
Imaging Flow Cytometry and High-Throughput Microscopy for Automated Macroscopic Morphological Analysis of Filamentous Fungi

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17.1 Introduction

Fungi exhibit cellular and macroscopic morphological variations in response to genetic as well as environmental influences. Morphological variations have been shown to be an indicator for assessing properties such as pathogenicity (Hnisz et al. 2011), efficiency of industrial bioprocesses (Posch et al. 2013), as well as ecological impacts (Pomati and Nizzetto 2013). Morphological characterization of fungi is highly dependent on the application. Whereas assessing pathogenicity might depend on the choice of color and cell size as indicators of underlying genetic variations, bioprocess applications have considered macro-

morphological attributes, such as affinity for pellet growth, rate of branching, and septation frequency as quality attributes for production processes (Krull et al. 2013; Posch et al. 2012; Krabben and Nielsen 1998). Choosing a collection of suitable methods for morphological characterization, consisting of image acquisition/processing and data analysis, depend on the variables to be measured. However, for all cases, the description of quantitative morphological features with sufficient statistical power is only possible by methods that allow for high-throughput analysis of multiple cells or cell populations in a given sample. Hence, to be of practical use, applied measurement methodologies should be robust, fast, and not subject to observer bias.

The presented acquisition methods, (1) imaging flow cytometry and (2) whole-slide microscopy are chosen based on the versatility of applications and statistical rigor respectively. Flow cytometry devices with imaging capability present a stand-alone, standardized technological platform for high-throughput sampling with diverse applicability. Combination of the flow cytometer signals, such as fluorescence intensity, with “in-flow” images, opens diverse research possibilities for assessment of the molecular and genetic basis for morphological variations. On the other hand, whole-slide microscopy in combination with automated image analysis has been shown capable of achieving statistically

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verified morphological characterization (Posch et al. 2012; Cox et al. 1998).

Despite the availability of several automatic image acquisition systems, required image processing and data analysis steps are often a limiting factor due to the absence of one-fit-all software packages. Here, in addition to presenting two automated image acquisition methods, we provide a step-by-step guide for implementation of simple Matlab scripts capable of performing common data analysis tasks such as computation of morphological variables as a first step towards implementation of more advanced data analysis algorithms such as regression, classification, and clustering. The ability to setup fast and reliable methods for automatic and high-throughput morphological analysis as well as being able to implement custom algorithms for efficient data analysis is not only a useful tool for mycological research, but will also improve our ability to design improved biotechnological processes.

17.2 Materials

Materials are grouped according to the choice of the image acquisition platform.

17.2.1 Microscopy

1. 1-mL-pipette-tips (cut tips to avoid size exclusion).
2. Lactophenol blue (Art. No.: 3097.1, Carl Roth, Germany).
3. Microscope slide (25×75 mm).
4. High Precision Microscope Cover Glasses (24×60 mm) (Art. No.: LH26.1, Carl Roth, Germany).
5. Brightfield microscope (Leitz, Germany) equipped with a 6.3 magnifying lense, 5 megapixel microscopy CCD color camera (DP25, Olympus, Germany), and a fully automated x-y-z stage (Märzhäuser, Austria).

6. Microscope control program analysis 5 (Olympus, Germany).
7. MATLAB 2013a including Image Processing Toolbox.

17.2.2 Imaging Flow Cytometry

1. Milli-Q water.
2. 1-mL-pipette-tips (cut tips to avoid size exclusion).
3. Flow cytometer, e.g., CytoSense (CytoBuoy, Netherland) equipped with a PixeLINK PL-B741 1.3MP monochrome camera.
4. Flow cytometer software, e.g., CytoClus (CytoBuoy, Netherlands).
5. MATLAB 2013a including Image Processing Toolbox (MATLAB, Image Processing Toolbox Release 2013a).

17.3 Methods

The workflow for morphological analysis consists of the choice of an image acquisition platform and implementation of subsequent image and data processing routines. Figure 17.1 provides an overview of the presented methodologies. All of the presented methods have been developed for the analysis of *Penicillium chrysogenum* grown in submerged cultures, but could be extended to other similar organisms.

