

Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms

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Introduction

Fluorescence in situ hybridization (FISH) with rRNA targeted oligonucleotide probes has developed into an invaluable molecular tool for the identification of microorganisms in the environment [1–3, 7, 9–11, 14, 15, 19, 22, 25]. However, applications may be hindered by restricted detection sensitivity. Most bacteria in aquatic habitats are small, slowly growing, or starving [20], and therefore may contain a low number of ribosomes per single cell. Since the fluorescence intensities of hybridized cells depend on the concentration of the probe target, i.e. the ribosomal RNA, hybridization signals were frequently below the detection limits or lost in high background.

Recently, a novel FISH protocol was developed for the reliable identification of small bacterial cells in marine plankton and benthos [21]. This approach is based on the use of horseradish peroxidase (HRP) labeled oligonucleotide probes and tyramide signal amplification, also termed catalyzed reporter deposition (CARD).

CARD was introduced more than a decade ago for immunoblotting and immunosorbent assays [6]. A few years later, Kersten et al. [18] and Raap et al. [24] reported great increase in FISH signal intensity following various CARD strategies. CARD is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity. HRP reacts with hydrogen peroxide and the phenolic part of labeled tyramide to produce a quinone-like structure bearing a radical on the C2 group [23]. This “activated” tyramide then covalently binds to tyrosine residues in the target cell. Each HRP conferred by a probe catalyzes the deposition of many labeled tyramides. This results in greatly enhanced FISH sensitivity as compared to probes with a single fluorochrome.

CARD in combination with nucleic acid probes is routinely used in histology and cytochemistry to localize specific nucleic acid sequences (DNA, RNA) in microscopic preparations of tissues, cells, and chromosomes [17, 28], and allows the detection of rare and even single-copy-number targets (mRNAs, genes) [8]. To date, a wide variety of research and diagnostic applications have been described,

making this technique an integral part of studies of gene mapping, gene expression, RNA processing and transport, the three-dimensional organization of the nucleus, tumor genetics, microbial infections, and prenatal diagnosis [27].

CARD-FISH procedure for aquatic bacteria

CARD-FISH is a multistep procedure, including: (1) preparation of the biological material, (2) nucleic acid probe selection, (3) hybridization with labeled probe, (4) cytochemical probe detection, and (5) microscopy. Preparation of the specimen for CARD-FISH involves routine fixation to obtain maximum retention of target nucleic acid sequences and morphology. Such fixatives are, for example, buffered (para)formaldehyde and/or ethanol. Usually, cells need to be concentrated and/or immobilized. Plankton samples may be filtered onto polycarbonate membrane filters, sediment samples can be attached to gelatin- or poly-L-lysine coated glass slides. Depending on the cell walls of the target organisms and the fixation, different permeabilization strategies need to be applied, such as treatment with various enzymes, diluted HCl or detergents, thereby facilitating access of the labeled nucleic acid probes and cytochemical detection molecules.

Three types of probes are mainly used for FISH, i.e. double-stranded DNA probes, single-stranded RNA (or DNA) probes, and oligonucleotide probes. For use in combination with CARD, these probes are labeled with a hapten (such as fluorescein, digoxigenin, biotin), which can then be detected immunochemically using an anti-hapten-antibody conjugated to a peroxidase. Oligonucleotide probes can also be directly linked to horseradish peroxidase (HRP).

Several reviews and protocols are available on the principles of hybridization [4, 29] as well as on methodological aspects and applications of FISH with rRNA-targeted oligonucleotides [2, 3]. Here we will focus on the use of oligonucleotide probes labeled with HRP. Those probes can either be purchased (e.g. www.biomers.net, Ulm, Germany) or labeled with a procedure described elsewhere [30].

Quality check of probes

Purified probe stocks are frequently delivered lyophilized. To determine the exact probe concentration the probe has to be reconstituted with 50 μl sterile water. The absorbance of the 1:100 diluted stock solution should be measured at 260 nm. Since both, the HRP and the probe contribute to the absorption maximum at 260 nm, the probe concentration has to be lowered by a correction factor (cf) of 0.276.

$$\text{OD}_{260} = \text{measuredOD}_{260} - \text{measuredOD}_{404} \times \text{cf}$$

After this correction, 1 $\text{OD}_{260\text{nm}}$ is equivalent to approximately 20 $\text{ng } \mu\text{l}^{-1}$ of single stranded DNA oligonucleotide. Furthermore the labeling of the oligonucleotide

should be checked. The absorption of the peroxidase is measured at 404 nm (A_{404}). Presuming optimal labeling the peak ratio (OD_{260} / OD_{404}) should be around 3.

