Supplementary Information for

Optical sectioning of unlabeled samples using bright-field microscopy

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Supplementary Text
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Extended Materials and Methods

Samples. Unless otherwise indicated, reagents were purchased from Sigma-Aldrich (St. Louis, MO). All optical imaging was performed at room temperature (22-24 °C).

Fungi. For OSBM imaging of fungal samples, 700 µL of Vogel’s medium was left to solidify at the bottom of a polymethacrylate fluorimeter cuvette. Conidia of *Trichoderma atroviride* wild-type strain (IMI 206040) were inoculated at the surface of the medium and left to incubate during 36 h at 28 °C. Next, 2 mL of liquid Vogel’s medium at 1% was poured in the cuvette, over the fungal colony. After an incubation over 24 h at 28 °C, the sample was taken to the microscope for measurements.

For the desiccated *T. atroviride* sample, fungal plugs were grown in Petri dishes on solid medium (Potato Dextrose Agar, Difco) at 28°C during 60 h, in the dark. Colonies were then transferred to a dry environment at room temperature and left to desiccate over four months. Dry medium supporting dry fungi was detached off the Petri dish and suspended between two coverglasses for optical microscope visualization. For electron microscopy observation, fungal samples were coated with gold over 30 s at 40 mbar pressure.

Blood vessel. A section of rat spinal cord was cleared using the CLARITY technique [14], without introducing any labeling. Animal procedures and protocols were approved by the Institutional Ethics Committee (Institute of Neurobiology, UNAM, Mexico) and in accordance with the ethical guidelines of the IASP and the NIH. Meningeal tissue was separated from the rest of tissue and placed in 2,2′ thiodiethanol (TDE) at increasing concentrations of 20%, 40% and 60%, during 24 h in each, at room temperature. Tissue was
placed in a glass fluorimeter cuvette (Hellma) at 60% TDE, and subject to microscopy experiments.

Onion cells. A common onion (*Allium cepa*) from the local grocery store was peeled, a strip of scale epidermis was removed and immersed in distilled water (dH₂O) inside a plastic cuvette and taken to the microscope (results shown in Fig. 3A). For the results shown in Movie S3, the sample was stained in a 0.001% (w/v) solution of propidium iodide in water for 15 min, followed by submerging in dH₂O for 15 min. The sample was then immersed in dH₂O inside a plastic cuvette and taken to the microscope. Microscopy measurements were performed within the first 2 h after preparations.

*Arabidopsis thaliana*. Seeds of *A. thaliana* (ecotype Col-0) were disinfected with 75% ethanol during 3 min under agitation, then with 20% chlorine during 7 min, and followed by 4 rinses with sterile dH₂O. The disinfected seeds were placed on 0.7% MS agarose plates (Murashige and Skoog medium supplemented with 1% sucrose, pH 5.7, and plant agar), and kept in the dark for 2 days at 4 °C. Finally, seeds are placed to germinate at 22 °C under a 16 h light/8 h dark regime. 4-days old seedlings were removed from the agar plate and transferred to a plastic cuvette filled with dH₂O for microscopy observation.

**Microscopy.** Light microscopy was performed using a LSFM home-made instrument whose construction and testing has been described in detail elsewhere [15]. Briefly, the LSFM setup consists of an illumination laser beam (λ = 488 nm or 594 nm) focused onto the sample by a 4X/0.13 (magnification/numerical aperture) microscope objective (N4X-PF, Nikon). The laser beam is scanned using twin galvanometric mirrors and shaped using telescope and scanning lenses, such that a light-sheet 5.6-μm thick (twice the beam waist, 2ω₀) and 100-
µm deep (twice the Rayleigh length) is formed at the sample plane. The detection arm is formed by a 10X/0.28 long-working distance (LWD = 34 mm) microscope objective (MY10X-803, Mitutoyo), a GFP 525/39 (MF525-39 Thorlabs) or dual band 524/29 and 628/33 (FF01-524/628-25 Semrock) emission filters, a tube lens TL2 (TTL200, Thorlabs) and a sCMOS camera (OrcaFlash 4.0-LT, Hamamatsu). An 800-µm travel range piezo electric stage (P-628.1CD, PI) displaces the detection objective axially, concomitantly with the light sheet. Instrument control is achieved using a multifunction data acquisition system (DAQ) (USB-6341, National Instruments) operated by a dual Xeon gold 5122 (3.60 GHz) workstation (Precision 7920, Dell). Automated control and acquisition are achieved by custom-made routines developed in LabView (National Instruments). For LSFM, specimen autofluorescence under excitation at \( \lambda = 488 \) nm (525/39 emission filter) was collected throughout, except for fluorescence data shown in Movie S3. For the fluorescence results shown in Movie S3, excitation at \( \lambda = 594 \) nm and 628/33 emission filter were used. Typical LSFM imaging parameters: laser intensity 1-3 mW, exposure time 200 ms, z-step 4 µm. Laser intensity was increased for fungal samples to 30 mW.

