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Towards an automated analysis of video-microscopy images of fungal morphogenesis

by

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Abstract

Fungal morphogenesis is an exciting field of cell biology and several mathematical models have been developed to describe it. These models require experimental evidences to be corroborated and, therefore, there is a continuous search for new microscopy and image analysis techniques. In this work, we have used a Canny-edge-detector based technique to automate the generation of hyphal profiles and calculation of morphogenetic parameters such as diameter, elongation rates and hyphoid fitness. The results show that the data obtained with this technique are similar to published data generated with manual-based tracing techniques and that have been carried out on the same species or genus. Thus, we show that application of edge detector-based technique to hyphal growth represents an efficient and accurate method to study hyphal morphogenesis. This represents the first step towards an automated analysis of video-microscopy images of fungal morphogenesis.

Keywords: automated analysis, fungal growth, hypha, hyphoid model, image analysis, morphogenesis, Saprolegnia, video-microscopy.

Resumen

La morfogénesis de los hongos es un área de estudio de gran relevancia en la biología celular y en la que se han desarrollado varios modelos matemáticos. Los modelos matemáticos de procesos biológicos precisan de pruebas experimentales que apoyen y corroboren las predicciones teóricas y, por este motivo, existe una búsqueda continua de nuevas técnicas de microscopía y análisis de imágenes para su aplicación en el estudio del crecimiento celular. En este trabajo hemos utilizado una técnica basada en un detector de contornos llamado "Canny-edge-detector" con el objetivo de automatizar la generación de perfiles de hifas y el cálculo de parámetros morfogenéticos, tales como: el diámetro, la velocidad de elongación y el ajuste con el perfil hifoide, es decir, el perfil teórico de las hifas de los hongos. Los resultados obtenidos son similares a los datos publicados a partir de técnicas manuales de trazado de contornos, generados en la misma especie y género. De esta manera demostramos que la aplicación de esta técnica para el trazado de perfiles en hifas en crecimiento es un método eficaz y preciso para el estudio de la morfogénesis de hifas. Este trabajo representa el primer paso en la automatización de análisis de imágenes de video-microscopía de morfogénesis de hifas.

Palabras clave: análisis automatizado, análisis de imágenes, crecimiento de hongos, hifa, modelo hifoide, morfogénesis, Saprolegnia, video-microscopía.

Introduction

Fungi are microorganisms that growth as tubular cells named hyphae. Hyphae extend by a vesicle-based process of apical growth and the mechanisms of this process appear to be the same among all groups of fungi in spite of their evolutionary origin (Bartnicki-Garcia & Lippman, 1969; Bartnicki-Garcia, 1973; Bartnicki-Garcia & al., 1989; Bartnicki-Garcia, 1990, 1996, 2000, 2002; Diéguez-Uribeondo & al., 2004). Apical growth involves a continuous transformation of the highly curved cell wall surface of the tip of the

hyphae to the milder curvature of the subapical region surface. Consequently, to understand the mechanism of the apical growth and fungal morphogenesis, it is important to have a precise knowledge of hyphal-tip-geometry.

Most of what is known on hyphal morphogenesis has been based on experimental evidences involving application of new microscopy and image analysis techniques. Thus, development of enhanced video-microscopy had made possible to provide real-time digital contrast and to study growing cells with high resolution (Bartnicki-Garcia & al., 1989; López-Franco & al., 1994). Manual measurements from microscopy image-frames have been performed by development of a Windows application name "fungus simulator" that interfaces with Argus-10 Hamamatsu® image processor (Bartnicki-Garcia & al., 1994). This application and additional measurements tracing options from commercial computer programs such as ImagePro Plus® for Windows® had allowed describing several crucial parameters of fungal morphogenesis with fine detail, i.e. elongation rates (López-Franco & al., 1994), growth directionality (Riquelme & al., 1998), describe the relation of a structure operating as vesicle supply center, i.e. the Spitzenkörper (Bartnicki-Garcia & al., 1995a,b; Reynaga-Peña & al., 1997), and identifying the nature of forces driving fungal cell wall expansion (Bartnicki-Garcia, 2002). Recently, two Windows®-based computer routines have been devised to make quantitative comparisons of hyphal shapes (Diéguez-Uribeondo & al., 2004).