Working solutions are prepared at a concentration of $50 \text{ ng } \mu\text{l}^{-1}$ and stored in small portions (50 to 100 μl) in the dark at $-20 \text{ }^\circ\text{C}$. Once thawed, the HRP labeled probes should be stored in the refrigerator, since repeated freeze-thawing will damage the peroxidase. At $4 \text{ }^\circ\text{C}$ the probe working solutions are stable for up to 6 months.

Procedures

Use powder free gloves, since the powder may cause background fluorescence in the microscopic preparations.

Synthesis of tyramide conjugates

The tyramide labeling procedure is modified from Hopman et al. [16].

Reagents

- Dimethylformamide
- Triethylamine
- Tyramine HCl
- Succinimidyl esters of 5- (and 6-) carboxyfluorescein, Alexa₅₄₆, Alexa₄₈₈, or Alexa₃₅₀ (Molecular Probes Inc., Eugene OR, USA)

Succinimidyl esters can hydrolyze rapidly, therefore all reagents have to be water free. The active dye stock as well as the tyramine HCl stock must be prepared a few minutes before use.

Solutions:

- Tyramine HCl stock:
 - 10 μl triethylamine
 - 1 ml dimethylformamide
 - 10 mg tyramine HCl
- Active dye stock
 - 1 mg succinimidyl ester (Alexa₅₄₆, Alexa₄₈₈)
 - 100 μl dimethylformamide
- or:
 - 5 mg succinimidyl ester (Alexa₃₅₀)
 - 500 μl dimethylformamide

or:

100 mg succinimidyl ester (5- (and 6-) carboxyfluorescein)

10 ml dimethylformamide

Reaction:

1. Add active dye ester in 1.1 fold molar excess to tyramine HCl stock solution:

100 μ l Alexa₄₈₈ stock + 25.2 μ l tyramine HCl stock

100 μ l Alexa₅₄₆ stock + 14.7 μ l tyramine HCl stock

500 μ l Alexa₃₅₀ stock + 193 μ l tyramine HCl stock

10 ml (5- (and 6-) carboxyfluorescein) stock + 3.3 ml tyramine HCl stock

Incubate for 6 to 12 hours at room temperature in the dark. Then, dilute reaction mixture with absolute ethanol to a final concentration of 1 mg active dye per ml. Dispense portions of 20 μ l and dessicate them in a freeze dryer or under vacuum at room temperature. Dessicated tyramides are stable for years if stored at -20°C . For use, tyramides are reconstituted in 20 μ l of sterile deionized water (MilliQ) (Millipore, Eschborn, Germany) or dimethylformamide containing 20 mg ml⁻¹ p-iodophenylboronic acid (IPBA). In our lab we dissolve tyramides labeled with Alexa₅₄₆, Alexa₄₈₈, or Fluorescein in dimethylformamide (final concentration 1 mg ml⁻¹) containing 20 mg ml⁻¹ IPBA. Water free dimethylformamide prevents a rapid hydrolysis of the IPBA, which can enhance the deposition of several fluorescently labeled tyramides [5], for details see section: Catalyzed Reporter Deposition. The Alexa₃₅₀ labeled tyramide should be dissolved in MilliQ water. Tyramides in dimethylformamide can be stored in the freezer, tyramides in aqueous solution should be stored in the refridgerator.

Sample processing

Cell wall compositions of bacteria and archaea from different environments may be diverse. Therefore one has to consider different permeabilization and fixation strategies for each cell type. Here we report protocols for marine planktonic archaea and bacteria, for marine benthic bacteria, and for freshwater samples dominated by actinobacteria.

To prevent high cell loss during the permeabilization procedure, the cells have to be attached onto polycarbonate filters, for example

with agarose. Although our procedure did not cause cell loss in any of our samples, we recommend that cell densities are checked before and after pretreatment and hybridization.

