Automatic cell segmentation from brightfield microscopy images of pseudohyphal cell-aggregates

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Abstract

Background: The automatic segmentation of pseudohyphal cell-aggregates from brightfield microscopy images for counting forming cells is a challenging task due to the heterogeneous optical appearances of the cells as they may lie on different focal planes. The current cell counting method is based on a time-consuming manual counting of stained cells on a hemocytometer and in most cases, it represents estimates of low statistical significance due to the effort needed to prepare and analyze many samples. In this work, we evaluated the effectiveness of a marker-controlled watershed algorithm for automatic segmentation of pseudohyphae from brightfield microscopic images. The cell heterogeneity problem was addressed by processing intracellular contents of focused and defocused cells to extract initial foreground markers for the watershed method. By properly segmenting cells of different classes within a pseudohypha allows increasing the number of cells analyzed contributing thus to more reliable estimates. To facilitate the evaluation of the proposal by acquiring images containing a diversity of cells' appearances, we utilized in situ microscopy, an imaging system used to capture images directly from suspensions. **Results:** The performance of the method was evaluated on 120 portraits of a yeast exhibiting a diversity of pseudohyphal morphologies. Automatic results were compared with manual references obtained by visual inspection of the images. Despite the simultaneous occurrence of a representative mixture of focused, over-, and under-focused cells, the method produced robust results with an average segmentation sensitivity, specificity, and accuracy of 76%, 89%, and 76%, respectively. On average, each microscopic image was processed within 3 s. **Conclusions:** Our approach was capable to segment pseudohyphae formed by cells exhibiting a large diversity of appearances. The application of a marker-controlled watershed algorithm as a simple, yet effective technique for segmenting pseudohyphae demonstrated satisfactory overall performance to support automated analysis of pseudohyphal cell-aggregates from brightfield images.

Background

Single-cell yeast suspensions are usually preferable in industrial yeast fermentation processes over cells in aggregates. Single cells exhibit a larger contact area with the nutrient medium, which in many cases helps to optimize the process. However, under deprivation of glucose or nitrogen [1, 2] or in the presence of fusel alcohols [3], it is commonly observed that *Saccharomyces cerevisiae* strains switch between cell division with separated daughter cells and the generation of pseudohyphae, a morphology that exhibits several chains of still attached daughter cells growing at arbitrary orientations.

Besides affecting fermentation time and efficiency, pseudohyphal yeast morphology may also impair centrifugation systems. The latter is an expensive and complex process aimed at recovering yeast cells for subsequent fermentation cycles [4]. Pseudohyphal morphology is characteristic of a very common rough type of yeast colony on solid media. If it is found in biofuel fermentation processes, it normally exhibits a higher tolerance with respect to osmotic and ethanolic stresses [5]. This biotype of cell yeast morphology is also called *snowflake* [6]. Studies have shown that a wild *S. cerevisiae* strain exhibiting pseudohyphal morphology is often associated with lower ethanol production and higher residual sugar

content in the fermented must [7, 8]. In contrast, in the brewing and chemical manufacturing industry, pseudohyphal yeasts have been exploited to change flavour and aroma in beverage products and to produce acetic acid, respectively [9].

Because pseudohyphal morphology is so relevant to many industrial processes, the delivery of morphological information at high rates can assist the personnel of a production process in implementing assertive actions within practical times.

Direct counting using a hemocytometer in conventional light microscopy is an inexpensive and easy-to-use technique. However, it is also laborious and time-consuming if used for enumeration of single cells, budding cells and pseudohyphal cell-aggregates.

Only a few proposals for analysis of yeasts exhibiting pseudohyphal morphology have been presented. As an example, a computational algorithm to detect both yeast and pseudohyphal form structures from a database of phase contrast microscopy images of the human pathogen *Candida glabrata*, a species that exhibits elongated pseudohyphal growth structures, was developed [10]. Another work combined image cytometry with fluorescent staining to determine several parameters of the yeast *Brettanomyces* during beer fermentation [9], a strain that also forms pseudohyphae. Using acridine orange and propidium iodide stains to identify live and dead cells, respectively, cells imaged on a dedicated counting chamber allowed measurements of cell concentration and viability of pseudohyphae. Despite improving the accuracy and efficiency of cell counting by highlighting stained-nuclei yeasts, large statistics from non-correlated samples are only possible by extracting, preparing and processing of many samples.