17.3.1 Sample Preparation

The importance of sound protocols for sample workup and conditioning prior to the actual process of image acquisition and evaluation is often underrated. Since factors such as pH-value and osmolarity are known to affect morphology, it has to be assured that sample preparation does not interfere with results from morphological analysis. Staining steps may be included in order to focus the image-based analysis on selected

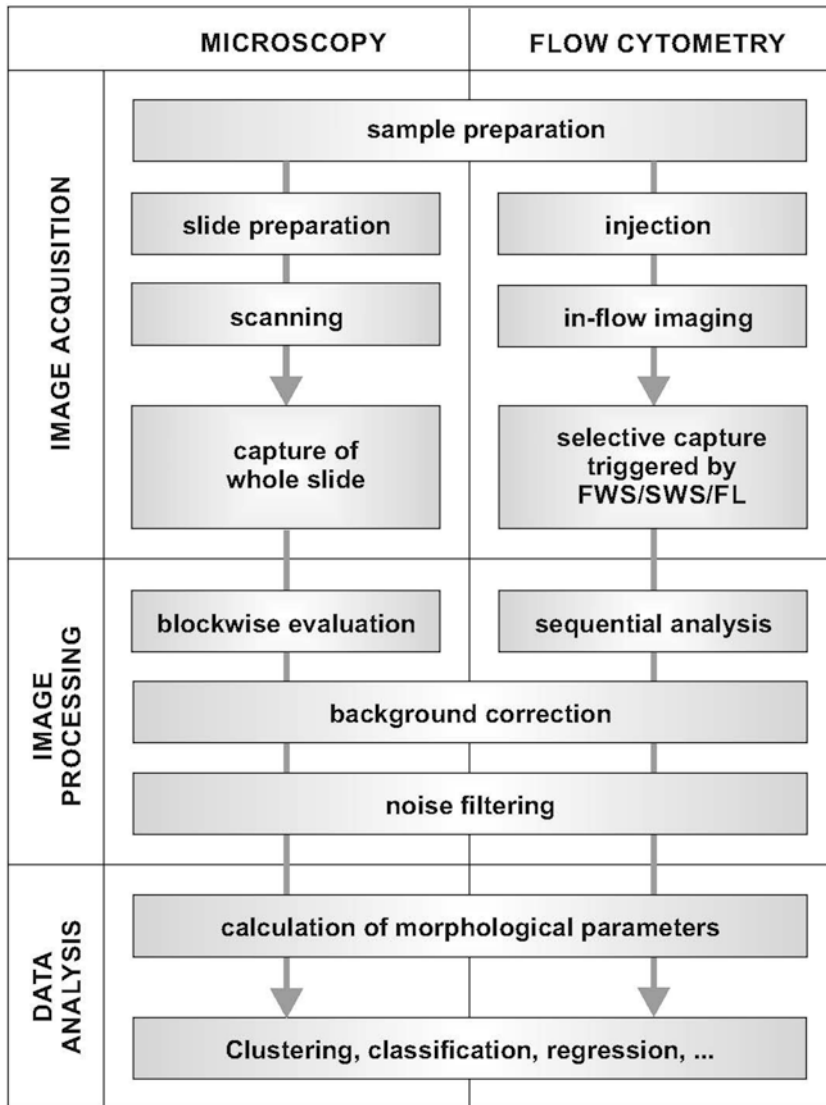


Fig. 17.1 Overview of the steps involved in presented methods

elements in the sample, which can be distinguished by specific structural or functional parameters of the fungal cell. Individual sample preparation procedures for the presented image acquisition methods are presented in Sects. 17.3.2.1 and 17.3.2.2.

17.3.2 Image Acquisition

The choice of the most suitable method for image acquisition depends on the nature of the sample and the analysis criteria with regard to throughput, accuracy, and investigated morphological

features. Selection of a specific method will also be influenced by the available equipment in most laboratories.