Cell fixation and filtration

1. Add buffered particle free (para)formaldehyde (final concentration 1% to 2% [v/v]) to the sample and fix at 4 °C for 24 hours, or at room temperature for 1 hour. This works well for marine plankton and benthos. For freshwater *Actinobacteria*, fixation in 50% (v/v) ethanol for 24 hours at 4 °C is recommended [26].
2. Filter the samples gently (pressure approximately 5 mm Hg) onto white polycarbonate filters (pore size 0.2 µm, GTTP, Millipore, Eschborn, Germany) and wash twice with 5 to 10 ml of particle free deionized water.
3. The filters can then be stored at –20 °C for several months or years.

Attachment

1. Prepare 0.1% (w/v) low gelling point agarose (gel strength should be approximately 1000 g cm⁻²) in MilliQ water.
2. Boil the agarose before each use in a microwave oven.
3. Fill the agarose in a petri dish and let it cool down to 40 to 35 °C.
4. Dip the filters with both sides into the agarose and put the filters face-down onto a clean glass plate or onto parafilm.
5. Let the filters dry at 20 to 40 °C for approximately 10 to 30 minutes.
6. To remove the filters from the glass plate, pipet ethanol (96% to 80% [v/v]) onto the filters and carefully peel them off.
7. Let the filters air dry on a paper tissue.

Inactivation of endogenous peroxidases

Some microorganisms, especially in anaerobic marine sediments, may carry peroxidases or proteins with pseudoperoxidase activity. This can be tested by incubating a filter section in amplification buffer containing H₂O₂ and fluorescently labeled tyramides. Cells with peroxidase activities will show bright fluorescence. For details see section: Catalyzed Reporter Deposition.

These enzymes have to be inactivated, for example by treatment with hydrochloric acid:

1. Incubate the filter sections in 50 ml of 0.01 M HCl for 10 minutes at room temperature.
2. Wash filters in 50 ml of 1 × phosphate buffered saline ([PBS] 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.6), then in 50 ml of MilliQ water. Filter sections can then be further processed or air dried and stored in a refrigerator.

Permeabilization with Lysozyme

1. Prepare 50 ml of a fresh lysozyme solution (0.05 M EDTA, pH 8.0; 0.1 M Tris HCl, pH 8.0; lysozyme [10 mg ml⁻¹], [Fluka, Taufkirchen, Germany]).
2. Incubate the filters in the lysozyme solution for 60 minutes at 37 °C.
3. Wash the filters twice in 50 ml of MilliQ water, then in 50 ml of absolute ethanol. Let filters air dry.

Permeabilization with Achromopeptidase

For CARD-FISH of freshwater Actinobacteria, cell walls need to be permeabilized with lysozyme, followed by a digestion with achromopeptidase (Enzyme Commission Number: 3.4.21.50, Sigma-Aldrich, Seelze, Germany) [26].

1. Prepare 50 ml of a fresh achromopeptidase solution (0.01 M NaCl, 0.01 M Tris HCl, pH 8.0, achromopeptidase [60 U ml⁻¹]).
2. Incubate filters in the achromopeptidase solution for 30 minutes at 37 °C.
3. Wash filters as described above.

This protocol might be suitable for other samples rich in Gram-positive bacteria. As an alternative to enzymatic digestion, cell walls can also be treated with diluted acid or detergents. These strategies may be useful, for example for the permeabilization of archaeal cells.

Permeabilization with hydrochloric acid

1. Incubate filters for 30 seconds in 50 ml of 0.1 M HCl.
2. Transfer filters to 50 ml of 1 × PBS (pH 7.6).
3. Wash filters as described above.

Permeabilization with sodiumdodecyl sulfate (SDS)

1. Incubate filters for 10 to 15 minutes in 50 ml of 1 × PBS (pH 7.6), containing 0.5% (w/v) SDS.
2. Wash filters as described above.

Pretreatment with dethylpyrocarbonate (DEPC)

For plankton samples from North Atlantic deep sea we use DEPC alone to permeabilize the cells and inactivate endogenous RNases and peroxidases.

1. Mix 50 µl DEPC with 50 ml 1 × PBS (pH 7.6) by vortexing.
2. Add to filter sections immediately and incubate at room temperature for 12 minutes
3. Wash filters as described above.

After permeabilization, the filters can be stored at –20 °C for several months.