Optical density measurements can automate the measurement of biomass without needing sampling. However, its accuracy bears on the assumption that a homogeneous culture of suspended single cells is under analysis. As a non-imaging approach, optical density lacks the capacity of assessing, for instance, a transient development of cell's morphology.

A suitable non-invasive method for real-time online monitoring of cell morphology is *in situ* microscopy, as described in [11]. An *in situ* microscope (ISM) is a light microscope adapted to capture images directly from suspensions. It has been used to monitor cell number, cell size distribution, and morphological characteristics of several specimens, including yeasts [12–20], filamentous bacteria [21, 22], Chinese hamster ovary (CHO) cells [23], and the viability of mammalian cells [24].

When observing pseudohyphal cell-aggregates through a light microscope, forming cells may exhibit different optical appearances as they may lie on different distances from the objective focal plane. Thus, only a fraction of the pseudohyphal structure may be imaged in-focus, an imaging condition usually chosen in routine microscopy as cellular morphology and internal structures of the specimen can be easily recognizable. As cells located outside the focal plane lead to defocused cells, rendering, ultimately, a cell unrecognizable under larger amounts of defocus, the number of forming cells may be underestimated when solely cells in-focus are considered by segmentation algorithms.

However, since defocused cells are still part of the pseudohyphae under analysis, more representative estimates with respect to the number of forming cells could be obtained if not only cells in-focus but also defocused ones could be considered.

In this work, intracellular contents of cells in-focus as well as those located above and below the focal plane were processed to create initial foreground markers for a marker-controlled watershed algorithm performs automatic pseudohyphal cell-aggregates segmentation from brightfield images. The markers extraction was carried out by applying the same sequence of morphological reconstructions along with conventional morphological operations, all adjustment-free parameters, to all pseudohyphae input images regardless of the forming cells' focus condition. The strategy was applied on microscopic images containing each pseudohyphae with a representative mixture of focused, over-focused, and under-focused cells. The performance of the segmentation algorithm was evaluated by comparing automatic results with manual references obtained by visual inspection of the images.

Results

2207 ISM images were acquired. An exemplary image is shown in Fig. 1.

Unlike dispersed *S. cerevisiae* single yeast cells, see *e.g.* [12], the present strain exhibits a varying amount of branched chains of cells with different orientations so that their optical appearance depends on their distance from the objective focal plane (Fig. 1). Cells located between the light source and the focal plane act as convex lenses and generate bright spots with smooth dark edges. On the other hand, cells located between the objective lens and the focal plane appear as dark, blurred spots [19, 25]. In this study, the first group of cells is termed *over-focused* and the second is termed *under-focused*.

As seen in Fig. 1, pseudohyphae with a representative mixture of focused, slightly over-focused and slightly under-focused cells were imaged. The coexistence of these different focusing conditions poses a challenge to the cell detection with fixed parameters like the threshold values and the cut-off frequencies in the filters. These parameters were optimized by experiment and kept constant during the whole experiment.

Fig. 1 Example of an ISM image showing pseudohyphae. Cell-aggregates are formed by cells imaged at different distances from the objective focal plane: in-focus cells are indicated by the thin arrow, whereas under-focused and over-focused cells are indicated by the arrowhead and large arrow, respectively. Scale bar is 10 μ m.

To test the performance of the cell segmentation technique, single-pseudohyphae portraits automatically cropped from ISM images were analyzed by the segmentation algorithm. The portraits were classified manually into four categories of pseudohyphae (Fig. 2).

Fig. 2 Categories of pseudohyphae classified manually according to the focus condition of the forming cells. a over-focused. b in-focus. c under-focused. d mixture of focused, over-, and under-focused. Scale

bar is 10 μm .

As shown in Fig. 2, the selected strain displays a diversity of pseudohyphal morphologies. The number of cells forming a pseudohypha varies. Individual cells exhibit elliptical shape and moderate size heterogeneity. The goal of this study is the automatic determination of the cell number per pseudohypha portrait.

Over-focused cell images show dark cell borders and a cell center much brighter than the rest of the cell, without exhibiting intracellular content. More complex textures inside cells can be observed when cells are in-focus. Halos are common around under-focused cells. Edges between conjoined under-focused and in-focus cells are hardly distinguishable.

