Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is an essential tool for the cultivation-independent identification of microbes within environmental and clinical samples. However, one of the major constraints of conventional FISH is the very limited number of different target organisms that can be detected simultaneously with standard epifluorescence or confocal laser scanning microscopy. Recently, this limitation has been overcome via an elegant approach termed combinatorial labeling and spectral imaging FISH (CLASI-FISH) (23). This technique, however, suffers compared to conventional FISH from an inherent loss in sensitivity and potential probe binding biases caused by the competition of two differentially labeled oligonucleotide probes for the same target site. Here we demonstrate that the application of multicolored, double-labeled oligonucleotide probes enables the simultaneous detection of up to six microbial target populations in a straightforward manner with higher sensitivity and less bias. Thus, this newly developed technique should be an attractive option for all researchers interested in applying conventional FISH methods for the study of microbial communities.

In medical and environmental microbiology, fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is a widely used technique for direct identification and quantification of microorganisms (24). However, several factors may prevent the successful identification of certain microorganisms with conventional FISH (1, 25). Among the most frequently encountered problems are weak or undetectable probe-conferred signals emitted from microorganisms that target molecule numbers below the FISH detection limit (9) or that are hybridized with probes with a poor in situ accessibility of the selected probe binding site (7, 29). Improved FISH techniques for ameliorating both problems have been developed. For example, if catalyzed reporter deposition (CARD)-FISH (14, 18) is applied, a 26- to 41-fold increased sensitivity (9) can be achieved, but the protocol is more complicated and needs to be specifically adapted for some microorganisms (8, 20), and multicolor imaging options of this technique are very tedious and limited (13, 19). Furthermore, several useful approaches have been developed to tackle the poor accessibility of certain probe target sites (6, 28, 30). In this context, double labeling of oligonucleotide probes (DOPE)-FISH (20) represents a recently introduced approach that maintains the elegant simplicity of conventional FISH but offers an approximately doubled signal intensity plus an increased in situ accessibility of probe target sites without affecting the specificity of the applied probes.

Another major limitation of FISH is the fact that only a few different target organisms can be simultaneously detected by applying probes labeled with different dyes. The major reasons for this are the use of band- or long-pass filters in fluorescence image acquisition and the excitation cross talk and emission bleed-through of suitable fluorophores (23). Therefore, in practice, not more than three different dyes can be conveniently used in parallel to examine the microbial population structure within a sample. If applied together with a carefully compiled hierarchical set of three different probes, this allows, in principle, the identification of up to seven microbial populations in situ in one assay (2). However, this approach imposes strong constraints on probe design and, for example, is not applicable if phylogenetically distantly related groups of target organisms should be detected in parallel.

These very limited multiplexing options of FISH were recently overcome by the introduction of combinatorial labeling and spectral imaging FISH (CLASI-FISH) (23). Here binary combinations of oligonucleotide probe-dye constructs were used for the simultaneous detection of up to 28 target organisms, resulting in unique mixed colors that were distinguished via spectral fingerprinting. However, as the CLASI-FISH approach exploits two probes for each target organism, hybridizing to the same binding site but each labeled with a different dye, these probes compete for the same binding site. Thus, for each probe, one would expect that the signal intensity is halved and hence the sensitivity of the assay is significantly reduced. Furthermore, it has been demonstrated that the very same probes possess different binding affinities to their target sites depending on the fluorescence dye used for labeling (20). Thus, the theoretically expected binding ratio of 50:50 for two probes with the same sequence but conjugated to different fluorophores might be shifted toward the more competitive probe-dye construct, complicating the identification of target organisms.

In this study, we experimentally demonstrate the above-mentioned signal reduction effects of CLASI-FISH and present an alternative and widely applicable DOPE-FISH-based approach, which offers straightforward identification of six taxa with a single FISH experiment.
RESULTS AND DISCUSSION