Thus, image analysis studies have been used as a tool to provide evidences to support or question previous mathematical models describing hyphal growth and shape (see review by Bartnicki-Garcia, 2002). The most plausible model is a two-dimensional model, the so-called "VSC model" (Bartnicki-Garcia & al., 1989; Bartnicki-Garcia & al., 2000; Bartnicki-Garcia, 2002), which explains the mechanisms of hyphal tip growth based on a simple mathematical equation $y = x \cot (x\ V/N)$. The equation relates the y of each x,y coordinate pair of the hyphal profile with the number of wall-building vesicles (N) randomly released from a vesicle supply center (VSC) per unit time, and the rate of advancement of the VSC (V). The relationship between the maximal diameter of a hypha and the VSC distance to the pole is defined by the equation $D = 2d\pi$, where D is the maximal diameter of the hyphae and d is the distance of the VSC to the apical pole. This allows standardizing the shape of all hyphae and make comparison of hyphal shapes among species. The regions of an idealized shape of an hypha can be divided into three main regions (Fig. 1): apical region from pole to 2d, subapical region from 2d to 20d and mature region beyond 20d (Bartnicki-Garcia, 1990; López-Franco & Bracker, 1996). Thus, research on hyphal morphogenesis is based on accurate descriptions of hyphal profiles and measurements of parameters such as location of apical pole and VSC, diameter at a certain distance to the pole, elongation rates, etc. New outstanding questions on hyphal tip morphogenesis are being addressed (see review by Bartnicki-Garcia, 2002).

The current techniques developed for these studies had clearly allowed retrieving relevant information. However, much efficient programs for analyzing and interpreting data could be achieved if new techniques, i.e. image and data processing, data based, etc., are incorporated to his studies and also automated in one application. Automated identification of hyphal contours instead of manual tracing represents the first step towards the development of an efficient user-friendly image analysis tool. Edge detection is a fundamental operation in image processing and, therefore, crucial to obtain this goal. Currently methods for edge detection are based on the application of wavelets operators (Placidi & al., 2003; Song & al., 2003; Turiel & al., 2003; Wang & al., 2003; Dai & al.,

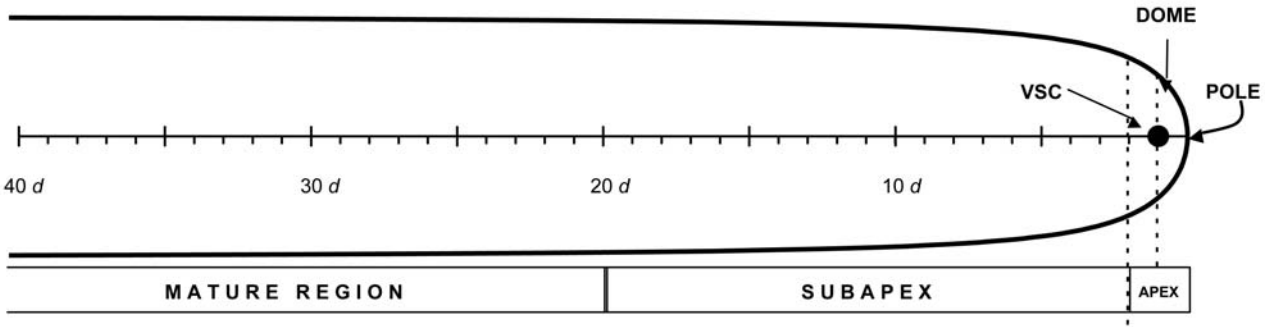

Fig. 1. Regions in the idealized shape of a hypha according to the hyphoid model (Bartnicki-García,1990; López-Franco & Bracker, 1996). The hypha is divided into three main regions: apical region from 0 to 2 d , subapical region from 2 to 20 d , and mature region beyond 20 d.

