C. CARD FISH

In environmental samples, single oligonucleotides carrying only one fluorochrome may not provide enough fluorescence signal to detect cells with **low ribosome content** (Pernthaler et al., 2002). **Polynucleotide** probes with a length of more than 100 nucleotides carrying several fluorochromes are an alternative (Trebesius et al., 1994;DeLong et al., 1999). However, these probes **lack specificity** for narrow target groups which are at the level of species and genera. An alternative labeling technique that increases fluorescence signals intensity uses **horseradish-peroxidase (HRP-) labeled oligonucleotides**. When using HRP-labeled probes, fluorescent staining results from a **secondary incubation** with **fluorescently labeled tyramide**. The specifically bound peroxidase molecules catalyze the deposition of these labeled reporter compounds within cells targeted by the HRP tagged probe. FISH signals are up to **20-fold brighter** with HRP labeled probes than with conventional single labeled probes (Schönhuber et al., 1997). However, cell permeabilization protocols need to be adjusted in order to enable the larger enzyme-labeled oligonucleotides to penetrate into the cells (Pernthaler et al., 2002).

Depending on the sample the protocols for CARD-FISH differ substantially. Generally there are two additionally steps in the protocols, embedding in agarose and tyramide incubation. The former is necessary to prevent cell loss due to the permeabilisation. It is also thought to have a cell stabilising effect. Tyramide incubation is needed for mediating the signal amplification process.

Here we present protocols for (1) plankton, (2) sediment, and (3) tissues.

NOTE: Please refer to page 8 for detailed information on the hybridization, washing and CARD amplification buffers. They apply to all three protocols.

Literature:

Pernthaler, A., J. Pernthaler, and R. Amann. 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Applied and Environmental Microbiology 68:3094-3101

Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger, and K.-H. Schleifer. 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Applied and Environmental Microbiology 60:3228-3235.

DeLong, E., L. Taylor, T. Marsh, and C. Preston. 1999. Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Applied and Environmental Microbiology 65:5554-5563.

Schönhuber, W., B. Fuchs, S. Juretschko, and R. Amann. 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Applied and Environmental Microbiology 63:3268-3273.

Thiele, S., B. Fuchs and R. Amann. 2010. Identification of microorganisms using the ribosomal RNA approach and fluorescence in situ hybridization. In Treatise on water science, volume 3, chapter 56. Ed. Frimmel, F. and P. Wilderer. Elsevie, Kidlington, UK.

1. CARD-FISH protocol for planktonic samples on membrane filters (according to Pernthaler et al., 2004)

please cite for this protocol: *Pernthaler, A., J. Pernthaler, and R. Amann. 2004. Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms, p. 711-726. In G. Kowalchuk, F. J. de Bruijn, I. M. Head, A. D. L. Akkermans, and J. D. van Elsas (ed.), Molecular Microbial Ecology Manual, 2nd 3.11 ed. Kluwer Academic Publishers, Dordrecht, Boston, London.*

Embedding

A. Boil low gelling point agarose (0.1%, gel strength should be approx. 1000 g cm^{-2}) and fill the agarose in a pre-warmed petri dish and let it cool down to 35-40°C.
B. Dip filter with both sides in the agarose and place it face-down (shiny side with bacteria down!) onto a parafilm covered, even surface (e.g. glass plate), let dry; Temperature for drying is not crucial, anything between 20 and 50°C is fine.
C. Remove filters from surface by soaking in 80 – 96% ethanol.
D. Let the filter air dry on a piece of tissue paper.

Permeabilization

(Permeabilization with lysozyme proofed to be the optimal method for most of the marine planktonic bacteria)

A. Incubate filter in 100 ml of fresh lysozyme solution in a small petri dish (10 mg ml^{-1} in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0) for 60 min. at 37°C.
B. Wash in excess MilliQ water.

(No ethanol washing is necessary at this point; proceed immediately to the inactivation of endogenous peroxidases.)

Inactivation of endogenous peroxidases

A. Incubate in 0.01 M HCl for 10-20 min. at RT. Wash filters well in excess MilliQ water and 96% ethanol and let the filter air dry on a paper.

