Chapter 2

Three-Dimensional Live Imaging of Filamentous Fungi with Light Sheet-Based Fluorescence Microscopy (LSFM)

Francesco Pampaloni, Laura Knuppertz, Andrea Hamann, Heinz D. Osiewacz, and Ernst H.K. Stelzer

Abstract

We describe a method for the three-dimensional live imaging of filamentous fungi with light sheet-based fluorescence microscopy (LSFM). LSFM provides completely new opportunities to investigate the biology of fungal cells and other microorganisms with high spatial and temporal resolution. As an example, we study the established aging model *Podospora anserina*. The protocol explains the mounting of the live fungi for the light sheet imaging, the imaging procedure and illustrates basic image processing of data.

Key words Advanced light microscopy, Light sheet-based fluorescence microscopy, Live cell imaging, LSFM, SPIM, DSLM, *Podospora anserina*, Ascomycetes, Mycology, Microbiology, Aging model, Autophagy

1 Introduction

Advanced fluorescence microscopy, including confocal microscopy [1, 2], is a standard tool to study filamentous fungi at cellular and subcellular levels [1–3]. Fluorescence microscopy is essential for in vivo studies of dynamic processes and provides spatially and temporally resolved information of organelles such as the endoplasmic reticulum, mitochondria, and the Golgi apparatus [2].

We describe a detailed step-by-step user guide for the live imaging of fungal mycelia and hyphae with the cutting-edge technology Light Sheet-based Fluorescence Microscopy (LSFM) [4]. As an example, we apply the protocol to the filamentous fungus *Podospora anserina*.

1.1 *Podospora anserina*

Podospora anserina is a filamentous fungus that is characterized by a limited, short lifespan [5, 6]. After germination of ascospores, the progeny of a sexual reproduction, the developing colony (mycelium) grows by extension at the tips of filamentous cells. After a strain-specific period of linear growth, growth slows down

Yolanda Markaki and Hartmann Harz (eds.), *Light Microscopy: Methods and Protocols*, Methods in Molecular Biology, vol. 1563, DOI 10.1007/978-1-4939-6810-7_2,

until it completely ceases and the mycelium dies at the periphery. This easily identifiable phenotype, the availability of mutant strains and the plethora of potential experiments make *P. anserina* an ideal model for the investigation of the basic mechanisms of aging [7–12]. Over the years, several specific *P. anserina* strains have been generated and molecular pathways have been identified, which are involved in the control of aging and life span [10], e.g., autophagy. For microscopic analyses of pathways related to autophagy, transgenic strains expressing fluorescent fusion proteins are available [8, 11, 13]. In our protocol, we employ the *P. anserina* GFP::PaATG8 strain. PaATG8 is the fungal homologue of the LC3 protein in mammalian cells. The GFP-tagged PaATG8 allows to monitor autophagosomal dynamics in live fungus [13].

1.2 Light Sheet-based Fluorescence Microscopy (LSFM)

Imaging dynamic processes in living organisms with fluorescence microscopy has developed into an essential tool in biology [14–16]. Time-lapse fluorescence microscopy provides information on cellular and subcellular processes over time in whole organisms and single cells. Three-dimensional imaging is essential to analyze the interplay of cells in multicellular organisms. The main challenges of fluorescence microscopy are minimizing phototoxic effects in live specimens, minimizing photobleaching and achieving a high three-dimensional recording speed. These challenges are addressed by spinning-disk confocal fluorescence microscopy [17], heavily optimized wide-field fluorescence microscopy (OMX) [18], and light sheet-based fluorescence microscopy (LSFM) [19, 20]. The youngest and most promising of these three techniques is LSFM. The principles of LSFM are described in Fig. 1. In LSFM, a laser light sheet illuminates the sample with an extremely low energy of about 2 μJ at 488 nm in the illumination plane [20, 21]. LSFM takes advantage of modern CCD and CMOS cameras, which provide a high recording speed. While in second-generation LSFMs such as the Digital Scanned Light Sheet-based Fluorescence Microscope (DSLM), a speed of six planes/second was achieved (one plane consisting of 2048 × 2048 pixels) [20], third-generation microscopes record >100 images per second [22]. A key advantage of LSFM over confocal microscopes is the high dynamic range of the images, which is essential for subsequent high-performance image processing. With LSFM, the long-term fluorescence imaging of zebrafish (*Danio rerio*) and fruit fly (*Drosophila melanogaster)* embryos for up to 72 h have been achieved without impairing embryonic development [20, 23]. The high speed of the light sheet microscope has allowed to follow the mitosis and migration in toto of more than 16,000 cells in the zebrafish embryo. LSFM has also been applied to study developing lateral roots in *Arabidopsis thaliana* [24], to investigate the development of *Tribolium castaneum* [25, 26], and, not the least, to study the behavior of three-dimensional cell cultures and multicellular spheroids [27–31].

