Review

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Methodological Aspects of Fluorescence *In Situ* Hybridization

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The current status of fluorescence *in situ* hybridization (FISH) is critically examined ten years after the initial application of fluorescently labeled, rRNA-targeted oligonucleotide probes as "phylogenetic stains" by Edward DeLong and co-workers in 1989 for the *in situ* identification of whole fixed bacterial cells in natural samples. The method has in the meanwhile found numerous applications including the identification and enumeration of bacteria in human feces. Still, however, the principal problems that need to be solved before a FISH assay is successfully applied have remained the same. These include e.g.: (i) Permeabilization of the cell envelope for free probe diffusion to the intracellular target molecules, mostly 16S rRNA, by a fixation protocol that preferentially also maintains the cell morphology. (ii) A selection of the probe target site that takes into consideration that not all rRNA sites are equally accessible. In this respect, the predictive power of a complete *in situ* accessibility map of 16S rRNA of *Escherichia coli* will be discussed. (iii) Low cellular ribosome content as found in many environmental samples automatically results in weak probe-conferred staining. Methods to increase the signal strength will be discussed together with issues of instrumentation and automation. Depending on the samples of interest and the questions to be addressed a high quality epifluorescence microscope with optional image analysis, a confocal laser scanning microscope or a flow cytometer may be the instruments of choice.

Key words: identification; fluorescence *in situ* hybridization; rRNA; flow cytometry

INTRODUCTION

Fluorescence *in situ* hybridization (FISH) with ribosomal RNA-(rRNA)-targeted nucleic acid probes is one part of the rRNA approach to microbial ecology *(23)*. This approach has in the last decade found more and more applications in the specific identification and enumeration of bacteria *(2)*. In combination with other techniques fluorescently labeled, rRNA-targeted oligonucleotide probes allow to study the structure and function of complex microbial communities *(1)*.

As a whole the rRNA approach to microbial ecology and evolution *(23)* encompasses the sequencing and hybridization of rRNA or rRNA genes (rDNA). In the first phase, the 16S rRNA sequences of defined bacterial strains are determined. Alternatively, there is an option for cultivation-independent rRNA gene sequence retrieval so that even hitherto uncultured bacteria can be examined. Based on the comparative analysis of the sequences for idiosyncrasies specific probes can be designed for species, genera or larger taxa in a directed way. Aspects of probe design have been described before *(35)*. In the computer age it should be very simple. Essentially tools like PROBE_DESIGN of the ARB program package developed by Wolfgang Ludwig and colleagues at the Technical University Munich *(37)* allow to mark with a mouse click one organism or a group of organisms. After few seconds the computer will come up with a couple of suggestions for potential probe target sites if these exist based on the current database. What the computer does is that it looks for a region where the 16S rRNA of the target organism(s) has differences to all non-targeted sequences. A complementary labeled oligonucleotide, the so-called probe, is ordered and everything is fine? In the ideal case, yes! But what happens in the ever increasing databases is the following: we are faced with numerous erroneous or partial rRNA sequences. Some of the old sequences from pure cultures or sequences directly retrieved from the environment might just consist of 200 nucleotides at the 5' end of the 16S rRNA or of 400 at 3'. Those sequences do not overlap and are no reliable foundation for probe design. A good 16S sequence coverage of the target group of interest together with a well-maintained database is the starting point of any successful *in situ* hybridization. Considering the ease and speed which sequencing techniques have reached today rRNA sequences should whenever possible be determined on almost full length in high quality (sequencing of both strands).

