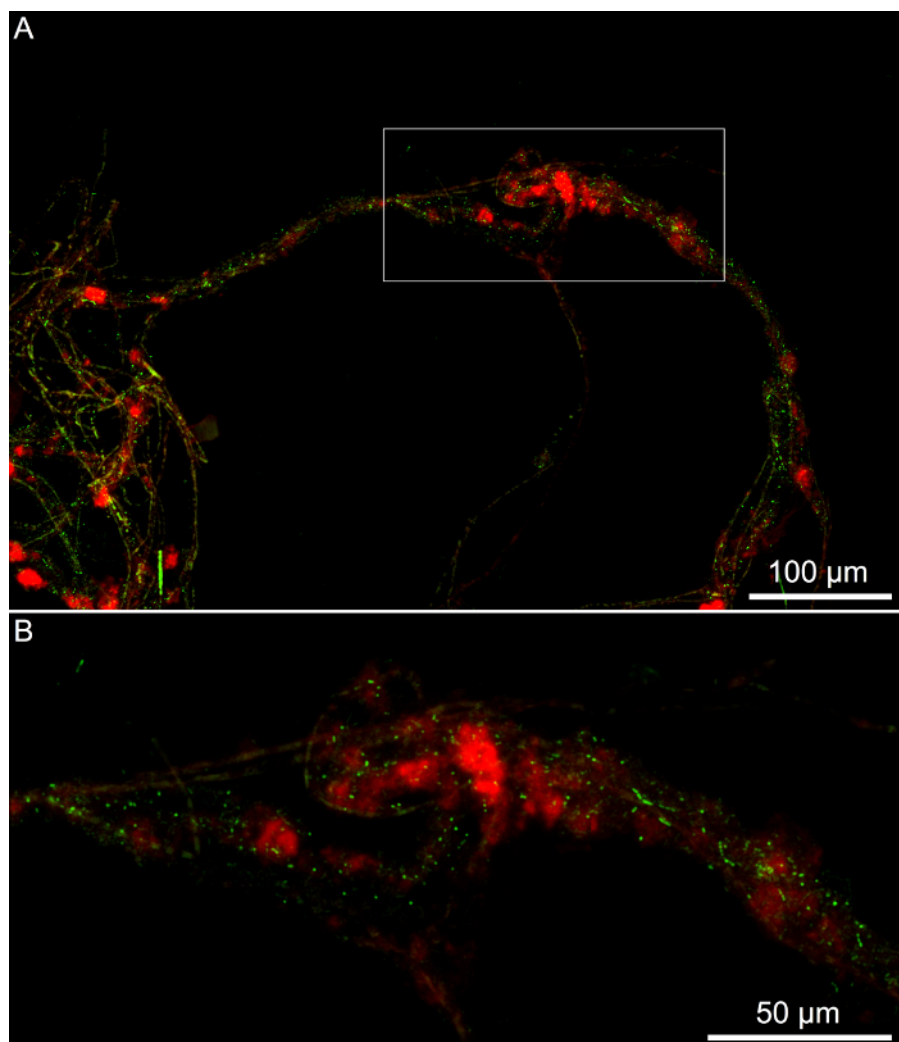


**Figure 2: BBc6 biofilm repartition on the fungal colony.** The sample was imaged after 18 hr of interaction at 10X magnification. The image was obtained via 2D maximum intensity projection of 3D confocal microscopy images. The grey grid on the figure depicts the mosaic positions. *L. bicolor* hyphae are stained with Congo Red (red) and bacteria are GFP-tagged (green). [Please click here to view a larger version of this figure.](#)

