Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes

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INTRODUCTION:

Fluorescence in situ hybridization (FISH) with rRNA-targeted probes is, amongst other things, a staining technique that allows phylogenetic identification of bacteria in mixed assemblages without prior cultivation (Fig. 1) by means of epifluorescence and confocal laser scanning microscopy, or by flow cytometry (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Amann *et al.*, 1990a; Amann *et al.*, 1990b; Amann *et al.*, 1996). FISH with polynucleotide DNA probes, and FISH with oligonucleotide probes targeted to mRNA has also been described (Trebesius *et al.*, 1994; Wagner *et al.*, 1998; DeLong *et al.*, 1999). This protocol will, however, exclusively focus on FISH with oligonucleotide probes for the purpose of bacterial identification, i.e. to analyze bacterial community structure, and to follow the spatial and temporal dynamics of individual microbial populations in their habitat (Alfreider *et al.*, 1996; Llobet-Brossa *et al.*, 1998; Murray *et al.*, 1998; Glöckner *et al.*, 1999; Simon *et al.*, 1999). Several reviews discuss numerous aspects and applications of the method (Amann *et al.*, 1995; Amann *et al.*, 1997).

In theory, each ribosome within a bacterial cell, containing one copy each of 5S, 16S and 23S rRNA, is stained by one probe molecule during the hybridization procedure, the high numbers of ribosomes per cell thus providing a natural signal amplification system (Fig. 2).The method is mainly based on the rapidly increasing set of bacterial small subunit (16S rRNA) rRNA sequences, which has been gathered during the last decade for the study of microbial phylogeny (Woese *et al.*, 1990; Ludwig and Schleifer, 1994). To a lesser extent, probes have been constructed that target the large subunit rRNA (23S rRNA) (Stoffels *et al.*, 1998); this is, however, still hampered by the comparatively small number of available 23S rDNA sequences.

FISH of bacteria has first been described more than a decade ago (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Amann *et al.*, 1990b), and celebrated as a breakthrough for microbial ecology. However, researchers initially encountered discouraging difficulties when applying the method to environmental samples other than from highly eutrophic systems. The majority of bacteria in aquatic habitats is small, slowly growing or starving, and the signal intensities of hybridized bacterioplankton cells were frequently below detection limit or lost in high levels of

background fluorescence. Accordingly, an early FISH protocol stated that there was "...a good deal of room for improvement of these techniques for practical field application." (DeLong, 1993). This still holds true to a certain extent, but several important advances, in particular new quantitative protocols (Glöckner *et al.*, 1996), brighter fluorochromes (Alfreider *et al.*, 1996; Glöckner *et al.*, 1996), commercial availability of probe labeling, advanced probe design software (Strunk *et al.*, 1999) and better instrumentation have made the method attractive also for the less "molecular" microbial ecologists.

FISH OF ENVIRONMENTAL SAMPLES ON MEMBRANE FILTERS

Principle:

Specificity of probe binding to the target site depends on the hybridization and washing conditions. Hybridization probes are added to a defined, stringency-determining buffer at saturation concentrations (5 ng μl^{-1}) to maximize probe binding. During hybridization the samples are incubated at elevated temperature in an airtight vessel saturated with water and formamide vapours of additional hybridization buffer to avoid concentration effects due to evaporation. The washing step is performed at a slightly higher temperature and serves mainly to rinse off excess probe molecules at conditions that prevent unspecific binding. The below protocols are focussing on FISH with monolabeled fluorescent probes on membrane filters and glass slides. This approach is routinely used in our lab for the analysis of bacterioplankton and sediment samples of unknown composition (Llobet-Brossa *et al.*, 1998; Pernthaler *et al.*, 1998), and has been optimized for the processing of numerous samples. Other hybridization strategies (indirect probe labeling, signal amplification systems; Amann *et al.*, 1992; Schönhuber *et al.*, 1997) may be more appropriate for specific application (e.g., FISH of autofluorescent cyanobacteria with horseradish peroxidase (HRP) labeled probes and Tyramide Signal Amplification (TSA); Schönhuber *et al.*, 1999), but also need a careful adaptation to the target microorganisms (e.g., cell wall permeabilization, fixation).