If you have problems with autofluorescence of your cells, an additional incubation in 3% H_2O_2 in water for 10 min @ RT may help.

Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Prepare a humidity chamber by inserting a piece of tissue paper in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer. Mix 300 µl hybridization buffer (see section 4) with probe working solution (50 ng DNA μl^{-1}) in a ratio 300:1 (i.e. 1 µl) to 100:1 (i.e. 3 µl) depending on sample.

2. Dip each filter completely into the hybridization solution and place filters face-up onto a parafilm covered glass slide; spread the rest of the solution evenly onto the filters. Close tube firmly and keep the tube in a horizontal position.
3. Incubate at 46°C for 2-3 h (coastal water) or overnight for oligotrophic/open ocean water samples.

Washing

Transfer filter to prewarmed (48°C) washing buffer (see section 4) by immersing the whole slide in the buffer => filter will swim off

1. Wash filters for 10 min. at 48°C.
2. Transfer filters to 1 x PBS (do not let filter run dry!) and incubate for 15 min at RT.
3. To remove excess liquid, dab filter on blotting paper, but **do not let filter run dry!**

CARD

1. Prepare a moisture chamber by inserting a piece of tissue paper in a 50 ml tube and soak it with 2 ml water.
2. Prepare a fresh solution of H_2O_2 (0.15% in PBS), keep it cool.
3. Mix amplification buffer with H_2O_2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient) and add fluorescently labeled tyramide [1 mg dye ml^{-1}] and mix well, keep in the dark (The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1000:1; if the signal is not sufficient: in-/decrease the ratio of added tyramide).
4. Dip filter completely in the amplification mix, place filter sections face-up on a parafilm covered glass slide and spread the rest of the amplification mix over the filters.
5. Put the slides into the humidity chamber and incubate at 46°C for up to 45 min in the dark.
6. To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 5-10 min at RT in the dark (or: 15 min at 46°C on a shaker).
7. Wash filters thoroughly in excess with deionized water. Therefore use a Büchner funnel and MQ water to create a gyre in the funnel. Then wash filters thoroughly twice in excess in 96% ethanol (1-2 min), let completely air dry in the dark before counterstaining with DAPI.
8. For counterstaining put filter sections on a glass plate, cover with 50 µl of DAPI solution, and incubate for 3 minutes; afterwards wash filter sections for several seconds in 80% ethanol to remove unspecific staining followed by rinsing in distilled H_2O and air-drying.
9. 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.
10. Double stained and air dried preparations as well as filters mounted on slides can be stored in the dark at -20°C without substantial loss of probe fluorescence.

2. CARD-FISH protocol for sediment samples on membrane filters (according to Ishii et al., 2004)

please cite for this protocol: *Ishii, K., M. Mußmann, B. J. MacGregor, and R. Amann. 2004. An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. FEMS Microbiology Ecology 50:203-212.*

Sonication and filtration

(Sonication protocol depends on sediment type and sampling depth, for some sandy sediments the following procedure turned out to be successful)

1. Put fixed sediment sample on ice and sonicate it 7 times for 30 sec with a 30 sec break in between with 1 pulse per second and an intensity of 20% (Sonopuls HD 70, Bandelin, Berlin, Germany)
2. Vortex sample and filtrate suitable amounts of the supernatant onto polycarbonate filter (pressure: -200 to 400 mbar)
3. Wash twice with sterile MQ or 1 x PBS pH 7.6
4. Let air dry *

Embedding

1. Boil low gelling point agarose [0.1%, gel strength should be approx. 1000 g cm^{-2}] and let it cool down to 35-40°C.
2. To avoid cross-contamination dip each filter with both sides in a separate portion of agarose and place it face-down onto a parafilm covered, even surface (e.g. glass plate), let dry at 20 – 50°C
3. Remove filters from surface by soaking in 80 – 96% ethanol

(Use a lot of ethanol for removal of the filter, if the filter sticks to the parafilm - use more ethanol)

4. Let the filter air dry on Kimwipes/ Whatman paper*

Permeabilization

(Permeabilization with lysozyme proofed to be the optimal method for most of the marine planktonic bacteria)

1. Incubate filter in fresh lysozyme solution [10 mg ml^{-1} in 0.05 M EDTA, pH 8.0; 0.1M Tris-HCl, pH 8.0] for 60 min at 37°C
2. Wash in excess MilliQ water

(No ethanol washing is necessary at this point, proceed immediately to the inactivation of endogenous peroxidases.)