The maximum number of images acquired per run, which influences the statistical robustness of a method, is one of the main differentiating factors of the two presented image acquisition systems. The flow cytometry system is capable of taking up to 150 images per run, each containing a single hyphal element, whereas scanning a microscope slide (at 63× magnification) can yield up to 900 images, each containing up to ten individual macromorphological fungal objects (such as pellets, mycelial clumps, and branched hyphae). Therefore, the microscopy-based system is at an advantage when it comes to recording a large number of images.

In both systems, discriminatory parameters (such as size and fluorescence intensity) may be utilized for confining the analysis to particles with given properties. However, in microscopy-based systems these parameters are not easily accessible, and the sorting process is carried out *post-hoc* (by analyzing the image and including or excluding certain structures). Flow cytometric analysis allows for in-situ control of image acquisition (by triggering acquisition based on the optical parameters of the particle in the flow cell). The signals required for selective acquisition are more readily available in-flow cytometry systems. Sample handling and mounting on the device is another important factor. In microscopy systems, it usually involves spreading the sample on glass slides, which is more tedious and less standardized compared to fully automated systems of sample injection.

The maximum size of particles being analyzed has traditionally been a limiting factor for flow cytometric analyses of fungal samples. The system presented here has been specifically designed to allow for analysis of biomass particles up to a size range of 1.5 mm, which is still in line with the requirements of many applications. However, a potential shortcoming of imaging flow cytometry is that the maximum possible exposure time is determined by the relatively fast transit of the fungal biomass element through the flow cell.

This may interfere with analysis of certain image features or stains that require prolonged signal integration times.

17.3.2.1 Microscopy

1. Dilute the sample with to about 1 g/L biomass dry cell weight. For pipetting, use cut pipette tips to avoid size exclusion effects.
2. Add 100 µL/mL lactophenol blue solution to stain the sample.
3. Transfer 50 µL of the stained sample to a microscope slide. Ensure homogenous sample distribution.
4. Place a high precision cover slip on the slide using tweezers. Avoid agglomeration of biomass elements and inclusion of air bubbles, dust, or debris. The use of a high precision slip ensures that the images remain in focus throughout scanning of the entire slide, eliminating the need for manually adjusting focus at each step.
5. Transfer the microscope slide to the automated microscope stage.
6. Adjust acquisition parameters (i.e. contrast, brightness, exposure time) based on illumination settings and desired image features.
7. Scan the whole slide. For analysis of *P. chrysogenum*, at 63× magnification, typically 600–800 images are taken (adjacently arranged in a grid pattern without overlap as shown in Fig. 17.2).
8. Measure multiple slide replicates (in our case 3, amounting to approx. 2,000 images) of a sample to account for variations in sampling and sample preparation steps.

17.3.2.2 Imaging Flow Cytometry

1. Sample is diluted with MQ water to a final concentration of approximately 10^4 cells/mL and stained if required. 5–10 mL of diluted sample is needed for one measurement.
2. Sample measurement is performed by the flow cytometer via the standardized routine.
3. Depending on the particular selection criteria for taking pictures, the in-flow images are recorded.