Equipment and supplies:

General:

- High quality epifluorescence microscope equipped with filter set for DAPI, FITC and CY3
- Dry-type incubator or hybridization oven
- Water bath
- Freezer
- Fridge

Fixation of plankton samples:

- 100 ml glass bottles
- plastic petri dishes (diameter, 5 cm)
- white polycarbonate membrane filters (diameter, 47 mm; pore size, 0.2 µm)
- cellulose nitrate support filters (diameter, 47 mm; pore size, $\geq$ 0.45 µm)
- filter towers for 47 mm membrane filters
- vacuum pump
- particle-free 35% (w/v) formaldehyde solution (formalin)
- 50, 80, and 96% (v/v) ethanol (only for TSA method)

Fixation and preparation of sediment samples:

- Microcentrifuge for 2 ml tubes
- vacuum pump
- ultrasonication probe
- 2-ml screw-top microfuge tubes
- 2-ml microfuge tubes
- plastic petri dishes (diameter, 5 cm)
- white polycarbonate membrane filters (diameter, 25 mm, pore size 0.2 µm)
- cellulose nitrate support filters (diameter, 25 mm, pore size $\geq$ 0.45 µm)
- filter tower for 25 mm membrane filters
- 1 x PBS
- ethanol
- 4% (w/v) formaldehyde solution

Hybridization on filter sections and counterstaining:

- 2-ml microfuge tubes
- 0.5-ml microfuge tubes
- 50 ml polyethylene tubes and rack
- blotting paper
- razor blades
- plastic petri dishes
- microscopic slides + cover slips
- CITIFLUOR mountant
- VECTA SHIELD mountant
- 1 M Tris / HCl, pH 7.4
- formamide
- 0.5 M EDTA, pH 8
- 10% (w/v) sodium dodecyl sulphate (SDS)
- 5 M NaCl solution
- 4´,6-Diamidino-2-Phenylindole (DAPI) dissolved in distilled H_2O, final concentration, 1 µg ml^{-1}
- 80% (v/v) ethanol

Hybridization with horseradish peroxidase (HRP) labeled probes and Tyramide Signal Amplification (TSA)

- 2-ml microfuge tubes
- 0.5-ml microfuge tubes
- 50 ml polyethylene tubes and rack
- blotting paper
- razor blades
- plastic petri dishes
- microscopic slides + cover slips
- CITIFLUOR mountant
- 1 M Tris / HCl, pH 7.4
- formamide
- 0.5 M EDTA, pH 8
- 10% (w/v) sodium dodecyl sulphate (SDS)

- 5 M NaCl solution
- 10% Blocking reagent (Roche)
- TSA™ Fluorescence system (containing fluorophore labeled tyramide and amplification diluent)
- TNT buffer (prepared according to the TSA™ Fluorescence system manual)

Assay: Fixation of plankton samples:

1. Add formaldehyde to water sample to a final concentration of 2-4% and fix for at least 1 but no longer than 24 hours.
2. A hand-operated vacuum pump and several autoclavable plastic filter towers that can be linked together for parallel sample processing make an inexpensive filtration apparatus for field work. Place the moistened support filter and polycarbonate filter into the filtration tower, and filter an appropriate volume of the fixed sample by applying gentle vacuum. Support filters may be utilized for several samples. For cell numbers of around 10^6 ml^{-1}, 10 ml of sample are sufficient.
3. After complete sample filtration, wash with 10-20 ml of sterile H_2O; remove H_2O by filtration.
4. Put membrane filter in petri dish and allow to air-dry.
5. Store at -20°C until processing. Filters can be stored frozen for several months without apparent loss of hybridization signal.

Assay: Fixation and preparation of sediment samples

1. Suspend 0.5 ml of freshly collected sediment in 1.5 ml of 4% formaldehyde solution in a 2-ml screw-top microfuge tube. Fix for 1 to 24 hours.
2. Centrifuge at 10.000 rpm for 5 minutes, pour off supernatant.
3. Add 1.5 ml of PBS and resuspend sample.
4. Repeat steps 2 and 3.
5. Centrifuge at 10.000 rpm for 5 minutes, pour off supernatant.
6. Add 1.5 ml of a 1:1 mix of PBS / ethanol and store sample at -20°C until further processing
7. Resuspend sample and transfer 20-100 µl of aliquot to 500 µl of a 1:1 mix of PBS / ethanol in a 2-ml microfuge tube

8. Sonicate aliquot for 20-30 seconds at low intensity using 1-second sonication pulses. If required, the sonicated sample can be further diluted.