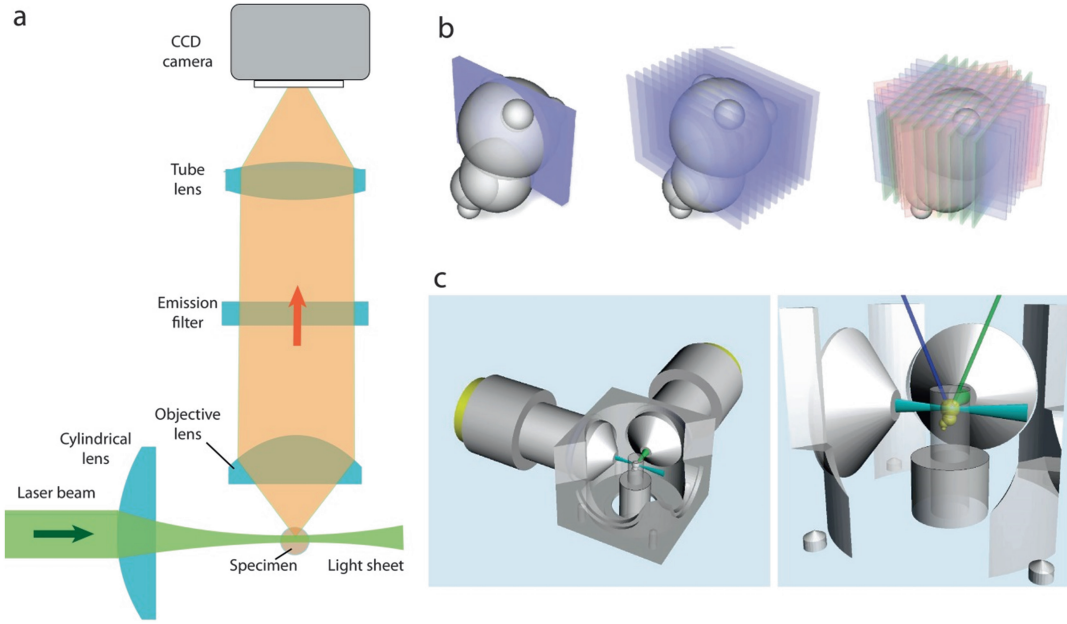

Fig. 1 Principles of Light Sheet-based Fluorescence Microscopy (LSFM). (**a**) Setup of a single plane illumination microscope (aka SPIM [19]). (**b**) Principles of LSFM imaging. *Left*: a single plane in the specimen is illuminated by a light sheet and overlaps with the focal plane of the detection lens. Hence, only the plane that is observed is also illuminated, resulting in lower photobleaching and lower phototoxicity. *Center*: by moving the specimen through the stationary light sheet, a three-dimensional stack of images is recorded. *Right*: by rotating the specimen multiple-views image stacks are obtained. Combining multiple different views of the specimen increases the resolution along the *z*-axis. (**c**) Close-up of a specimen chamber, showing both the illumination and detection objective lenses oriented at a 90° angle with respect to each other

2 Materials

2.1 Chemicals and Small Laboratory Equipment

1. *Low-melting point agarose aliquots*: prepare a 1 % solution of low-melting point agarose (gel point 26–30 °C, e.g., Sigma A9414) in PBS. Aliquot the agarose solution in 2 ml vials. Store at 4 °C.
2. *5 × 5 mm coverslips* with thickness between 0.06 and 0.08 mm (Wagner & Munz GmbH, Munich, Germany, http://www.wagnermunz.com/).
3. *Sharp angled-tip precision forceps* (e.g., Excelta SKU 50-SA, http://www.excelta.com/).
4. Bent coverglass forceps with enlarged rectangular tips (e.g., Leica Biosystems 38DI11102, http://www.leicabiosystems.com).
5. *Clear transparent nail polish.*
6. *Disposable scalpel.*
7. *Laboratory pipettes (P200, 20–200 µl, yellow tips).*

2.2 P. anserina Strains and Culture Media

1. *P. anserina Gfp::PaAtg8 strain* constructed from the wild-type strain 's' [5].
2. *Standard cornmeal agar* (BMM) [6].
3. *M2 agar* [9].

2.3 Custom Equipment

2.3.1 Sample Holder for the LSFM

The function of the LSFM holder is to provide a stable support for the sample and mechanically connect it with the xyzθ translational/rotational stage of the microscope. We describe a basic sample holder design that can be adapted to the LSFM stage available to the user. Our holder has been designed for the *monolithic Digitally Scanned Light Sheet Microscope* used in our laboratory (mDSLM, *see* Fig. 1c and [24]).

The holder consists of a stainless steel rod carved at one edge in order to support a coverslip, as shown in Fig. 2. The holder can be fabricated by any mechanical workshop. An alternative fabrication method is 3D printing, which is a reasonable approach when testing various holder configurations (*see* **Notes 1–3** for tips and tricks). In any case, the material hast to be biocompatible and endure repeated autoclaving. For machined holders we recommend stainless steel or a hard plastic material such as Delrin™ (polyoxymethylen, POM).

The 5 mm × 5 mm glass coverslips that supports the *P. anserina* sample is glued to the specimen holder by a droplet of transparent

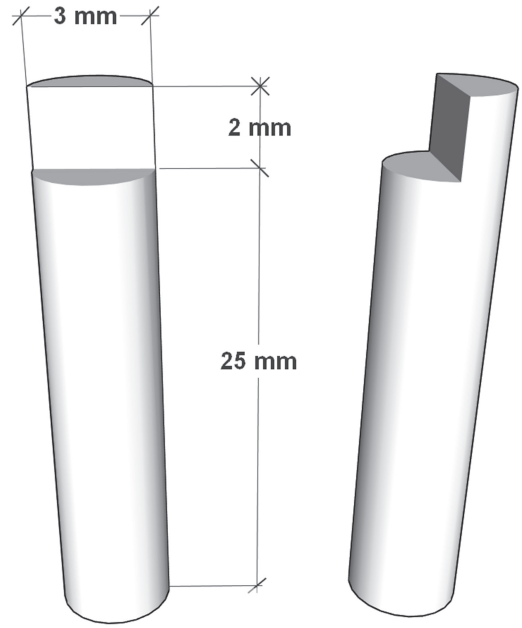

Fig. 2 Custom sample holder for LSFM. The LSFM holder connects the sample with the xyzθ translational/rotational stage of the light sheet microscope. The holder is custom built in a mechanical workshop or 3D-printed (*see* **Note 1–3**). Possible materials are stainless steel or hard plastic, such as POM