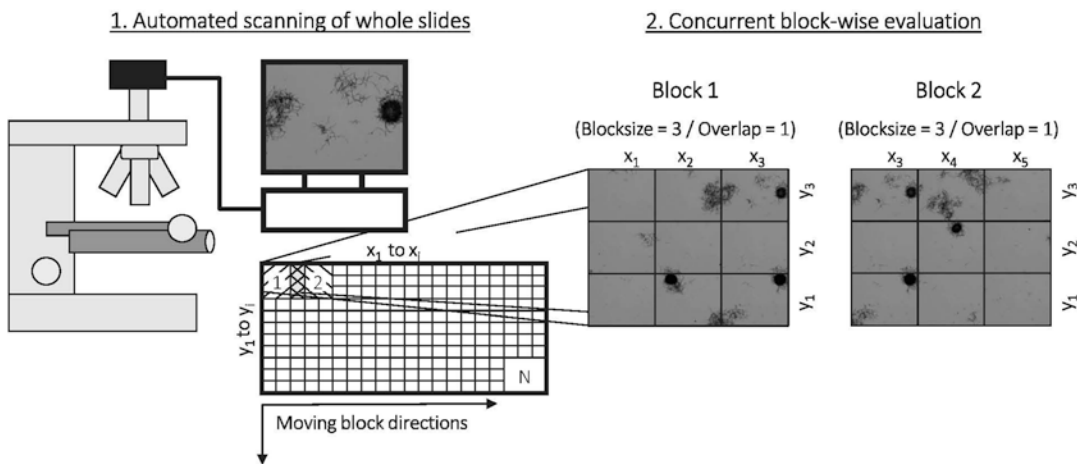


Fig. 17.2 Implemented workflow for automated morphological analysis. Step 1 is controlled by the image recording software Analysis5; step 2 by the evaluation routine implemented in Matlab. (From Posch AE, Spadiut O,

Herwig C. A novel method for fast and statistically verified morphological characterization of filamentous fungi. *Fungal Genet Biol.* 2012 Jul;49(7):499–510 with permission.)

17.3.3 Image Processing and Data Analysis

Following the acquisition of a series of images, either via whole-slide microscopy or imaging flow cytometry, images are analyzed using custom-built Matlab scripts. In the following sections, the introduced programming functions (such as *imread* and *im2double*) either refer to inbuilt functions of MATLAB or are part of the MATLAB Image Processing Toolbox (MATLAB, Image Processing Toolbox Release 2013a).

17.3.3.1 Image Processing for Whole-Slide Microscopy

In contrast to Imaging Flow Cytometry where only one hyphal biomass element is captured on an image, in whole-slide microscopy, each image often contains more than one biomass element. It is therefore required to either combine all images prior to evaluation or evaluate a set of multiple images sequentially because if only one image were to be evaluated at a time, the biomass elements spanning an image border would not be evaluated correctly. An iterative evaluation method of overlapping composite image blocks allows for the evaluation of all biomass elements

by using a combination of 4 or 9 microscope images at each iterative evaluation step.

1. Export/store all images of a slide in a folder
2. Implement the following Matlab functionalities in a script (m-file):
 - (a) Create an index of block numbers and be able to choose a set of n images (4 or 9) that are adjacent to each other. Implement a loop functionality that traverses through all images in a blockwise fashion as illustrated in Fig. 17.2.
 - (b) Open the set of 4 or 9 microscope images corresponding to a block using the *imread* function (MATLAB, Image Processing Toolbox Release 2013a) and combine them into one image .
 - (c) Convert the images from RGB format to grayscale using the *rgb2gray* function (MATLAB, Image Processing Toolbox Release 2013a). Further convert the image values to double precision using the *im2double* function (MATLAB, Image Processing Toolbox Release 2013a).
 - (d) Convert the images to binary (black/white) using the *im2bw* function (MATLAB, Image Processing Toolbox Release 2013a). Adjust the threshold value based on particular light conditions.

- (e) Apply mean and median filters to enhance image quality.
 - (f) Convert to binary (black/white) using the *im2bw* function. Use a threshold value according to the camera settings of the microscope.
 - (g) Identify connected objects using the *bwconncomp* function (MATLAB, Image Processing Toolbox Release 2013a) and append to list of objects (excluding border-touching elements and eliminating duplicates stemming from overlapping composite image blocks)
 - (h) Go back to step b and evaluate the next block (repeat until all images have been analyzed)
 - (i) Save the list of objects as a MAT file for subsequent data analysis steps.
3. Find the length of a pixel (magnification) in the camera software. This will depend on the resolution of the installed camera. Square this value to get the area [μm^2] corresponding to each pixel.