9. Place cellulose nitrate support filters beneath the membrane filters to improve the distribution of cells. Add 15-20 µl of aliquot from the sonicated sample to 2 ml of distilled water and filter this volume onto the membrane filters.

10. Air-dry filtered preparations and store in petri dishes at -20°C until hybridization.

Assay: Fixation for the TSA method:

1. Fix the water sample by adding the same volume of ethanol and store at –20°C until further treatment. Alternatively, fresh water samples may be immediately subjected to fixation and subsequent filtration onto membrane filters.

2. For the preparation of membrane filters, filter an aliquot (the volume depending on the density of the cells of interest; see above) of the fixed or fresh sample.

3. A second fixation (dehydration) is performed in the filtration apparatus by applying increasing concentrations of ethanol : Cover the filter with 1 ml of 50% ethanol and incubate for 3 minutes, then remove liquid by filtration. This is repeated with 80% and 96% ethanol.

4. Filters are air dried and can be stored at –20° for several weeks.

Assay: Hybridization of cells on membrane filters:

1. Prepare 2 ml of hybridization buffer in a microfuge tube:

Stock reagent	Volume	final concentration in hybridization buffer
5M NaCl	360 µl	900 mM
1 M Tris / HCl	40 µl	20 mM
formamide	% depending on probe	
distilled H_2O	add to 2 ml	
10% SDS (added last to avoid precipitation)	2 µl	0.01%

2. For the hybridization mixtures add 2 µl of probe working solution to 18 µl of hybridization buffer in a 0.5-ml microfuge tube; keep probe solutions dark and on ice.

3. Cut sections from membrane filters with a razor blade. A 47 mm diameter filter should allow the preparation of 16 – 20 individual hybridizations. Label filter sections with a pencil, e.g., by numbering them.

4. Put filter sections on glass slides (upside facing up); several filter sections can be placed on one slide and hybridized simultaneously with the same probe.

5. Put a piece of blotting paper into a polyethylene tube and soak it with the remaining hybridization buffer.

6. Add hybridization mix on the samples and place the slide with filter sections into the polyethylene tube (in a horizontal position).

7. Incubate at 46°C for at least 90 min (maximal: 3 hours)

8. Prepare 50 ml of washing buffer in a polyethylene tube:

Stock reagent	Volume	final concentration in hybridization buffer
5M NaCl	depending on % formamide in hybridization buffer (Tab. 1)	900 mM
1 M Tris / HCl	1 ml	20 mM
0.5 M EDTA	500 µl	5 mM
distilled H_2O	add to 50 ml	
10% SDS (added last to avoid precipitation)	50 µl	0.01%

9. Quickly transfer filter sections into preheated washing buffer and incubate for 15 min at 48°C (water bath).

10. Pour washing buffer with filter sections into a petri dish. Pick filter sections and rinse them by placing them into a petri dish with distilled H_2O for several seconds, then let them air-dry on blotting paper.

11. For counterstaining put filter sections on a glass plate, cover with 50 µl of DAPI solution, and incubate for 3 minutes. The side holding bacterial cells should face up. Afterwards wash filter sections for several seconds in 80% ethanol to remove unspecific staining, followed by rinsing in distilled H_2O and air-drying.

12. Samples are mounted in a 4:1 mix of Citifluor and Vecta Shield. Vecta Shield contains a superior antibleaching reagent, but quenches DAPI fluorescence. The filter sections have to be completely dry before embedding, otherwise a fraction of cells will detach during inspection.

13. Double stained and air dried preparations as well as filters mounted on slides can be stored in the dark at -20°C for several days without substantial loss of probe fluorescence.

14. Probe-conferred fluorescence fades much more rapidly than DAPI fluorescence in the microscopic image, and UV excitation will also bleach the CY3 signal. For counting, it is, therefore, safer to first quantify specifically stained cells in green excitation, and subsequently all cells from the same field of vision in UV excitation. At least 500 DAPI-stained cells should be counted in plankton samples to obtain a counting error < ±5% (Fig. 3).

