

# Benchmarks

## The fluorescent dyes TO-PRO-3 and TOTO-3 iodide allow detection of microbial cells in soil samples without interference from background fluorescence

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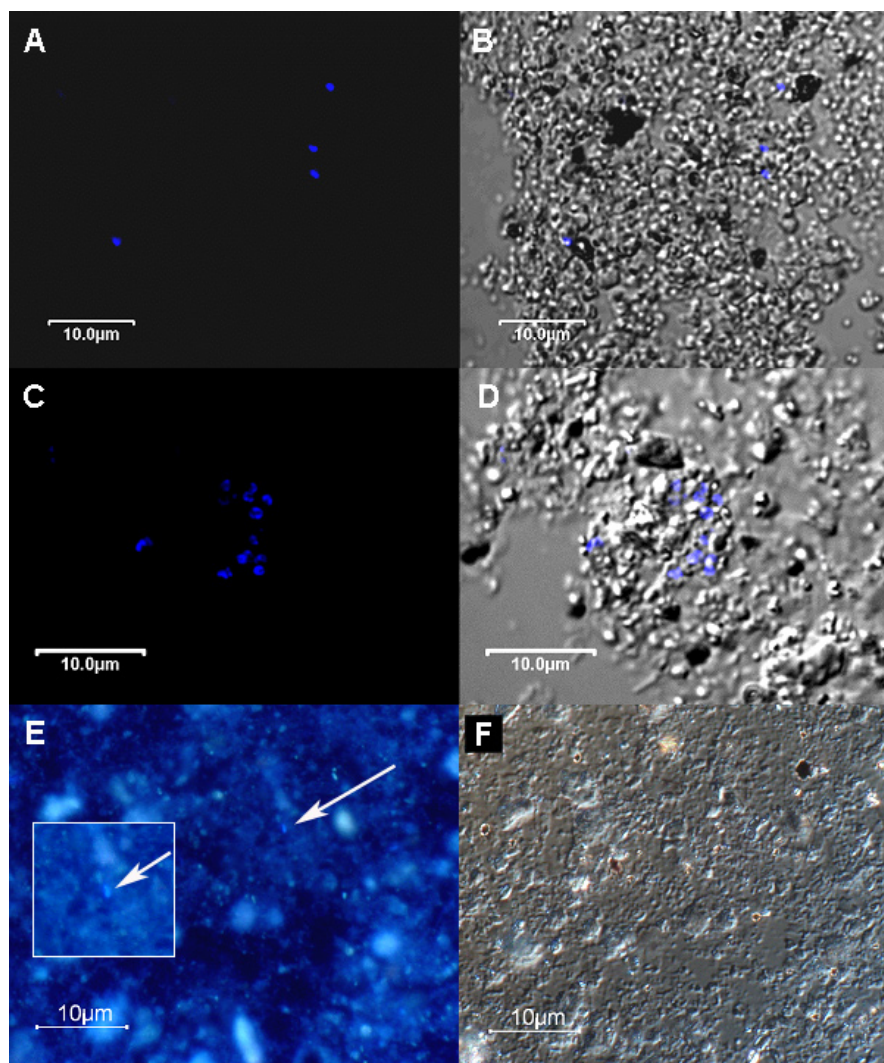
Visualization of microorganisms in soils and sediments using fluorescent dyes is a common method in microbial ecology studies, but is often hampered by strong nonspecific background fluorescence that can mask genuine cellular signals. The cyanine nucleic acid binding dyes TO-PRO-3 and TOTO-3 iodide enabled a clear detection of microbial cells in a mineral soil, while nonspecific background was greatly reduced compared with commonly used dyes. When used as counterstains for fluorescence in situ hybridization (FISH), both cyanine dyes allowed identification of microbial cells despite strong background from nonspecifically bound probes. TO-PRO-3 and TOTO-3 are easy to use and represent superior alternatives for detecting microorganisms in soil environments.