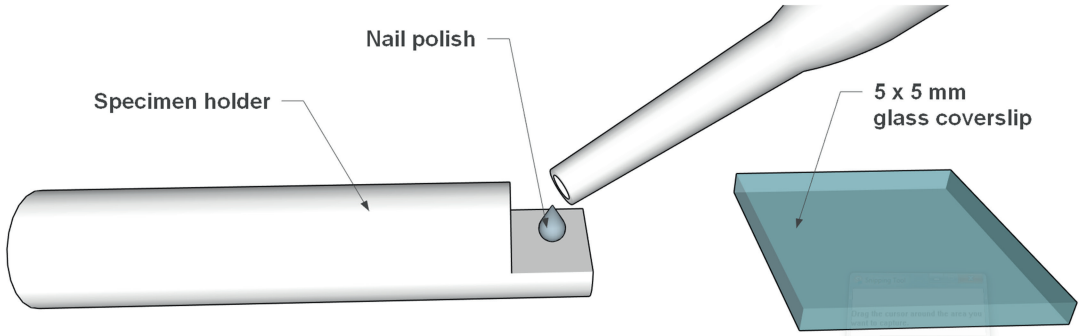

Fig. 3 Attachment of the glass substrate on the sample holder. A tiny droplet of nail polish is deposited on the carved part of the holder with a pipette and a 5 mm × 5 mm glass coverslip is firmly attached for a few seconds

nail polish. Nail polish is an effective, cheap, and quickly hardening glue for mounting samples in light microscopy. Once dried it has no harmful effects on biological specimens immersed in common culture media.

As shown in Fig. 3, a tiny amount of nail polish is deposited on the carved part of the LSFM holder by using a pipette or a brush. Next, the coverslip is rapidly but gently attached to the holder by using flat-tip coverslip forceps. The nail polish is allowed to dry for at least 30 min while the holder lies horizontally with the sample pointing upwards, to ensure a positioning parallel to the holder's longitudinal axis.

The appearance of the assembled holder, ready for mounting the sample, is shown in Fig. 4.

2.4 Imaging Equipment

1. Various suitable LSFM implementations are described in detail in [19, 32, 33] (SPIM), [20] (DSLM), [24] (monolithic DSLM, mDSLM). Commercial LSFMs are now available from the companies Zeiss (LightSheet Z.1, http://www.zeiss.com/microscopy/en_us/products/imaging-systems/lightsheet-z-1.html), Luxendo (http://luxendo.eu/), and 3i (https://www.intelligent-imaging.com/index.php).
2. Long working-distance water-dipping objective lenses with high numerical aperture (e.g., Carl Zeiss Plan-Apochromat 63x/1.0 W, 421,480–9900-000).

2.5 Software for Image Processing

Image processing software package (e.g., Fiji—Fiji is Just ImageJ, a distribution of ImageJ, http://fiji.sc/).

3 Methods

3.1 P. anserina Cultivation

The *P. anserina Gfp::PaAtg8* strain expresses an N-terminal fusion of the GFP protein to PaATG8, the fungal homologue to mammalian LC3, a well-known marker of autophagy. Labeling of ATG8

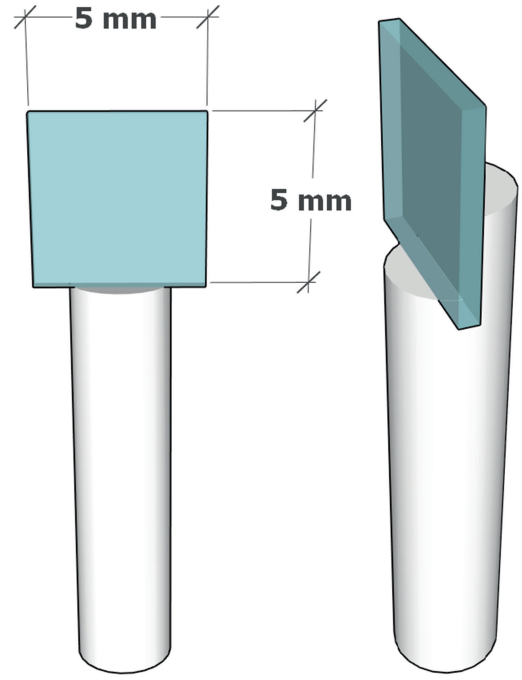

Fig. 4 Assembled sample holder. The final holder ready for mounting the sample

allows the observation of the translocation of the fusion protein from the cytosol to the autophagosome to the vacuole, where the final degradation of the autophagosome takes place in fungi. For the cloning procedure *see* [13].

1. Isolate monokaryotic spores from asci of a *Gfp::PaAtg8* cross.
2. Germination of isolated spores takes place by cultivation on standard cornmeal agar (BMM) supplemented with 60 mM ammonium acetate.
3. Incubate the spores at 27 °C in the dark for 2 days.
4. Culture freshly isolated *P. anserina* strains on M2-agar. See for the exact composition of the M2-medium.

3.2 Sample Mounting

A 2 ml low-melting point agarose is put in a heater at 65 °C–70 °C until the agarose is completely liquid. Shortly before imaging, a 5 mm × 5 mm slab of M2-agarose with the cultured mycelium growing is cut with a sharp-tip scalpel (Fig. 5). In our setup, the mycelium is grown on a thin layer (<1 mm) of M2-agarose deposited on a glass slide. The glass slide is placed inside a 100 mm petri dish also containing water-soaked tissue paper. This avoids drying of the agarose during the culture.

