

Standards for Bacterial Identification by Fluorescence In Situ Hybridization Within Eukaryotic Tissue Using Ribosomal rRNA-Based Probes

To the Editor:

The article "Molecular Characterization of Rectal Mucosa-associated Bacterial Flora in Inflammatory Bowel Disease" by Maria Mylonaki, Neil B. Rayment, David S. Rampton, Barry N. Hudspeth, and Jonathan Brostoff published in the journal (2005;11[5]:481–487) is not scientifically sound.

The authors performed fluorescent in situ hybridization (FISH) using a set of 16s RNA-based Cy5 probes and found high concentrations of *Bifidobacteria* (mean 15 bacteria/1-mm epithelial surface) in normal control subjects and increased intracellular *Escherichia coli* and *Clostridia* in inflammatory bowel disease patients. The authors assure us that accurate morphometric measurements were made of each biopsy, that "the total mucosal area was calculated." Individual bacterial numbers were expressed as numbers of bacteria per millimeter of epithelial surface. Actually, the Cy5 fluorochrome that the authors used is dark red and cannot be perceived with the human eye. Because the only possible way to monitor the fluorescence is with a camera, the microscopy with Axioplan 2 must be performed "blindly," and morphology can be evaluated only after pictures are taken. However, Cy5 fades quickly, which makes simultaneous searching for optimal location within the biopsy and taking usable pictures difficult. The weak signal of Cy5 makes it impossible to perform orienting shots at low ($< \times 400$) magnifications to obtain an overview of the biopsy. An inevitable random focusing

over the biopsy surface does not allow any morphometric measurements. The following evaluation of microphotographs depends on photoprocessing software. For each setting of photographs, the investigator must choose individually the brightness, contrast, and so forth at which he or she can best evaluate the findings. This adjustment necessarily manipulates the results. The counting of edited signals by 2 observers does not change anything.

In fact, none of the figures presented by the authors shows signals that should be identified as bacteria. Figure 1A shows ≈ 100 irregular fluorescent clouds that do not have a bacterial morphology and are not DAPI counterstained. The range the authors give for *Bifidobacteria* is 4 to 56 cells/1-mm biopsy surface. That means that maximally 25 *Bifidobacteria* can be seen within a single microscopic field at a magnification of $\times 400$. It should be easy for the authors to use arrows to point out what they regard as bacteria in Figure 1A.

The negative control, Figure 1B, shows nothing and therefore cannot be

interpreted at all. Normally, each FISH probe, especially Cy5-labeled probes, produces a marked background staining by binding nonspecifically to human tissues. This unspecific background fluorescence makes the human tissue morphology clearly perceivable at fluorescence microscopy and eases the orientation. The phenomenon is general, and the unspecific background fluorescence contrasting human tissues is obvious in Figures 1C through I. In Figures 1A and B, the background fluorescence of the human tissue is absent. The authors do not explain why. Because epithelial morphology cannot be perceived, it is impossible to say from what area Figures 1A and B were taken and how the microphotographs were made. Clearly, they were not made from the biopsy tissue or its surrounding area. All of the other panels claiming to present intracellular bacteria (C, E, G, H, I) demonstrate typical biases of unspecific Cy5 binding to inflammatory cells.

Unlike hybridization signals specific for bacteria, these signals cannot be washed off at any temperature, even at

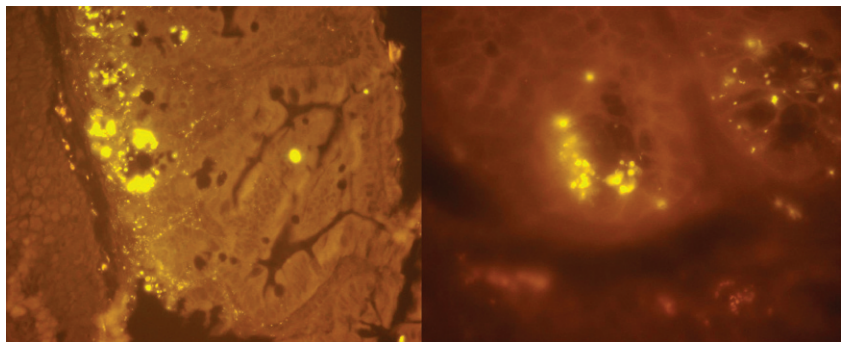


FIGURE 1. Unspecific binding of the Eub338 Cy3 probe to the eukaryotic inflammatory cells at original magnification $\times 400$ (left) and $\times 1000$ (right) showing morphologically extreme diverse structures, some of which resemble bacteria. The photographs were made after the sections were washed at 90°C for 10 min. Despite rigorous washing far above the melting point, the signals preserved their high fluorescence. The demonstrated signals could not be counterstained with DAPI; they hybridized positively with Ec1531 (*E coli*) and Gam42a (*Gamma proteobacteria*) but also with Fprau (*Fusobacterium prausnitzii*), PF2 (*Fungi*), and Lab158 (*Lactobacilli*) probes. The fluorescence was much lower when Bac303 (*Bacteroides*), Erec482 (*Eubacterium rectale-Clostridium coccooides*), and nonsense probes were used, which may lead to false interpretation when pictures are evaluated separately. (For reasons of length, figures illustrating the latter statements are not shown.)

