Quantitative imaging and statistical analysis of fluorescence in situ hybridization (FISH) of *Aureobasidium pullulans*

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Abstract

Image and multifactorial statistical analyses were used to evaluate the intensity of fluorescence signal from cells of three strains of *A. pullulans* and one strain of *Rhodosporidium toruloides*, as an outgroup, hybridized with either a universal or an *A. pullulans* 18S rRNA oligonucleotide probe in direct or indirect FISH reactions. In general, type of fixation (paraformaldehyde or methanol-acetic acid) had no apparent effect on cell integrity and minimal impact on fluorescence. Permeabilization by enzyme treatment for various times, though needed to admit high 

1. Introduction

Within the past decade, highly specific molecular methods of detection (Olsen et al., 1986; Holben and Tiedje, 1988; Atlas et al., 1992) have been combined with sensitive nucleic acid labels (Stahl and Amann, 1991; Leitch et al., 1994) to produce a powerful approach to visualizing target sequences, chromosomes, nuclei, cells, or tissues of interest (Olsen et al., 1986; DeLong et al., 1989; Burggraf et al., 1994; Leitch et al., 1994; Amann, 1995; Amann et al., 1995). When the label is a fluorochrome, or an intermediate compound ultimately detected by fluorescence microscopy, the technique is known as fluorescence in situ hybridization (FISH; Stahl and Amann, 1991; Leitch et al., 1994; Amann, 1995). In brief, the direct form of FISH involves the following steps (Stahl and Amann, 1991; Leitch et al., 1994; Amann, 1995): (i) fixation of the sample, followed by permeabilization of cell walls and membranes by enzymes or detergents where necessary to facilitate entry of the probe or detection reagents (the latter pertains to indirect FISH, below); (ii) labelling of probe; (iii) prehybridization and hybridization procedures to promote duplex formation between the complementary nucleic acid strands of labelled probe and unlabelled target; (iv) washing, mounting, and
observation. Indirect FISH involves introducing an invisible label (e.g. digoxigenin or biotin) which is then visualized by a reporter molecule (e.g. fluorescent antibody conjugated to anti-digoxigenin, or avidin-FITC, respectively) after hybridization (Zarda et al., 1991; Li et al., 1993; Leitch et al., 1994). Though more complicated and subject to artifacts, one advantage of indirect FISH is the potential for signal amplification by multiple rounds of reacting antigen with labelled antibody (Zarda et al., 1991; Leitch et al., 1994).

FISH originated in biomedicine (Tkachuk et al., 1991; Trask, 1991) but has found increasingly wide application in environmental bacteriology (Giovannoni et al., 1988; Stahl and Amann, 1991; Amann, 1995; Amann et al., 1995; Fischer et al., 1995; Langendijk et al., 1995). Its utility in mycology has been demonstrated (Bertin et al., 1990; Uzawa and Yanagida, 1992; Li et al., 1993, 1997) but remains largely unexploited. As part of research to develop FISH for quantification of the yeast-like fungus *Aureobasidium pullulans* (de Bary) Arnaud directly on leaf surfaces (Li et al., 1996, 1997), we examined certain key variables related to fungal applications of FISH. These included the influence on fluorescence signal strength of: (i) the type of fixative; (ii) cell wall permeabilization; and (iii) direct vs. indirect labelling. We present evidence here for yeast-like blastospores of four strains of fungi that fixation in methanol-acetic acid vs. paraformaldehyde did not have a major influence on cell integrity or signal strength whereas exposure for various time periods to the cellulolytic enzyme Novozym™ did. The direct FISH procedure was easier and faster to conduct than the indirect, gave a higher fluorescence signal, was amenable to amplification by multilabelling, and was not associated with artifacts.

2. Materials and methods

2.1. Fungal strains, storage, and preparatory procedures

Three strains of *A. pullulans*, ATCC 30393, ATCC 28998, and *Rhodosporidium toruloides* Banno Y1091 (anamorph, *Rhodotorula glutinis*) as an outgroup control, were used. The origin of the *A. pullulans* strains is described elsewhere (Li et al., 1996, 1997); *R. toruloides* Y1091 was obtained from the USDA Northern Regional Research Laboratory, Peoria, IL. Cells were stored in 15% glycerol at –80°C. For experiments, working cultures were established on potato dextrose agar (Difco, Detroit, MI) and transferred to Brown’s balanced liquid medium (Brown et al., 1973) for 20–40 h at 25–27°C with agitation (100 cycles per min) prior to fixation.