Next, the square slab is gently removed with a sharp angled-tip forceps and placed on the sample holder coverslip, on which a

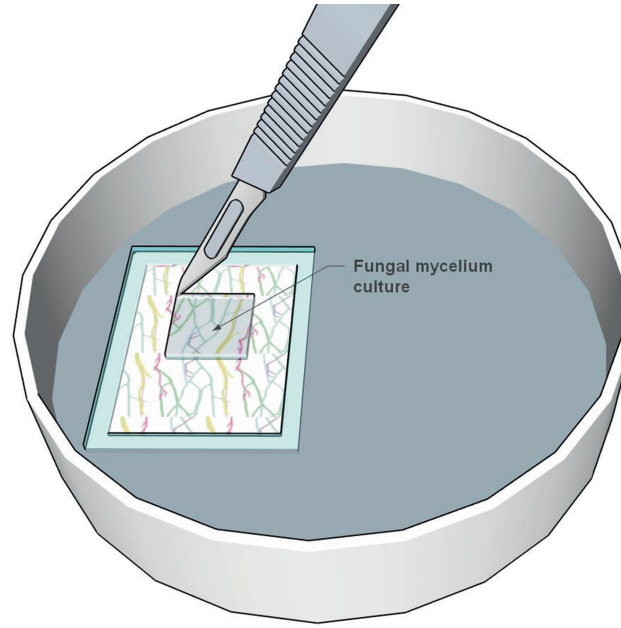

Fig. 5 Cutting the mycelium sample from the fungal culture on agar gel. A roughly 5 mm × 5 mm square slab is cut from the fungal culture by using a sterile scalpel

droplet of liquid low-melting agarose is deposited (Fig. 6a, b). The agarose ensures a stable adhesion between the glass substrate and the sample.

Figure 6c displays the real sample observed under a stereomicroscope.

3.3 LSFM Imaging

Figure 7 shows the position of the sample holder in the LSFM chamber. For imaging it is essential that the coverslip surface with the sample is oriented at an angle of 45° with respect to the illumination axes of both the illumination and the detection paths of the light sheet microscope, as depicted in Fig. 7a, b.

An illumination/detection angle of 45° ensures an optimal imaging of the nearly flat coverslip-mounted sample in the LSFM. In order to record a three-dimensional image stack, the sample is translated stepwise along the *z*-axis, parallel to the detection optical axis (Fig. 7a, arrow). A typical *z*-axis step size for the imaging of *P. anserina* is 0.5 μm. The stack recording procedure is schematically shown in Fig. 8.

In order to resolve subcellular structures in the hyphae, high-numerical aperture (NA) water-dipping objectives are recommended. We employed a Carl Zeiss Plan-Apochromat 63×/1.0 water dipping lens, which resolves tiny organelles such as individual autophagosomes. Objective lenses with low magnification and high NA (e.g., Nikon N16XLWD-PF, 16×, NA 0.80) are ideal,

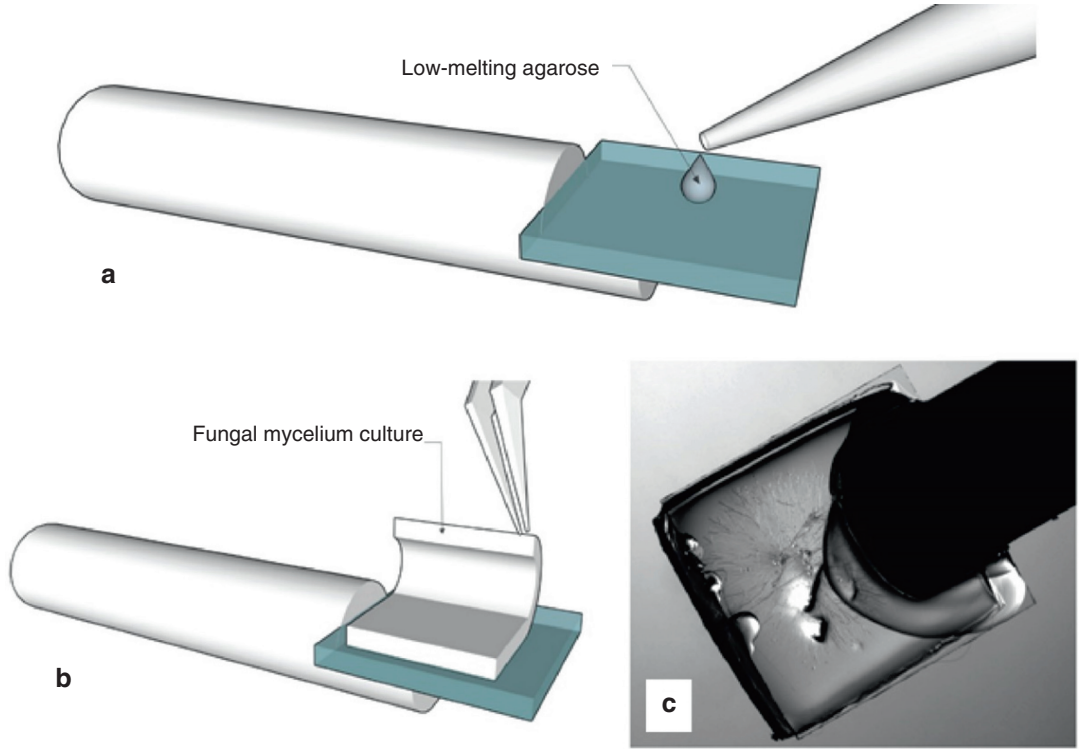

Fig. 6 Mounting the sample on the LSFM-holder. (**a**) A droplet of low-melting point 1 % agarose is pipetted on the glass coverslip. (**b**) The fungal culture cut slab is placed on the coverslip with a forceps. (**c**) A photograph of the real mounted sample recorded with a stereomicroscope (from Ref. [13]). The mycelium and hyphae are visible on the coverslip (magnification 10×)