Fluorescence microscopy with nucleic acid-specific dyes, such as 4',6-diamidino-2-phenylindole (DAPI) or acridine orange, is used widely to visualize microbial cells in environmental samples independently of their physiological state or phylogenetic placement (1). However, inefficient staining, nonspecific binding to sample components, and autofluorescence of

mineral particles under UV excitation often interfere with efficient cell detection, in particular in soil and sediment samples (2). Novel high-affinity stains, such as SYBR Green, Sytox, or Syto dyes, promise better signal-to-background ratios due to their increased fluorescence when bound to DNA (3–5). Nevertheless, the general issue of nonspecific background

fluorescence remains problematic for many sample types. Excitation of nucleic acid-specific dyes with monochromatic light (e.g., in confocal laser scanning microscopy; CLSM) can reduce such background fluorescence, and CLSM is now often used for direct cell counts and fluorescence in situ hybridization (FISH) applications in environmental studies (2,6). However, many commonly used dyes require excitation in the UV range, for which confocal microscopes often are not properly equipped. In contrast, the monomeric TO-PRO-3 iodide (TP3; 642/661 nm excitation/emission peaks) and the dimeric TOTO-3 iodide (TT3; 642/660 nm) can be excited with a standard CLSM He-Ne 633 nm laser. While not assessed in the present study, they can also be used with light microscopes that are equipped for detection of red fluorescence (630–700 nm). These cyanine dyes display a high affinity for double-stranded nucleic acids in fixed cells, fluorescing strongly as DNA-dye complexes but only weakly prior to DNA intercalation. TP3 is widely applied to stain DNA in eukaryotic cells (e.g., for flow cytometry, DNA quantification, or as a FISH counterstain) (7–9). Even though reports on using TP3 to stain prokaryotic cells are limited to flow cytometry of pure cultures (10,11), the weak fluorescence of unbound and nonspecifically bound dye molecules and the lower levels of autofluorescence with high wavelength excitation make TP3 and TT3 promising alternatives for the visualization of microbial cells in natural ecosystems. Here we present our results on the use of these dyes as both general nucleic acid stains for detecting microorganisms in mineral-rich soils and as FISH counterstains.

Initially, pure microbial cultures were stained with both dyes to evaluate optimal dye concentrations and staining conditions. Samples were analyzed by CLSM, and staining efficiency was determined by visual comparison of signal intensities and numbers of stained cells (see the Supplementary materials). Fixed cells of pure cultures (*Escherichia coli*, *Sulfobacillus* sp., *Sulfolobus metallicus*, and the SM1 euryarchaeon) (12) showed clear fluorescent signals when stained for 15 min with 1–5  $\mu$ M TP3 or TT3 in 0.01 M Tris-HCl, pH 7.2. Nonspecific background fluorescence was not detected, and signal intensities increased with increasing dye concentrations up to 4–5  $\mu$ M (see Supplementary Figure S1). In general, signal intensity for TT3 appeared slightly lower compared with TP3, and addition of SDS to the staining buffer resulted in slightly



**Figure 1. Application of TP3 for the visualization of microbial cells in mineral-rich soil.** (A–D) CLSM micrographs of samples stained with TP3, small, coccoid cells visible. (A and B) Same field of view shown; (A) TP3 fluorescence; (B) fluorescence and DIC overlay. (C and D) Same field of view shown; (C) TP3 fluorescence; (D) fluorescence and DIC overlay. (E and F) Epifluorescence micrograph, same field of view shown; (E) DAPI, putative cell indicated by arrow (magnification in insert); (F) DIC and DAPI overlay. Scale bars, 10.0  $\mu\text{m}$ .

reduced fluorescence. On the other hand, TP3 showed higher sensitivity to photobleaching, which agrees with published results for eukaryotic cells (13,14).

The suitability of TT3 and TP3 for detecting cells within mineral-rich samples subsequently was tested using hydrothermally degraded soils with small particle-sizes and high clay contents (from Mt. Hood, Oregon, USA) (15). When stained with DAPI or SYBR Green I (data not shown for latter), these soils showed strong nonspecific background fluorescence of mineral particles with cell-like sizes and shapes. To evaluate the suitability of TP3 and TT3, sterile soil samples were spiked with bacterial and archaeal cells prior to staining (see the Supplementary material). After incubation for 1 h, fluorescent cells

were clearly visible within and distinguishable from mineral particles, while nonspecific background fluorescence was absent (data not shown). Stained, unspiked control samples did not display any signals or background fluorescence, indicating that signals observed in the spiked samples corresponded to cells and not mineral particles. Despite its higher sensitivity to photobleaching, TP3 showed superior signal intensities, allowing improved detection of cells compared with TT3, and was subsequently used to stain unspiked Mt. Hood samples. Again, nonspecific background fluorescence was very low, and endogenous cells were clearly visible and distinguishable from sample particles. Small, single coccoid cells (Figure 1, A and B) and cell clusters associated with

