[34] Fluorescent Labels, Confocal Microscopy, and Quantitative Image Analysis in Study of Fungal Biology

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Introduction

Microscopy in the study of fungi has been applied basically in two contexts. First, the fungus in isolation can be the subject of investigation, in which case studies typically have focused on cell dynamics, morphogenesis, cytochemistry, and organelle or spore structure and development. Within the past decade, laser scanning confocal microscopy (LSCM) has become

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a powerful tool in the analysis of subcellular detail such as that just noted.[1–4] It combines laser, computer, and imaging technologies to enable optical rather than mechanical sectioning of specimens. The second context pertains to the relationship between a fungus and its substratum. Examples include host-fungus interactions and the extent or spatial pattern of colonization. LSCM can reconstruct multiple, in-focus image planes, rendering them in three dimensions. Thus, it has been used to obtain sharp images of fungi within a host or colonizing highly contoured or rugged surfaces such as the phylloplane or rhizoplane of plants.

Fluorescence is the usual mode for LSCM and many natural and synthetic fluorescent probes (i.e., fluors, fluorophores, or fluorochromes) are available. Fluorescent labels have great utility because they are relatively photostable and sensitive. They are also potentially highly specific when linked to various macromolecules (e.g., proteins, nucleic acids). The main shortcoming of fluorescence-based procedures is related to noise, i.e., nonspecific fluorescence contributed by background-contaminating substances such as host cells, debris, or reagents. Operationally, the goal is usually to maximize signal-to-noise ratios.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported[5] in 1994 as a marker and has since been expressed in its native or mutated state in prokaryotes and diverse eukaryotes, including fungi. It is a 238 amino acid protein, the fluorophore of which is formed by posttranslational cyclization and oxidation of the amino acids Ser-65, Tyr-66, and Gly-67. GFP has revolutionized cell biological research (trafficking; intracellular signaling; gene expression) and has proven to be a useful label to follow cell movement within organisms or in the environment because of its stability, versatility, and ability to function solely with the stimulus of UV or blue light and oxygen, i.e., without cofactors or substrates.[6]

Another major area of recent technical advance considered in this article is quantitative image analysis. In theory, it allows for the rapid and relatively objective enumeration and classification of fungal propagules meeting specific descriptions. Briefly, this procedure involves the manipulation of pictorial information. Its role in biological investigation is accelerating with the advent of modestly priced computers powerful enough to handle pictorial

[1] J. B. Pawley, ed., "Handbook of Biological Confocal Microscopy," 2nd Ed. Plenum, New York, 1995.
[2] Y. H. Kwon, K. S. Wells, and H. C. Hoch, Mycologia **85,** 721 (1993).
[3] K. J. Czymmek, J. H. Whallon, and K. L. Klomparens, *Exp. Mycol.* **18,** 275 (1994).
[4] B. Matsumoto, ed., "Methods in Cell Biology," Vol. 38. Academic Press, New York, 1993.
[5] M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, *Science* **263,** 802 (1994).
[6] M. Chalfie and S. Kain, eds., "Green Fluorescent Protein: Properties, Applications, and Protocols." Wiley-Liss, New York, 1998.

data.' In the context of fungal biology, the information is typically microscopic and is related to the size, shape, and quantity of various cell components, cells, or cell aggregates such as hyphae. Thus, the initial image usually begins in optical form and is converted subsequently through an analog (electrical) stage by a video camera to a digital form for computer processing.[7] For example, an image of continuous gray tones is converted to a rectangular array of discrete points of brightness. Each point is known as a pixel or picture element and has an associated digital value representing its intensity. Interpretation of the digital array of quantitative information is controlled by the observer who, through different analysis operations, adjusts the image for enhanced contrast.[7] The enhanced image is then categorized by various criteria related to size, shape, brightness, and so on.

We are interested in the growth and ecology of the cosmopolitan, yeast-like fungus *Aureobasidium pullulans* on leaf surfaces. In these studies, we have developed various microscopic techniques, including quantitative fluorescence *in situ* hybridization using rRNA-specific probes, vital staining, and the tracking of and quantification of GFP-expressing transformants. Essential to this work is validation of these methods by various techniques with both confocal and conventional microscopy. The protocols described here for DNA staining and detection of actin by the probes of small (phalloidin) and large (antibody) molecular size are the outgrowth of these goals. They also demonstrate the power and versatility of the techniques.

Green Fluorescent Protein Expression in Fungi

Green fluorescent protein has proven to be a valuable aid for identifying the subcellular location of proteins and for monitoring gene expression. To date, GFP has been expressed successfully in various fungi or fungus-like organisms, including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Dictyostelium discoideum, Aspergillus nidulans, Podospora anserina, Ustilago maydis, Candida albicans,* and *A. pullulans.* In several instances, expression has been monitored in situ. For example, GFP can be visualized in corn plants and mouse kidney cells infected with transformants of *U. maydis*[8] and *C. albicans,*[9] respectively. In our laboratories, GFP expression has proven useful for the quantitative detection of *A. pullulans* transformants on leaf surfaces.[10] We describe later the methodology for

[7] G. A. Baxes, "Digital Image Processing: Principles and Applications." Wiley, New York, 1994.