Assay: Hybridization with HRP labeled probes and TSA:

1. The hybridization mixture described above is slightly changed by replacing the 400 µl of distilled H_2O with the same volume of 10% blocking reagent (2% final concentration). This volume is reduced accordingly, if the concentration of formamide should exceed 60%. The 2 µl probe working solution should contain 100 ng of HRP-labeled probe instead of fluorescently labeled oligonucleotides.

2. The next steps follow the assay of hybridization with fluorescently labeled probes except that the temperatures of hybridization and of washing are lowered to 35°C. This is required because of the instability of the enzyme at higher temperatures and is also reflected in different NaCl concentrations for washing buffers (Tab. 2).

3. After washing and rinsing in distilled H_2O, transfer the filter sections directly to TNT buffer and equilibrate for 15 minutes at room temperature.

4. In the meantime, prepare substrate solution by mixing 1 volume of 40% dextrane sulfate in water, 1 volume of amplification diluent, and 1/50 volume of tyramide

solution (the latter two are supplied with the TSA kit). About 20 µl of the substrate solution is needed per filter section.

5. Remove excess liquid by briefly putting filters on blotting paper, but transfer them to a glass slides before they become completely dry. Cover them with substrate solution.

6. Put the slide in another polyethylene tube, the blotting paper this time soaked by distilled water, and incubate for 30 to 90 minutes.

7. Rinse the filter pieces carefully in TNT buffer, immerse them in the same buffer and heat to 55°C for 15 minutes.

8. Rinse them in distilled H$_2$O and let them air dry.

9. Cells can be counterstained with DAPI as described above or directly embedded with Citifluor mountant.

Troubleshooting:

- Depending on the type of sediment, it might be necessary to also adapt the aliquot size prior to sonication. If too much sediment is suspended, sonication will lead to incomplete detachment of cells from particles.

- Do not attempt to determine absolute cell counts from filters after hybridization, but only the percentage of hybridized cells. The distribution of cells on sections of a 47 mm diameter membrane filter is never as even as on a small filter, resulting in a higher error of the total DAPI counts. We find that, following our procedure, 80-90 % of the initial bacterial cell numbers are recovered after hybridizations of bacterioplankton on membrane filters. This fraction may, however, depend on the type of sample and should be verified experimentally.

- Hybridization stringency may also be adjusted by temperature rather than by the chemical compositon of buffers. We find that it is more convenient to keep incubator and water bath at one set temperature, so that several people can hybridize their samples in parallel irrespective of the probes they are using.

- Due to the size of the HRP molecule, accessibility of probes to the cells may be discriminating. This is, e.g., reflected in the preference for ethanol fixation rather than fixing with the crosslinking agents paraformaldehyde or formaldehyde. The probability that not all organisms can be detected under the same conditions increases with the phylogenetic diversity of the target group. So it is recommended to use the signal amplification method only for probes with a

- restricted target group for which fixation and hybridization conditions can be readily achieved.

- On white polycarbonate filters background fluorescence after DAPI staining is always somewhat worse than on black membrane filters. Black filters, however, show high levels of background fluorescence at green excitation. Use shorter DAPI staining time and / or longer ethanol washing to improve background. Make sure that hybridized filters have been thoroughly rinsed in distilled water before DAPI staining.

- Sometimes there can still be high or uneven background also in green excitation. To the untrained eye, a number of objects might appear to be hybridized bacteria, in particular autofluorescent debris (usually without DAPI signal) and cyanobacteria. Unhybridized filter sections should , therefore, be inspected sporadically to estimate the background level of "false positives".

- Different fluorochromes are available for the TSA method. Among the kits one can choose between coumarin-, fluorescein-, tetramethylrhodamine, cyanine 3-, and cyanine 5-labeled tyramides. For the detection of cyanobacteria, fluorescein was superior to tetramethylrhodamine, as the difference between intrinsic autofluorescence and signal intensity was higher at blue excitation. For other applications different fluorescent dyes might be most suitable.

- It has been reported that a 1 to 4 hour pre-incubation of environmental samples with the antibiotic chloramphenicol (100 µg ml^{-1} final concentration) in combination with an image intensifyer equipment may result in significantly higher FISH detection rates in coastal marine sample (Ouverney and Fuhrman, 1997). We could not verify these results in our samples using standard epifluorescence microscopy.