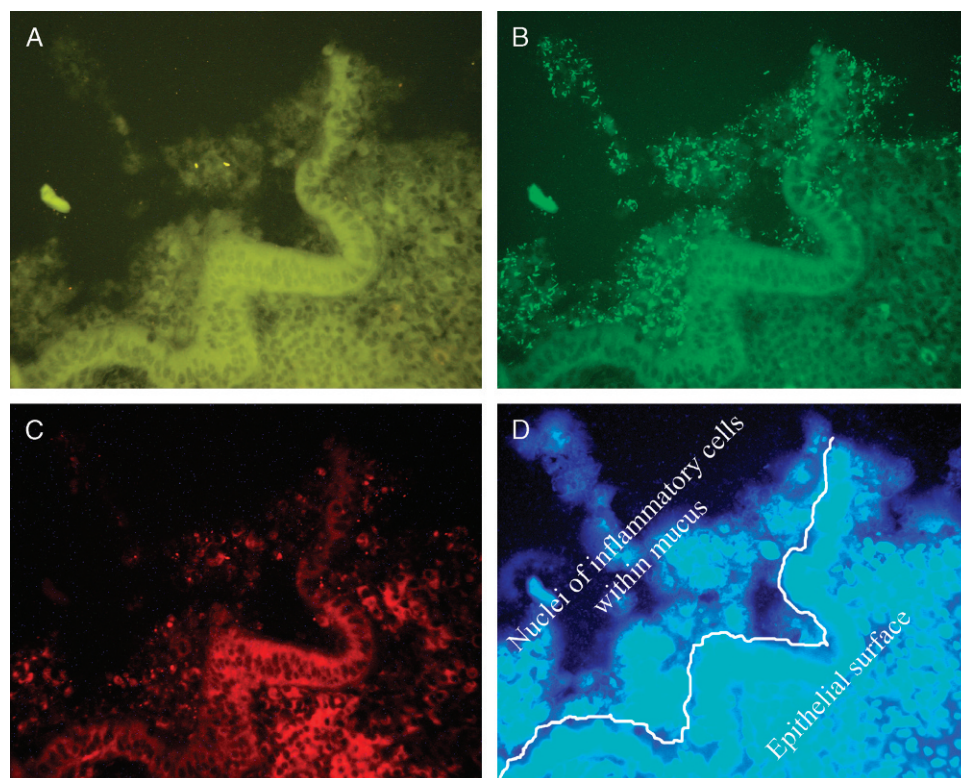


FIGURE 2. The same microscopic field (original magnification $\times 400$) as Figure 1 demonstrating hybridization with the following: A, Bif164 Cy3 probe (*Bifidobacteriaceae*, orange); B, Eub338 FITC probe (all bacteria, green); C, HGC Cy5 (*Actinobacteria*, red); and D, DAPI counterstain (unspecific DNA stain, blue fluorescence). The photographs were not manipulated and the real color appears here. Note the clearly perceivable morphology of both Bif164 Cy3-positive bacteria that can be definitively located within mucus of the epithelial layer (seen in background fluorescence). Bacteria hybridizing with the Bif164 probe also positively hybridize with Eub338 and HGC probes. In DAPI stain, multiple nuclei of inflammatory cells can be seen within mucus attached to the epithelial layer. For intensive fluorescence of human inflammatory cells attached to the mucosa and low concentration of Bif164-positive cells, it is impossible to assign both Bif164 signals to DAPI signals with bacterial morphology in these series; however, because the Bif164 signals have a typical rod morphology and simultaneously hybridize with Eub338 and HGC probes, the probability is high that the signals indeed represent *Bifidobacteriaceae*. Many signals in HGC Cy5 hybridization (arrows) have no counterpart in Eub338 hybridization or DAPI counterstain and are definitively of nonbacterial origin.

90°C; they have irregular morphology; they cannot be counterstained with DAPI; and they can be simultaneously hybridized with different unrelated bacterial FISH probes. These biases often are seen within different eukaryotic tissues of healthy people and patients. We observed and documented them in tonsils, vaginal epithelia, nasal epithelia, brain, testes, gallbladder, pancreas, and intestines, as shown in Figure 1. Because most investigators using FISH know and can easily recognize these signals, we never published the data.

Use of new methods such as FISH affords experience in FISH microscopy

and adherence to evaluation standards. FISH signals within complex eukaryotic tissues, which generate a vast number of unspecific fluorescence phenomena, must fulfill the following basic criteria for bacterial identification:

1. They must have a morphological form typical for bacteria.
2. They must be detectable in DAPI counterstain, at least in some regions that are not overshadowed by fluorescence of eukaryote cell nuclei.
3. They must hybridize positively with at least 1 other group- or species-specific FISH probe related to the probe of interest.