[8] T. Spellig, A. Bottin, and R. Kahmann, Mol. Gen. Germ. **252,** 503 (1996).

[9] B. P. Cormack, G. Bertram, M. Egerton, N. A. R. Gow, S. Falkow, and A. J. P. Brown, Microbiology **143,** 303 (1997).

[10] A. J. Vanden Wymelenberg D. Cullen, R. N. Spear, B. Schoenike, and J. H. Andrews, *Bio Techniques* **23,** 686 (1997).

detection and quantification of this fungus in isolation or associated with the phylloplane. We also discuss how GFP can be combined with other fluorescent tags.

There are three basic requirements for GFP expression in fungi: a reliable transformation system; functional transcriptional control elements to drive expression; and the appropriate GFP-encoding gene. With regard to transformation, a wide range of selectable markers has been described,[11] most commonly complementation of auxotrophic markers or conferral of drug resistance. Resistance to hygromycin B is conferred by a phosphotransferase-encoding gene, *hph*, and this dominant marker has been used for several fungi expressing GFP, including *A. pullulans*.[8,12] Transformation of *A. pulluluns*, like most fungal systems, involves polyethylene glycol-induced aggregation of protoplasts in the presence of plasmid DNA.[10,12] Protoplasts are prepared by treating germlings with a commercial lytic enzyme, Novozym, in an osmotically stabilized buffer, e.g., 1 *M* sorbitol. Digestion conditions often require minor alterations because enzyme lots vary.

Hygromycin-resistant colonies appear within 5 days, and at least in the case of *A. pullulans* transformed with pTEFEGFP, these may appear yellow-green without UV irradiation.[10] Transformation of *A. pullulans*, like the vast majority of filamentous fungi, involves the stable integration of plasmids within the genome. The copy number and integrative context of plasmid insertions can be ascertained by Southern blot hybridization of genomic DNA. Another common feature of fungal transformation is high frequencies of cotransformation. Thus, the GFP expression plasmid pTEFEGFP can be introduced by simultaneously transforming with a separate plasmid (pDH33)[13] carrying the selectable marker *hph*.

With respect to transcriptional control, both constitutive and regulated promoters have been used for GFP expression in fungi. Regulated expression has been obtained with promoters of the *C. albicans* maltase gene *MAL2*[9] and the pheromone-inducible gene *mfal*[8] of *U. maydis*. For constitutive expression, we[10] and others[8] have found the translational elongation factor promoter *TEF* to be especially useful (Fig. 1). Both translational fusions[14] and direct promoter fusions[10] (Fig. 1) have produced fully active GFP.

[11] J. R. Fincham, *Microbial. Rev.* **53,** 148 (1989).
[12] D. Cullen, V. Yang, T. Jeffries, J. Bolduc, and J. H. Andrews, J. *Biotechnol.* **21,** 238 (1991).
[13] T. Smith, J. Gaskell, R. Berka, D. Henner, and D. Cullen, Gene **88,** 259 (1990).
[14] C. Lehmler D. Zickler, R. Debuchy, A. Panvier-Adoutte, C. Thompson-Coffe, and M. Picard, *EMBO J.* **16,** 12 (1997).

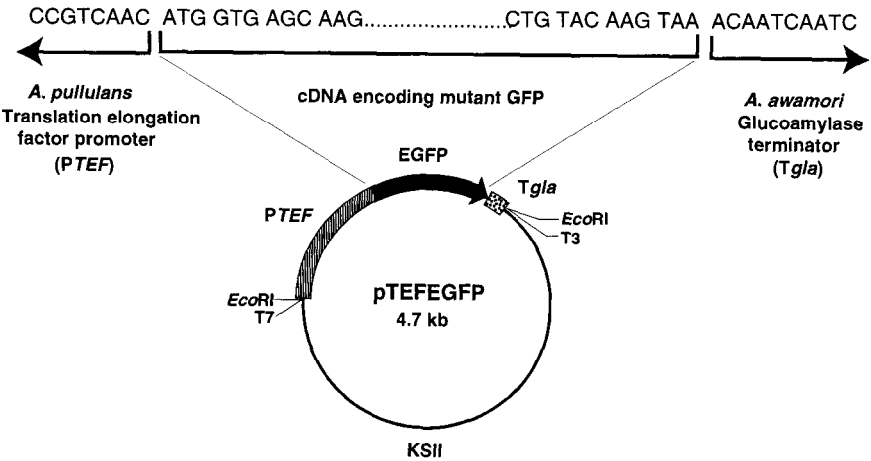

FIG. 1. The expression vector pTEFEGFP. A red-shifted, engineered mutant GFP cDNA (pEGFP) is fused to the TEF promoter from *Aureobasidium pulluluns* and the glucoamylase terminator from *Aspergillus awamori* The entire expression cassette is ligated into pBluescript II KS(-) and the sequence of junctions is confirmed by dideoxy sequencing. Partial sequence of the relevant regions *is* shown.