- Prepare only small aliquots of probe working solutions (50-100 µl). Use of a repeatedly frozen and thawed probe working solutions may cause the appearance of numerous brightly fluorescent particles at green (CY3) excitation which do not show any signal in UV (DAPI) excitation. In addition, hybridization signals become dim and background is high. For HRP labeled probes, do not freeze working solutions but store them in the fridge. Stock solution aliquots can be stored in the freezer, but once an aliquot is thawn to prepare working solutions, keep it at 4°C.

- Watch out for black cats and other signs of bad luck during hybridizations.

PROBE DESIGN AND TESTING

Principle:

Presently, the major challenge of using FISH in environmental samples is no longer the staining technique itself, but rather the design and application of new probes within the context of a particular unknown microbial assemblage. This approach, making use of FISH as but one in a suite of molecular biological techniques, has been termed "full cycle rRNA analysis" (Amann *et al.*, 1995). It refers to the initial construction of a rRNA clone library from environmental DNA after polymerase chain reaction (PCR) amplification with specific primers of 16S (23S) rDNA genes, comparative sequence analysis, subsequent probe design and the meaningful application of such new probes in the original habitat. Sequence information for probe construction may alternatively be obtained from bacteria isolated from the habitat by traditional means, e.g. by dilution techniques (Schut *et al.*, 1993), but also from environmental RNA after reverse transcription into DNA or PCR reamplification of excised individual bands obtained during denaturing gradient gel electrophoresis (DGGE) community fingerprinting (Rosselló-Mora *et al.*, 1999).

The melting behaviour of oligonucleotide probes depends on temperature, the composition of the hybridization buffer, oligonucleotide sequence (G +C content), and on probe length. There are several strategies to determine stringent hybridization conditions for a newly designed probe, i.e. conditions that only allow hybridization of the probe to a perfectly matching template. They all rely on the experimental testing of the probe-target vs. probe-non-target stability. It would go beyond the scope of this chapter to provide detailled protocols for the various techniques used for probe testing, therefore here only one method based on image analysis (Neef *et al.*, 1996) is described.

Equipment:

- appropriate software for probe construction, e.g., the program package ARB, released by the Technical University of Munich (Strunk *et al.*, 1999). This program runs on different UNIX-based operating systems. It should work on a large database of currently >10,000 aligned 16S rDNA sequences, and additional sequences can easily be imported from various sources. The major drawback of

ARB is a rather minimalistic online help system. The "Probe Design Tool" of ARB allows to search for specific oligonucleotides of user-defined length and G + C - content in a set of selected sequences, and to match these oligonucleotides with the ARB database. Potential probe candidates can then be BLASTed to GENBANK (http://www.ncbi.nlm.nih.gov/BLAST/) in order to check them against all currently available sequences.

- Teflon-coated multi-well slides
- Mix of a 0.01% CrK(SO$_4$)$_2$ / 0.1% gelatin solution
- reagents and equipment for FISH (see above)
- image analysis system: light-sensitive video or slow-scan CCD camera mounted on an epifluorescence microscope, linked to a PC or Macintosh computer; image analysis software, e.g. the freely distributed program *NIH Image*
- (alternatively: flow cytometer)
- (alternatively: reagents and equipment for membrane hybridizations)

Probe design and synthesis:

1. Preferably, full 16S rDNA sequences should be available when constructing specific probes. Partial sequences greatly limit probe design by reducing the number of potential target regions. In addition, partial sequences allow no decision about the specificity of existing probes that target to unsequenced regions of the respective rRNAs.

2. Not all sites within the ribosome are equally well accessible for FISH, but may be blocked e.g. by rRNA quartery structure. The accessibility of the 16S rDNA of *E.coli* for oligonucleotide probes has recently been mapped by quantitative hybridization and flow cytometry (Fuchs *et al.*, 1998). This colour-coded map and a list of normalized hybridization intensities can probably not be directly transferred to other organisms, but due to the high evolutionary conservation of the ribosome it should give hints for probe design, and probe target sites promising high signal intensities can be selected.