Cells were washed twice in phosphate-buffered saline (PBS) (0.05 M NaPO₄, 0.15 M NaCl (pH 7.2)), and fixed either overnight at room temperature in freshly prepared depolymerized 3% paraformaldehyde in PBS or for 4 h at room temperature in freshly made methanol-acetic acid (MAA 3:1 v/v). Following fixation, the paraformaldehyde-fixed material was washed three times in 0.1% diethyl pyrocarbonate (DEPC)-treated, autoclaved distilled water (DADW) (Leitch et al., 1994), dehydrated in 25, 50 and 70% ethanol, and stored in 70% ethanol at –20°C until prepared for hybridization. All aqueous solutions were made with DADW. The methanol-acetic acid-fixed cells were washed three times in 70% ethanol and stored as above. For observation, cells were washed three times in DADW for 5 min each, placed in 10 μl drops on gelatin-subbed Superfrost-Plus™ slides (Fisher Scientific, Pittsburgh, PA) and air dried.

2.2. Cell wall permeabilization

Preliminary experiments were conducted to determine the effect of cell wall digestion on uptake of compounds lower or higher in molecular weight than the FISH reagents. To accomplish this, the cytoplasmic protein actin was selected as a target for localization by phalloidin-FITC ($M_w \sim 1.2$ kDa) or FITC-labelled anti-actin antibody ($M_w \sim 156$ kDa). *A. pullulans* cells were grown, fixed in paraformaldehyde, stored, washed, and mounted on slides as described above. Slides were exposed to Novozym™ 234 (cell wall lysing enzyme prepared from *Trichoderma harzianum*; Calbiochem, La Jolla, CA; 1 mg/ml; Alfa et al., 1993) in PBS for 0, 1, 2, 4, 6, 10, 30, and 45 min. To arrest digestion, slides were washed at 0–4°C in DADW (three changes of 2 min each) and then air-dried. The 0-time, undigested controls
were subjected to the same regimen but with PBS in place of the enzyme solution.

For the phalloidin treatments, slides were exposed in darkness to phalloidin-FITC (Sigma, St. Louis, MO; 10 μg/100 ml) in 10 mM PIPES buffer, pH 7.0, for 1 h at room temperature (Alfa et al., 1993). Then they were washed twice in PBS, rinsed in DADW, air dried, mounted in VectaShield (Vector, Burlingame, CA), and stored in the dark at 4°C until imaged. For the antibody treatments, slides were exposed overnight in a moist chamber at room temperature to rabbit anti-actin antibody (Sigma) diluted 1:100 in 100 mM PIPES buffer, pH 7.2, containing 2% donkey serum. Then they were washed three times as above, 10 min per change, and incubated overnight in darkness in anti-rabbit donkey serum conjugated with FITC (Sigma) prepared as for the primary reagent. The slides were washed, mounted, and stored as described above. Controls consisted of slides where the primary antibody was omitted. Two slides were assessed by epifluorescence microscopy (see below) for each permutation of phalloidin or antibody treatment × digestion time and the experiment was conducted twice. Based on the results of these pilot studies, the digestion periods selected for the comparisons reported here were 4 and 8 min (with 0 min as an undigested control).

2.3. Probes, labels, and whole-cell hybridization

The following rRNA-targeted oligonucleotide probes were used: (i) Ap665 designed for A. pululans (Li et al., 1996, 1997) and labelled either with five molecules of fluorescein (4 FITC on 5’ end; 1 FITC on 3’ end; Clontech Laboratories, Palo Alto, CA) or with one fluorescein molecule (5’ end; Promega, Madison, WI); and (ii) U519, a universal oligonucleotide (Giovannoni et al., 1988) complementary to a sequence within the 16S-like rRNA in all known organisms, labelled either with multifluorescein as above or with a single biotin molecule on the 5’ end (Promega). Probes were stored dry in the dark at −20°C. The major factorial design (see Section 2.4) compared the multifluoresceinated Ap665 and U519 probes in direct FISH and the biotinylated U519 probe in indirect FISH. Separate experiments explored the effect of uni- vs. multi-labelled Ap665.

The prehybridization, hybridization, and post-hybridization conditions for the directly (fluorochrome) labelled probes have been described (Li et al., 1997). Controls for this experimental set consisted of: (i) hybridization with unconjugated probes followed by hybridization with FITC-conjugated probes; and (ii) omission of conjugated probes from the hybridization set (autofluorescence control). For the indirectly (biotin) labelled probe, prehybridization involved 50% formamide but otherwise was as described for the direct system (Li et al., 1997). Hybridization was as described (Li et al., 1997) but with a biotinylated label (Promega; 50 ng/20 μl hybridization solution) instead of a fluorochrome marker. Posthybridization was with avidin-FITC (Vector; 20 μg/ml PBS, pH 8) for 4 h in a PBS-saturated moist chamber. The slides then were washed in three changes of PBS (10 min each), followed by a rinse in DW and air drying. All slides were mounted in VectaShield. Controls for this experimental set consisted of: (i) testing for the non-specific binding of avidin-FITC (by omitting the biotin-conjugated probe from the hybridization mixture); and (ii) testing for the specificity of the biotin–avidin reaction by blocking avidin-FITC binding by applying unconjugated avidin (50 μg/ml) followed by unconjugated biotin (50 μg/ml) followed by the avidin-FITC detection step.