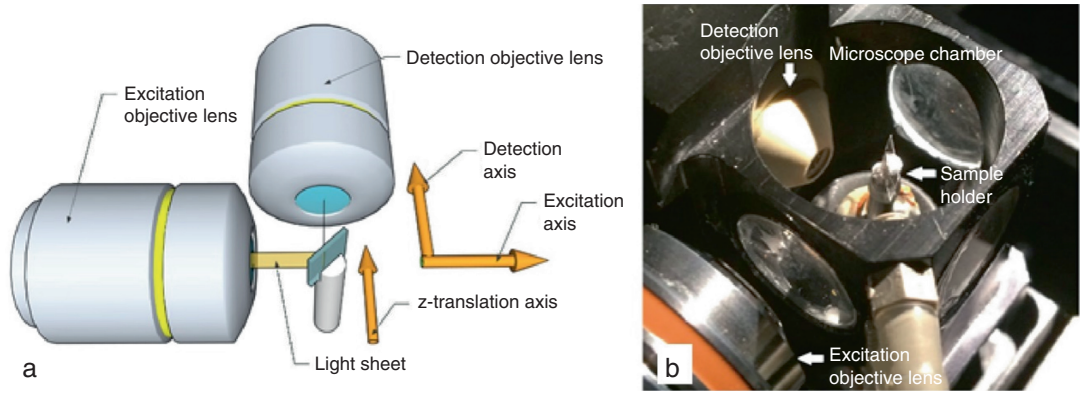

Fig. 7 Positioning the mounted sample in the LSFM. The mounted sample is inserted in the light sheet microscope (in this case an mDSLM). (**a**) Note that the coverslip surface is oriented at an angle of 45° with respect to the illumination and detection optical axes (*arrows*). (**b**) An actual implementation, including the microscope chamber, the excitation objective lens, the detection objective lens and the mounted sample. During imaging, the chamber is filled with culture media

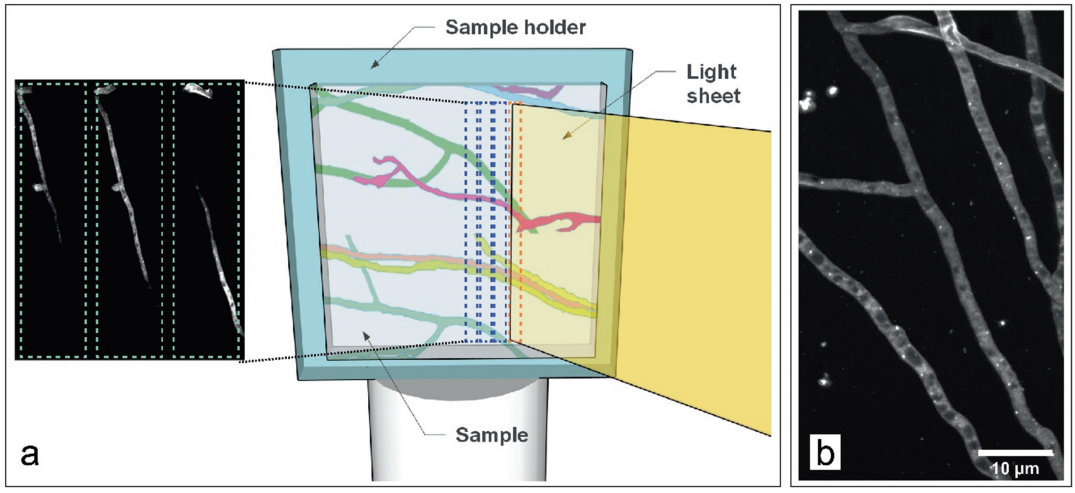

Fig. 8 Recording a three-dimensional stack of the sample. The sample, positioned at 45° is translated along the *z*-axis. (**a**) Left, *z*-slices of isolated hyphae are shown. The slices are recorded with a 0.5 µm step size. (**b**) Maximum projection of 15 slices showing the mycelium hyphae

since they combine high spatial resolution, long working distance and a large field-of-view. As illumination lens we used a Carl Zeiss Plan-Neofluar 5×/0.16, which produces a light sheet with a thickness of ~2 µm.

3.4 Results

The montage in Fig. 9 shows the individual slices of a stack, recorded at increasing depth in the sample with 0.5 µm steps. A single hypha in the mycelium is imaged.

By taking advantages of the high scanning speed of LSFM, the shape and spatial orientation of the hyphae is directly obtained (Fig. 10a–c). This provides new insights into fungal growth patterns. Moreover, the high resolution of the 63×/1.0 objective lens allows the detection of single autophagosomes. Due to the high signal-to-noise ratio and the high dynamic range of a camera, the autophagosomes can be readily segmented by applying the segmentation algorithms available, e.g., in the image processing software *Fiji* (Fig. 10b).

The light sheet microscope's high recording speed is exploited for the time-lapse imaging of rapid subcellular events. In the example shown in Figs. 11 and 12, the displacements of one individual autophagosomes are tracked in three-dimensions.

4 Notes

1. 3D printing (aka additive manufacturing) is a suitable alternative to conventional machining for the production of custom LSFM holders. It is particularly useful for prototyping and