The choice of GFP clone is critically important to success. Several laboratories have reported that the wild-type cDNA will not yield a functional GFP in fungi.[8–10] Functional forms of GFP usually feature two mutations, (Ser-65 → Thr and Phe-64 → Leu), which dramatically increase fluorescence and frequently alter the codon bias to those codons preferred by higher vertebrates and by some plants. In the case of yEGFP, all codons are optimal for *C. albicans.*[9] The commercially available clone EGFP1[15] (CLONTECH, Inc., Palo Alto, CA) works well in *P. anserina*[16] and in *A. pullulans*[10] (Fig. 1).

Fluorescence Microscopy and GFP

Regardless of the type of fluorescence microscopy (LSCM or conventional epifluorescence), it is important to match the fluorochrome used to the illumination spectra and filters available. Several GFP variants are available and must be chosen carefully for LSCM. Few instruments are presently equipped with lasers with output lines in the UV range that can excite either wild-type GFP at its major peak of 390 nm or the UV-optimized

[15] T. T. Yang, L. Cheng, and S. R. Kain, *Nucleic Acids Res.* **24,** 4592 (1990).

[16] V. Berteaux-Lecellier, G. Steinberg, K. M. Snetselaar, M. Schliwa, R. Kahmann, and M. Bölker, *EMBO J.* **17,** 1248 (1998).

variant at 395 nm. Wild-type GFP can be excited at its secondary peak of 470 nm with the 488-nm line of either argon or argon/krypton lasers, although this is fairly inefficient. The red-shifted excitation mutants, such as EGFP, GFP-S65T, and RSGFP, possess excitation maxima of 488, 489, and 490 nm, respectively, which closely match the 488-nm line, and have emission spectra that closely match fluorescein isothiocyanate, allowing the use of configurations for that fluorochrome. Also, these red-shifted mutants possess much higher fluorescence (4- to 35-fold) than the wild-type, allowing more efficient detection.[17]

GFP is very stable after formation and can withstand fixation with either formaldehyde or glutaraldehyde, although it might be advisable to avoid the latter due to its tendency to produce autofluorescent aldehyde groups in tissues. Fixation in methanol/acetic acid (3 : 1) destroys fluorescence, although it is retained when methanol is used alone.[18] Fluorescence is best in a range from pH 7.2 to pH 8.0 with most common buffers, i.e., phosphate-buffered saline (PBS) 50 m*M*, pH 7.4, 0.8% NaCl, piperazine-*N*,*N'*-bis (2-ethananesulfonic acid) (PIPES); and *N*-[2-hydroxyethyl]piperazine- *N'*-[4-butansulfonic acid] (HEPES) 50 m*M*, pH 7.2. In our hands, *A. pullulans* EGFP transformants with a *TEF* promoter are extremely bright and do not photobleach appreciably in room light, although this should be determined with each system. Cautionary notes against the use of nail polish in sealing preparations have appeared,[17] but we have found that Revlon Epoxy 1000 has not caused any harm. It can be used to seal coverslips for periods of up to 1 month when slides are stored in the dark at 4°.

Laser Scanning Confocal Microscopy

The following section describes conditions and equipment that have performed well for us. We use a Bio-Rad (Hercules, CA) MRC-1024 confocal microscope equipped with an argon/krypton laser illumination source. The microscope and laser are controlled with Bio-Rad Laser Sharp 24-bit software. The MRC-1024 allows up to three colors of fluorochrome to be imaged either simultaneously or sequentially. In the simultaneous mode, all fluorochromes are illuminated at the same time, and collection of each channel (color) is also done concurrently. In the sequential imaging mode, each fluorochrome is illuminated only by the appropriate laser line and the

[17] S. A. Endo and D. W. Piston, *in* "Green Fluorescent Protein: Properties, Applications, and Protocols" (M. Chalfie and S. Kain, eds.), p. 271. Wiley-Liss, New York, 1998.

[18] W. W. Ward, in "Green Fluorescent Protein: Properties, Applications, and Protocols" (M. Chalfie and S. Kain, eds.), p. 4.5. Wiley-Liss, New York, 1998.

corresponding filters for that fluorochrome are changed via a filter wheel. Each image is collected, then the next laser line is used to illuminate the next fluorochrome and its image is stored, and so on. In addition, a z-series (depth) may be acquired by moving the stage focus by a step between each image in either of these collection modes.

The sequential collection mode allows for a more precise adjustment of the laser intensity and photomultiplier (PMT) gain and threshold. This enables better compensation for differences in the intensity of different fluorochromes and adjustment to compensate for spectral overlap ("cross talk") of emission signals to each PMT ("bleed through"). The disadvantage of this collection strategy is the slower speed of data collection and more exposure of the specimen to excitation illumination that could photobleach specimens. "Cross talk" compensation in the simultaneous mode can be implemented by adjusting the PMT gain and sensitivity of the different channels. This adjustment, however, can be difficult when the brightness of differing channels is great and the spectral overlap of colocalizing signals is high. Older instruments, such as the Bio-Rad MRC-600, are not capable of the consecutive detection of multicolor signals, and signals of greatly different strengths present problems in channel overlap.