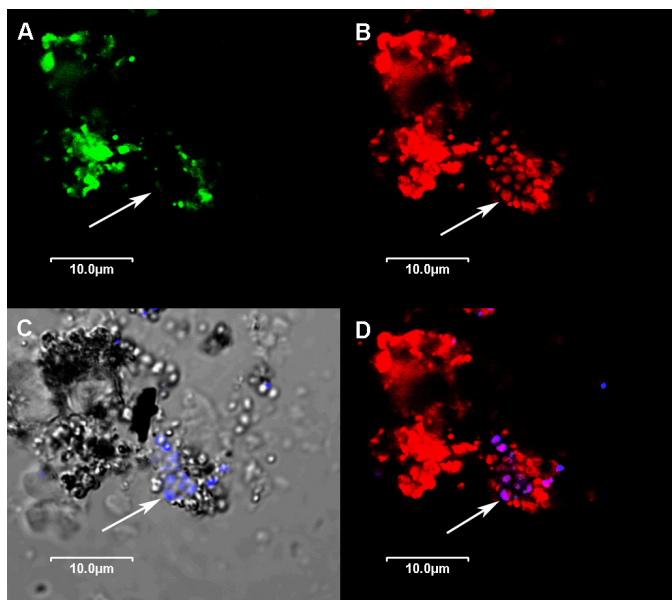
mineral structures (Figure 1, C and D) were identified, whereas staining with DAPI resulted in nonspecific background fluorescence, masking any cellular signals present (Figure 1, E and F).

Both dyes were also tested as DNA counterstains for microbial FISH. TP3 and TT3 signals were clearly visible in bacterial and archaeal cultures hybridized with domain-specific probes (data not shown). The counterstaining did not influence FISH signals, but laser excitation of the probes resulted in photobleaching and reduced signal intensities, in particular for TP3. In spiked and unspiked Mt. Hood samples, cells were identified by positive TT3 staining after hybridization and counterstaining, despite very strong background fluorescence of nonspecifically bound FISH probes and autofluorescence of minerals that masked cellular FISH signals. Furthermore, it was possible, using image overlay, to detect putative archaeal FISH signals that would have been overlooked without TT3 counterstaining (Figure 2).

To the authors' knowledge, application of TP3 and TT3 to directly visualize prokaryotic cells in environmental samples has not been reported previously. Our experiments show that TP3 and TT3 are promising for the detection of microorganisms in soils with high particle loads. The weak fluorescence of unbound or nonspecifically bound dye molecules and the use of high-wavelength monochromatic excitation light significantly reduced nonspecific background. Samples can be stained directly with a simple protocol, while aggressive pretreatment is not required (16). These properties make TP3 and TT3 superior to other commonly used dyes for visualizing microorganisms in complex samples such as soils and, presumably, sediments. The dyes also show potential to be applied in FISH applications in which the number of mineral particles nonspecifically stained by the FISH probe are not excessively high, and far-red excitation and emission will allow combination with many commonly used fluorochromes with shorter excitation maxima.

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**Figure 2. Application of TT3 as a FISH counterstain in mineral-rich soil.** CLSM micrograph labeled with EUB-Mix (Alexa488, green), ARCH-Mix (Cy3, red), and TT3; same field of view shown; arrow pointing to a group of cells. (A) FISH (EUB-Mix). (B) FISH (ARCH-Mix). (C) Overlay of DIC and TT3 fluorescence. (D) Overlay of FISH (ARCH-Mix) and TT3 fluorescence, colocalization of ARCH-Mix and TT3 visible in purple. Scale bars, 10.0 µm.

during sample collection, and Russell Field for particle size analysis.

## Competing interests

The authors declare no competing interests.

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