The estimate of the number of pseudohyphae was based on the number of portraits generated by the algorithm. From 6690 pseudohyphae from manual counting performed directly in the 2207 ISM images, the algorithm generated 6632 portraits. Table 1 displays the manual classification results from the portraits. The algorithm detection error of 4% (*i.e.*, 244 missing pseudohyphae) was due to much blurred structures (probably from pseudohyphae located far away from the over-focused plane) and moderate background intensity heterogeneities erroneously detected as pseudohyphae.

The distribution was determined by manual classification of 6446 pseudohyphae portraits from 2207 ISM images.

Segmentation of pseudohyphae and of their individual cells

Fig. 3 shows an example of the output at each stage of the cell segmentation algorithm.

Fig. 3 Stages of the image processing of a typical hypha-portrait. a Input portrait. *b* High-pass filtered image. *c* Smoothed high-pass filtered image. *d* Opening-by-reconstruction. *e* Opening-closing by reconstruction. *f* Regular morphological opening *e. g* h -minima transform computed in the complement of *f*. *h* Watershed image overlaid on the input image. The detected cells were pseudo-colored for visualization purposes. Portraits correspond to 250×250 pixels.

Compared to the input image in Fig. 3a, the high pass filtering enhanced contrast of intracellular content and edges between conjoined cells and background (Fig. 3b). However, this step also increased background noise, which was significantly suppressed by applying a moderate low-pass filtering (Fig. 3c).

Organelles generate image objects interpreted as foreground markers by the watershed algorithm. Opening-closing by reconstruction followed by regular morphological opening helped to smoothen intracellular regions without impairing significantly the cell contours (Fig. 3 d-e-f). During this process, grayscale irregularities over the entire image are also smoothened to avoid under- and over-segmentations. As undesired regional minima may still exist after this morphological filtering, these are

suppressed by applying a *h*-minima transform, whose effect is illustrated in Fig. 4. The *h*-value was determined by trial and error, aiming at minimizing segmentation errors as assessed manually on one hundred portraits.

Fig. 4 Effect of the h-minima transform on the segmentation performance. a input image to the watershed transform with a reference line for measuring the grayscale profile. *b* red curve: original grayscale profile along the line shown in *a*, blue curve: grayscale profile after the *h*-minima transform: all regional minima with depth smaller or equal to 2 (the adopted *h*-value) were removed (as shown by the large arrow), while the height of the remaining regional minima was increased by 2 (pointed by the thinner arrows). Wherever blue curve segments are identical with red segments, they are shown only in blue color. *c* watershed segmentation of the input image without processing with the *h*-minima transform. The over-segmentation occurred due to the existence of two catchment basins inside a single cell. *d* watershed segmentation of the input image previously processed by the *h*-minima transform. Portraits correspond to 250 × 250 pixels.

Finally, the watershed-transform segmented pseudohyphae forming cells as watershed regions (Fig. 3h and 4d). Hereby it is a merit of the segmentation technique that the bright center spots within cells (shown in Fig. 3g) can be avoided. They were not segmented because the catchment basins within them had been flattened out by the previous morphological operations and *h*-minima transform (Fig. 4d).

To evaluate the influence of the focusing conditions on the segmentation performance, the algorithm was applied to the four pseudohyphal categories (Fig. 5).

Fig. 5 Examples of segmentations on pseudohyphal formed by cells imaged at different focusing conditions. a over-focused. *b* in-focus. *c* under-focused. *d* mixture of focused, over-, and under-focused cells. In each group of images, the first and second columns correspond, respectively, to the input portrait and the segmented cells overlaid on the input image. Arrowheads point to cells with missing contours, whereas arrows point to missing cell edges between conjoined cells. The segmented cells were pseudo-colored for visualization purposes. Portraits correspond to 250 × 250 pixels.

The overall performance of the segmentation algorithm was evaluated by comparing its results with the portraits segmented manually. Table 2 shows the results in detail.

The algorithm was applied to portraits containing pseudohyphae at different focusing conditions. The number of cells in pseudohyphae, as determined by the algorithm, is shown as mean standard deviation of true positives, false negatives, and false positives. For each focusing condition, 30 single-pseudohypha portraits were segmented by the algorithm and subsequently examined manually.