Another image acquisition technique that can be applied is Kalman average filtering,' which is an image averaging technique that allows the removal of electronic noise. Kalman filtering is especially useful for acquiring dim images.

The image acquisition parameters for our work have been generally as follows: Objectives either 60× NA 1.40, plan-apochromatic oil, or 40× NA 1.30, plan-apochromatic oil (Nikon, Melville, NY); Kalman averaging of three or four images per focal step; z-series focal step 60× objective 0.5 μm, 40× objective 1.0 μm; consecutive collection mode with appropriate filters for EGFP (FITC set) and for propidium iodide, and rhodamine phalloidin and antibodies (TRITC set) (see following sections). For the TEFEGFP transformant on leaf surfaces, we use the fluorescein isthiocyanate (FITC) set to acquire the GFP signal, whereas the Texas Red set is used to acquire the autofluorescence signal for leaf chlorophyll.[10]

Images are saved as *z*-series stacks (see Fig. 2) and merged *z*-series projections of each color channel (green and red), allowing individual images from each signal to be displayed and analyzed. Postacquisition processing can be done with the freeware program Confocal Assistant v3.10 from (ftp://ftp.genetics.biorad.com/Public/confocal/cas/) or Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA) using Bio-Rad image conversion "plug-ins" for Photoshop (ftp://ftp.genetics.biorad.com/Public/confocal/photoshop/).

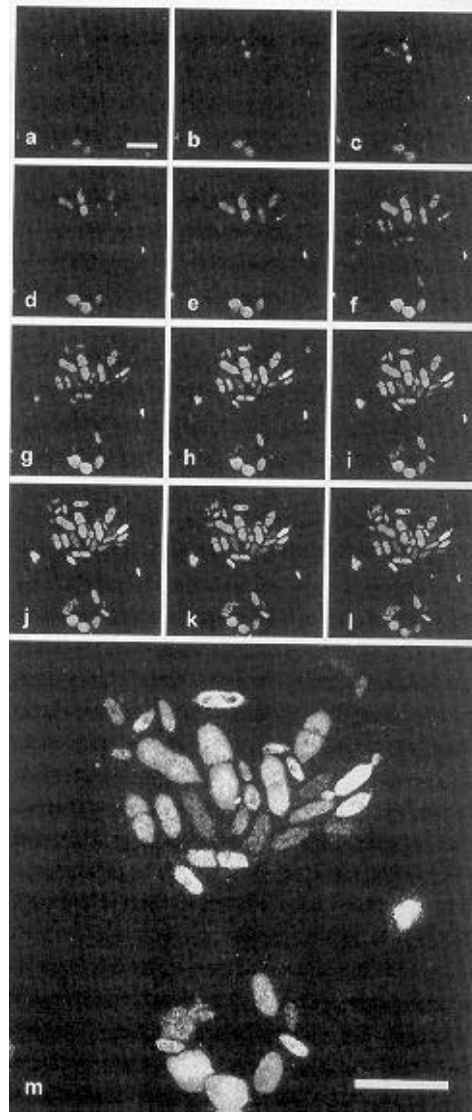

FIG. 2. GFP expression in *A. pullulans;* *z*-series image consisting of 12 0.5-μm steps (a-l); m is the composite image. Bars: 20 μm.

Multicolor LSCM: Use of GFP with Other Fluorescent Labels

Localizing GFP Concurrently with Nuclear Staining by Propidium Iodide

To a midlog phase culture grown in any standard fungal growth medium for 18-20 hr at 26–28°, with shaking, add 1/10 volume freshly prepared 30% formaldehyde in PBS, replace on shaker, and fix for 2-4 hr at room temperature.

Formaldehyde 30%. Caution: Make immediately prior to use in a fume hood. Formaldehyde is a carcinogen!

1. Place 3 g of paraformaldehyde (EM Sciences, Ft. Washington, PA) in approximately 5 ml PBS, 50 m*M*, pH 7.4, heat with stirring to 70° (do not allow to boil), add 0.5 ml of 5 *M* NaOH, allow solution to clear, and adjust pH to 7.4. Make up to 10 ml with PBS. Cool to room temperature before use.
2. Pellet cells by centrifugation at 5000 rpm for 2 min in a microfuge. Wash the cells three times with PBS. We have found that processing cells in suspension produces a lower level of background fluorescence than drying cells on slides prior to staining.
3. Suspend the cells in 1% Triton X-100 in PBS. Incubate for 3-5 min at room temperature. This permeabilizes the cell walls.
4. Wash by centrifugation three times with PBS.
5. Incubate with 10 μg/ml RNase A (Sigma, St. Louis, MO) in PBS at pH 7.2 in a 37° water bath for 1 hr. This step removes RNA that would also stain with propidium iodide.
6. Wash with PBS three times at room temperature.

Carry out the following steps under reduced light to prevent fading.