Double hybridization of *E. coli* with two probes binding to the same target site reduces signal intensities in a dye-dependent manner. In an initial pure-culture experiment with probe EUB338 and *E. coli* as the target organism, the influence of double hybridization of the same rRNA target site with two probes labeled with different dyes was examined. For this purpose, we hybridized *E. coli* separately with the EUB338 probe monolabeled with Cy3, Cy5, and Fluos. Subsequently, we mimicked the CLASI-FISH approach (23) and used two differentially labeled derivatives of the probe in the same hybridization experiment (Cy3-Fluos, Cy3-Cy5, and Cy5-Fluos) and compared the probe-confferred signal intensities between both experiments. If Cy3- and Cy5-labeled probes were used for double hybridization, the obtained signal intensities for each dye were about 50% reduced compared to the FISH experiment without probe competition (Fig. 1A). This finding is fully consistent with previously published data demonstrating that if two differentially labeled probes are used in a competitive manner and dye-associated effects influencing the probe binding affinity are excluded, the intensity of fluorescence conferred by these probes depends linearly on their molar fraction in the probe mixture (9). As Cy3 and Cy5 show similar properties concerning their molecular weights and chemical structure, it is not surprising that no label-derived effects on the competitiveness of the respective probes were detected. In contrast, the competition experiments with Cy3-Fluos-labeled and Cy5-Fluos-labeled probes revealed that cyanine-labeled probes preferentially bind to the target site (Fig. 1A). Thus, compared to the signal intensity of the Fluos-labeled probe EUB338 probe applied alone, the signal inten-
sity of the Fluos-labeled EUB338 probe is reduced to below 40% when applied together with the Cy5-labeled EUB338 probe derivative and is even further reduced to about 25% when used in combination with a Cy3-labeled EUB338 probe. This effect reflects the stronger binding of Cy dye-labeled probes to RNA that has been described previously (20) and can be reversed if the less-competitive probe is used in a higher concentration in the hybridization mixture (Fig. 1B).

In summary, the CLASI-FISH approach leads, in the best case, to a loss of probe-conferred signal intensities of 50% for both dyes of the binary combination, if the dyes possess similar properties. Thus, the detection limit of the CLASI-FISH technique for microbes with low cellular ribosome content is significantly reduced. However, this problem could be solved if future CLASI-FISH applications were to be based on probes labeled with two identical dyes (23). The observed shift in the probe binding ratio, as a consequence of a difference in competitiveness of certain probe-dye constructs, is more problematic. In particular, cells with low ribosome content might not display the intended color mix but instead might appear single colored and thus would not be correctly identified. It did not escape our attention that neither Cy dyes nor Fluos have been used in the original CLASI-FISH approach (23). However, the chemical structures and properties of the applied dyes in that study were highly diverse, and thus it would be necessary to test all binary dye combinations in well-controlled experiments analogous to those described above in order to exclude dye combination-conferring biases or to select suitable dye combinations.

**Multicolor DOPE-FISH.** Due to the above-described limitations of multicolor FISH based on double hybridizations with binary mixtures of differently labeled probes, we evaluated whether double labeling of the same oligonucleotide probe with two different dyes could be a suitable alternative. For this purpose, a set of experiments with probe EUB338 and *E. coli*, analogous to those described above, were performed. Theoretically, one would expect that probe-conferred signal intensities obtained after hybridization with a probe labeled with two different dyes would be comparable to those measured in the control experiment with the respective monolabeled probe. Indeed, the Cy5-Fluos- and Cy5-Cy3-double-labeled probe signal intensities for each dye were comparable to those obtained from hybridization with the Fluos- and Cy5-single-labeled probe derivatives, indicating that quenching effects can be neglected for these dye combinations (Fig. 2). This is not surprising, since the emission maxima of Fluos (521 nm) and Cy3 (570 nm) are well separated from the absorption maximum of Cy5 (649 nm). Concerning the Fluos-Cy3 combination, the situation is different with the emission maximum of Fluos being quite close to the absorption maximum of Cy3 (550 nm). As a consequence, the photons emitted by the Fluos dye are partially quenched by Cy3 in double-labeled probes, leading to an approximately 20% reduction in Fluos signal intensity (Fig. 2), which is, however, much less pronounced than in the competition experiment of Fluos-labeled probes with Cy3-labeled probes (Fig. 1).

In summary, dual-colored DOPE probes are an easy-to-apply alternative for multicolor FISH experiments, which lead to signal intensities between 75 and 104% for each dye compared to the use of conventional monolabeled FISH probes and thus outperform the use of two differentially labeled probes competing for the same target site.

