B. FISH with fluorescently monolabeled oligonucleotide probes

This section describes the hybridization with fluorescently monolabeled probes. Two formats are available: (1) the hybridization of cultured organisms on glass slides and (2) the hybridization of fixed cells on membrane filters

For extended protocols please cite: *Fuchs, B. M., J. Pernthaler, and R. Amann. 2007. Single cell identification by fluorescence in situ hybridization, p. 886-896. In C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. Marzluf, T. M. Schmidt, and L. R. Snyder (ed.), Methods for General and Molecular Microbiology, 3rd ed. ASM Press, Washington, D.C.*

Literature:

Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Systematic and Applied Microbiology 15:593-600.

Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F.-O. Glöckner, A. Wille, and R. Psenner. 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Applied and Environmental Microbiology 62:2138-2144.

Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K.-H. Schleifer. 1996. An in situ hybridization protocol for detection and identification of planktonic bacteria. Systematic and Applied Microbiology 19:403-406.

1. Hybridization of cultured organisms on glass slides *(Manz et al., 1992)*

In a quick, but non quantitative, protocol fixed cells are transferred onto gelatin coated slides and incubated in a moisture chamber with a buffer containing an oligonucleotide probe. After a short washing the cells are embedded in antifading reagent for microscopic visualization.

1. heat a 0.01% $CrK(SO_4)_2$ (prevents fouling of gelatin) / 0.1% gelatin solution to 65°C; dip precleaned multi-well slides in this solution; let air-dry;
2. spot 2-20 µl of fixed cell suspension (depending on cell density) in the wells of the gelatin-coated slide; let air-dry, then dehydrate for 3 minutes each in 50, 80 and 100% ethanol;

3. prepare hybridization buffer: see table 1;

Stock reagent	Volume	final concentration in hybridization buffer
5 M 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	2 µl	0.01%
(add SDS last to avoid precipitation)		

Table 1: Standard hybridization buffer

Note: The formamide concentration is dependent on the probe used and determines the stringency of the hybridization. Hybridization stringency may also be adjusted by temperature rather than by the chemical composition of buffers. We find that it is more convenient to keep incubator and water bath at one set temperature and to modulate the stringency by adding formamide.

4. for the hybridization mixtures add 1 volume of probe working solution (50 ng DNA μl^{-1}) to 9 volume of hybridization buffer in a 0.5-ml microfuge tube; keep probe solutions dark and on ice;

Note: The probe is fluorescently labeled and can be e.g. purchased from biomers.net (Ulm, Germany). The probe stock usually comes dried in a tube and needs to be diluted with sterile water according to the manufacturer's instructions. Alternatively, measure a small aliquot in the photometer and use the formula: 1 OD ~ 20 ng DNA μl^{-1} (see webpage).

5. prepare hybridization vessels from 50 ml polyethylene tubes: insert a piece of blotting paper into a polyethylene tube and soak it with the remaining hybridization buffer; use separate tubes for each concentration of formamide;
6. add 10 µl of hybridization mix to the samples in each well and place the slide into the polyethylene tube (in a horizontal position);
7. incubate at 46°C for at least 90 min (maximum: 3 hours);
8. prepare 50 ml of washing buffer (see table 2) in a polyethylene tube and preheat in a 48°C water bath

Stock reagent	Volume	final concentration in hybridization buffer
5 M NaCl	concentration depending on % formamide in hybridization buffer (see table A in appendix)	
1 M Tris / HCl	1 ml	20 mM
0.5 M EDTA (only if ≥ 20% formamide in hybridization!)	(500 µl)	5 mM
distilled H_2O	add to 50 ml	
10% SDS	50 µl	0.01%
(add SDS last to avoid precipitation)		

Table 2: Standard washing buffer

Note: The stringency in the washing buffer is achieved by adjusting the NaCl concentration. This avoids the use of excess amounts of formamide.

9. quickly rinse the slide carefully with a bit of washing buffer, transfer slide into preheated washing buffer and incubate for 25 min at 48°C (water bath);
10. rinse slide with distilled H_2O, let air-dry;
11. for counterstaining cover each well with 10 µl of a 1 µg ml-1 DAPI solution, and incubate for 3 minutes; rinse slide with distilled H_2O let air-dry;
12. samples are mounted in a 4:1 mix of Citifluor (Citifluor Ltd, London, U.K) and Vecta Shield (Vector Laboratories, Inc., Burlingame, CA); Vecta Shield contains a superior antibleaching reagent, but quenches DAPI fluorescence; the wells 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 mounted 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 probe signal; for counting, it is, therefore, safer to first quantify probe stained cells and subsequently all cells from the same field of vision in UV excitation;

2. Hybridization of fixed cells on membrane filters *(Glöckner et al., 1996)*

1. Cut sections from membrane filters with a razor blade and label filter sections with a pencil, e.g., by numbering them.
2. Put filter sections on glass slides (cells facing up!), several filter sections can be placed on one slide and for simultaneous hybridization with the same probe.
3. Prepare 2 ml of hybridization buffer in a microfuge tube (360 µl 5 M NaCl, 40 µl 1M Tris/HCl, formamide % depending on probe, add water to 2 ml, add 2 µl SDS (10%)).
4. Remove an aliquot of 20 µl per filter piece into a separate cap and add 2 µl probe working solution (concentration: 50 ng DNA $µl^{-1}$) per filter piece.
5. Prepare moisture chamber by putting a piece of blotting paper into a 50 ml polyethylene tube and soaking it with the remaining hybridization buffer without probe (see above).
6. Carefully cover the filter section with the hybridization mix 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 (maximum: 3 hours).
8. Meanwhile prepare 50 ml of washing buffer in a polyethylene tube (X ml 5M NaCl, depending on formamide concentration in the hybridization buffer (see table A in Appendix), 1 ml 1 M Tris/ HCl, 500µl 0.5M EDTA (if formamide concentration of the hybridization buffer was ≥20%), add to 50 ml with water, 50 µl 10% SDS).
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 app. 50 µl of DAPI solution (1 µg ml^{-1}), and incubate for 3 min. Afterwards wash filter sections subsequently for 1 min. in distilled H_2O and for 1 min. in 80% ethanol to remove unspecific staining. Let air-dry.
12. Samples are mounted in a 4:1 mix of Citifluor and Vecta Shield. The filter sections have to be completely dry before embedding, otherwise part of the cells might 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.

Notes:

- 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.
- Therefore, do not attempt to determine absolute cell counts from filters after hybridization, but only the percentage of hybridized cells. Additionally, 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.
- At least 500 DAPI-stained cells should be counted per hybridized filter piece to reduce the counting error <5% (see also: Pernthaler, J., A. Pernthaler, and R. Amann. 2003. Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. Applied and Environmental Microbiology 69:2631-2637.).

Appendix

Table A: NaCl concentration in the washing buffer according to % formamide of the hybridization buffer (depending on probe).

	Washing at 48°C	
% formamide in hybridisation buffer	[NaCl] in M endconcentration	µl 5 M NaCl in 50 ml
0	0.900	9000
5	0.636	6300
10	0.450	4500
15	0.318	3180
20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	-	-
70	-	-