In situ hybridization in a strict sense defines a localization technique in which labeled probe molecules bind to target nucleic acids in cells that are thereby identified at the sites where they lived. In a somehow wider

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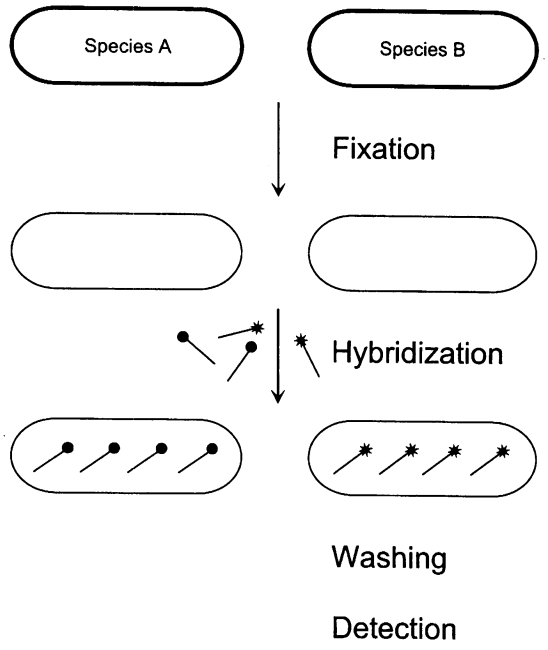

Fig. 1. Principal steps of fluorescence *in situ* hybridization. The dots and asterisks indicate two different fluorescent dye molecules that are linked to two specific oligonucleotide probes.

definition microbiologists are using the term to describe the detection of target nucleic acids within whole fixed cells even though early attempts were made to discriminate between true *in situ* and whole cell hybridization *(5)*. Ribosomal RNA is not the only target for *in situ* hybridization, but, if the identification of the organism is the purpose, for obvious reasons the 16S rRNA is the most common target molecule. Its stability and high copy number makes rRNA a much easier target than, e.g., mRNA. Furthermore, a large number of more than 20,000 16S rRNA sequences covering almost all validly described species and many environmental sequences have by now been determined. This does, however, not mean that *in situ* mRNA detection in single cells has not yet been achieved (e.g., Refs. *15, 16, 40*). Still, however, this technique is far from the routine applications rRNA-targeted *in situ* hybridization probes have found in microbiology. The basic steps of fluorescence *in situ* hybridization are outlined in Fig. 1.

There are distinct differences between the results which may be obtained by *in situ* or whole cell hybridization and those that are measured by other hybridization techniques. FISH yields cell numbers together with data e.g. on cell sizes, frequency of dividing cells and cellular rRNA contents. Furthermore, the exact three-dimensional distribution of defined bacteria with regard to other species or physico-chemical gradients can be monitored at a resolution of individual bacterial cells.

In the last decade my group has mainly focussed on the visualization, identification and enumeration of microorganisms by *in situ* hybridization. Here, I would like to critically examine the current status of *in situ* hybridization ten years after the initial application of fluorescently labeled, rRNA-targeted oligonucleotide probes as "phylogenetic stains" by DeLong and coworkers in 1989 *(9)* for the *in situ* identification of whole fixed bacterial cells in natural samples.

MATERIALS AND METHODS

Detailed protocols for *in situ* hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes have been published *(3, 21)*. The advantages of using Cy3-labeled oligonucleotides have been discussed by Glöckner and coworkers *(14)*. Signal amplification by the use of horseradish peroxidase labeled probes and tyramide signal amplification (TSA) has been described *(32)*. Methodological aspects of probe optimization, sensitivity and specificity testing can be found elsewhere *(22, 36)*.

RESULTS AND DISCUSSION

FISH has in the meanwhile found numerous applications *(2)* including the identification and enumeration of bacteria in human feces which is the focus of this conference. Still, however, the principal problems that need to be addressed before FISH can be successfully applied have remained the same. These are:

Permeabilization/Fixation

The permeabilization of the cell envelope for free diffusion of nucleic acid probe to the intracellular target nucleic acids by a cell fixation protocol that preferentially maintains the cell morphology is the first important step. For that, cell compounds like the cell wall, membranes and, if present, capsular material or other extracellular polymeric substances need to be made permeable for the probe molecules. This will be the easier the smaller the probe molecules are. Oligonucleotides are in this regard better than polynucleotides and small fluorescent labels with a molecular weight of below 1,000 dalton better than large enzyme labels like horseradish peroxidase *(6, 32)*. Furthermore, since intact membranes are generally impermeable for standard oligonucleotides a fixation/permeabilization is required. Permeabilization is usually achieved by treatment of the sample with crosslinking aldehyde solutions (paraformaldehyde, formalin) and/or denaturing

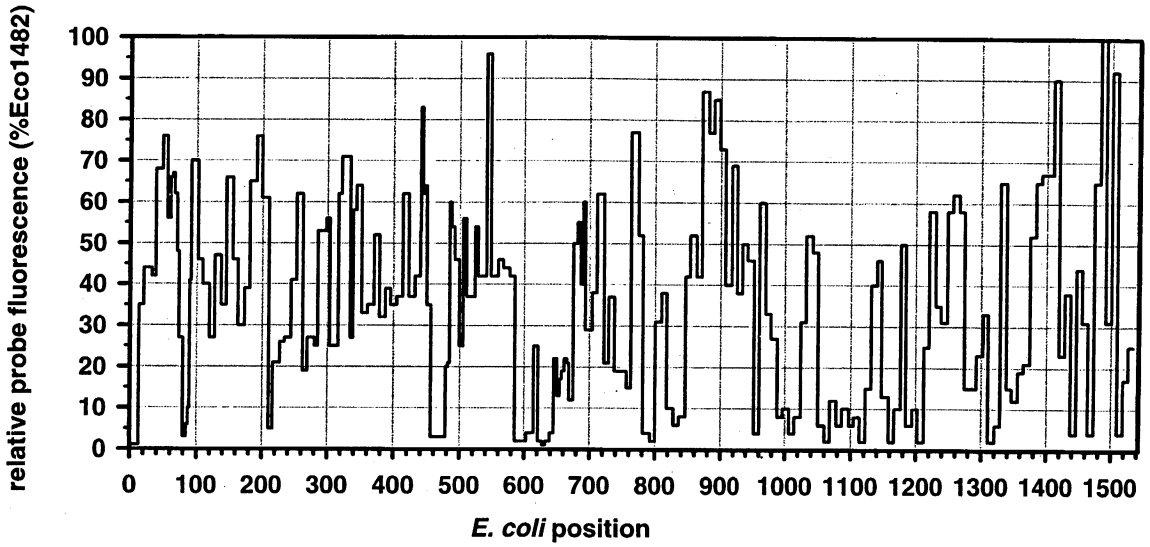

Fig. 2. *In situ* accessibility of the 16S rRNA of *E. coli* for fluorescently labeled oligonucleotide probes. Modified from Fuchs et al. *(12)*.

alcohols (for detail see Ref. *3*). Even though several fairly general fixation protocols have been described *(2, 3)* care should be taken that the procedure is optimized for the target cells so that neither the morphological integrity is compromised nor cell walls become so strongly cross-linked that probe penetration is hindered. Thick-walled Gram-positive bacteria need other fixation protocols than Gram-negative bacteria *(8, 10, 30)*. Furthermore, diffusion will require a certain time which is dependent on the distance between probe and target. Therefore, larger aggregates need either to be dispersed, e.g., by sonication *(20)* or sectioned to preserve the natural organization (e.g., Refs. *28, 33*).

Whether fluorescently labeled oligonucleotide probes can reach target molecules may conveniently be checked by hybridization with a complementary domain- or group-specific oligonucleotide probe.