In summary, the mean specificity values (always > 0.88) indicate satisfactory segmentation for all focusing conditions investigated. The accuracy values over all categories show that 77% of the pseudohyphal structures were analyzed correctly.

The average processing time of the algorithm was 3 s per ISM image on an Intel PC, Quad-CPU, 2.66 GHz, 4.0 GB RAM.

Discussion

Because pseudohyphal cell-aggregates are typically heterogeneous with respect to the number and appearance of forming cells, estimating the number of cells in pseudohyphae from images acquired at a single focal plane may not provide reliable information about broadening pseudohyphal structure. By properly processing focused and defocused cells within the same pseudohyphal structure made it possible to quantify larger numbers of forming cells, contributing thus to improve estimates of the number of cells in pseudohyphae. However, while the generation of pseudohyphal cell-aggregates information from brightfield microscopy images relies on accurate analysis of many non-correlated images, automated pseudohyphal cell-aggregates segmentation posed a challenge due to changes in the appearance of the imaged forming cells, as they may lie on different planes of focus.

In applications where the lack of intracellular information is not an issue, the easiest way to segment brightfield images using conventional image processing is based on the detection of over-focused cells through thresholding-based techniques since the cells exhibit a bright spot of focused light which is sharply imaged on a darker background [19, 25]. Because defocused brightfield microscopy produces contrast-enhanced images, this image acquisition strategy has been applied on several specimens, including yeast cells [14], mammalian cells [26], human cells [27], and CHO cells [23].

However, as pseudohyphae formed by chains of cells growing at arbitrary orientations, as in the case of the present work, may lie on different distances from the objective focal plane, forming cells may exhibit different appearances in the acquired image. Therefore, reliable estimates of the number of cells in pseudohyphae require the acquisition and proper segmentation of many non-correlated images.

To automate image analysis of pseudohyphae from brightfield microscopic images we applied a standard marker-controlled watershed algorithm. Before applying the watershed method, the input images were preprocessed with the aim to extract initial foreground markers, ideally with a one-to-one correspondence between markers and forming cells. To this end, we exploited cell's body contents, a common feature observed in most acquired images, provided cell-border exhibit enough contrast against the background. In this process, the same sequence of morphological reconstructions along with conventional morphological operations, all adjustments-free parameters, was applied in every input image to homogenize intracellular contents, no matter their appearance. By inverting the modified image so that formerly dark cell borders become bright structures helped to obtain more realistic cell shapes in the segmented image. Over-segmentations were minimized by calculating the h -minima transform in the inverse image; however, it is worth mentioning our difficulty in empirically select the h -value to perform satisfactory segmentation over all classes of pseudohyphae due to their heterogeneous appearance.

Inspection of the values of sensitivity, specificity and accuracy revealed that the segmentation algorithm performed best on over-focused cells. The reason is that these cells are displayed with large contrast. In

contrast, the performance of the algorithm is less in cases of unfocussed or focused cells due to over- and under-segmentations. Over-segmentation occurred due to higher-contrasted intracellular contents that were wrongly segmented as single cells. Under-segmentation occurred mainly because edges between conjoined cells were hardly discernable when they are in-focus and therefore many cell groups were incorrectly segmented as single cells. In the case of under-focused cells, over-segmentation occurred mainly due to the lack of contrast between cell border and background. Bright halos, artifacts observed mainly in under-focused yeasts, generated false positives. This effect was significantly suppressed by applying the h -minima transform. Similarly, most intensity irregularities in the background were not segmented as cells. A moderate number of cells, mostly cells in focus, were not recognized (*i.e.*, falsely negative) because cells' contours were missing so that the inside part of the cells was falsely regarded as background. Some false negatives were caused by under-focused cells overlying other cells in the pseudohyphal structure.

In contrast to comparable approaches using, *e.g.*, the fluorescence capacity of an image cytometer to detect stained cells with higher accuracy, our approach utilized brightfield images. This imaging modality is a crucial prerequisite to facilitate the development of automated analysis of many non-correlated samples as it requires no time-consuming steps to prepare samples with fluorescence labels.