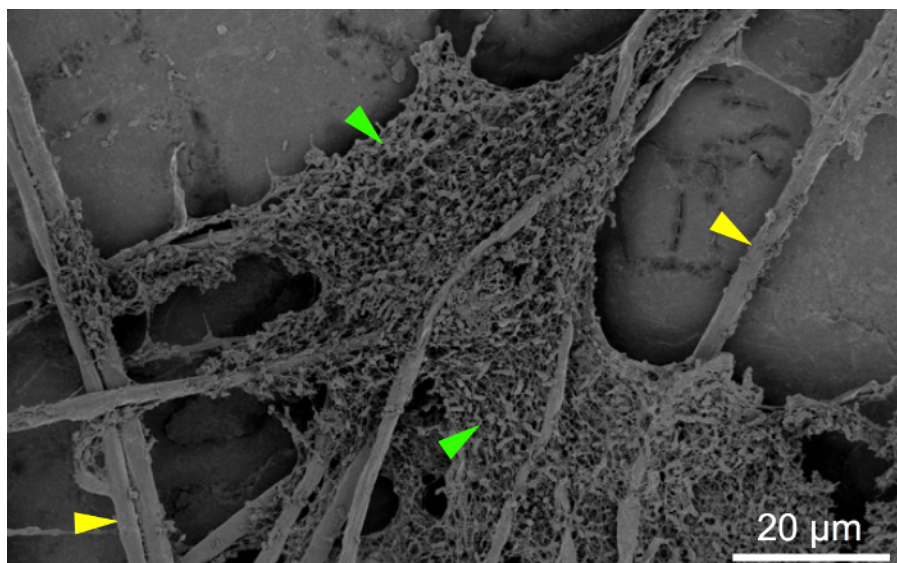




**Figure 3: Early and late stages of BBc6 biofilm formation on *L. bicolor* hyphae.** This image was obtained via 2D maximum intensity projection of 3D confocal microscopy images. Imaging was performed at 40X magnification. **(a)** Biofilms repartition after 2 hr of interaction. **(b)** Biofilm repartition after 14 hr of interaction. **(c)** Enlargement of the white rectangle in (a). **(d)** Enlargement of the white rectangle in (b). Fungal hyphae are stained with Congo Red (red) and bacteria are GFP-tagged (green). [Please click here to view a larger version of this figure.](#)



**Figure 4: Matrix staining of BBc6 biofilm on *L. bicolor* hyphae.** (a) Biofilms after 16 hr of interaction. (b) Enlargement of the white rectangle in (a). Imaging was performed at 40X magnification, and these images were obtained via 2D maximum intensity projection of 3D confocal microscopy images. Bacteria are GFP-tagged (green), fungus is stained with Fun1 (dark green), and proteins are stained with S. Ruby (red). [Please click here to view a larger version of this figure.](#)



**Figure 5: SEM imaging of BBC6 biofilms on *L. bicolor* hyphae.** SEM Imaging was performed at 2360X, EHT: 1kV. Before imaging, the sample was slowly freeze-dried in the SEM chamber and coated with platinum. Green arrows point to bacterial biofilms and yellow arrows point to fungal hyphae. [Please click here to view a larger version of this figure.](#)

## Discussion

Bacterial biofilms are retrieved in many environments and have been studied since the 1950s, leading to the development of a number of methods to analyze them<sup>24</sup>. Classical methods to quantify and monitor biofilms include micro-titer assays and, the most widely-used method, crystal violet (CV) staining. These methods are fast, low cost, and easy to handle<sup>25</sup> and are particularly useful to quantify total biofilm biomass or to perform viability and matrix quantification assays. On the other hand, "omics" methods are also useful in biofilm studies, allowing for quantitative and functional analyses of biofilms<sup>26,27</sup>. Despite the advantages of micro-titer plate and "omics" methods, several essential features of biofilms cannot be captured with these techniques, hindering a complete understanding of this process. Such features include matrix structures, bacterial colony architectures, cell/cell interactions, and colonization patterns, which are key data for understanding both the functioning of biofilms and the dynamics of their formation. Despite the capacity of microscopy to capture these features, microscopy analysis of bacterial biofilms on filamentous fungi are still scarce. This is mainly due to the growth of filamentous fungi, which often forms colonies of thick, complex, tridimensional networks. Formation of bacterial biofilms on fungi is common in diverse environments and is significantly involved in various fields<sup>4</sup> (e.g., medicine, agriculture, and environment); hence, it is critical to develop new methods to facilitate their investigation. To this end, we combined a method to generate very thin fungal colonies with microscopic imaging of the bacterial biofilms. In addition, we proposed a set of microscopy tools to qualitatively analyze those biofilms. The success of the method relies upon the ability to produce very thin hyphal colonies and to apply the appropriate dyes. These points are discussed below.

Due to the complex structures of the biofilms, understanding their function requires a multi-scale approach<sup>28,29</sup>. Distribution patterns of the biofilms, bacterial colony architecture, and matrix structure and composition are analyzed at different scales (i.e., meso-scale and micro-scale). Moreover, nanoscale resolution allows access to the cell/cell physical interactions and the nano-structure of the matrix. Thus, the developed method easily enables a multi-scale analysis of the bacterial biofilms formed on the fungal colony.

In most studies, LSCM analyses of biofilms are limited to the micro-scale, the meso-scale usually being performed by optical coherence tomography<sup>30,31,32</sup>. The method presented here enables both micro- and meso-scale analyses by LSCM. It demonstrates the utility of combining both analyses in the same region of the sample and even on the same image using new-generation confocal microscopes with high resolution (Figure 3). Thus, issues linked to compiled data gathered at different scales with different methods are here avoided.