Preparation of hybridization buffer and washing buffer

Solutions

- 5 M NaCl
- 1 M Tris HCl, pH 8.0
- formamide, molecular biology grade (Fluka, Taufkirchen, Germany)
- 0.5 M EDTA, pH 8.0
- 20% [w/v] SDS
- 10% [w/v] Blocking Reagent (Roche, Basel, Switzerland)

For the preparation of hybridization buffer, pipet in a 50 ml tube:

- 3.6 ml 5 M NaCl
- 0.4 ml 1 M Tris HCl
- 20 µl SDS
- x ml MilliQ water (See Table 1)
- x ml formamide (See Table 1)
- 2.0 ml Blocking Reagent

Add 2.0 g 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.

Table 1. Volumes of formamide and water for 20 ml of hybridization buffer

% formamide in hybridization buffer	ml formamide	ml water
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

For preparation of washing buffer pipet in a 50 ml tube:

- 0.5 ml EDTA
- 1.0 ml Tris HCl
- x μ l NaCl (volume see Table 2)
- add MilliQ water to a final volume of 50 ml
- 25 μ l SDS

The NaCl concentration in the washing buffer, as well as the formamide concentration of the hybridization buffer determine the stringency of the hybridization at a certain temperature.

Hybridization

Solutions

- HRP labeled oligonucleotide probe [50 ng μ l⁻¹] (www.biomers.net, Ulm, Germany)
 - hybridization buffer (as described above)
 - washing buffer (as described above)
1. Cut filters in sections (for example, about 16 sections can be cut out of a filter with a diameter of 47 mm). Label sections with a lead pencil, since other markers might contain fluorescent compounds.
 2. Mix hybridization buffer and probe (300 : 1). Place filter sections in a reaction vial (0.5 ml to 2 ml, depending on the number of sections)

Table 2. Volumes of 5 M NaCl in 50 ml of washing buffer with corresponding formamide concentration in the hybridization buffer. The Na⁺ concentration is calculated for stringent washing at 37 °C after hybridization at 35 °C.

% formamide in hybridization buffer	μl of 5 M NaCl
20	1350
25	950
30	640
35	420
40	270
45	160
50	90
55	30
60	0
65	0
70	0

and pipet the hybridization mix onto the sections. At least 2/3rd the total volume of the reaction vial should be filled with buffer. Hybridize on a rotation shaker (appr. 10 rpm) for 2 to 15 hours at 35 °C.

3. For stringent washing, prepare washing buffer and preheat at 37 °C. Wash sections after hybridization for 5 minutes in 50 ml of washing buffer.

Do not let the filter sections run dry, this will reduce the activity of the HRP.

Catalyzed reporter deposition (cytochemical probe detection)

Solutions

- 20× PBS (pH 7.6)
- 10% Blocking Reagent (Roche, Basel, Switzerland)
- 5 M NaCl
- 30% [v/v] H₂O₂
- fluorescently labeled tyramide [1 mg ml⁻¹]
- PBS

Preparation of Amplification buffer

Pipet in a 50 ml tube:

- 2 ml 20 × PBS
- 0.4 ml Blocking Reagent
- 16 ml NaCl
- add sterile MilliQ water to a final volume of 40 ml

Add 4 g dextran sulfate. Heat (40 to 60 °C) and shake until the dextran sulfate has dissolved completely. The amplification buffer can be stored in the refrigerator for several weeks.

1. To equilibrate the probe delivered HRP, remove the sections from the washing buffer and incubate them in 50 ml PBS for 15 minutes at room temperature.
2. Prepare fresh 100 × H₂O₂ stock by mixing 1000 µl of 1 × PBS with 5 µl of 30% H₂O₂.
3. Mix 1000 µl of amplification buffer with 10 µl of the 100 × H₂O₂ stock and 1 to 5 µl of fluorescently labeled tyramide.
4. Put filter section in a reaction vial and pipet the amplification buffer with the tyramide onto the sections. Incubate for 10 to 15 minutes in the dark at temperatures between 37 °C and 46 °C (FISH signal intensity increases with temperature).
5. Remove excess liquid by dabbing filters onto blotting paper. Wash sections in 50 ml of PBS for 5 to 15 minutes at room temperature in the dark.
6. Wash sections in 50 ml of deionized water, then in 50 ml of absolute ethanol. Let sections air dry. The filter section can now be counterstained (e.g. with the DNA stain 4',6'-diamidino-2-phenylindol, DAPI, see also Table 3). Microscopy is performed after embedding in Citifluor (Citifluor Ltd., London, U.K.). Sections could also be stored at -20 °C until further processing.