In Situ Accessibility of Probe Target Sites

Not all target sites on the 16S rRNA are equally accessible. Signals after *in situ* hybridization of one and the same batch of permeabilized cells with oligonucleotides with identical labeling may range from very bright to almost invisible. A systematic study on the accessibility of 16S rRNA target sites was long missing. Recently, we performed one with more than 200 oligonucleotide probes (mostly 18-mers) on whole fixed cells of *Escherichia coli* DSM 30083^T *(12)*. Two overlapping sets of adjacent oligonucleotides were designed to cover the full length of the 16S rRNA. The probes were all labeled with carboxyfluorescein and signal intensities of hybridized cells were quantified by flow cytometry. Care was taken that the signal intensity of cells was solely dependent on the *in situ* accessibility of probe target sites. The brightest signal resulted from probe Eco1482, complementary to positions 1482–1499. Fluorescence was 1.7 times brighter than that of the standard bacterial probe EUB338 and 44 times brighter than that of the worst probe, Eco468 (see Fig. 2). The distribution of probe-conferred cell fluorescence in six arbitrarily set brightness classes (I–VI: 100–81%, 80–61%, 60–41%, 40–21%, 20–6%, 5–0% of Eco1482, respectively) was as follows: I, 4%; II, 14%; III, 21%; IV, 29%; V, 19%; VI, 13%. A more detailed analysis of the helices 6, 18 and 23 with additional probes demonstrated that already a shift of the target region by only a few bases could result in a decline of cell fluorescence from >80% to <10%. Interestingly, some of the most variable target sites are difficult targets. Considering the high evolutionary conservation of 16S rRNA the *in situ* accessibility map of *E. coli* should facilitate a more rational selection of probe target sites for other species as well. Currently, we are performing a similar study on 23S rRNA which, with a length of approximately 3,000 as compared to 1,500 of the 16S rRNA, offers twice as many potential target sites.

Assay Sensitivity

Low cellular ribosome content as found in many environmental samples automatically results in weak probe-conferred staining. Here, methods to increase the signal strength become important. This problem should

always be considered in the context of possibilities to visualize weak signals and to discriminate them from non-specific probe binding and background fluorescence.

When discussing the sensitivity of *in situ* hybridization one has first to realize that, even though an individual cell can be identified, this cell first needs to be brought into the microscopic field of observation. In a marine sediment containing $>10^9$ cells/cm^3 and a quite high fraction of autofluorescent particles the detection limit might not be better than 0.1% or 10^6 cells/cm^3. However, given a relatively clean water sample and the right set-up for cell concentration it should also be possible to detect < 1 cell/cm^3.

Another frequently encountered problem is that bacterial cells from the environment might have low signals upon *in situ* hybridization with fluorescently monolabeled oligonucleotide probes *(2)*. Necessarily, the fluorescence conferred by a rRNA-targeted probe will be sensitive to changes in the cellular rRNA content of the target cells. A linear correlation between growth rate of *Salmonella typhimurium (31)* and cellular ribosome content has been shown before. This correlation does also apply to other bacteria *(26, 41)* and might be the reason why small, starving cells with little to no growth are so difficult to detect by FISH with rRNA-targeted probes. On the other hand, if this correlation is really true for cells in the environment then it should be possible to determine or, at least, to estimate *in situ* growth rates of individual cells based on quantitation of probe-conferred fluorescence. This has been attempted for sulfate-reducing bacteria in a biofilm by digital microscopy *(26)*. However, there is a large difference between the highly controlled growth conditions in Schaechter's experiments *(31)* and those experienced by environmental bacteria which might have to cope with rapid changes in the physical and chemical environment. Since ribosome synthesis is energetically quite costly, ribosome degradation as rapid first response to the slowing of the growth rate would be very wasteful. Indeed, during periods of starvation of up to several months bacteria maintain cellular ribosome pools in excess of their current needs *(11, 39)*. Consequently, in strongly fluctuating environments the cellular ribosome content should not be used to estimate actual growth rates. Nevertheless, the FISH signal of a cell is ecologically meaningful since it reflects the potential of the cell to synthesize protein and, thereby, is a good indicator for viability.