This combination of analyses gave access concomitantly to the biofilm repartition on the fungal colony, the bacterial colony architecture along the developing biofilm, and the matrix structure. The meso-scale analysis showed a heterogenic distribution of the bacterial biofilms on the fungal colonies (Figures 2 and 3). This observation would not have been possible with protocols that only permit imaging of a small portion of the fungal colony, which is not necessarily representative of the entire colony. Thus, while often neglected, the meso-scale analysis can give precious information about biofilm distribution patterns.

Finally, the developed method can be used to analyze samples with different microscopy techniques, including scanning electron microscopy. Here, SEM was used to reach the nano-scale and to obtain the bacterial spatial organization within the biofilm. It performed very well with the thin fungal colonies, while SEM only permitted surface imaging. In contrast to LSCM, SEM, however, required sample dehydration and, most often, coating with a conductive metal. This dehydration process might alter biological structures when it is not properly executed and may require optimization. Here, sample dehydration using slow lyophilization was used<sup>33</sup>. Nevertheless, applying both LSCM and SEM to the samples will allow the performance of correlative microscopy at the same location of the sample.

Despite the advantages described above, some limitations exist. Firstly, it may not be applicable to all kind of fungi. Indeed, this culturing method is developed for fungi spreading radially on the surface of solid media. This method may not be suitable for fungi forming mainly aerial hyphae (e.g., *Fusarium* sp.) or for micro-aerobic fungi spreading mainly inside agar. Moreover, fungi degrading cellophane may be problematic as well

(e.g., *Trichoderma* sp.). Secondly, it is important to note that the staining strategy is a critical point and the choice of the stain must be made carefully, as the stain must not disturb the biofilm. For example, we noticed that Calcofluor White caused partial biofilm disruption (data not shown), likely due to the high pH of this stain. Also, some dyes produced heterogeneous staining (e.g., Congo Red), while others produced homogeneous staining (e.g., cell wall staining with WGA lectin), giving a heterogeneous image quality. Moreover, it is important to be aware that some dyes might not be fully specific. For example, WGA stains not only fungal cell walls but also N-acetylneuraminic acid in gram-positive bacterial cell walls and adhesins produced by gram-positive and -negative bacteria during biofilm formation<sup>34,35</sup>. Therefore, using fluorescent protein-tagged bacteria and/or fungi is recommended to avoid multiple staining. If multiple dyes are used, they must not chemically interfere, and their emission spectra should not overlap.

Meso-scale analyses require a large scanned area, and therefore, LSCM may be time-consuming (40 min to 1 hr, depending on the sample thickness) and bottleneck the analysis of a large number of samples. Nonetheless, adjustments can be made depending on the type of data required. It is possible to decrease acquisition time and image size by altering the image quality. For example, high resolution is not necessary to analyze the biofilm general repartition.

Finally, some limitations need to be considered when choosing to display Z- stack data as 2D or 3D projections. Two-dimensional projections are a good way to summarize data, but depth information is lost, and overlapped structures become hidden. On the other hand, 3D projections allow the visualization from different points of view, but they often render poorly in case of spatial complexity.

In conclusion, we have reported a method for the characterization of bacterial biofilms on hyphae at the structural level. The methodology can be extended to other applications. Indeed, this method allows the performance of functional or chemical characterization of bacterial biofilms forming on fungal hyphae. Due to the great variety of existing fluorescent reporter systems, LSCM analysis can be used for multiple purposes<sup>29</sup>. For example, the fluorescence microscopy could be used to monitor pH gradient<sup>36</sup> or molecule diffusion in biofilms<sup>37</sup>. Additionally, the method allows for community analysis in multispecies biofilms. For example, fluorescence *in situ* hybridization targeting specific bacterial groups is particularly useful to study specific bacterial repartition in multispecies biofilms<sup>38,39</sup>. Last, numerous fluorescent dyes can be used to characterize the matrix composition of the biofilms<sup>21</sup>. Here, proteins were targeted using Sypro, which stains a large range of proteins, among them matrix proteins (Figure 4), but other dyes allow for the visualization of other important matrix constituents, such as exopolysaccharides or extracellular DNA. Interestingly, all these analyses could be performed at the meso-scale using the described method. Since LSCM can be performed on living samples, it is also possible to achieve time-lapse imaging using, for example, coverwell chambers, particularly suitable for thin fungal colonies. This option is particularly interesting, as biofilm formation is a complex, dynamic process. Finally, for a quantitative purpose, the reported method may improve the accuracy of automatic quantitative analysis by making this quantification possible on meso-scale images. This may overcome biofilm heterogeneity and statistical issues<sup>29</sup>.

## Disclosures

The authors have nothing to disclose.

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