Although the automated cell segmentation method allowed the analysis of many non-correlated pseudohyphal cell-aggregates images, the proposal has limitations: while applying the watershed method to all classes of forming cells was a key consideration in obtaining more representative estimates, precise size and shape information about over-focused cells might be not extracted from the segmented images. Due to the convex-lens effect producing a bright spot in the cell center, the apparent size of over-focused cells may differ from their actual size. Likewise, cell shape might be hardly determined in over-focused cells since their edges are less defined.

Despite the demonstrated capability of our straightforward segmentation approach to support automated analysis of pseudohyphae based on a label-free imaging modality, the proposal still has a large potential for applying more sophisticated image analysis algorithms aiming at improving segmentation accuracy. As an example, one could, *e.g.* use the overall image contrast and brightness as additional parameters allowing machine learning techniques to adapt the cell segmentation algorithm to the image conditions.

Conclusions

In this work, we evaluated the effectiveness of an image analysis approach based on a standard marker-controlled watershed method to automate cell segmentation for cell counting in pseudohyphal cell-aggregates. The proposal was designed for segmenting forming cells from brightfield microscopy images of pseudohyphae imaged at different levels of focus and produced satisfactory accuracy, sensitivity, and specificity levels when compared to manual evaluation. The overall segmentation performance over a wide variety of cells' appearances showed that the approach supports analysis of pseudohyphal cell-aggregates from brightfield images in a fully automated fashion. By incorporating

different classes of cells within the same pseudohypha into the process of estimating the number of forming cell, this work contributed with an alternative approach towards to solve the problem of providing more representative estimates. Improved pseudohyphal cell-aggregates estimates are particularly important for enabling a better insight into biological processes and, ultimately, to improve their performance.

Methods

Microorganism, culture medium and growth conditions

A rough-colony yeast strain of *S. cerevisiae* displaying pseudohyphae was isolated from a fuel ethanol facility in São Paulo State, Brazil, and utilized in this experiment. The strain (originally termed 'strain 52') was identified by the sequencing of the D1/D2 region of the large subunit (26S) rRNA and deposited at the culture collection 'Coleção de Culturas Tropical' of 'Fundação André Tosello', Campinas—SP—Brazil under the code CCT7787. This yeast strain was utilized in previous studies of fermentation characteristics [5, 7, 8].

The yeast strain was stored in YPD medium (10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone and 20 g/L agar) in slants at 4°C. For analysis, 10 mL of YPD broth in Falcon tubes were inoculated with two loops of the yeast cells and cultivated at 160 rpm and 30°C for 24 h. After 24 h, the yeast suspension was centrifuged at 580*g* for 10 minutes and the resulting yeast pellet was suspended in saline solution (0.85% NaCl) for the microscopic observations.

The experiment was conducted at room temperature in an unbaffled glass vessel (250 mL working volume) equipped with the *in situ* microscope and a magnetic stirrer (150 rpm).

Image acquisition

Microscopic images were acquired directly from the suspension by using a custom-built high-resolution (0.5 μm) *in situ* microscope developed at the Mannheim University of Applied Sciences, Mannheim, Germany [11, 24]. Essentially, it consists of a transmitted brightfield microscope that is directly coupled to moving suspensions to capture micrographs of the suspended objects.

Illumination is generated by a LED (DieMOUNT, Wernigerode, Germany) peak-wavelength at 650 nm, nominally 4 mW at 20 mA, peak current approximately 2 A, pulse-length 0.5–10 μs . The light is guided through an optical fiber to provide direct light microscopy. The micrographs were acquired by a digital camera (SCA1400–17gm, Basler, Ahrensburg, Germany, CCD-size 8.98 mm $\times$ 6.71 mm, 1392 1040 pixels, 6.6 8.8 mm², pixel size 6.45 6.45 m², bitmap, 8 bits) through an objective lens (40 $\times$, NA = 0.75, object field 0.16 $\times$ 0.22 mm², water immersion).

The images were acquired immediately after the yeast suspension was prepared.

Image analysis

An image analysis algorithm was implemented using the MATLAB Image Processing Toolbox (MathWorks, Ismaning, Germany). Essentially, the algorithm comprises two main functions: (i) detect pseudohyphae in ISM images, and (ii) segment individual cells within the pseudohyphae to estimate the number of cells within each pseudohypha. All steps involved in the algorithm are described as follows.

The first step is segmenting pseudohyphae from the input ISM image to create portraits containing single pseudohyphae. Due to the illumination by a thin light fiber, the ISM images suffer from some vignetting, *i.e.*, its periphery is darker than its center [24]. This effect is compensated by using the intensity mean of the first 30 images for brightness normalization of the entire ISM image.