17.3.3.2 Image Processing for Imaging Flow Cytometry

Image acquisition via imaging flow cytometry eliminates the need for blockwise evaluation because each image captures only one hyphal biomass element. In rare cases where the biomass elements is not captured wholly and touches the image border, the image can be discarded automatically.

1. Export images from the Cytosense software (using the *export all images* function) to a common folder
2. Perform following activities in a Matlab script (m-file). Programming functions (such as *rg2gray* and *im2double*) either refer to inbuilt functions of MATLAB or are part of the MATLAB Image Processing Toolbox (MATLAB, Image Processing Toolbox Release 2013a).
 - (a) Open an image and a background image.
 - (b) Convert both images from RGB format to grayscale using *rgb2gray* function. Also convert the image data format to double precision using the *im2double* function.
 - (c) Subtract the background image from the actual image being analyzed.
 - (d) Convert to binary (black/white) using the *im2bw* function. Use a threshold value according to the camera settings of the flow cytometer. For our settings values between 0.04 and 0.1 work best.
 - (e) Apply mean and median filters to enhance the image quality and remove noise.
 - (f) Convert to binary (black/white) again. Use a threshold value according to the camera settings of the flow cytometer. For our settings values between 0.4 and 0.6 work best.
 - (g) Detect all connected objects using the *bwconncomp* function. Ideally, if the right pre-processing parameters are chosen, each image should result in one connected object only. If additional small elements are found, i.e. due to noise, the largest element should be chosen automatically using the *bwconncomp* function to output the area of the connected objects.
 - (h) Append the largest connected object to a list for later processing and iterate steps a–g until all images of a flow cytometer run have been analyzed.
 - (i) Save the list of objects as a MAT file for subsequent data analysis steps.
3. Compute the length and area of each pixel from the scale bar on one of the output images. The resolution (number of pixels) of each exported image should be the same for each analyzed sample.
 - (a) Count the number of pixels in the scale bar of an image by cropping the image, loading it in Matlab, and then using the *length* function. For instance: Length of scale bar=670 pixels=450 μm .
 - (b) Length of a pixel=450/670=0.67 $\mu\text{m}/\text{pixel}$.
 - (c) Area of a pixel=0.67 \times 0.67=0.45 $\mu\text{m}^2/\text{pixel}$.

17.3.3.3 Data Analysis and Calculation of Morphological Variables

The output of either method (microscopy or imaging flow cytometry) will be the same type of Matlab structure, namely a collection of binary

images of individual hyphal elements. Subsequent data analysis steps will be the same for both image acquisition methods. Here, we describe the procedure for deriving a set of commonly-used morphological variables using easy-to-implement Matlab routines. For statistical verification of calculated morphological data, the reader is referred to Posch et al. (2012).

Area

Area of the 2d slice through the hyphal element is proportional to the size of the hyphal element. This property can be calculated according to the following procedure.

1. Use the *regionprops* function to get a list of all region properties.
`properties=regionprops(input_bw, 'all');`
2. In the resulting structure, access the “Area” matrix using “`props.Area`.”
3. Multiply the pixel number by the “area of each pixel [$\mu\text{m}^2/\text{pixel}$]” (calculated in previous steps) to get the area of the hyphal element in units of μm^2 .
4. The properties structure contains a collection of variables such as circularity and roughness which can be used for a variety of tasks, such as classification of hyphal elements.

Equivalent Diameter and Major Axis Length

The “equivalent diameter” parameter is a scalar that specifies the diameter of a circle with the same area as the region. Similarly, the “major axis length” specifies the length of the major axis of the ellipse that has the same normalized second central moment as the region. Both parameters provide an appropriate measure of the size of the biomass elements and are calculated by the *regionprops* function, which returns these values in units of pixels. Multiplication by pixel length (calculated in previous steps) returns the *EquivDiameter* and *MajorAxisLength* in units of μm .

Classification of Hyphal Elements

Each fungal object can be classified into distinct morphological classes, such as pellets, large and small clumps, branched and unbranched hyphae.