All experiments were conducted twice and, except where noted otherwise, the trends were consistent for both replications.

2.4. Microscopy: image analysis and statistical analysis

The instrumentation and general operating conditions for microscopy and image analysis are described in Li et al. (1997). Images were collected under constant conditions of illumination, brightness, contrast and gamma, and integration time as controlled by the cooled charge-coupled device (CCD) video camera (DEI-470; Optronics Engineering, Goleta, CA) and Targa + 64 frame grabber (Truevision, Indianapolis, IN). The Optimas 5.2 software (Bothell, WA) was calibrated for intensity measurements with ImaSpeck™ fluorescent beads (Molecular Probes, Eugene, OR). Mean fluorescence intensity readings were obtained as electronic images on a per
cell basis by averaging intensity of pixels per cell (total pixels intensity/number of pixels per cell) and then by field. Twelve to 16 400 × microscope fields (50 to ~415 cells per field and at least 1000 cells in total, depending on strain and experiment) were then averaged to obtain mean cell fluorescence values which were plotted as a function of strain, fixation method, digestion time, and probe.

Analysis of variance (ANOVA), based on the general linear model (GLM) from MiniTab 11.0 (Minitab, Inc., 1996), was conducted on log_{10} transformed data of mean cell fluorescence values. The need for this transformation was determined by preliminary inspection of plots of variance vs. mean signal strength. Factors considered were experiment (2), strain (4), fixative (2), and digestion time (3). The model was a split plot with time nested within combinations of experiment, isolate, and fixative. The whole-plot error term was the aggregate of all interactions including experiment but excluding time; the sub-plot error was the aggregate of all interactions including both experiment and time. In the analysis of U519 indirect, data were used only for the 4 and 8 min treatments because all of the 0 min data resulted in 0 calibrated intensity.

3. Results

3.1. Permeabilization

The effect of cellulolysis on cytochemical localization of actin is shown in Fig. 1. Staining of actin (which occurred in localized rather than generalized fashion) by fluorescent antibodies was negligible without cell wall digestion, increased with digestion time to 4–6 min, and decreased thereafter. In contrast, lysis up to 2–4 min had only a minor effect on detection of actin by FITC-phalloidin (Fig. 1) and signal strength decreased precipitously with longer digestion. The localized staining pattern was similar to that for the fluorescent antibodies (not shown). The same trends were apparent when the experiment was repeated.

3.2. Epifluorescence signal from probed cells

Fig. 2 and Table 1 summarize the overall influence of digestion time and fixation method on epifluorescence signal intensity from the four yeast strains probed by Ap665-direct (Fig. 2A), U519-direct (Fig. 2B), and U519-indirect (Fig. 2C). The P-values in Table 1 reflect evidence from the data against a null hypothesis of no factor effect (smaller P-values indicative of stronger evidence). An example of the form of the data and the ANOVA tables from which these summaries were compiled is shown in Fig. 3 and Table 2.

The most striking and consistent result is that while digestion decreased signal in the case of the direct probes (Fig. 2A and B; Fig. 3) it increased intensity for the indirect probe (Fig. 2C), presumably by facilitating entry of the avidin-FITC detection system (cf. Fig. 1; M_w of the avidin-FITC complex is ~68.7 kDa while that of the multilabelled fluorescein probes is ~10.7 kDa). The fixation scheme did not influence results with Ap665 but MAA appeared preferable to paraformaldehyde for U519. There was a significant interaction between strain and fixation for U519 indirect (Table 1). U519-probed cells from all four stains fluoresced brightly, whereas appreciable signal from Ap665-probed cells was restricted to (undigested) A. pullulans; the control, R.
Fig. 2. Summary of multifactorial FISH experiments involving 3 probes (panels A, B, C) × 4 fungal strains (ATCC 30393; ATCC 28998; CBS 105.22; Y-1091) × 2 fixatives (paraformaldehyde or methanol-acetic acid) × 3 cell wall digestion regimens (0, 4, 8 min) × 2 experiments (open vs. closed symbols). Each data point is the mean per cell fluorescence quantified electronically from images of 50 to 285 cells per field.

denise (Y-1091; Fig. 2A), did not fluoresce above background levels. The effect of experiment was statistically significant for Ap665 (Table 1); this is most evident in distribution of the fluorescence data from experiment replication 1 vs. replication 2 for the three A. pullulans strains fixed in paraformaldehyde (Fig. 2A).