Thereafter, the local variance [28] of the normalized image is computed in order to find objects of interest. By applying an intensity threshold to the variance image, a binary image containing segmented objects as groups of connected white pixels on a black background is created. Structures touching the image border are removed by applying border cleaning operator. Morphological dilation using a 3-pixel linear structuring element followed by holes filling operation is performed in the remaining objects. Objects smaller than 1500 pixels are also removed. Another morphological dilation (line-shaped, 7 pixels) followed by holes filling is carried out. Objects smaller than 800 pixels in the resulting image are removed. A morphological erosion (disk-shaped, 3 pixels) followed by removing objects smaller than 350 pixels and image cleaning border creates the final binary image.

Afterwards, the centroid of the objects in the final binary image is computed and this information is utilized to crop 250 250 pixel-sized micrographs—called *portraits* - from the original ISM image. As the generated portraits may still contain more than one object each, a segmentation using a Sobel operator [28] is performed. To generate portraits containing only one objects centralized inside the portrait, the following steps are performed: morphological dilation (line-shaped, 7 pixels), followed by holes filling and discarding of objects smaller than 1000 pixels. The area of the objects inside the portrait is computed and the largest object is selected. Afterwards, its centroid is computed, and this information is used to centralize the selected object within the portrait.

The second step is to segment individual cells within each single-pseudohypha portrait. To this task, this study utilized a marker-controlled watershed transform [29], one of the most used segmentation techniques for separating touching objects. This method considers a grayscale image as a topographic surface where the intensity of each pixel represents the elevation at this point. In this interpretation, “catchment basins” correspond to dark regions surrounded by bright structures. The segmentation process can be visualized by the idea that the basins are “flooded” starting from certain seed-pixels or pixel regions which are designated as “markers”. Here, we use minima in the cell-bodies as markers. The flooding stops at ridge lines where water coming from different basins would meet, separating adjacent catchment basins as the objects of interest. In this way, foreground and background pixels are generated. If the cell-borders exhibit enough contrast against the background or against neighboring cells in hypha, it

suggests itself to exploit them as ridge lines between catchment basins in the watershed algorithm. The foreground objects should have a one-to-one relationship to individual cells, no matter whether these are single cells or cells in hyphae. For this to happen, each cell should only get one marker, *i.e.*, possess one local minimum. Therefore, the cell portraits must be preprocessed in order to eliminate noise and multiple local brightness minima within the cell-bodies.

Related to this task, the input portraits are preprocessed as follows:

First a reduction of high frequency noise is done by averaging over 3×3 neighborhoods [28]. Then, the brightness inside portraits is normalized. High-pass filtering is used to enhance the object border and a final smoothening is applied.

Opening-by-reconstruction [28] is the first procedure aiming at extracting foreground markers from the cell's body by homogenizing intracellular contents. However, there are still some remaining inhomogeneities that can generate multiple local minima and thus several markers within a single cell. In order to avoid over-segmentation by multiple markers, these inhomogeneities are flattened by applying an opening-closing by reconstruction [28]. As result, the intracellular region is further smoothened without altering the overall shape of the object. The last steps before applying the watershed operation are:

1. regular morphological opening (square-shaped, 6 pixels) for further smoothening,
2. inverting the image so that the formerly dark cell borders become bright and can perform as ridge lines,
3. border cleaning to get rid of adherent objects to the image borders,
4. h-minima transform [30] in order to obtain only one minimum per cell for a well-defined marker.

Finally, the watershed operation is applied and the number of cells forming the object (a single cell or a pseudohypha) in a portrait is determined as the number of watershed regions inside that portrait. Please see Fig. 3 for details.

Performance evaluation

To evaluate the performance of the segmentation, a visual inspection is performed on each single-pseudohyphal portrait and the segmentation results are classified as true positive (TP), false positive (FP), false negative (FN) or true negative (TN).

Complete cell bodies, the largest fragment of over-segmented cells (cells divided into many parts) and one cell for each case of under-segmentation (cells divided into too few parts) are classified as TP. Halo artifacts and intensity irregularities in the image background are classified as FP. Missed cells and remaining cells in cases of under-segmentation are classified as FN. Image background is classified as TN. The interpretation of the listed terms is adapted from [31].