3. Ideally, a probe should show perfect base matching only with the target sequence(s), and have more than one base mismatch with the homologous region of non-target microorganisms. In order to minimize hybridization to such sites the base mismatches between probe and non-target rRNA should not be situated at the 3'- or 5'- ends of the oligonucleotide, but rather at a more central

position. In addition, mismatches must be weighted according to their probability of undergoing non Watson-Crick pairing with the base at the respective target position (e.g., a G – T mismatch is weaker than a G – A mismatch).

4. The G + C content of an oligonucleotide influences duplex stability and therefore its melting behaviour. Usually it should range between 50 and 60 % for 18 - to 24 - base oligonucleotides. If the G + C content is too high or the probe is too long, stringent hybridizations may no longer be obtainable at our suggested hybridization temperature (see above).

5. Readily labeled probes can be purchased from various companies. The indocarbocyanine fluorescent dye CY3, linked to the 5' –end of the oligonucleotide to our knowledge is the brightest commercially available, routinely used label for FISH probes. A epifluorescence filter set specifically designed for CY3 excitation should be used in combination with the dye for optimal results.

Principles of probe testing:

1. Probe stock are frequently delivered lyophilized. Suspend in 100 µl sterile H_2O. To determine probe concentration measure the absorbance of a 1:100 diluted stock solution at 260 nm (1 A_{260} ≈ 20 µg ml^{-1} DNA). This info is usually also supplied by the manufacturer, but it is still useful to perform this measurements in order to check the quality of the labeling. The dye CY3 shows maximum absorbance around 550 nm, and the ratio of A_{260} / A_{550} should be approximately 1 for a monolabeled 18-mer. Aliquots of working solutions are prepared at concentrations of 50 ng $µl^{-1}$ and stored in the dark at -20°C. Lyophilized HRP-labeled probes are suspended in sterile H_2O, too. For calculating the concentration it has to be taken into account that the enzyme itself contributes to the measured absorbance at 260 nm. Therefore the measured A_{260} has to be lowered by $k \times A_{404}$ with the correction factor $k = 0.276$. Presuming optimal labeling the ratio of the measured A_{260} and A_{404} should be around 3. Aliquots of the stock solution can be stored in at –20°C, working solutions prepared at concentrations of 50 ng $µl^{-1}$ should be stored at 4°C (see also above).

2. The "gold standard" of testing new probes for FISH is hybridization of isolates that show no and one mismatch with the oligonucleotide, respectively, at increasing levels of stringency. The simplest way of establishing stringency is by changing the concentrations of formamide in the hybridization buffer (in steps of 5 %) at one

fixed incubation temperature rather than by changing hybridization temperature. Change of fluorescence intensities of individual cells can then be quantified, e.g., by computer-assisted image analysis (Neef *et al.*, 1996) or by flow cytometry (Fuchs *et al.*, 1998). The adequate hybridization stringency is often the highest concentration of formamide in the hybridization buffer that does not result in loss of fluorescence intensity of the target cells. At this concentration, hybridization to the non - target organism should no longer occur. As a rule of thumb, an 18 - base oligonucleotide with a G+C content between 50 - 60 % will start to dissociate at a concentration of approximately 30 – 40 % of formamide in the hybridization buffer when hybridized in our buffer at 46°C. Alternatively, probes can be radiolabeled and hybridized to extracted rRNAs that have been blotted onto nylon membranes (Stahl and Amann, 1991).

3. Frequently new probes will be designed to target bacterial groups that are only known from their rDNA sequences. In this case it is not possible to test these probes on isolates. Two strategies are available to confirm probe specificity: (i) the rDNA to be tested can be transcribed in vitro into RNA, which is then blotted on a nylon membrane and hybridized to the radiolabeled oligonucleotide at increasing levels of formamide (e.g., Pernthaler *et al.,* 1998); (ii) FISH with the new probe is carried out on directly on environmental samples. The relative abundance of detected cells and the average cell brightnesses are determined at different formamide concentrations by counting and image analysis. At too low stringency levels cell counts may be higher because non-target populations are also detected. If stringency is, however, too high, mean cell brightness of the target population will rapidly decline (Fig. 4).

4. To quickly evaluate the brightness of a probe and thus its sensitivity in environmental samples it may be helpful to hybridize bacteria from a stationary phase culture of the target organism and compare signal intensities with those of a hybridization with a general bacterial or universal probe.