The tyramide signal amplification can be enhanced by the addition of salts [5]. The deposition of tyramides labeled with Cy3, fluorescein, Alexa₃₅₀, Alexa₄₈₈ and Alexa₅₄₆ is enhanced by presence of NaCl. Preferably, concentrations of NaCl in the amplification buffer range from 2 M to saturation. p-iodophenylboronic acid (IPBA, 20 mg IPBA per 1 mg of tyramide) will also enhance the CARD-FISH signal. This works

Table 3. CARD-FISH procedure for North Atlantic deep sea picoplancton.

Fixation & Filtration	Fix plankton samples with 1% buffered paraformaldehyde (pH 7.6) for 10 to 20 hours at 4 °C Filter samples onto polycarbonate filters (pressure: 5 mm Hg) Wash twice with 10 ml of sterile MilliQ water Let air dry*
Embedding	Dip filter in 0.1% low gelling point agarose Place filters face-down onto parafilm, let dry at 46 °C Remove filters from glass surface by soaking in 80–96% ethanol Let air dry*
Permeabilization	Incubate in 50 ml of fresh 0.1 % diethylpyrocarbonate in 1 × PBS (pH 7.6) at RT for 12 minutes Wash twice in 50 ml of MilliQ water, wash in 50 ml of absolute ethanol Let air dry*
Hybridization	Place 10–20 filter sections in a 0.5 ml reaction vial Mix 300 µl of hybridization buffer with 1 µl of probe working solution [50 ng µl ⁻¹] and add to filter sections Incubate at 35 °C for 2 to 15 hours on a rotation shaker. Wash filters in prewarmed washing buffer (5 minutes, 37 °C) <i>Don't let filter run dry</i>
CARD	Incubate in PBS for 15 minutes at RT To remove excess liquid, dab filter on blotting paper, <i>but don't let run dry</i> Mix 1000 µl amplification buffer [1 × PBS, pH 7.6; 10% dextran sulfate; 0.1% blocking reagent; 2 M NaCl] with 10 µl of freshly prepared H ₂ O ₂ stock [0.15%], then add 4 µl of fluorescein labeled tyramide [1 mg ml ⁻¹], mix, and add to filter sections in a petri dish & incubate at 46 °C for 15 minutes in the dark To remove excess liquid, dab filter on blotting paper Wash in PBS for 5 to 10 minutes at RT in the dark Wash twice in 50 ml of MilliQ water, wash in 50 ml of absolute ethanol let air dry* Preparations can now be counterstained, embedded and examined under the microscope

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

well for tyramides labeled with fluorescein, Alexa₄₈₈ and Alexa₅₄₆, but not for tyramides labeled with Cy3 and Alexa₃₅₀. For other fluorescent labels one will need to test either of the salts as well as the combination of both.

A step-by-step protocol for CARD-FISH is given in Table 3.

Troubleshooting

High background fluorescence.

The cause might be:

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- Too high tyramide concentration. Either decrease the tyramide concentration, or increase the blocking reagent concentration.
- Too high probe concentration. If the background is covered with tiny fluorescent dots, check the probe concentration, $0.2 \text{ ng } \mu\text{l}^{-1}$ is plenty.
- Too short washing after CARD. Prolonged washing in deionized water and/or several changes with fresh water may help.

Low signal intensity

The cause might be:

- Low ribosome content of the target cells. Increase the tyramide concentration or the temperature during the tyramide signal amplification. A prolonged hybridization time (up to 15 hours) may also help.
- Too low tyramide concentration.
- The probe delivered HRP has too low or no activity. Check the probe. The probe should be thawed only once and should not be stored in the fridge for more than 6 months. Check the pH of the PBS, it should be around 7.6. Check the H_2O_2 concentration and its age. Check the reactivity of the tyramide.
- The HRP is not coupled to the probe.
- The HRP-labeled probe cannot penetrate the cell wall. Try different permeabilization protocols.
- Sometimes even the most talented scientist has a bad day. Don't give up trying!