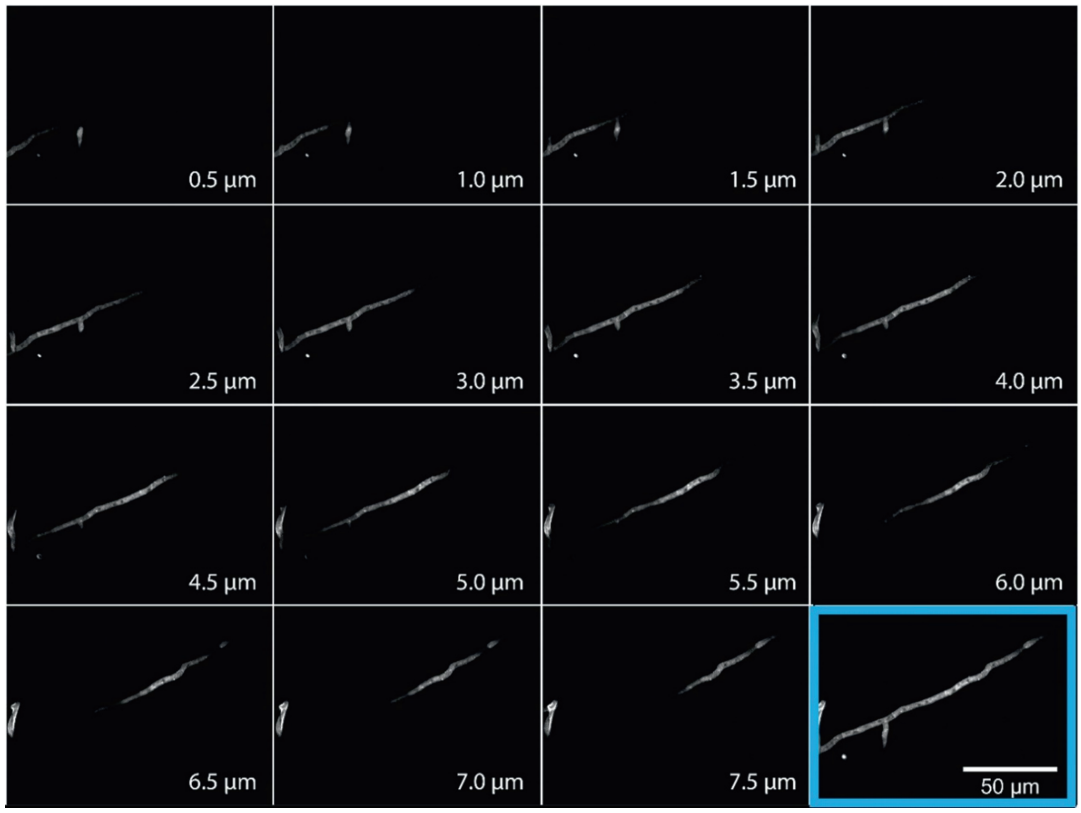

Fig. 9 Three-dimensional image stack of a single hyphae of *Podospora anserina* recorded with an mDSLM. The recording depth is shown in each slice. The highlighted image at the bottom right is a maximum projection of the stack slices and shows the entire hyphae. Acquisition parameters: detection lens CZ Plan-Apochromat 63×/1.0 W, excitation lens Plan-Neofluar 5×/0.16, laser line 488 nm, laser intensity 2.5 mW, exposure time 100 ms, emission filter 525/50. Marker: GFP-tagged Atg8

testing different configurations of a holder. A further advantage of 3D printing is that it allows the construction of shapes that are not achievable with a conventional milling machine or a lathe. Professional printing services are available (e.g., Shapeways, www.shapeways.com). CAD-files can be uploaded on the website of the 3D printer service and the printed parts are usually shipped within few days.

2. The 3D parts can be designed with CAD software packages that are downloadable for free under determined license restrictions. Examples are Inventor 2014 and Autodesk 123Design (both from Autodesk, San Rafael, CA), or Sketchup (https://www.sketchup.com). The CAD files of the designed parts can be converted to a file format that is interpretable by the 3D printer (a typical file format is .stl).
3. The biocompatibility of the material should be tested before using 3D-printed parts in experiments. Studies assessing this issue have been performed, see for instance [34].

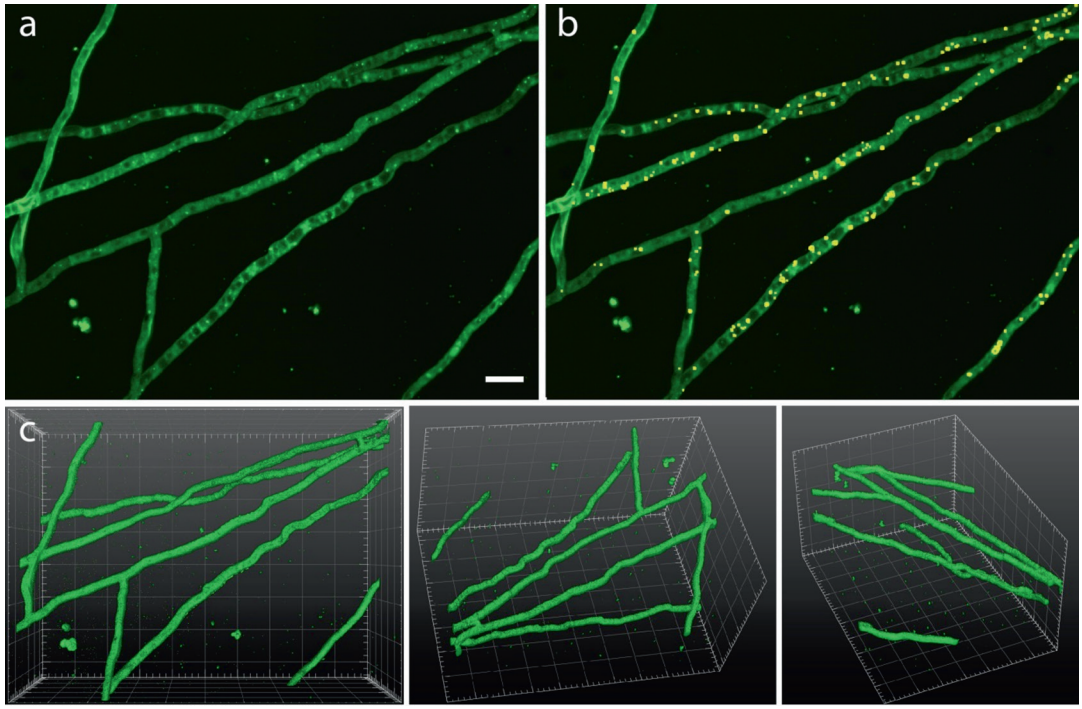

Fig. 10 Three-dimensional imaging of individual hyphae of *Podospora anserina* with a light sheet microscope (mDSLM) The specimen is a 20-day-old wild-type *P. anserina* culture, expressing Gfp-tagged Atg8. (**a**) Maximum projection of the image stack. Scale bar 10 μm. (**b**) Segmented autophagosomes (*yellow dots*) superimposed to the maximum projection. (**c**) Three-dimensional spatial orientation of the hyphae from three different points of view, showing the highly entangled architecture. The squares are 10 μm × 10 μm. The 3D rendering has been performed with the "3D Viewer" plugin of Fiji. Imaging parameters: the stack was composed by 192 slices. The slice spacing was 0.5 μm. Acquisition parameters: detection lens CZ Plan-Apochromat 63×/1.0 W, excitation lens Plan-Neofluar 5×/0.16, laser line 488 nm, laser intensity 2.5 mW, exposure time 100 ms, emission filter 525/50