The decision for such classification is based on a combination of morphological parameter and simple if/else rules. Combination of classification and area can be used to derive the area fraction of each class for a sample, such as area fraction of pellets or large clumps (Fig. 17.3).

Hyphal Growth Unit

The hyphal growth unit (HGU), defined as the average length of a hyphae supporting a growing tip, is used for studying the growth kinetics and morphology of filamentous organisms. It can be given by the equation:

$$\text{HGU} = \frac{L_t}{N_t}$$

where L_t is total mycelial length, and N_t is the total number of tips. The procedure for calculating the total mycelial length and the total number of tips is as follows:

1. Starting from the binary image of the previous steps, apply the *bwmorph* function with the “skel” method. The operation “skel” removes pixels on the boundaries of objects but does not allow objects to break apart. The pixels remaining make up the image skeleton. Use “inf” as the n (input) parameter, which causes the skeleton to have a width of 1 pixel.
`T1=bwmorph(input_bw,'skel',inf);`
2. Apply the *bwmorph* function with the “shrink” method in order to eliminate tips which are smaller than a specified length. Use the minimum tip size [pixels] as the n (input) parameter.
`T2=bwmorph(T1,'shrink',2/pxl_length);`
3. The total hyphal length is calculated by considering the length of a rectangle having the same area and perimeter as the hyphal object (Cox et al. 1998).

$$\text{Total length} = \frac{\text{Perimeter} + \sqrt{\text{Perimeter}^2 - 16 \times \text{Area}}}{4}$$

4. Apply the *bwmorph* function with the “end-point” method in order to identify all of the endpoints.
`T3=bwmorph(T2,'endpoints');`

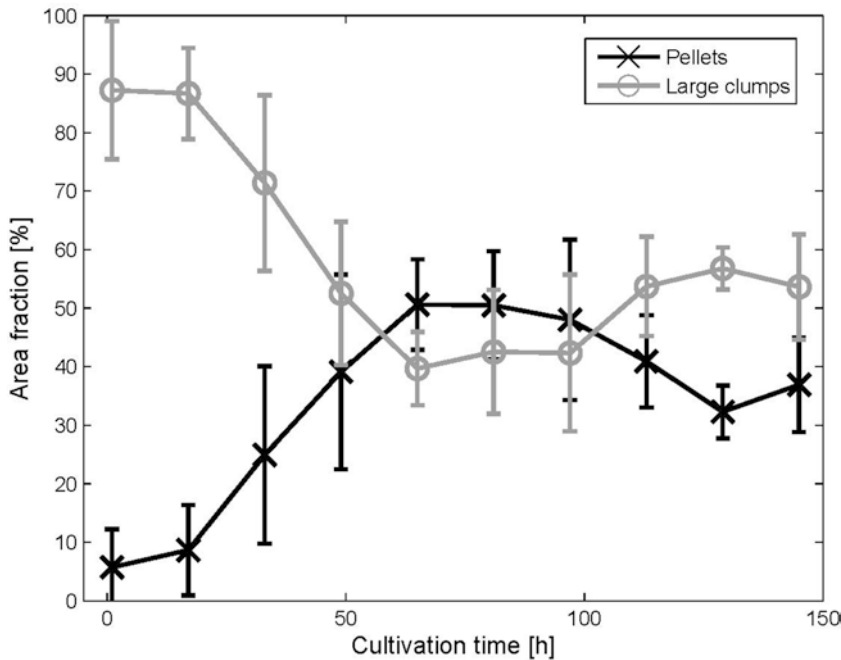


Fig. 17.3 Time course of area fraction of pellets and large clumps over a *P. chrysogenum* fed-batch cultivation using the whole-slide microscopy method

5. Apply the `bwconncomp` function to find all connected objects in the resulting image. The `NumObjects` property of the resulting structure is equivalent to the number of tips.
6. Dividing total length by number of tips results in the hyphal growth unit parameter.