The bright field part of the microscope uses a conventional setup that affords Köhler illumination in the coherent regime (see Fig. 1A), consisting of a high-power light emitting diode (LED) illumination source (\( \lambda = 445 \) nm, bandwidth (FWHM) = 23 nm, SOLIS-445C, Thorlabs), followed by a collector lens (\( f = 60 \) mm) and a LWD air condenser lens (0.78 NA, CSC2001, Thorlabs), all mounted on a rigid microscope body (CFB1500, Thorlabs). To provide space for sample manipulation, a \( 2f' \) relay lens was used to image the condenser illumination plane onto the specimen plane. Bright field was aligned for Köhler illumination, and the condenser iris diaphragm was adjusted to limit the numerical aperture of the
condenser to $NA_{\text{condenser}} = 0.05$ for all samples, except for fungi in liquid ($NA_{\text{condenser}} = 0.15$). BF imaging parameters for $z$-stacks: exposure time 200 ms, $z$-step 4 μm. Images were acquired at 16-bit depth, $2048 \times 2048 \text{ px}^2$ or binned to $1024 \times 1024 \text{ px}^2$, and illumination intensity was adjusted such that background intensity in images was 30,000-40,000 counts. Acquired images were stored for off-line processing.

**Electron microscopy.** Scanning electron microscopy was performed using an ESEM Quanta 250 FEG system (FEI, Hillsboro, OR, USA), operating at 10 keV.

**Digital image processing.** All digital image processing is performed using FIJI [16] plugins. The following steps, depicted in Figure 1D, are implemented sequentially: “FFT highpass” (step $i$), “smooth and z-gradient” (steps $ii$-$v$), “white top hat” (step $vi$).

(i) Large spatial structures are removed from raw images by applying the FFT-based **Bandpass Filter** ($\text{filter\_large} = 1$ or 2 px; $\text{filter\_small} = 0$ px) on each $x$-$y$ image of the $z$-stack, yielding $I_{\text{FFT,xy}}$. To obtain stacks of $xz$ and $yz$ slices, the stack $I_{\text{FFT,xy}}$ is subject to **Reslice** from top and left, producing $I_{\text{FFT,xz}}$ and $I_{\text{FFT,yz}}$, respectively. **Bandpass Filter** ($\text{filter\_large} = 1$ or 2 px; $\text{filter\_small} = 0$ px) is applied to each $I_{\text{FFT,xz}}$ and $I_{\text{FFT,yz}}$, and the two resulting stacks are resliced back to $x$-$y$ views and averaged. (ii) Background is subtracted on a frame-by-frame basis, by computing the mean count value of a ROI where no sample is present and subtracting this value from all pixel-counts in the frame. (iii) To reduce computation time, images acquired at $2048 \times 2048 \text{ px}^2$ are binned by a factor of 2×2. Images acquired at $1024 \times 1024 \text{ px}^2$ are not modified in this step. (iv) The smoothing filter **Gaussian Blur 3D** ($x = 2, y = 2, z = 1.5$) is applied to reduce noise. (v) To better localize scattering structures, we obtain the intensity gradient along $z$ by performing pairwise frame subtraction in the $z$-stack,
using $\Delta z = 4$ frames. (vi) A White Top Hat filter along the $z$-axis is applied to improve localization of scattering structures, available in *Morphological Filters (3D)* with parameters “operation=[White Top Hat] element=Z-Line x-radius=1 y-radius=1 z-radius=2”. For the hyphal sample a final filter is applied to remove small particles (below 40 px$^2$), helping to reduce background noise. For the onion sample, the $xy$ OSBM image stack was rotated by 3 deg counterclockwise (Fig. 3A, left) to align the $xz$ cross section to the main axis of the cells. No further filters are applied to the resulting images. The plugins *XYZ Max Projection* and *Volume Viewer* are used to produce MIP images and 3D renderings, respectively. Image processing is fully automatic, with typical computation time $\sim 3$ min ($\sim 25$ min) for $1024 \times 1024 \times 260$ voxels ($2048 \times 2048 \times 260$ voxels) image stacks, using our workstation. The limiting step in processing time is the FFT-based bandpass filter. The filter sequence was found applicable to any BF image stack and the parameters quoted optimize OSBM results (by comparing against LSFM); adjustment to these parameters may be required for new images.