7. Incubate in 5 μg/ml propidium iodide (Sigma) in PBS for 10-15 min at room temperature. *Caution:* Propidium iodide is a carcinogen!
8. Wash in PBS, two changes, at room temperature.
9. Place drops of cell suspension on Super-Frost (Fisher Scientific, Pittsburgh, PA) microscope slides and allow to air dry at room temperature.
10. Rinse slides briefly with distilled water and mount in an antifade compound such as VectaShield (Vector Labs, Burlingame, CA), coverslip, and seal with nail polish (see earlier note). Sealing of coverslips is desirable to prevent movement when using oil-immersion objectives. Store slides prior to examination in darkness at 4° Examine as described earlier for LSCM (Fig. 3).

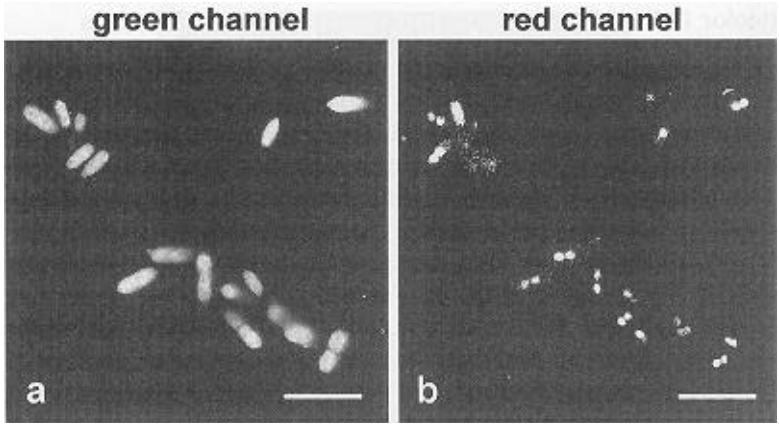

FIG. 3. GFP expression in the cytoplasm of *A. pullulans* cells in the green image channel (a) and DNA staining with propidium iodide of the nuclei in the red channel (b). These images consist of a nine image *z*-series of 0.5-μm steps merged into a single plane. Cells are pretreated with RNase; otherwise the red signal would have colocalized with the green in the cytoplasm. Bars: 20 μm.

Note: If a UV laser is available, the RNase treatment can be omitted, and the propidium iodide step (7) can be replaced with 5-10 μg/ml. 4',6-diamidino-2-phenylindole (DAPI), which stains only DNA. *Caution:* DAPI is a suspected carcinogen!

Staining of Actin with Rhodamine-Conjugated Phalloidin in GFP-Expressing Cells

1. Fix cells with formaldehyde as described earlier, substituting PBS with 50 m*M* PIPES buffer, pH 7.4.
2. Wash three times in PIPES buffer by centrifugation at 5000 rpm for 2 min at room temperature.
3. Permeabilize with 1% Triton X-100 in PIPES for 2-5 min at room temperature.
4. Wash in three changes of PIPES.

Perform the following steps under reduced light to prevent fading.

5. Incubate in rhodamine-phalloidin conjugate (Sigma), 10 μm/ml in PIPES, for 1-2 hr at room temperature. *Caution:* Phalloidin is extremely toxic, work in fume hood, wear laboratory coat, gloves, mask, and eye protection!
6. Wash in three changes of PIPES.

7. Place a drop of cell suspension on a Super-Frost microscope slide and air dry at room temperature.
8. Rinse slide briefly in distilled water and air dry, mount in VectaShield and coverslip, and store slides in the dark at 4° prior to examination (Fig. 4).

Staining of Actin with Rhodamine-Conjugated Antibodies in GFP-Expressing Cells

If one wishes to detect actin and avoid the hazard of toxic phalloidin, immunologic detection may be employed.

1. Fix cells with 3% formaldehyde in PIPES buffer as described earlier.
2. Wash suspension by centrifugation at 5000 rpm for three times at 5 min each at room temperature.
3. Digest the cell wall using Novozym 234 (Calbiochem, San Diego, CA), 0.5 mg/ml in PIPES buffer for 2-10 min at room temperature. Monitor the cell wall digestion by phase-contrast microscopy, halting digestion by washing in ice-cold PIPES when approximately 10-20% of the cells show a decrease in refraction. Alternatively, digest several batches of cells for differing periods of time, process, and select those with the best staining.
4. Wash three times in PIPES.