17.4 Illustrative Examples

17.4.1 Imaging Flow Cytometry

The advantage of the imaging flow cytometry method lies in its ability to combine classical morphological analysis with flow cytometer detector signals. Equipped with appropriate staining methods, it would be possible to differentiate hyphal elements on a functional level (i.e. pathogenicity, live/dead), and these subpopulations can then be analyzed with respect to macroscopic morphological differences. In comparison to whole-slide microscopy, an advantage of this method lies in the fact that overlapping and touching elements are eliminated since the

flow cytometer adjusts the flow such that every image contains only one biomass element.

As an example for calculation of common size parameters, Fig. 17.4 depicts a hyphal element and the corresponding calculated values. Figure 17.5 shows an example of skeletonization used for calculation of total hyphal length, number of tips, and HGU. Figure 17.6 shows an image of a pellet and the corresponding detector signals of the flow cytometer which can be used as a criteria for capturing images at specific signal levels. Lack of detailed focus for larger particles is a clear drawback of the in-flow image acquisition method as seen by the example of Fig. 17.6. Further optimization of the method may result in images with improved quality.

17.4.2 Whole-Slide Microscopy

Application of the described whole-slide microscopy method has been previously shown to significantly enhance characterization of bioprocesses (Posch et al. 2013). As an example, classification

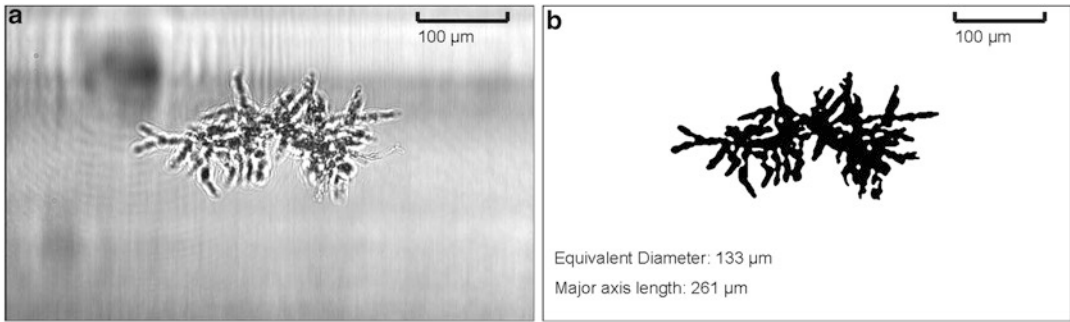


Fig. 17.4 (a) Image of a branched hyphae captured by the in-flow camera. (b) Calculation of the equivalent diameter and major axis length of a biomass element

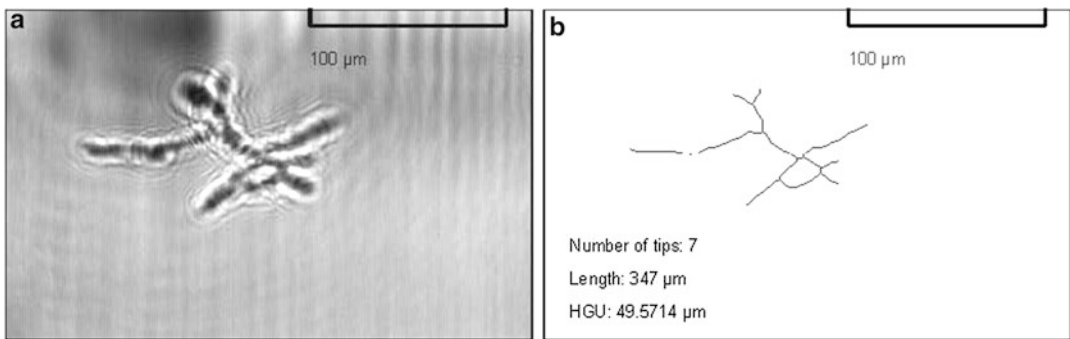


Fig. 17.5 (a) Image of a branched hyphae captured by the in-flow camera. (b) Calculation of the number of tips, total length, and hyphal growth unit from an image taken

in the flow cell of the flow cytometer is performed via skeletonization of the image

