A. Fixation

The fixation of the sample is one of the most critical steps in the protocol. A good fixative should preserve the cell morphology while concomitantly permeabilizing all cells for the labeled oligonucleotide. Standard fixatives are aldehydes and alcohols.

Formaldehyde is reacting slowly, therefore the fixation can be finetuned according to the specific needs. For many microorganisms, good results are achieved by fixation at final concentrations between 1% - 4% formaldehyde over night at 4°C. Alternatively 1-2 h at room temperature may also suffice for good fixation. After the fixation a dehydration series of 50, 80, and 96% ethanol may help to permeabilize cells for FISH.

Literature:

Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology 56:1919-1925.

Roller, C., M. Wagner, R. Amann, W. Ludwig, and K.-H. Schleifer. 1994. In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. Microbiology 140:2849-2858.

Llobet-Brossa, E., R. Rosselló-Mora, and R. Amann. 1998. Microbial community composition of wadden sea sediments as revealed by fluorescence in situ hybridization. Applied and Environmental Microbiology 64:2691-2696.

Glöckner, F. O., B. M. Fuchs, and R. Amann. 1999. Bacterioplankton composition in lakes and oceans: a first comparison based on fluorescence in situ hybridization. Applied and Environmental Microbiology 65:3721-3726.

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.

The **quality of the formaldehyde solution** is critical. Ideally it should be freshly prepared from paraformaldehyde. We often store fresh prepared PFA under a nitrogen atmosphere.

- 4% PFA preparation:
    1. pour 2 g of paraformaldehyde (PFA) powder in 50 ml phosphate buffered saline (PBS; 130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) or a similar buffer (use mask for weighing PFA – irritant if inhaled)
    2. heat to app. 60° C (must not boil!), until suspension is clear (app. 1/2 h); if not, add some drops of 1N NaOH
    3. check pH and adjust to pH 7.0
    4. filter through 0.2 µm filter and place on ice

    Usable for up to 1 week, and up to 6 month if stored dark under nitrogen atmosphere at RT.

Formalin, a concentrated 35-37% formaldehyde solution, can be readily used as well, if the solution is fresh (means, no precipitates at the bottom of the bottle). Below you find protocols for fixing pure cultures with (1) Gram-negative, (2) Gram-positive cell wall, for (3) planktonic cells and for (4) sediment/soil samples.

1. Fixation of pure cultures with Gram-negative cell wall *(Amann et al., 1990)*

1. harvest cells during logarithmic growth by centrifugation of an aliquot (ca. 2 ml) in microcentrifuge, (10 min. at 4000 x g)
2. discharge supernatant and resuspend cells in 750 µl PBS (145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.4)
3. fix by adding 250µl of a 4% PFA fixative (1% final concentration)
4. incubate for 1 (for thick walled cells) to 24 h (for fragile cells) at 4°C
5. pellet cells by centrifugation (10 min. at 4000 x g), discharge supernatant
6. thoroughly resuspend fixed cells in 500 µl PBS
7. repeat step 5 and 6
8. add 500 µl absolute ethanol and resuspend cells thoroughly
9. at this stage samples can be stored at -20°C for several months

2. Fixation of pure cultures with Gram-positive cell wall *(Roller et al., 1994)*

1. harvest cells during logarithmic growth by centrifugation of an aliquot (ca. 2 ml) in microcentrifuge, (10 min. at 4000 x g)
2. discharge supernatant and wash cells in PBS
3. pellet cells by centrifugation (10 min. at 4000 x g), discharge supernatant
4. add 500 µl PBS, resuspend cells thoroughly
5. add 500µl cold, absolute ethanole, mix
6. at this stage samples can be stored at -20°C for several months

No PFA used (pure ethanole fixation) because the thick cell wall of the Gram-positive bacteria can become completely impenetrable, otherwise.

3. Fixation of planktonic samples *(Glöckner et al., 1999)*

1. Add formalin (37% formaldehyde) to a water sample to a final concentration of 1-3% and fix for 1-24 h at 4°C; needs to be optimized for new sample types.
2. Place a moistened support filter (0.45 µm pore size, cellulose nitrate, 47 mm diameter; Sartorius, Germany) and a membrane filter (0.2 µm pore size, white polycarbonate, 47 mm diameter; Millipore, Eschborn, Germany; shiny side up!) into a filtration tower; filter a known 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 is generally sufficient.
3. After complete sample filtration, wash with 10-20 ml of sterile H_2O; remove H_2O by filtration, put the membrane filter in a plastic petri dish, cover and allow air-drying.
4. Store at -20°C until processing; filters can be stored frozen for several months without apparent loss of hybridization signal.

4. Fixation of sediment / soil samples *(Llobet-Brossa et al., 1998)*

1. Fix sediment samples with fresh formaldehyde (end concentration 1 - 4%) for 1 -2 at RT or max 24 hours at 4°C;
2. centrifuge at 16.000 g for 5 minutes; pour off supernatant and resuspend sample with 1 X PBS pH 7.6;
3. repeat step 2 twice;
4. store sediment sample in a 1:1 mix of PBS / ethanol at -20°C or -80°C until further processing;