Multi-color CARD-FISH

Sometimes it may be necessary to localize two or more target species simultaneously, for example to clarify the distribution of microorganisms within a dense community (e.g. biofilm, symbiosis) [7, 12, 13]. For multi-color CARD-FISH with oligonucleotide probes directly linked to HRP, the hybridization and cytochemical detection of different targets have to be performed in sequence. After the first hybridization and detection of the probe, the probe delivered HRP has to be inactivated (e.g. by acid, heat, or H_2O_2). Then the next hybridization with another probe and another fluorochrome for probe detection can be performed. Choose appropriate fluorophores for multi-color FISH.

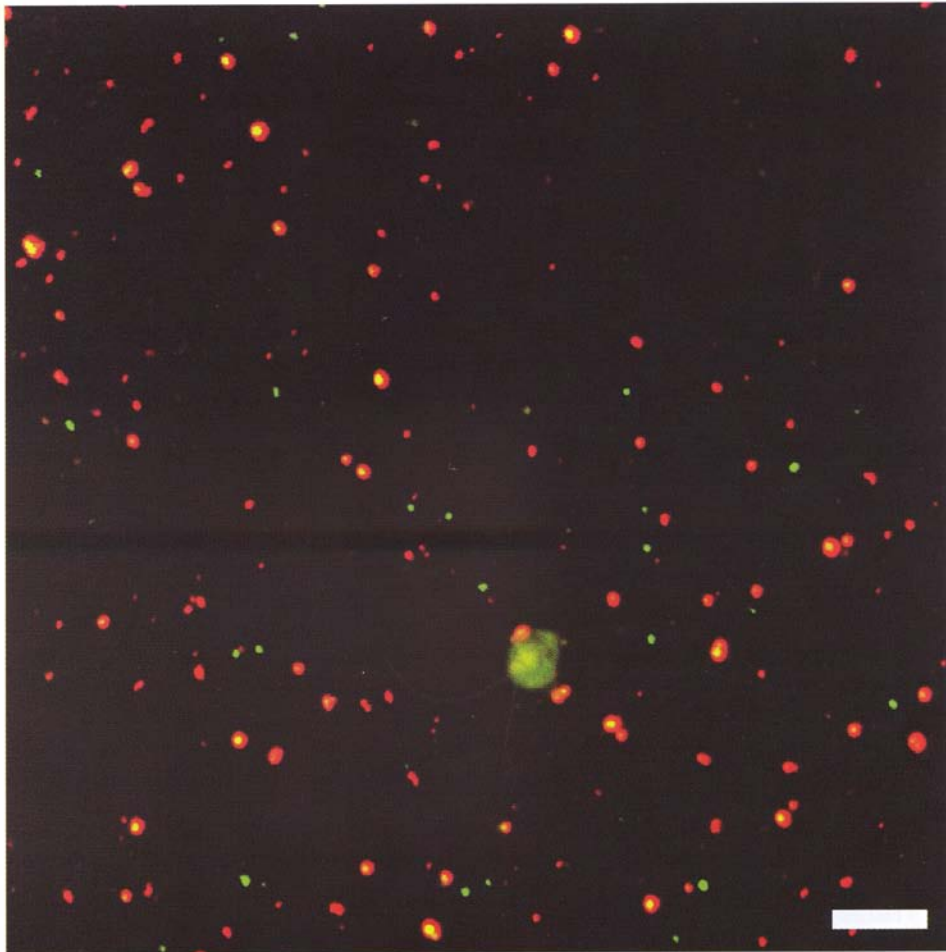


Figure 1. Photomicrograph of CARD-FISH stained picoplankton from North Atlantic surface water, taken with a confocal laser scanning microscope using an Helium-Neon laser (633 nm) for Cy5 and an Argon laser (488 nm) for Alexa₄₈₈ excitation. Emissions of both dyes were recorded separately, and images were combined afterwards. Red: cells hybridized with a probe against *Bacteria*, CARD with Cy5-tyramide; green: cells hybridized with a probe against *Euryarchaea*, CARD with Alexa₄₈₈-tyramide. Scale bar: 10 μm .

For this purpose, Alexa dyes are superior. In our lab we combine Alexa₄₈₈, Alexa₅₄₆ and Alexa₃₅₀ labeled tyramides with good results (Fig. 1).

1. Inactivate the probe delivered peroxidase from the first hybridization by incubating the filter sections in 0.01 M HCl for 10 minutes at room temperature.
2. Wash sections twice with 50 ml of MilliQ water.

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3. A second hybridization followed by a second CARD using another fluorescently labeled tyramide can now be performed as described above.

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