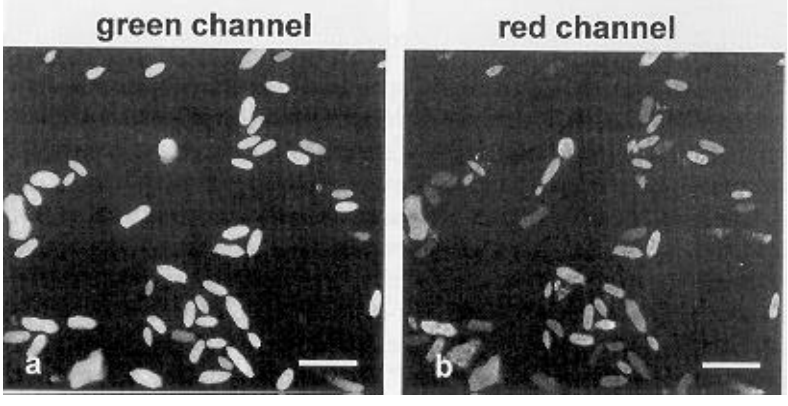

FIG. 4. GFP localization in the green channel (a) with phalloidin-rhodamine staining (red channel) of actin (b) in *A. pullulans*. In this case, the GFP and actin colocalize in the cytoplasm. The signals, however, differ greatly in brightness; with the sequential detection mode, laser power was varied between 1.0% for the bright signal of green GFP and 30% for the weaker red signal of the phalloidin-rhodamine. The difference of these signals would have made compensation for "bleed through" difficult if the simultaneous detection mode were used. This image is a merged z-series of nine images at 0.5-μm steps. Bars: 20 μm.

5. Incubate cells in blocking buffer consisting of PIPES buffer with 2% bovine serum albumin (Sigma) and 2% normal donkey serum (Sigma) for 1 hr at room temperature with agitation.
6. Incubate with rabbit antiactin antibody (Sigma) 1: 100 in the just-described blocking buffer overnight at room temperature with agitation.
7. Wash in blocking buffer, three changes of 15 min each.

Perform the following steps in reduced light to prevent fading.

8. Incubate with goat antirabbit rhodamine-conjugated antibody (Sigma) 1: 50 in blocking buffer for 6 hr to overnight at room temperature with agitation.
9. Wash with PIPES buffer, three times, for 1.5 min each at room temperature, dry on Super-Frost slides, rinse with distilled water, mount with VectaShield, and store in the dark at 4° prior to examination (Fig. 5).

Note: More drastic methods of permeabilization must be employed for antibody staining than for tagged phalloidin because of the higher molecular weight of antibodies. If allowed to proceed too long, Novozym digestion can completely permeabilize the cell wall and cause loss of both the GFP and the cytoplasmic antigen. Digestion is empirical, subject to considerable variability, and must be monitored carefully by microscopy.

Conventional Epifluorescence Microscopy and Digital Deconvolution

The recent increase of processor speeds of personal desktop computers and the decrease in cost of both hard drive storage devices and random access memory, coupled with computer control of image acquisition, allow an alternative to confocal microscopy.

A standard epifluorescence microscope equipped with a computer-controlled *z*-stepper motor and filter wheels to control excitation and emission wavelengths is used with a cooled charge-coupled device (CCD) video camera. This equipment provides a z-series of images of low light fluorescence that is stored on a hard drive. The z-series is then processed by deconvolution software to "reassign" out-of-focus light in the images.[19,20] This technique eliminates the need for point scanning and the pinhole aperture needed in the confocal system to exclude the out-of-focus light. Thus dimmer images can be collected without as much exposure to the

[19] G. Sluder and D. E. Wolf, eds., "Methods in Cell Biology," Vol. 56. Academic Press, New York, 1998.

[20] R. Rizzuto, W. Carrington, and R. A. Tuft, *Trends Cell Biol.* **8,** 288 (1998).