4. They should not cross-hybridize with FISH probes specific for unrelated bacterial groups and species.

The negative hybridization with a nonsense probe in a separate set of hybridization as performed by the authors gives usable results in pure bacterial communities. When applied to complex eukaryotic tissue, the nonsense probe always generates positive fluorescence signals that are difficult to distinguish from bacteria and can be correctly interpreted only when differently labeled specific bacterial probes are simultaneously used. The signals generated with unrelated probes should not be identical

when photomicrographs of the same microscopic field are overlaid.

An example of correct evaluation is presented in Figure 2, which demonstrates FISH of a rectal biopsy section from a patient with ulcerative colitis. Two morphologically definite bacterial rods of *Bifidobacteriaceae* (hybridization is performed with Bif164 Cy3) can be seen in Cy3 orange fluorescence (Fig. 2A) and can be assigned within a mucosal bacterial biofilm (Fig. 2B) to universal bacterial signals hybridizing with the Eub338 FITC probe (all bacteria, green signals) and HGC Cy5 probe (Fig. 2C; *Actinobacteria*, red signals). Note that many other Cy5 signals marked with arrows can be seen within mucus and within biopsy tissue in Figure 2C but they have no counterpart either with Bif164 or Eub338 hybridization or in the DAPI counterstain when pictures are overlaid. Therefore, they are obviously of a nonbacterial nature.

FISH of bacteria in eukaryotic tissues that is based exclusively on Cy5 images and uses no DAPI counterstain or simultaneous hybridizations with other related and unrelated FISH probes to verify the fluorescence signals is unacceptable for scientific research.

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Mylonaki et al reply:

We were interested to read Dr Swidsinski's comments on our article on mucosa-associated flora in the journal (2005;11[5]:481–487). Like Kleessen et al (2002) and, more recently, Kuehbach et al (2006), we used a Cy fluorochrome-based method for our FISH analysis. We were kindly donated Cy5-labeled probes and were able to maximize the signal-to-noise ratio using a filter block selected from the Zeiss range in a manner similar to that used by Ferri et al (2000). Aware that viewing

the samples with fluorescence microscopy is impractical because the signal from Cy5 under these conditions fades rapidly, we used phase contrast microscopy (for surface-associated images) and DIC (differential interference contrast; for lamina propria images) combined with Axiovision/KS300 software to take and record both tissue coordinates and epithelial/lamina propria measurements without exposing the sample to Cy5 excitation wavelengths. Once saved to the computer, the fluorescent image was overlaid, using multichannel zvi (Zeiss vision imaging), onto the phase or DIC image, and a precise assessment made as to the location of the signal. There is nothing random or blind about these measurements as coordinates are recorded and used in the multichannel evaluation of the sample. This analysis can be made at magnifications as low as $\times 200$ if a more global assessment is required, but at magnifications of $\times 400$ and above, accurate identification of signal is obvious (Fig. 1).

Dr Swidsinski asserts that none of the figures presented in our article show bacteria. For reasons of space and expense, we were restricted in the numbers of photomicrographs that we could publish, but, as shown in Figure 1C and D, higher-magnification pictures confirm that the signals shown in Figure 1A and in Figure 1 in the May 2005 article have the morphology of bacteria. Our counts were made by 2 experienced observers, one of whom was trained as a microbiologist, and were based on morphological criteria. Counterstaining with DAPI was positive, but was not included in the article because we believed that the images would have added little to readers' interpretation. To optimize the staining protocol and minimize nonspecific background staining, we conducted a series of preliminary studies involving stringent washes at different ionic strengths and temperatures. Like Dr Swidsinski, and as stated in our article, we confirmed that our bacterial signals hybridized positively with the universal bacterial probe, EUB338. Unfortunately, however, at the

time of these studies we had no access to group- or species-specific FISH probes related to the probe of interest. Use of nonsense probes was suggested by one of the original reviewers: When using serial sections and a multichannel zvi, it is possible to subtract hybridization signals to confirm probe specificity and eliminate nonspecific signals. Finally, artifactual or false identification of our Cy5 FISH signals as bacteria would not explain the different results we observed for different organisms within the patient groups studied; examples include the increased numbers of epithelium-associated *Bifidobacteria*, *Lactobacillus* and *Bacteroides* compared with other organisms in control subjects (see Table 3 in the original article), and the increase in *Escherichia coli* compared with other bacteria in the lamina propria in patients with inflammatory bowel disease (Table 4 in the original article). Nor would artifact account for the fact that from gentamicin-treated biopsies from patients with ulcerative colitis (but not controls) we have now isolated and serotyped *E coli* (by implication intracellular; unpublished data).

Dr Swidsinski goes further in asserting that Figures 1A and B in the May 2005 article "were not made from the biopsy tissue or its surrounding." Our intention in Figure 1A in the article, as indicated in its legend, was to illustrate *Bifidobacteria* apposed to the surface of the mucosa; our intention in Figure 1B was to demonstrate, using the nonsense probe for *Bifidobacteria*, the lack of staining for bacteria. All of these pictures were originally submitted to the journal in color, but they were printed in black and white, with a subsequent substantial loss of quality. We agree that in the journal, as opposed to the original images, it is impossible to confirm the source of the images as being mucosa. The color micrographs shown here illustrate these points. Identifying bacteria with arrows was considered unnecessary in our original submission.

We agree with Dr Swidsinski that great care and experience is required