Protocol for standard FISH and DOPE-FISH for prokaryotes

(slightly modified from Amann, 1995, note, for other modifications or other microorganisms like eukaryotes, consult special literature or check labscript from FISH course www.microbial-systems-ecology.de/fish_course_new.html

General comments: Once cells have been fixed (see e.g. fixation protocol http://www.microbial-systems-ecology.de/lab_issues.html#fish), they can be used for FISH. The procedure is quite straightforward and can be performed either on microscope slides, other devices like membrane filters, or in solutions in tubes:

Brief outline for FISH on microscope slides or on membrane filters attached to microscope slides (more detailed information on page 2):

- Simply pipette your fixed sample on suitable microscope slides (coated or non-coated, depending on your cells, see http://www.microbial-systems-ecology.de/lab_issues.html#fish), allow to dry.
- Dehydrate the microscope slide in a series of different EtOH solutions.
- Prepare a hybridization solution containing the nucleic acid probe, apply it to the cells on the microscope slide.
- Hybridize at appropriate temperature (normally 46 °C, for about 90 min up to 96 h).
- Wash your microscope slide to remove excess/unbound nucleic acid probe (normally at 48 °C, for about 10-20 min.).
- Mount and microscope or store in the fridge before you can microscope your sample.

Brief outline for FISH in solutions (or in other formats like cut filter pieces) in tubes: Instead of drying your fixed sample on a microscope slide, pipette your fixed sample into a tube and proceed as described above for microscope slides, with the following modifications:

- The amount of probe solution and washing hybridization must be increased so that the sample is constantly immersed during all "wet-steps".
- All washing steps are done via centrifugation.

Reagents (advice, prepare 0,5 to 1 Liter autoclaved solutions of all, these can be stored for longer periods. Use aliquots in e.g. 50 ml falcon tubes for daily work):

- 5 M NaCl
- 1 M Tris/HCl pH 8,0
- Sterile ddH_2O or MQ (ultra pure water)
- Formamide of high quality (e.g. Merck, # 1.09684.1000), properly stored in the refrigerator at dark. Do not use old solutions. Commercial formaldehyde solutions may also be used but be aware of the quality.
- 10 % (w/v) SDS (do not autoclave, but dissolve SDS in a autoclaved bottle with sterile water)
- 0,5 M EDTA pH 8, 0
- Fixed sample
- Controls (probes and appropriate reference cells
- Oligonucleotide probes (singly labeled, or DOPE-labeled, see e.g. Stoecker et al. 2010) can be provided from e.g. Biomers http://www.biomers.net (store at -20° C, dark, as aliqouted working solutions, see concentrations in step 5, page 2).

Detailed step by step procedure (for FISH on microscope slides/in tubes):

1) Plan your FISH experiment (select appropriate samples, probes and controls, use e.g. the tables suggested on www.microbial-systems-ecology.de/lab_issues.html#fish (select "Tables for FISH experiments).

2) Check list to get started:

   a. Turn on the hybridization oven (**46 °C**) and the water bath (**48 °C**). Check that the temperatures are stable and correct.

   b. Check that the EtOH series (50, 80, ≥96%) solutions, FISH reagents, probes with suitable fluorochromes and if appropriate, DNA stains, are available.

   c. Use sterile pipette tips and sterile reagent tubes. Use new sterile falcon tubes for the washing buffer. Used/dish-washed falcon tubes can be used for the moisture chamber.

   d. Other material: Teflon coated microscope slides (with or without additional coating), racks (2 ml, 50 ml), tissue.

   e. Prepare special waste bin to collect liquid waste with formamide, and solid waste contaminated with formamide (e.g. pipette tips, tissue).

3) Pipette fixed cells (1-20 µl (depending on the cell concentration, and/or if the sample is to be quantified.

   - If the cell concentration of your sample is low, you can concentrate the sample on the well by simply adding subsequently more cells after drying) on a teflon coated microscope slide (e.g. Marienfeld Laboratory Glassware, Germany, www.marienfeld-superior.com/): allow to air dry, either shortly (~ 20 min) at 45-60°C, or over night (comment, some samples do show better adhesion if more time is allowed for the drying).