Assay: FISH of pure cultures on multi- well glass slides for probe testing

1. Heat the Cr K$(SO_4)_2$ /gelatin solution to 65°C, soak multi-well slides for 2 min and air dry them.

2. Harvest cells during logarithmic growth, fix aliquot with formalin (2% final concentration) for 1 - 24 h.

3. Centrifuge cells (at 14000 rpm)

4. pour off supernatant, add 1 ml of PBS and resuspend cells

5. repeat steps 3 and 4

6. Centrifuge cells (at 14000 rpm), pour off supernatant, add 1 ml of a 1:1 mix of PBS / ethanol and resuspend cells; at this stage samples can be stored at -20°C for several months.

7. Spot 2-20 µl of fixed cell suspension on gelatin-coated slide and air-dry.

8. Prepare hybridization buffers with different formamide concentrations as described above

9. Prepare hybridization vessels from polyethylene tubes as described above; use separate tubes for each concentration of formamide; the buffer reservoir in each air-tight vessel must be the correct hybridization buffer

10. For each hybridization (well) mix 1 µl of probe working solution and 9 µl of hybridization buffer.

11. Hybridize and wash as described

Assay: Cell brightness quantification by image analysis:

1. Hybridize fixed cells on multi-well slides at different concentrations of formamide.

2. Capture gray scale images in all samples at one fixed exposure condition; depicted cells must contain no overexposed pixels.

3. Prefilter gray scale image to enhance cell edges, e.g. by "Unsharp Masking": subtract a repeatedly low- pass filtered ("blurred") image from the original and subsequently multiply the resulting image by an appropriate factor (10 - 20).

4. Threshold the processed gray scale image to produce a binarized image containing only objects and background

5. Set a lower and upper levels of accepted object size in the binarized image to eliminate pixel noise and clustered cells.

6. Determine the cell brightnesses of all objects in the mask image by overlaying it with the original gray scale image. Average the mean object brightnesses of several images (5-10) to determine means and standard deviations per image.

Troubleshooting:

- To increase the specificity of probes that show only one mismatch with non-target sequences, it may be required to construct one or several competitor oligonucleotides. Such competitors are designed to perfectly match with the non-target sequence at the homologous site, and they are subsequently synthesized without fluorescent label. During hybridization, the competitor is added to the buffer at an equal concentration as the labeled probe. This strategy has, e.g., been successfully applied to construct a pair of 23S rRNA-targeted probes that discriminate between the beta and gamma subclasses of the *Proteobacteria* (Manz *et al.*, 1992).

- One may encounter unresolved bases (N, R, Y, K, ...) rather than mismatches at the target region of database sequences from non-target organisms. In this case, it is impossible to know if such sequences will be detected by the probe. However, after careful comparison with closely related sequences some intelligent guesses about the likelihood of accidental base identity may be possible (e.g., is the position conserved or highly variable etc.). If possible, check probe specificity experimentally.

- Sometimes we observe that FISH signal increases when adding some formamide to the hybridization buffer. We assume that formamide helps to enhance probe accessibility. Therefore during initial testing probes should be used with at least 10% of formamide in the hybridization buffer.

- A "quick and dirty" way to decide if newly designed probes are working at all (i.e. if the target site is in principle accessible) is to perform FISH of environmental samples at increasing levels of formamide followed by the counting of hybridized cells at different stringencies and a subjective estimate of signal intensity (e.g., in 3 arbitrary brightness classes).

- Frequently one cannot find perfect probes. Probe design represents a search for compromise of advantages (e.g., full group-coverage) and drawbacks (e.g. outside-group hits). One should also bear in mind that 15 to 30-base oligonucleotide probes are as reliable as the database from which they are constructed. The influx of new rRNA sequence information to the databases may eventually require a re-evaluation of probe specificity and even redesign of probes that can no longer be regarded specific. For the reliable detection of bacterial lineages or phylotypes without cultivated representatives, it is, therefore,

advantageous to construct at least 2 parallel probes to different positions of the target 16S rRNA(s).