There have been several attempts to combine short term incubation of environmental samples with nutrients and/or antibiotics and FISH in attempts to increase the ribosome content of environmental cells. Oligotrophic drinking water biofilms were incubated for 8 hr with a mixture of carbon sources and an antibiotic preventing cell division *(17)* prior to FISH in a modification of the direct viable count technique *(18, 19)*. The number of cells detectable by FISH increased from 50 to 80%, clearly demonstrating viability of the majority of the cells. In a similar approach marine water samples have been incubated for approximately 1 hr with chloramphenicol *(24)*. Again an increase in detection yield has been observed from 75 to almost 100%. It should be noted here that even though both studies described precautions taken to prevent changes in total cell number or microbial composition during the incubation of the samples the treatments had effects, e.g., on the cellular ribosome content. They can therefore, in a strict sense, not be regarded as *in situ* hybridizations and it might in any case be helpful to also investigate parallel samples after direct fixation.

Recently, technical improvements have been reported that result in more sensitive FISH. This includes the use of more sensitive fluorescent dye molecules such as the carbocyanine CY3 *(17)*, dual labeling of oligonucleotide probes *(13, 41)* or the use of multiple labeled polynucleotide probes *(38)*, the application of the tyramide signal amplification *(32)* or detection of the probe-conferred fluorescence by highly sensitive cameras (e.g., Refs. *13, 27*) or confocal laser scanning microscopy (e.g. Ref. *7*). Still, however, a certain fraction of those particles that are based on their binding of the DNA stain DAPI *(25)* and their cell morphology identified as cells are not detected by these methods. Problems with cell permeabilization and the fact that even the most general bacterial 16S rRNA probes do not bind to all bacteria are just two of several possible explanations.

Automation

Another important current limitation of FISH is the lack of automation. Usually cells are still counted manually. It has to be stressed that FISH is the method of choice when numbers of individual cells or cell localization are required. However, without automated, accurate counting the FISH examination of multiple samples with multiple probes will remain infeasible.

One of the instruments that could potentially allow to circumvent this limitation of FISH is the flow cytometer. In it, suspended cells individually pass an observation point at which they are usually analyzed by a laser for light scattering and fluorescence. After FISH

of bacteria the scatter gives data on cell size, shape and sometimes even on internal cell structures, the fluorescence analysis should allow for discrimination of FISH-positive and negative cells *(4)*. The combination of FISH and flow cytometry has, e.g., been successfully used for the enumeration of specific bacterial populations in activated sludge *(42)*. Even though initially developed for blood analysis good research flow cytometers now have cell size limits down to 0.2 μm. Unfortunately the sensitivity usually is not as high as in good microscopes and in oligotrophic environments the detection of the FISH signal may become limiting. In other samples like soils, sediments or feces the suspension of cell clumps and the removal of particles that might block the inlet of the flow cytometer might be the problem. Another advantage of flow cytometry is its sorting capability. One can do sorting at a speed of one thousand cells per second and enrichments up to 1,000 fold can be reach in a single run. This allows for the directed molecular analysis also of less frequent cells *(34, 43)*.

Conclusion

I would like to end by stressing that the accurate, cultivation-independent molecular quantification of specific bacterial populations in complex communities remains to be a challenging task. Considering, however, the known inaccuracy of the cultivation-based quantification of many bacteria together with its slowness and labor-intensity molecular biological assays will be the technology of the future. In this respect, it is important to remember the principal difference between the non-linear, usually only semi-quantitative PCR-based approaches and the linear hybridization techniques. The former might be more easily automated but the later promise more accurate results. Considering the many potential pitfalls in the application of a technique like FISH it is wise to initially use two different methods for quantification in parallel like, e.g., slot blot hybridization based on extracted rRNA *(29, 36)* and FISH. Thereby, the most suitable method for a particular monitoring task can be found. However, if accurate cell numbers need to be determined, if the homogeneity of a population with respect to, e.g., cell size and ribosome content or the spatial distribution are of interest FISH will likely be the method of choice.

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