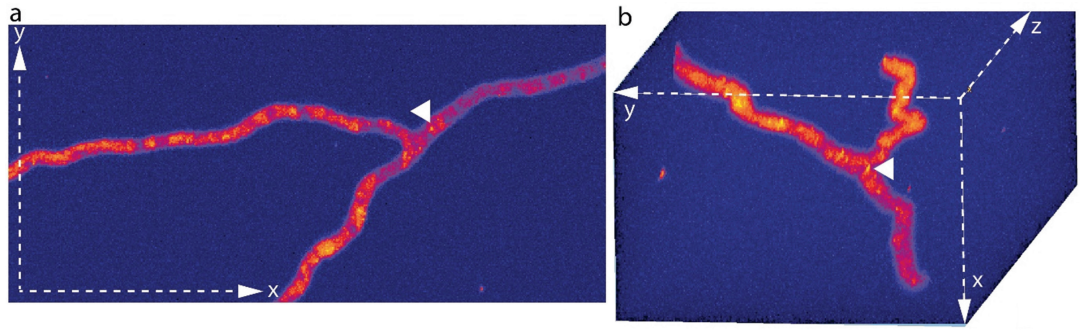

Fig. 11 Three-dimensional time-dependent localization of autophagosomes in the hyphae. (**a**) Maximum projection of one stack. The *white arrow* points at one single autophagosome within the hypha. (**b**) The same stack as a maximum projection rotated in space to highlight the shape of the hyphae from another point of view. The *white arrow* points to the same autophagosome in (**a**). The look-up table "blue orange icb"(In Fiji: Image/Lookup Tables/blue orange icb) was applied to highlight the autophagosomes. The acquisition parameters are the same as in Fig. 10. The data was processed with the "3D project" command of Fiji

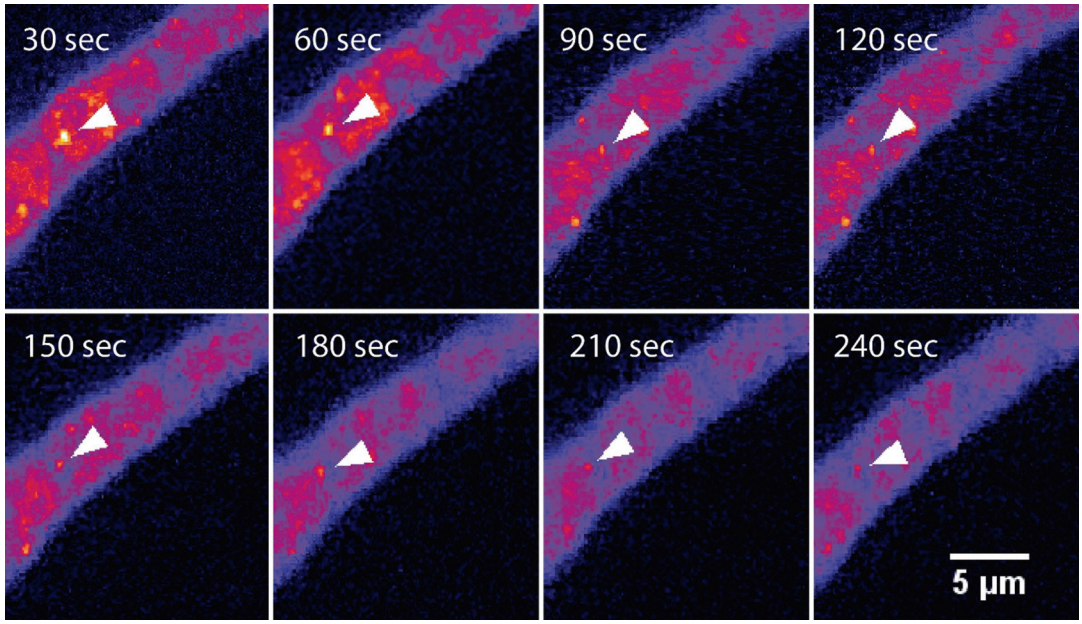

Fig. 12 Time-dependent localization of autophagosomes in the hyphae. Zoom-in of the region around the autophagosome in Fig. 11a. The time sequence shows the movements of the autophagosome. Each frame represents the maximum projection of one complete three-dimensional image stack. Stack were recorded every 30 s. The look-up table "blue orange icb" was applied to better highlight the autophagosomes. Acquisition parameters as in Fig. 10

Acknowledgments

The authors thank the LOEWE initiative of the Federal State of Hessen (Ub-Net, speaker Ivan Dikic) and the Deutsche Forschungsgemeinschaft (DFG, Os75/15-1, and Excellenzcluster für Makromolekulare Komplexe, CEF-MC II, EXC 115, speaker Volker Doetsch) for financial support.