**Comparison of BF imaging with a ground truth.** This subsection refers to Fig. 1C. To perform in-focus imaging tests, we used a quasi-2D phase object. A colony of the filamentous fungus *T. atroviride* was grown on solid medium and left to dry over several months, resulting in collapsed cell wall structures with height variations $\sim 1$ μm (estimated from electron microscopy imaging). The dry mycelium footprint presents little light absorption, providing an excellent phase sample to perform tests on BF imaging. Observing this sample through the BF microscope at low magnification (10× objective), images display the familiar change in contrast (from positive to zero to negative) when scanned axially (tens of micrometers) through focus. We set the axial position of exact focus ($z = 0$) when images
Next, imaging of samples within the depth of field (DOF) of the BF microscope was performed. As a guide, we consider two different measures of the DOF: the physical depth of field\(^1\) (DOF\(_1\) = \(\lambda n/\text{NA}_{\text{objective}}^2\)), and the setting accuracy (DOF\(_2\) = \(\lambda/(4n\sin^2(u/2))\)), where \(u\) is the half angle of the cone of light captured by the objective lens) that corresponds to the DOF when a thin detector is used\(^2,3\). The DOF increases significantly (up to a factor of two) as the numerical aperture of the condenser (\(\text{NA}_{\text{condenser}}\)) decreases toward zero (the coherence of illumination increases)\(^1\). We use \(\text{NA}_{\text{condenser}} \sim 0.1\); therefore, we consider twice the DOF values provided by the above formulas as the permissible range (centered around \(z = 0\)) to be “within focus”. For the dry fungal sample (Fig. 1C in the main text) parameters are: \(\text{NA}_{\text{objective}} = 0.25\), \(\lambda = 450\ \text{nm}\), \(n = 1\); resulting in \(2 \times \text{DOF}_1 = 14.4\ \mu\text{m}\) and \(2 \times \text{DOF}_2 = 14.2\ \mu\text{m}\). To determine the allowed sample in-focus range, we simply take the geometrical average of DOF\(_{1,2}\). In this case, \(z \in [-7.1\ \mu\text{m}, 7.1\ \mu\text{m}]\) is the allowed in-focus range.

Figure 1C in the main text shows an image of the dry fungal sample at position \(z = -6.0\ \mu\text{m}\), after pixel intensities in the raw image were multiplied by -1 and a sharpening filter was applied. Individual filaments, hyphal cell wall, and septa are clearly observed. To determine the accuracy of this BF image, we observe the same sample using scanning electron (SEM) microscopy (Fig. 1C). The SEM image has a little contrast, evidencing a sample with limited height variations at the surface (estimated: \(\sim 1\ \mu\text{m}\)). Despite this limitation, an excellent correspondence between BF and SEM images is found.

**Parameter considerations for OSBM.** Expected extensions to OSBM imaging include applicability at higher magnification, and further exploration of parameters such as illumination characteristics (e.g. source wavelength and spectral range, \(\text{NA}_{\text{condenser}}\)).
microscopy components (e.g. camera bit depth), and optical sample properties (e.g. refractive index of surrounding medium). As an example, using an illumination source of narrow spectral range helps increase BF image contrast of weakly scattering objects under coherent illumination. Future improvements should consider minimizing sample optical absorption by choosing an appropriate wavelength of the illumination source (use of infrared light for biological specimens). In addition, knowledge of the expected pPSF could be included to improve OSBM imaging, for example by means of an iterative algorithm based on minimizing the residual between the raw BF image and the OSBM image convolved with the pPSF.

**SI Movies**

**Movie S1.** False color, OSBM image of the *T. atroviride* sample shown in Fig. 2C of the main text. Field of view, 1495×1495×1016 μm³.

**Movie S2.** False color, OSBM image of the blood vessel sample shown in Fig. 2F of the main text. Field of view, 1495×1495×1128 μm³.

**Movie S3.** Set of y-z MIP image overlays of OSBM (green) and LSFM (red) for a skin onion sample. OSBM sections consistently show cell shape, even in places where the fluorescence signal is weak or absent. Each MIP image corresponds to a ROI 36.5 μm × 2111 μm × 1008 μm in volume, obtained for different x-positions. Field of view, 2111×1008 μm²; separation between images, Δx = 73 μm.

**Movie S4.** 3D viewing of the OSBM result for the plant root sample shown in Fig. 3B of the main text. Field of view, 1495×1495×1008 μm³.

**SI References**

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