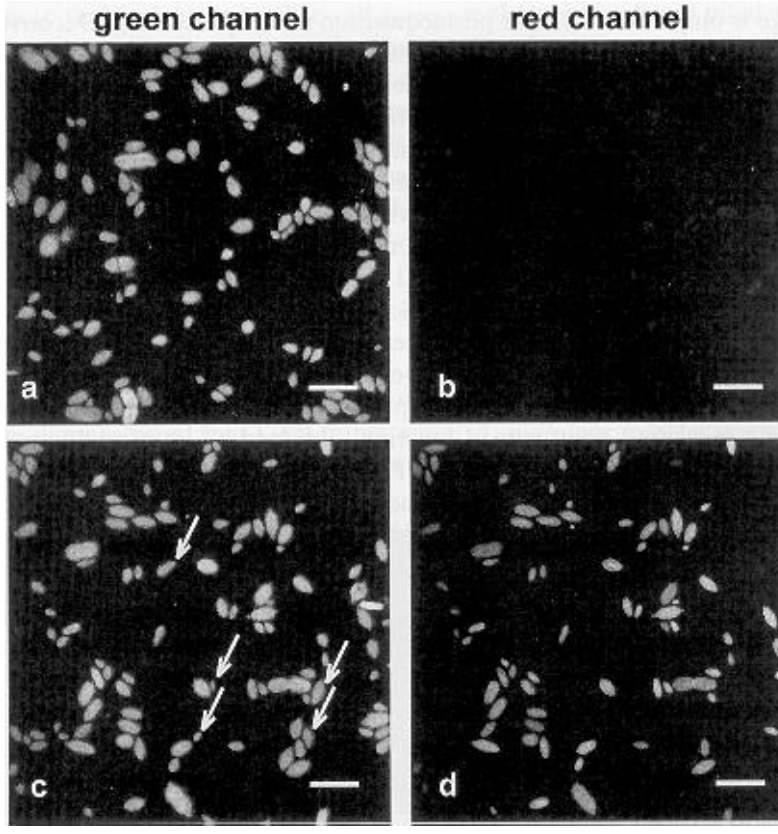

FIG. 5. Immunological detection of actin in GFP-expressing cells by rhodamine-conjugated antibodies. (a and b) Cells were not digested with cell wall-permeabilizing enzymes; note the lack of staining of actin in the rhodamine (red) channel caused by the inability of the antibodies to penetrate undigested cells walls. (c and d) Cells digested for 8 min with Novozym 234. The actin stains with the rhodamine conjugate, demonstrating that antibodies are able to penetrate permeabilized cell walls. Arrows show examples of cells staining in the green (GFP) but not in the red (rhodamine) channel, indicating that not all cells have permeabilized sufficiently to allow uptake of the labeled antibody. Merged *z*-series of 13 images at 0.5-μm steps. Bars: 20 μm.

excitation wavelengths, allowing more efficient imaging and less photobleaching compared with LSCM. There is no limit to the excitation of the fluorochromes due to the lack of appropriate laser lines, so fluorochromes exciting from the UV to infrared can be obtained from a single light source. With proper attention to filter selection, several GFP mutants can be monitored either simultaneously or sequentially, allowing multiparameter information to be obtained. A disadvantage is that the reconstructed "in focus"

image is only obtained after postacquisition image processing. Deconvolution algorithms may be based on either measured point-spread function for the specific optical elements of the microscope or estimated from values for lens numerical aperture, mounting media refractive index, and the illumination wavelength used to acquire the image prior to deconvolution.

Our system is an Olympus BX-60 (Olympus America, Lake Success, NY) epifluorescence microscope with a mercury HBO-100 arc lamp, equipped with a cooled CCD camera DEI-470 (Optronics Engineering, Goleta, CA). A Ludl focus motor (Ludl Electronic Products, Ltd., Hawthorne, NY) controlled by Volume Scan (VayTech, Fairfield, IA) software is used to obtain the z-series image of the specimen. Deconvolution is performed by Microtome Digital Deconvolution Software (VayTech) by nearest neighbor estimation or by processing of groups of three images using a measured point-spread function, obtained by imaging fluorescent PS-Speck microspheres (Molecular Probes, Eugene, OR). In our system, filter changes are done manually, although filter wheel control of the excitation and emission wavelengths could be accomplished by software from either the filter wheel supplier or the image analysis software. Software programs can be written to integrate filter wheel excitation/emission wavelength changes, image averaging to eliminate temporal noise, z-step acquisition, and time-lapse acquisition of data.

Quantitative Image Analysis of Fungal Cells on Leaves

As discussed earlier for LSCM, we describe here the approach that has worked well in our hands. Leaves supporting naturally occurring or inoculated fungal populations should be fixed in 3% formaldehyde in PBS, pH 7.4, for 18-20 hr at 4°. If leaf disks (ca. 13 mm diameter) are used, fixation time can be reduced, if desired. The leaf material is then washed in three changes of PBS for 1-2 hr each at room temperature and mounted in VectaShield with the surface of interest uppermost. To prevent deformation of the leaf, the coverslip can be supported by placing 5-μl drops of nail polish at the corners. Slides are sealed with nail polish and stored in the dark at 4° pending examination.

We routinely process images from GFP-expressing cells by merging green channel (GFP) *z*-data into a single 8-bit gray-scale image consisting of pixel intensities ranging from 0 (black) to 255 (white). z-data can be acquired from either LSCM or deconvolution equipment, both as described earlier. Image analysis software (Optimas v6.2, Bothell, WA) is used to apply a gray-scale threshold to include all cells. Any touching cells are separated by applying progressive erosion filters to the image. When the cells are deemed separated, a nonmerging dilation filter is applied to restore

them to their original size. The cell areas are then outlined and filled using binary filters. Finally, the processed image is analyzed for the total number of cells, individual cell areas, and total area coverage of the cells. From these data the percentage area coverage of the leaf and average cell size can be calculated; statistical information relating to certain cell population parameters (mean, SD, range, etc.) can also be calculated (Minitab v.12, State College, PA) and displayed. Figure 6 illustrates data acquired by LSCM; Figure 7 is from conventional epifluorescence microscopy combined with deconvolution.