2004; Duccottet & al., 2004; Gleich & al., 2004; Junxi & al., 2004). Traditional used operators for edge detection were based on Fourier series. However, one of the main advantages of wavelets is that they are well suited for approximating data with sharp discontinuities and easily characterize local regularity, which is an important property for biological images (Graps, 1995). Wavelets have been mainly developed in fields of mathematics, quantum physics, electrical engineering, and seismic geology (Graps, 1995), and have been mainly applied to image compression, turbulence, human vision, radar, and earthquake prediction. The applications of wavelets represent an unexplored and exciting field. Wavelet techniques have not been throughly worked out in applications such as image analysis of biological systems.

In this work, we describe the application of an edge detector, i.e. Canny's edge detector, equivalent to detecting modulus maxima in a wavelet transform (Mallat, 1992), to video-microscopy images of hyphal growth. Thus, the goal of this study was to developed the initial requirement of an automate application for image analysis of video-microscopy images. This may allow obtaining crucial measurements for studying hyphal morphogenesis such as elongation rates, hyphal shape and diameter in real time and in an efficient and rapid way.

Material and methods

Cell images

The fungal strain used in the experiment was *Saprolegnia parasitica* Coker (SpT) kindly provided by Dr. Kenneth Söderhäll, Department of Comparative Physiology, University of Uppsala, Sweden. Fungal strains were maintained at 22 ± 2 °C in potato dextrose agar (PDA), at pH 5.5. For microscopic observations and recordings, fungi were inoculated in Petri dishes containing thin layers ca 0.5 mm of potato dextrose agar (PDA). For the final analysis, we grew the fungi on PDA, a medium where branching was less frequent and longer lengths of primary hyphae could be measured. The inoculum consisted of a ca 5 mm plug excised from a colony edge, and placed in the center of agar layer. The fungi were allowed to grow for 24-36 h before observations. Colonies that had grown at least 2 cm on PDA were selected for analysis. Before observation, a drop of Difco potato dextrose broth was added to the edge of the colony and carefully covered with a square cover slip (22 × 0.1 mm thick: Carolina Biological Supply Co). For the study, only randomly selected hyphae from the growing edge of young colonies were used.

Video-microscopy

Petri dishes were placed on the stage of an Olympus Vanox microscope and hyphal growth monitored with bright-field optics (40× objective and 25× WF eyepiece) (American optical). Hyphal shapes were imaged with a Hamamatsu® C2400-07 video camera; images were enhanced with an Argus-10 digital processor (Hamamatsu® Photonic Systems, Bridgewater, NJ), recorded on S-VHS videotapes and displayed on a 12-inch black and white monitor (Sony® Model PVM-122). The analogical-video sequence consisting in 30 frames per second was digitalized into AVI-files using a JVC HR-XVS20 video player connected to a computer by a mvDELTA-BNC frame grabber (http://www.matrix-vision.com/products/hardware/mvdelta.php?lang=en). The analogical signal was processed by a plugin of the software I+Solex® (Image Solex Inc.) and converted into a video sequence on an AVI format. The image format used was 8 bits gray-scale.

Image processing

Image processing of the digitalized video sequences was performed in the following steps: (1) Selection of area of analysis. This consisted in selecting sequences of video with straight growing hyphae and that hyphal tip was focused in the entire sequence. (2) Edge detection. The edge of the profile of interest was the limit of the hyphae. The criterion to define the limit of the hyphae was the inner boundary of the cell wall. The methodology followed to detect the limits was a method similar to the Canny-Deriche edge operator (Canny, 1986) described in http://bigwww.epfl.ch/demo/jedgedetector/index.html. Basically, the processing consisted in a smoothing (using a Gaussian smoothing operator), gradient computation, a non-maximum suppression, and a hysteresis threshold. (3) Diameter calculation, the algorithm used was programmed to detect the apex of the hyphae. The diameters were calculated at a distance of 1d, 2d, and 5d from the apex. These distances could be change according to experimental samples. The Java based computer program I+Solex® (Image Solex Inc.) was used for this purpose.

Data analysis

The x,y coordinate pairs were obtained as txt files. Diameter and elongation rate calculations were processed and graphed with Microsoft Excel. Hyphal profiles were studied with two computer routines designed to study diameter fluctuations and percentage of hyphoid fitness, i.e. "diameter tool" and "hyphoid fitness" (Diéguez-Uribeondo & al., 2004) and available at http://www.is-si.com/fungal-morphogenesis/oomycetes.htm.