APPLICATIONS:

During recent years FISH has been successfully applied in freshwater, coastal and offshore marine planktonic habitats, as well as in coastal sediments. It has been shown that the fraction of bacteria detectable by FISH corresponds well with the abundance of active cells as determined by microautoradiography in coastal marine bacterioplankton (Karner and Fuhrman, 1997). Group-specific probes for different subclasses of the *Proteobacteria* have, e.g., been utilized to study the composition of lake snow (Weiss *et al.*, 1996) and of the microbial assemblages in the plankton and winter cover of a mountain lake (Alfreider *et al.*, 1996), to demonstrate fundamental differences in the composition of freshwater and marine bacterioplankton (Glöckner *et al.*, 1999), or to show vertical zonations of the microbial communities in muddy and sandy Wadden Sea sediments (Llobet-Brossa *et al.*, 1998). The seasonal dynamics of members of major phylogenetic lineages and of several individual bacterial phylotypes have been studied in lake bacterioplankton (Pernthaler *et al.*, 1998). Blooms of members of the Cytophaga / Flavobacterium cluster have been described at the Antarctic marginal ice zone during a *Phaeocystis* bloom (Simon *et al.*, 1999). Archaeal seasonal abundances and vertical distributions in Antarctic coastal waters were followed by FISH with oligonucleotide and polynucleotide probes (Murray *et al.*, 1998; DeLong *et al.*, 1999). FISH was also applied in laboratory and field studies to prove that protistan predation can shift community composition in freshwater bacterioplanktonic assemblages (Pernthaler *et al.*, 1997b; Šimek *et al.*, 1997; Šimek *et al.*, 1999). Other applications of FISH with rRNA-targeted oligonucleotides in, e.g., soil or wastewater treatment systems, are too numerous to be listed here. An overview of early applications can be found in Amann *et al.* (1995).

CONCLUSIONS:

The low fluorescence intensities of hybridized aquatic bacteria still represent a partially unresolved problem of the method presented here. Due to low signal levels, it is, e.g., presently not feasible to use flow cytometry, the ideal tool for directly counting FISH-stained cells, in plankton samples. Microscopically, a variable fraction of cells detected by general nucleic acid staining (e.g., DAPI) are also visualized by FISH with more general probes. We find that, depending on the season, 30% to >80% of DAPI-stained objects in the German Bay hybridize with the general bacterial probe EUB338 (J.P. *et al.*, unpubl. data). Depending on the philosophy of the researcher, the FISH-undetectable fraction has been dismissed as dead "ghosts" (Zweifel and Hagström, 1995), or classified as living, non-growing bacteria. Relatively new groups of bacteria that were only recently found to be abundant in some environments by cultivation-independent methods, are however not detected by the bacterial probe EUB338 (Daims *et al.*, 1999). There is furthermore ample evidence that a fraction of the cells not hybridizing with a general bacterial probe (EUB338) are members of the archaea (DeLong *et al.*, 1994; Fuhrman and Ouverney, 1998). We believe that eventually even those cells will be detected with improved FISH techniques: Multiple-labeled polynucleotide probes have been described (Trebesius *et al.*, 1994; DeLong *et al.*, 1999) that promise to overcome the problem of low signal intensity in oligotrophic open ocean environments. However, the synthesis and testing of such probes is much more technically demanding than FISH with

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Table 1: Concentrations of NaCl in washing buffer (48°C) at different concentrations of formamide in hybridization buffer (46°C).

% formamide in hybridization buffer	mM NaCl in washing buffer
0	900
5	636
10	450
15	318
20	225
25	159
30	112
35	80
40	56
45	40
50	28
55	20
60	14
65	10
70	7
75	5
80	3.5

Table 2: Concentrations of NaCl in washing buffer (35°C) at different concentrations of formamide in hybridization buffer (35°C).

% formamide in hybridization buffer	mM NaCl in washing buffer
20	145
25	105
30	74
35	52
40	37
45	26
50	19
55	13
60	9
65	8
70	5

FIGURE LEGENDS:

Figure 1: Members of an uncultivated phylotype of beta Proteobacteria in the plankton of a mountain lake. Left panel: DAPI-stained cells, right panel: same microscopic field, bacteria detected by FISH

Figure 2: Schematic depiction of oligonucleotide binding

Figure 3: Effect of increasing cell counts on the precision of FISH results (plankton sample).

Figure 4: Melting curve equivalent of a probe targeted to an uncultivated freshwater bacterial phylotype, determined from hybridizations of environmental samples at increasing levels of formamide and subsequent image analysis (mean $\pm$ SE).