References

1. Hickey, P. C., Swift, S. R., Roca, M. G. & Read, N. D. (2004) Microbial imaging. Methods Microbiol 34, Elsevier, p. 63–87
2. Hickey PC, Read ND (2009) Imaging living cells of *Aspergillus* in vitro. Med Mycol 47(Suppl 1):S110–S119
3. Dahms, T. E. S., Czymmek, K. J. (eds) (2015) Advanced microscopy in mycology. Springer. doi: 10.1007/978–3–319-22437-4
4. Stelzer EHK (2015) Light-sheet fluorescence microscopy for quantitative biology. Nat Methods 12:23–26
5. Rizet G (1953) Impossibility of obtaining uninterrupted and unlimited multiplication of the ascomycete *Podospora anserina*. *C. R.* Hebd Seances Acad Sci 237:838–840
6. Esser K (1974) *Podospora anserina*. In: King, RC (ed) Handbook of Genetics. Plenum Press, New York, 531–551
7. Osiewacz H (1996) Genetic analysis of senescence in *Podospora anserina*. In: Bos CJ (ed) Fungal Genetics. Marcel Dekker, New York, 317–335
8. Hamann A, Krause K, Werner A, Osiewacz HD (2005) A two-step protocol for efficient deletion of genes in the filamentous ascomycete *Podospora anserina*. Curr Genet 48:270–275

9. Osiewacz HD, Hamann A, Zintel S (2013) Assessing organismal aging in the filamentous fungus *Podospora anserina*. Methods Mol Biol 965:439–462
10. Wiemer M, Grimm C, Osiewacz HD (2016) Molecular control of fungal senescence and longevity. In: Wendland J (ed) The Mycota I, growth, differentiation and sexuality, 3rd edn. Springer, Switzerland, 155–181
11. Knuppertz L, Osiewacz HD (2016) Orchestrating the network of molecular pathways affecting aging: Role of nonselective autophagy and mitophagy. Mech Ageing Dev 153:30–40
12. Silar P, Lalucque H, Vierny C (2001) Cell degeneration in the model system *Podospora anserina*. Biogerontology 2:1–17
13. Knuppertz L, Hamann A, Pampaloni F, Stelzer E, Osiewacz HD (2014) Identification of autophagy as a longevity-assurance mechanism in the aging model *Podospora anserina*. Autophagy 10(5):822–834
14. Gitai Z (2009) New fluorescence microscopy methods for microbiology: sharper, faster, and quantitative. Curr Opin Microbiol 12:341–346
15. Kentner D, Sourjik V (2010) Use of fluorescence microscopy to study intracellular signaling in bacteria. Annu Rev Microbiol 64:373–390
16. Thorn K (2016) A quick guide to light microscopy in cell biology. Mol Biol Cell 27:219–222
17. Toomre D, Pawley J (2006) Disk-scanning confocal microscopy, Handb Biol confocal Microsc. Springer, New York, pp. 221–238
18. Carlton PM, Boulanger J, Kervrann C, Sibarita JB, Salamero J, Gordon-Messer S et al (2010) Fast live simultaneous multiwavelength four-dimensional optical microscopy. Proc Natl Acad Sci 107(37):16016–16022
19. Huisken J, Swoger J, Del Bene F, Wittbrodt J, Stelzer EHK (2004) Optical sectioning deep inside live embryos by selective plane illumination microscopy. Science 305:1007–1009
20. Keller PJ, Schmidt AD, Wittbrodt J, Stelzer EHK (2008) Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. Science 322:1065–1069
21. Keller PJ, Stelzer EH (2008) Quantitative in vivo imaging of entire embryos with digital scanned laser light sheet fluorescence microscopy. Curr Opin Neurobiol 18(6):624–632
22. Liu Z, Keller PJ (2016) Emerging Imaging and Genomic Tools for Developmental Systems Biology. Dev Cell 36:597–610
23. Keller PJ et al (2010) Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. Nat Methods 7:637–642
24. Maizel A, von Wangenheim D, Federici F, Haseloff J, Stelzer EHK (2011) High-resolution live imaging of plant growth in near physiological bright conditions using light sheet fluorescence microscopy. Plant J 68: 377–385
25. Strobl F, Schmitz A, Stelzer EHK (2015) Live imaging of *Tribolium castaneum* embryonic development using light-sheet-based fluorescence microscopy. Nat Protoc 10:1486–1507
26. Strobl F, Stelzer EHK (2014) Non-invasive long-term fluorescence live imaging of *Tribolium castaneum* embryos. Development 141:2331–2338
27. Pampaloni F, Ansari N, Stelzer EHK (2013) High-resolution deep imaging of live cellular spheroids with light-sheet-based fluorescence microscopy. Cell Tissue Res 352:161–177
28. Pampaloni F, Berge U, Marmaras A, Horvath P, Kroschewski R, Stelzer EH (2014) Tissue-culture light sheet fluorescence microscopy (TC-LSFM) allows long-term imaging of three-dimensional cell cultures under controlled conditions. Integr Biol 6(10):988–998
29. Pampaloni F, Chang B-J, Stelzer EHK (2015) Light sheet-based fluorescence microscopy (LSFM) for the quantitative imaging of cells and tissues. Cell Tissue Res 360:129–141
30. Pampaloni, F., Richa, R., Ansari, N., & Stelzer, E. H. (2015). Live spheroid formation recorded with light sheet-based fluorescence microscopy. Advanced fluorescence microscopy: methods and protocols. 43–57.
31. Pampaloni F, Reynaud EG, Stelzer EHK (2007) The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 8:839–845
32. Greger K, Swoger J, Stelzer EHK (2007) Basic building units and properties of a fluorescence single plane illumination microscope. Rev Sci Instrum 78(2):023705
33. Huisken J, Stainier DYR (2007) Even fluorescence excitation by multidirectional selective plane illumination microscopy (mSPIM). Opt Lett 32(17):2608
34. Hyde J, MacNicol M, Odle A, Garcia-Rill E (2014) The use of three-dimensional printing to produce in vitro slice chambers. J Neurosci Methods 238:82–87

http://www.springer.com/978-1-4939-6808-4

Light Microscopy
Methods and Protocols
Markaki, Y.; Harz, H. (Eds.)
2017, XI, 285 p. 73 illus., 62 illus. in color., Hardcover
ISBN: 978-1-4939-6808-4
A product of Humana Press