Conclusion

As a case study of the biology of a fungus in its natural habitat, we have demonstrated that GFP-transformed *A. pullulans* may be imaged *in situ* by either confocal or conventional microscopy and by the use of single or

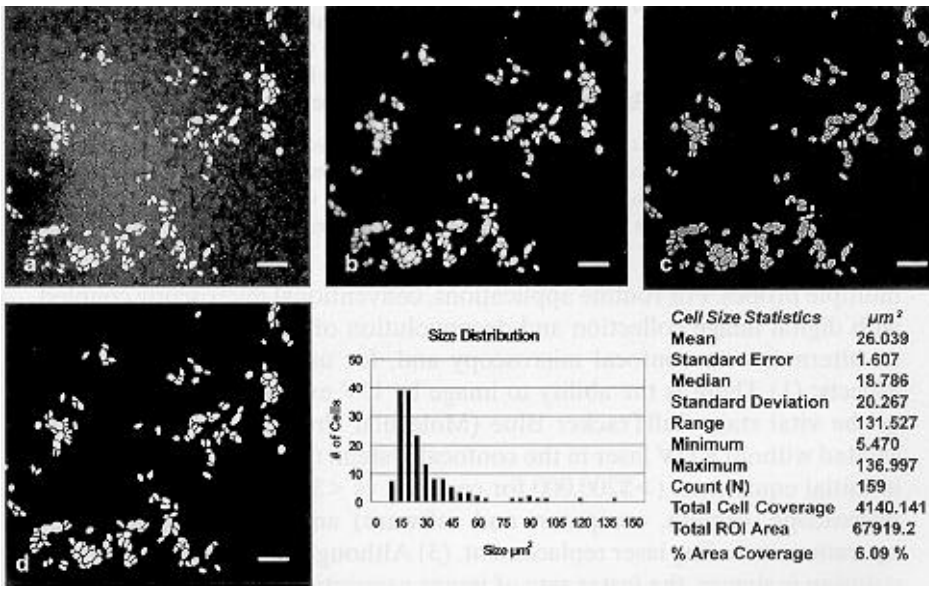

FIG. 6. Apple seedling leaf inoculated with GFP-expressing *A. pullulans* cells and incubated for 96 hr at high (>90%) humidity. (a) A combined green (GFP-expressing cells) and red (chlorophyll of leaf) image of 28 sections merged at 1.0-μm steps with the outline of leaf epidermal cells in the background. (b) The merged image of the green signal alone representing the fungal cell component of the image (a). Image (c) is the image of (b) following erosion, dilation, and filling of the outlined cells. Image (d) is the binary image of (c) subjected to image analysis. Quantitative and statistical output derived from the image analysis software and Minitab v12 software is shown. ROI, region of interest. Bars: 20 μm .

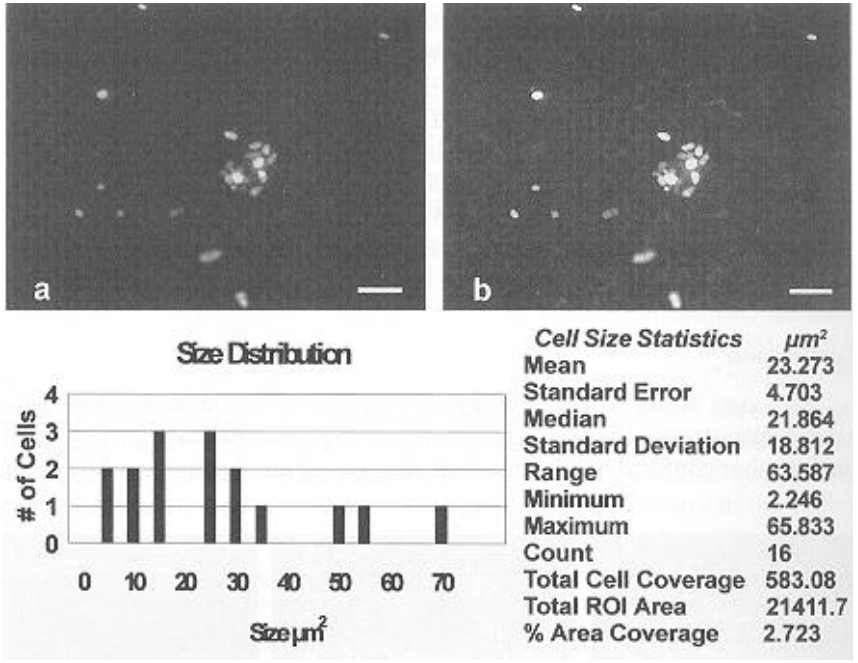

FIG. 7. GFP-transformed cells on a leaf as seen by conventional epifluorescence microscopy with a 40×, NA 0.75 neofluor objective and a *z*series of five images with 1.0-μm spacing. (a) A merged series without haze removal; (b) the same series deconvolved with Microtome software. Image analysis was performed on (b), using the technique as in Fig. 6. Bars: 20 μm.

multiple probes. For routine applications, conventional microscopy coupled with digital image collection and deconvolution of the image stacks offers an alternative to confocal microscopy and, for us, is superior in several aspects: (1) There is the ability to image by UV excitation of such probes as the vital stain CellTracker Blue (Molecular Probes), which cannot be excited without a UV laser in the confocal system. (2) Costs are lower, both in initial equipment (>$200,000 for confocal vs <$60,000 for conventional microscope, camera, computer, and software) and ongoing expenses of operation, including laser replacement. (3) Although postacquisition deconvolution is slower, the faster rate of image acquisition by wide-field microscopy reduces photobleaching of the specimen and allows imaging deeper into specimens with less light loss.

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