Considering the optimum treatment permutation for each probe, by electronic quantification the
image analysis enabled us to compare and analyze statistically the relative magnitude of several experimental variables on signal strength in FISH. With rare exception (Bertin et al., 1990), these have not been addressed previously for fungi; some of the factors have been explored in bacterial studies but, even for bacteria, there are few quantitative assessments (Poulsen et al., 1993; Langendijk et al., 1995) to date, and we know of no multifactorial analyses of variance in either discipline.

Our major result is that while cell wall digestion was necessary to detect a fluorescence signal in the indirect biotin-avidin FITC system, it degraded cells and actually was associated with reduced fluorescence with the directly labelled probes. Fixation serves in part to permeabilize cells (DeLong et al., 1989; Bertin et al., 1990; Leitch et al., 1994; Amann, 1995) but additional treatment with detergents or lytic enzymes may be necessary (Alfa et al., 1993; Li et al., 1993; Leitch et al., 1994; Trebesius et al., 1994; Amann, 1995; Amann et al., 1995). Our results with the \( M_w \) markers for actin detection are consistent with those of Bertin et al. (1990) and suggest that the direct, fluorochrome labelled probes are small enough to penetrate cells without further permeabilization, whereas digestion is needed to admit the larger molecules (avidin-FITC) of the indirect detection system (Alfa et al., 1993). Without

Table 1
Significance (P-values) for experimental factors influencing signal strength of probes in FISH

<table>
<thead>
<tr>
<th>Factor</th>
<th>Probe and target</th>
<th>U519 direct all strains</th>
<th>U519 indirect all strains</th>
<th>Ap665 direct all strains</th>
<th>Ap665 direct A. pullulans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion time</td>
<td></td>
<td>&lt; 0.0001 (2,16)</td>
<td>&lt; 0.0001 (1,8)</td>
<td>&lt; 0.0001 (2,16)</td>
<td>0.002 (2,12)</td>
</tr>
<tr>
<td>Fixation</td>
<td></td>
<td>0.086 (1,7)</td>
<td>0.008 (1,7)</td>
<td>n.s.* (1,7)</td>
<td>n.s. (1,5)</td>
</tr>
<tr>
<td>Strain</td>
<td></td>
<td>n.s. (3,7)</td>
<td>0.003 (3,7)</td>
<td>0.031 (3,7)</td>
<td>n.s. (2,5)</td>
</tr>
<tr>
<td>Experiment</td>
<td></td>
<td>n.s. (1,7)</td>
<td>n.s. (1,7)</td>
<td>0.052 (1,7)</td>
<td>0.004 (1,5)</td>
</tr>
<tr>
<td>Strain ( \times ) fixation</td>
<td></td>
<td>n.s. (3,7)</td>
<td>0.008 (3,7)</td>
<td>n.s. (3,7)</td>
<td>n.s. (2,5)</td>
</tr>
<tr>
<td>Strain ( \times ) time</td>
<td></td>
<td>0.072 (6,16)</td>
<td>0.0002 (3,8)</td>
<td>0.068 (6,16)</td>
<td>n.s. (4,12)</td>
</tr>
<tr>
<td>Fixation ( \times ) time</td>
<td></td>
<td>n.s. (2,16)</td>
<td>n.s. (1,8)</td>
<td>n.s. (2,16)</td>
<td>n.s. (2,12)</td>
</tr>
<tr>
<td>Fixation ( \times ) time ( \times ) strain</td>
<td></td>
<td>n.s. (6,16)</td>
<td>&lt; 0.0001 (3,8)</td>
<td>n.s. (6,16)</td>
<td>n.s. (4,12)</td>
</tr>
</tbody>
</table>

*Degrees of freedom for numerator, denominator follow the P-values in parentheses.

n.s., not significant (\( P \geq 0.10 \)).
digestion, our indirect avidin-FITC probe gave artifacts similar to the digoxigenin-anti-DIG Fab system of Trebesius et al. (1994) where the fluorescent antibodies bound to target at the bacterial cell periphery resulting in a halo effect. Digestion largely overcame this problem for us but was accompanied by loss in signal strength and generalized cell degradation with digestion time. This was likely caused by proteases and nucleases accompanying the cellulases in the relatively crude enzyme preparations. We have achieved some success in mitigating these effects by treating Novozym™ with bentonite (data not presented; cf. Ruiters and Wessels, 1989; Alfa et al., 1993). Alternatively, shorter digestion periods, if experimentally feasible, or more dilute enzyme preparations might be beneficial. Thus, as noted by Amann et al. (1995) for bacteria, digestion is a delicate compromise between rendering the rRNA target accessible within cells while minimizing loss of cell integrity and possible loss of target.