**Multicolor DOPE-FISH for the simultaneous visualization of six phylogenetically distinct sponge symbiont populations without spectral unmixing.** In a proof-of-principle experiment, the multicolor DOPE-FISH approach was applied to visualize six different microbial symbiont populations in a marine sponge. These animals are well known to harbor dense and diverse microbial communities with substantial ecological and biotechnological importance (21). Sponge-associated microbes include bacteria and archaea that possess different degrees of host specificity (22) and can comprise up to 40% of the sponge tissue volume. At the time of this report, members of more than 25 different bacterial phyla and 3 archaeal phyla (11, 17, 26, 27) had been detected in sponges. However, due to the rather low ribosome content of certain microbes thriving in sponges (10) and the considerable high background fluorescence often observed, sponge tissue represents a challenging material for FISH analyses of inhabiting microbes. Consequently, any loss in sensitivity of the FISH assay has to be avoided. For the multicolor DOPE-FISH application, we decided to employ the marine sponge *A. alata* due to its high microbial abundance and diversity revealed in previous studies by transmission electron microscopy (TEM), 16S rRNA (gene) clone library analyses, and amplicon pyrosequencing (10, 17). Using six specific DOPE-FISH probes uniquely labeled with different dyes, six phylogenetically distinct microbial symbiont populations—members of the candidate phylum “Poribacteria,” the phylum *Chloroflexi*, the genus *Nitrosira*, the *Deltaproteobacteria*, the *Gammaproteobacteria*, and the *Archaea*—could be simultaneously detected in a single hybridization event (Fig. 3A). *Archaea*, *Gammaproteobacteria*, and *Poribacteria* clearly dominated the microbial biomass (Fig. 3A and B), whereas the *Deltaproteobacteria*, *Chloroflexi*, and *Nitrosira* were less abundant (Fig. 3A and C). Generally, this observation is in good accordance with previous findings: *Archaea*, *Poribacteria*, and *Gammaproteobacteria* are often found as numerically dominant players in microbial sponge
communities (10, 15–17). Furthermore, Kamke and coworkers reported low abundances of Nitrospira- and Deltaproteobacteria-related clones in DNA- and RNA-based clone libraries generated from A. alata (10).

As a consequence of differences in the cellular ribosome content of the different target populations, the optimal adjustment of the CLSM settings for image recording was quite challenging. The rather low probe-conferred signal intensity of, for instance, the Nitrospira-like target cells (detected by a Cy3-Cy5-double-labeled probe), required a high detection sensitivity for the Cy3 and Cy5 channels. This again led to overexposure for three other distinct target populations detected by probes carrying a Cy3 or Cy5 dye as well (Poribacteria, Cy3-Fluos; Gammaproteobacteria, Cy3; Archaea, Cy5) but displaying a higher ribosome content. Regardless

FIG 3 Multicolor DOPE-FISH analyses of microbial sponge symbionts. (A) FISH of tissue from the marine sponge A. alata with rRNA-targeted probes specific for Poribacteria (Por1130, yellow), Nitrospira (Ntspa662, pink), Chloroflexi (GNSB941, cyan), Deltaproteobacteria (Delta495a, green; larger green areas are sponge autofluorescence), Gammaproteobacteria (Gam42a, red), and domain Archaea (Arch915, blue). (B) Poribacteria (Por1130, yellow), Gammaproteobacteria (Gam42a, red), and domain Archaea (Arch915, blue). Autofluorescence of sponge tissue is displayed in green. (C) Nitrospira (Ntspa662, magenta), Chloroflexi (GNSB941, cyan), and Deltaproteobacteria (Delta495a, green; larger green areas are sponge autofluorescence). For all panels, CLSM images were recorded separately for each color channel and subsequently superimposed to obtain the final composite image. Bars, 10 μm.
of which approach is chosen (multicolor DOPE-FISH or CLASI-FISH), such conditions, with target populations differing in their cellular ribosome content, require careful consideration regarding the design of the applied probe-dye constructs, but any loss in sensitivity (as a result of two probes competing for the same binding site) or skew in the dye-conferred signal ratio (as a result of different competitiveness of certain probe-dye constructs) will further aggravate the simultaneous detection of different target populations under such difficult conditions.

In summary, we were able to demonstrate that multicolor DOPE-FISH is a straightforward stand-alone approach for the simultaneous detection of up to six microbial populations in a single FISH experiment. If combined with spectral unmixing, as impressively applied in a recent CLASI-FISH study (23), the use of oligonucleotide probes labeled with different binary combinations of dyes will almost certainly further enhance the potential of multicolor FISH for the analyses of samples with elevated levels of background fluorescence and/or for microbes with a low cellular ribosome content.

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REFERENCES