Inactivation of endogenous peroxidases

1. Incubate in H_2O_2 (0,15% in methanol) for 30 min at RT

2. Alternative: incubate in 0.1 M HCl for 30-60 sec at RT, wash in 1 x PBS, incubate in H_2O_2 (3% in MQ) for 10 min at RT
3. Wash filters well in excess MilliQ water
4. Wash 96% ethanol and let the filter air dry on Kimwipes/ Whatman paper*.

Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Mix hybridization buffer (see section 4) with probe working solution [50 ng DNA μl^{-1}] in a ratio 300:1;
2. Dip each filter completely into the hybridization solution and place filters face-up onto a parafilm covered glass slide; spread the rest of the solution evenly onto the filters; close tube firmly and keep the tube in a horizontal position (e.g. in an appropriate rack)
3. Incubate at 46 °C for 2-3 h (coastal water) or overnight for oligotrophic/open ocean water samples

Washing

Transfer filter to the washing buffer (see section 4) by immersing the whole slide in the prewarmed washing buffer => filter will swim off

1. Wash filters in prewarmed washing buffer (10 min, 48°C)
2. Transfer filters to 1 x PBS (do not let filter run dry!) and incubate for 15 min @ RT
3. To remove excess liquid, dab filter on blotting paper, but **do not let filter run dry**

CARD

1. Prepare a fresh solution of H_2O_2 (0,15% in PBS), keep it cool
2. Mix amplification buffer with H_2O_2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient)
3. Add fluorescently labeled tyramide [1 mg dye ml^{-1}] and mix well, keep in the dark

(The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: in-/decrease the ratio of added tyramide.)

4. Dip filter completely in the amplification mix, place filter sections face-up on a parafilm covered glass slide and put this e.g. into a petri dish; spread the rest of the amplification mix over the filters and incubate at 46°C for up to 45 min. in the dark!
5. To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 5-10 min @ RT in the dark
6. Wash filters thoroughly in excess MQ and twice in 96% ethanol (~1 min), let completely air dry in the dark before counterstaining with DAPI
7. To keep the background fluorescence low, it is important to wash the filters in large volumes of water and ethanol

* Filters may now be stored in the freezer without apparent loss in CARD-FISH signal intensity. RT: room temperature

3. CARD-FISH protocol for paraffin-embedded samples on slides (according to Blazejak et al., 2005, modified)

please cite for this protocol: *Blazejak, A., C. Erseus, R. Amann, and N. Dubilier. 2005. Coexistence of Bacterial Sulfide Oxidizers, Sulfate Reducers, and Spirochetes in a Gutless Worm (Oligochaeta) from the Peru Margin. Applied and Environmental Microbiology 71:1553-1561.*

Attention: This protocol is for a hybridization temperature of 35°C (= add 20% more formamide in hybridization buffer than for hybridization at 46°C!)

Prehybridization treatments for paraffin-embedded samples

- Incubate slide as follows (in following order of steps:)

1. RotiHistol® 100% 10 min RT
2. RotiHistol® 100% 10 min RT
3. RotiHistol® 100% 10 min RT
4. Ethanol 95% 10 min RT
5. Ethanol 80% 10 min RT
6. Ethanol 70% 10 min RT
7. HCl 0.2 M 12 min RT
8. TrisHCl 20 mM 10 min RT
9. Proteinase K 0.5 µg ml^{-1} 5 min 37°C
10. TrisHCl 20 mM 10 min RT

- Let air dry

(Incubations except for step 7, 9 and 10 might be shortened to 6 min.)