We conclude that, at least for fungal cells similar to A. pullulans or R. toruloides blastospores, digestion is not necessary and is in fact disadvantageous for FISH with directly labelled probes.

Fixation, needed to stabilize cells for the hybridization process (Stahl and Amann, 1991), is a compromise between promoting permeability and stabilizing structure (Moench et al., 1985; Leitch et al., 1994; Amann, 1995). There are two broad categories of fixatives, cross-linking (e.g. formaldehyde) and precipitating (e.g. methanol), and we used a representative of each. In general, the former group renders good morphology and stability but reduces tissue permeability; the latter may enhance permeability but tissue and target integrity can be poor (Leitch et al., 1994; Amann, 1995). Differences between the two fixation regimens were seen only for the universal probe system and ranged from marginal ($P = 0.05$ for U519 direct) to a highly significant ($P = 0.001$ for U519 indirect) enhancement of signal by methanol. As noted, there was also a significant interaction between fixative and strain. These results may simply be an artifact of smaller observed error for the indirect system, or they could reflect the permeabilizing effect of MAA in addition to Novozym™, more so in some strains than other strains (some strains produced more extracellular polysaccharide than others).

That experiment as a variable appeared statistically significant for Ap665 implies that, notwithstanding the overwhelming impact of digestion on intensity, performance of this probe was somewhat variable across experiments, i.e. through time. This may be attributable to experimental noise. Alternatively, maybe Ap665 was more sensitive than the other probes to variation in digestion rates between the two experiments.

Although fluorescence intensity varied by strain, U519 reacted with all strains, as expected, and Ap665 reacted with all A. pullulans strains, but not with R. toruloides, the negative control. Overall, the universal probe resulted in a stronger fluorescence signal than the A. pullulans probe though in visual as opposed to electronic comparisons the differences were often not noticeable. The discrepancy may have been due to disparities in the intensity of labelling due to synthesis or purification protocols affecting degree of substitution (i.e. ratio of fluorescein molecules to oligonucleotide molecules; Trebesius et al., 1994; Promega Corp., 1996). Alternatively, the target site sequences hybridizing with Ap665 may have been less accessible than those for U519 (cf. 1). The direct procedure appeared preferable to indirect

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**Fig. 4.** Electronic signal intensity of A. pullulans ATCC 30393 cells fixed in paraformaldehyde and probed by Ap665 labelled with either five FITC molecules (left) or one FITC molecule (right). Data are means + S.D. per cell fluorescence for populations of 150 to ~415 cells per field.
detection (Fig. 2). Apparently either multilabelled avidin does not perform as efficiently as a multilabelled oligonucleotide, perhaps due to quenching, or some of the target may be lost during the digestion step. The indirect 'sandwich' technique, involving two or more rounds of amplification (Leitch et al., 1994), has performed well in some situations (Zarda et al., 1991; Lim et al., 1996) but contributed primarily noise in our system in part due to endogenous biotin or biotin-like compounds (data not shown). In the direct method, signal was enhanced fourfold by using five fluorescein tags and in theory it could be further strengthened by targeting several multi- or mono-labelled probes to different regions within the rRNA molecule, among other possibilities (Amann, 1995; Amann et al., 1995). Limits on
multilabels would be set by synthesis capabilities or
self-quenching of adjacent fluorescein molecules,
and on multiple probes by availability of specific
target sites.

In conclusion, we note that our results have
particular relevance to FISH applied to microbes in
their natural environments and on their natural
substrata rather than on glass slides. Such surfaces
are typically difficult to manipulate and may be
avtelfluorescent. In these situations it is important to
maximize the signal:noise ratio while minimizing
autofluorescent. In these situations it is important to
maximize the signal:noise ratio while minimizing
specimen manipulation to avoid artifacts or loss of
target cells. A case in point is FISH performed on
leaf (Li et al., 1997) or root (Assmus et al., 1995)
surfaces to quantify fungal or bacterial populations.
If digestion were used on the walls of epiphytic
microbes it could displace the microbial cells or
expose host tissues that might non-specifically bind
the probe. Direct FISH avoids these possible compli-
cations.

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