Experimental validations

To test the validity of the edge detector applied, several morphogenetic parameters calculated with the profiles obtained with this technique were compared to published data on the same parameters and on the same fungal genus (López-Franco & al., 1994) and species (Diéguez-Uribeondo & al., 2004) that were obtained with manually tracing techniques. Ten hyphal profiles from different time frames were analysed.

The parameters used for these comparisons were diameter, elongation rates, and concordance to the theoretical hyphal shape, i.e. hyphoid fitness. The increase in diameter from the pole towards the end of the hypha was studied by using the computer routine "diameter tool". The elongation rates were calculated as described above. The calculation of the hyphoid fitness requires a previous step consisting in the straightening of the actual profile of the hyphae. This process is carried out by the computer routine "hyphoid fitness" (Diéguez-Uribeondo & al., 2004).

Results

The process of image analysis is summarized in Fig 2. The source of images used for testing the edge detector was a sequence of 764 images equivalent to a period of growth of about 28 s of videotaping (Figs. 2a-c). The profiles generated by the edge detector approximated the inner boundary region of the fungal cell wall as shown in Figs. 2d-f. The hyphal profiles and the lengths of the diameter at a distance from the pole of 1d, 2d and 5d, are shown in Figs. 2g-i. The validation of the technique used was done by studying the hyphal morphogenetic parameters: diameter, hyphoid fitness and elongation rate.

Diameter

The computer routine "diameter tool" allowed studying the variation of diameter of hyphal profiles. The profiles obtained with the edge detector approximate the theoretical hyphoid shape of a hypha (Fig. 3). The profiles automatically obtained with the edge detector exhibited similar diameter properties to profiles obtained using manual-based tracing applications (Diéguez-Uribeondo & al., 2004). The progressive increase in diameter from the pole towards the mature region of the hyphal tube was not entirely even but was punctuated by irregular fluctuations (Fig. 3). The amplitude of the fluctuations ranged between ca. 0.1 to 0.6 µm (1% to 5 % of the mean diameter in the hyphal region).

The possibility of obtaining automated hyphal profiles from this edge detector technique, and conse-

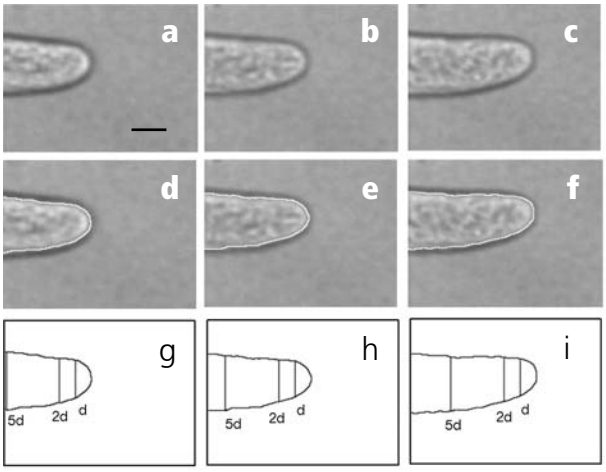

Fig. 2. Process of application of a Canny's based-edge detector to generate hyphal profiles. **a-c,** source of images corresponding to time frames (**a,** frame 1; **b,** frame 315; **c,** frame 764); **d-f,** the obtained hyphal profiles overimposed to the corresponding images of the hyphae at same time frames as above; **g-i,** generated profiles for each image frame and the calculation of their corresponding diameters at fixed distances from the pole 1d, 2d, and 5d. (Bar = 5 µm).

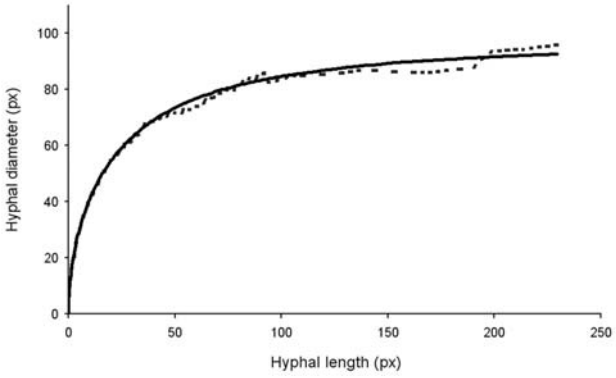

Fig. 3. Diameter variation from the pole towards the mature region of the hypha in the Canny's edge detector-based profile (----) and in the theoretical shape of the hypha (—). (1 px = 0.1384 µm).