The performance of the segmentation algorithm is quantified by computing the sensitivity (SE), specificity (SP), and accuracy (ACC):

[Due to technical limitations, this equation is only available as a download in the supplemental files section.]

List Of Abbreviations

ISM: *In situ* microscope; TN: True Negative; TP: True Positive; FP: False Positive; FN: False Negative; CHO: Chinese hamster ovary; YPD: Yeast extract, Peptone, Dextrose; LED: Light-emitting Diode; NA: Numerical Aperture; rpm: Revolutions per minute;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

VLB and OMJr designed the research idea. VLB wrote the manuscript, and all authors edited the manuscript. VLB wrote the code. SRCA prepared the cell experiments. HS and PW were the major contributors in writing and in providing comments and guidance on the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Distribution of single-pseudohypha portraits

Focal condition of individual cells within pseudohyphae	Number of detected pseudohyphae
Only over-focused (<i>o</i>)cells	1629
Only in-focus (<i>i</i>)cells	93
Only under-focused (<i>u</i>)cells	821
Contains <i>o</i> , <i>i</i> and <i>u</i> cells	3903
Total	6446

Table 2 Overall performance of the cell segmentation algorithm

Parameter	Focal condition of pseudohyphal forming cells			
	Over focused	In focus	Under focused	Mixture of focal conditions
Cells per pseudohypha by manual counting	4.6 ± 1.4	5.4 ± 2.0	6.7 ± 2.9	9.2 ± 3.0
Cells per pseudohypha by automatic counting	4.2 ± 1.3	5.5 ± 2.4	7.0 ± 3.4	9.4 ± 2.9
Total number of cells counted manually	139	162	201	277
Over-segmented cells	2	47	21	36
Under-segmented cells	9	17	4	11
True positives	102	118	148	180
False positives	8	8	8	4
True negatives	30	30	30	30
False negatives	17	40	55	97
Sensitivity	87%	76%	73%	69%
Specificity	88%	89%	88%	93%
Accuracy	85%	77%	75%	70%

Figures

Figure 1

Example of an ISM image showing pseudohyphae. Cell-aggregates are formed by cells imaged at different distances from the objective focal plane: in-focus cells are indicated by the thin arrow, whereas under-focused and over-focused cells are indicated by the arrowhead and large arrow, respectively. Scale bar is 10 μm .

Figure 2

Categories of pseudohyphae classified manually according to the focus condition of the forming cells. a over-focused. b in-focus. c under-focused. d mixture of focused, over-, and under-focused. Scale bar is 10 μm .

Figure 3

Stages of the image processing of a typical hypha-portrait. a Input portrait. b High-pass filtered image. c Smoothed high-pass filtered image. d Opening-by-reconstruction. e Opening-closing by reconstruction. f Regular morphological opening. g h-minima transform computed in the complement of f. h Watershed image overlaid on the input image. The detected cells were pseudo-colored for visualization purposes. Portraits correspond to 250×250 pixels.

Figure 4

Effect of the h-minima transform on the segmentation performance. a input image to the watershed transform with a reference line for measuring the grayscale profile. b red curve: original grayscale profile along the line shown in a, blue curve: grayscale profile after the h-minima transform: all regional minima with depth smaller or equal to 2 (the adopted h-value) were removed (as shown by the large arrow), while the height of the remaining regional minima was increased by 2 (pointed by the thinner arrows). Wherever blue curve segments are identical with red segments, they are shown only in blue color. c watershed segmentation of the input image without processing with the h-minima transform. The over-segmentation occurred due to the existence of two catchment basins inside a single cell. d watershed segmentation of the input image previously processed by the h-minima transform. Portraits correspond to 250×250 pixels.

Figure 5

Examples of segmentations on pseudohyphal formed by cells imaged at different focusing conditions. a over-focused. b in-focus. c under-focused. d mixture of focused, over-, and under-focused cells. In each group of images, the first and second columns correspond, respectively, to the input portrait and the segmented cells overlaid on the input image. Arrowheads point to cells with missing contours, whereas

arrows point to missing cell edges between conjoined cells. The segmented cells were pseudo-colored for visualization purposes. Portraits correspond to 250×250 pixels.

Supplementary Files

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