- Encircle each section with an immunostaining Pen (wax) to ensure that the solutions (hybridizations) do not run into each other.

Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue to the bottom of a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Cover sections with hybridization buffer (see section 4), insert slide into preheated humidity chamber, close tube firmly and keep the tube in a horizontal position (e.g. in an appropriate rack), incubate for 20 min at 35°C
2. remove buffer before the application of the new solution
3. Mix hybridization buffer with probe working solution [50 ng DNA μl^{-1}] in a ratio 100:1 and apply onto sections
4. For each section 50 µl are sufficient
5. Incubate at 35°C for 3 h in horizontal position

Washing

1. Remove hybridization buffer by dabbing the slide onto tissue; be careful to avoid mixing of different probes
2. (Quick) briefly dip the slide in first washing buffer (see section 4)
3. Wash the slide in second washing buffer for 15 min at 35°C
4. Incubate slide in 1xPBS pH 8.0, shaking at RT for 15 min

Depending on background signal the incubation may last up to 1 h

CARD

1. Prepare a new humidity chamber by inserting a tissue to the bottom of a 50 ml tube and soak with MQ
2. Prepare a fresh solution of H_2O_2 (0,15% in PBS), keep it cool
3. Mix amplification buffer with H_2O_2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient)
4. Add fluorescently labeled tyramide [1 mg dye ml^{-1}] and mix well, keep in the dark

The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: in-/decrease the ratio of added tyramide.

5. Apply amplification mix onto sections and incubate at 37°C for 20 min in the humidity chamber, keep dark!
6. To remove excess liquid, dab slide on blotting paper and incubate in 1 x PBS for 15 min at RT in the dark
7. Wash slide in MQ for 1 min at RT in the dark, let completely air dry before counterstaining with DAPI

4. Preparation of buffers for CARD-FISH (for all three protocols)

Hybridization buffer (final volume 20 ml)

3.6 ml 5 M NaCl
0.4 ml 1 M Tris HCl, pH 8.0
20 µl SDS (20% w/v)
x ml formamide (depending on probe, see Appendix, table 1)
y ml sterile dH_2O (depending on probe, see Appendix, table 1)
2.0 ml Blocking Reagent (10%, Roche, Basel; prepare according to manufacturer's instructions)
2.0 g of dextran sulfate

Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. Small portions of the buffer can then be stored at –20°C for several months.

Washing buffer (produce freshly when needed, final volume 50 ml)

0.5 ml 0.5 M EDTA, pH 8.0 (only if ≥ 20% formamide is used in hybridization)
1.0 ml 1 M Tris HCl, pH 8.0
x µl NaCl (depending on probe and hybridization temperature, see Appendix, table 2 and 3)
add dH_2O to a final volume of 50 ml
25 µl SDS (20% w/v)

The NaCl concentration in the washing buffer, as well as the formamide concentration of the hybridization buffer determines the stringency of the hybridization at the selected temperature.

CARD amplification buffer (final volume 40 ml)

2 ml of 20 × PBS, pH 7.6 (important for proper enzyme function)
0.4 ml Blocking Reagent (10%, see above)
16 ml 5 M NaCl
add sterile dH_2O to a final volume of 40 ml
add 4 g of dextran sulfate

Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. The amplification buffer can be stored in the refridgerator for several weeks.

Appendix

Table 1: Volumes of formamide and water for 20 ml of hybridization buffer		
% formamide in hybridization buffer	ml formamide	ml water
0	0	14
5	1	13
10	2	12
15	3	11
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

Table 2: NaCl concentration in the washing buffer for washing at 48°C after hybridization at 46°C		
% formamide in hybridization buffer	[NaCl] in M (final concentration)	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	-	-

Table 3: NaCl concentration in the washing buffer for washing at 35°C after hybridization at 35°		
% formamide in hybridization buffer	[NaCl] in M final concentration	µl 5 M NaCl in 50 ml
20	0.145	1350
25	0.105	950
30	0.074	640
35	0.052	420
40	0.037	270
45	0.026	160
50	0.019	90
55	0.013	30
60	0.009	0
65	0,008	0
70	0,005	0