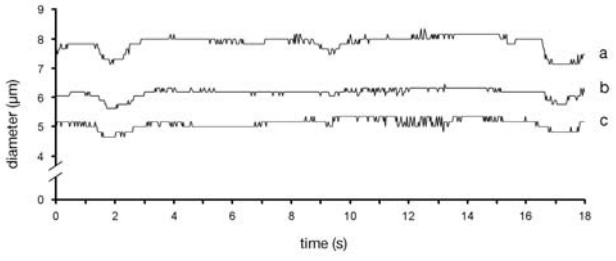

Fig. 4. Diameter fluctuations at fixed distances to the pole of the hyphae (µm): **a,** 5d; **b,** 2d; **c,** 1d.

quently x,y coordinate pairs, allowed an easy calculation of changes in diameter at a fixed distance from the pole and during short periods of time, e.g. 1/30 s. The diameter at a fixed distance from the pole fluctuated irregularly during growth. This was observed at three regions of the hypha corresponding to the apical region and subapical region diameters. The fluctuations in diameter were simultaneous and proportional in intensity in the three regions of the hypha (Fig. 4).

Hyphoid fitness

The computer routine "fitness tools" was used in order to compare shape differences among the edge detector profiles and the hyphoid theoretical profile (Fig. 5). The percentages of hyphoid fitness were of 97.88%, 97.93%, and 97.91% at distances from the pole of 1d, 2d and 5d, respectively. This is similar to previously described hyphoid fitness for *S. parasitica* hyphal profiles that: 1) were grown and videotaped under the same conditions, 2) obtained with manual-based applications (Diéguez-Uribeondo & al., 2004).

Elongation rate fluctuations

Under our experimental conditions, the mean elongation rate was 0.24 µm/s (Fig. 6). The elongation rate was not steady but pulsated continuously (Fig. 6), as was shown before for hyphae of different fungal species with manual tracing techniques (López-Franco & al., 1994). These pulsate growth could be observed in all time periods studied, i.e. 1, 2 s. However, when we forced the calculation of the elongation rates to the limit of the technique, i.e. 1/30 s, we could only detected a minimum progression of the pole of ca. 1 pixel (0.1384 µm). When elongation rates were calculated for this time period, i.e. 1/30, we observed displacements both forward and backwards of the advancing hyphal pole (Fig. 6c).

Discussion

In this study, a Canny's-edge detector based technique was applied to quantify and characterize the profiles of fungal hyphae and to relate this to other parameters of crucial relevance in fungal morphogenesis studies. Video-microscopy image sequences that were previously studied with manual-based tracing techniques were used to test the validity of our approach. The profiles of the hyphae and their morphogenetic parameters and properties, i.e. diameter variation, hyphoid fitness, and pulsated growth, obtained with the edge detector were as accurate as those obtained with manual-based techniques.

The application of edge detection–based techniques on fungal growth represents the first step towards an automated generation of hyphal profiles and quantification of morphogenetic parameters. This is important because it provides a more efficient way of carrying out studies on morphogenesis and also allows the possibility of retrieving data at real-time. Currently, morphogenetic studies involve several manual-based computer programs, which made this type of studies very tedious and time consuming. The possibility of retrieving data at real time will also facilitate studying cell growth by simplifying the selection of video sequences and the comparison of fluctua-

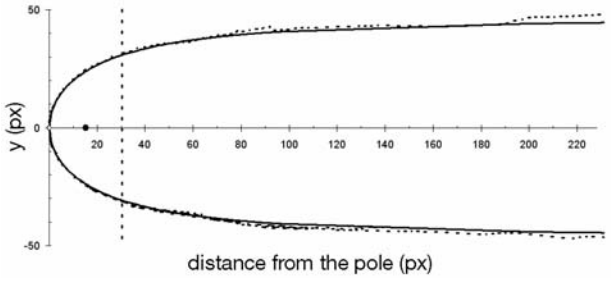

Fig. 5. Concordance of the hyphal profile generated with an edge detector (----) to the theoretical hyphal shape (—), i.e. hyphoid fitness. The calculation of the hyphoid fitness requires a previous step consisting in the straightening of the actual profile of the hyphae. This process is carried out by the computer routine "hyphoid fitness" (Diéguez-Uribeondo & al., 2004).