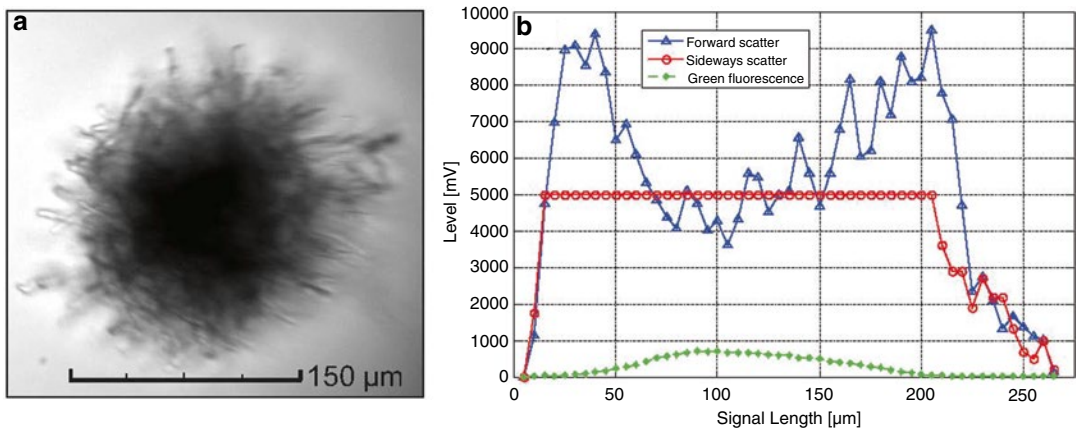


Fig. 17.6 (a) Image of a pellet captured by the in-flow camera. (b) detector signals of the flow cytometer which can be used as a criteria for capturing images at specific signal

levels. The sideways scatter detector has become saturated due to the limitations of the device. The size of the pellet can be easily calculated using the forward scatter signal

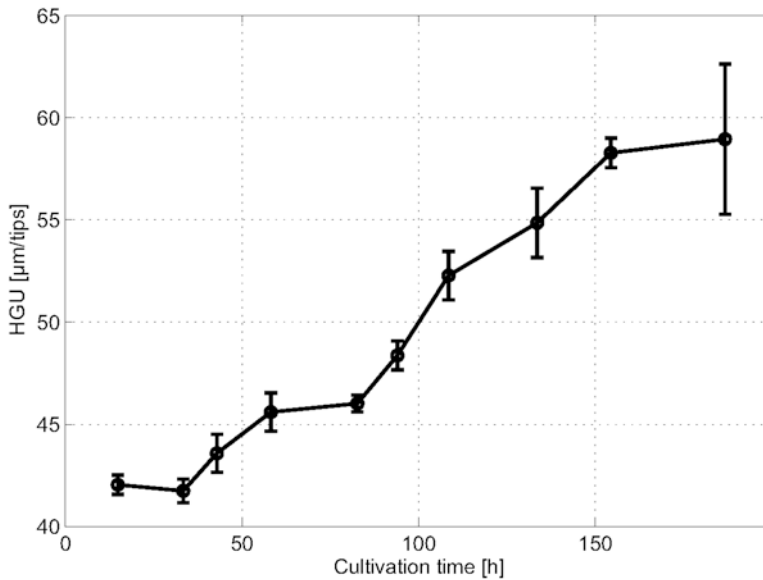


Fig. 17.7 Time course of hyphal growth unit (HGU) over a *P. chrysogenum* fed-batch cultivation using the whole-slide microscopy method

of biomass into distinct morphological classes according to a rule-based method can provide the time course of morphological variations over a typical industrial cultivation process of *P. chrysogenum* (Fig. 17.3). The dynamics of pellet growth and subsequent breakage provides insights for optimization of rheological properties of the culture. The time course of the hyphal growth unit over process time (Fig. 17.7) can serve for assessing the effects of process conditions, such as agitation speed, on the branching behavior of the organisms.

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