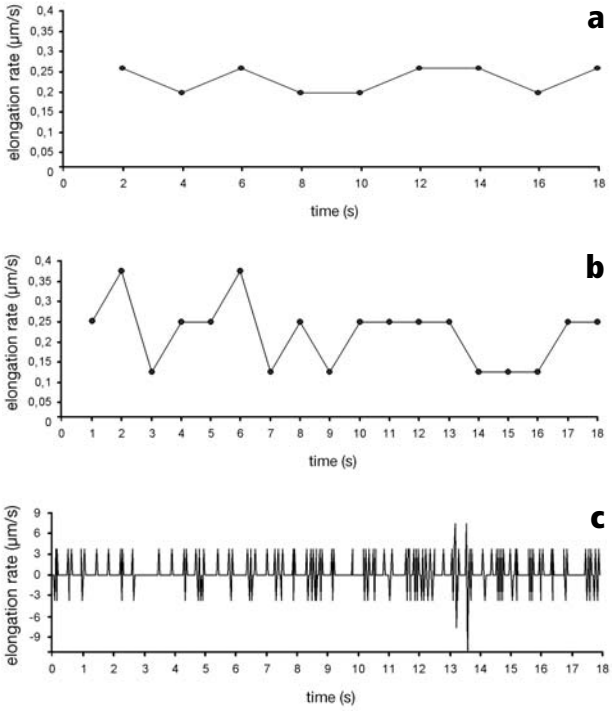

Fig. 6. Pulsed growth of fungal hyphae: **a,** at 2 s growth intervals; **b,** at 1 s growth intervals; **c,** at 1/30 s growth intervals.

tions of several parameters at the same time, i.e. diameter and elongation rates, diameters at different distances from the pole, movements of VSC and their consequences on hyphal shape, etc.

However, this technique, at its current development, has some drawbacks detailed as follows: (1) The technique is limited to sequences of fungal growth in a straight axis. Hyphae often meander during growth (Riquelme & al., 1998; Bartnicki-Garcia, 2002) and, consequently, the position of the hyphal pole changes and the calculation of all pole-based parameters. New functions efficiently detecting the pole and axis of growth need to be developed for fully automated analysis of any growing hyphae. (2) Because hyphae grow, the characterization of its growth is also limited by the optical field of view. This needs to be moved and, therefore, the corresponding coordinates require to be adjusted. Thus, studies on extended cell growth will require the used of motorized microscopes and the development of programs allowing the automatic movement of the platform of the microscope and the correction of coordinates. (3) Errors in edge detection can occur as consequence of manual focusing of hyphal growth**.** These focusing variations may be responsible for negative values of elongation rates at times intervals of 1/30s, and/or for the observed simultaneous variations of diameter at fixed distances from the pole. Focusing variations may be due to a probable meandering growth of hyphae in the z axis. The improvement of image focusing by incorporating new autofocusing techniques will allow testing whether some of the observed properties, i.e. negative elongation rates, simultaneous variations of diameter at different distances from the pole are due to artifacts of the technique or represent true biological events such as retractile type of growth, or variations in elongations rates respectively. These aspects will be also the subjects of future studies.

Currently, there is a need for development of automated techniques for image analysis. The development and application of these techniques in fungal morphogenesis represents an important challenge for researchers in this field. These techniques will ease the generation of accurate morphometric and morphogenetic data and will allow studying properties of growth at much shorter intervals of time than in previous studies based on manual tracing techniques. This work represents the first attempt to automate edge detection in video-microscopy of fungal cells that may allow automating many studies that are currently being based on manual, tedious and dispersed techniques.

Acknowledgments

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