   - If you are doing FISH in solutions in tubes, pipette your cells into tubes. Centrifuge and remove supernatant.

4) In the meantime, prepare the hybridization buffer (page 5) and store it at room temperature (use within next hour(s)). Use the safety hood and gloves when pipetting the formamide.

5) Dehydrate the fixed, dried cells on the microscope slide in an increasing ethanol series (1-5 min each in 50, 80 and ≥96 % ethanol (denatured EtOH, diluted with distilled water).

   If you are doing FISH in solutions in tubes, pipette EtOH into tubes. Incubate and centrifuge, remove supernatant. Proceed with next washing step. Remove the supernatant after the last washing procedure.

6) In the meantime, thaw the fluorochrome labeled oligonucleotide probes (diluted to appropriate working solutions with appropriate pH, generally ~30 ng/µl for Cy3 and Cy5 labeled probes, or ~50-100 ng/µl for FLUOS labeled probes). Protect them from light with e.g. alu foil. Vortex and spin down the probe solution.

7) Pipette 8-10 µl of the well mixed hybridization buffer onto the wells, without scratching the teflon surface. If you are doing FISH in solutions in tubes, pipette the hybridization buffer into the tube.

8) Add 1 µl of each working solution of probe, without scratching the teflon surface. Note, if two or more probes are applied simultaneously, consider whether it might be necessary to adjust probe concentrations (i.e. use a higher probe concentration of the working solutions).

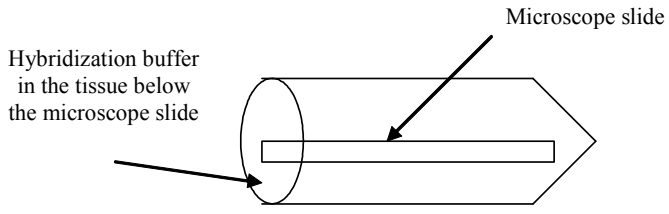
   If you are doing FISH in solutions in tubes, pipette the probes into the tube.

Comment on steps 7-8): If you are preparing a large amount of slides using the same probes, you may simplify your work by preparing a master mix (hybridization reagents and probes) in one tube, and then apply the master mix on each well on the microscope slide.

Advice: It is recommended to include "one extra" sample for your master mix. For example: *If you wish to do FISH on 6 samples distributed in 6 wells, pipette into a small tube: 7x8 µl hybridization buffer and then 7x1 µl working solution of probe. Mix and spin down. Distribute 8 µl of master mix on each well.*

9) Prepare a 50 ml hybridization moisture chamber (e.g. a 50 ml Falcon tube) by folding a piece of tissue into the tube and pouring the rest of the hybridization buffer onto the tissue. If you are doing FISH in solutions in tubes, simply place the tube with the hybridization buffer and probes into the hybridization oven (there is no need for a moisture chamber).

10) Immediately transfer the microscope slide into the hybridization tube and place the tube horizontally in a rack, and then place the rack in the oven (**46° C**).



11) Incubate the hybridization chamber with the microscope slide in the hybridization oven (**46°C**) for minimum 60 min up to 96 h. The average hybridization time is ~ 90 min.

12) In the meantime, prepare the washing buffer (page 6) and preheat this buffer at **48°C** (water bath).

13) Rinse the microscope slide with the washing buffer and incubate the microscope slide in the washing buffer for 10-20 min in a preheated water bath (**48°C**). If cells are to be quantified, the washing procedure should be standardized for all processed samples. If you are doing FISH in solutions in tubes, wash via centrifugation. Act quickly to avoid too much cooling.

14) Remove the washing buffer with cold, distilled water (avoid cell detachment), carefully remove excess water with tissue paper (don't remove the samples!) and dry the microscope slide as soon as possible carefully with compressed air. If you are doing FISH in solutions in tubes, remove the water via centrifugation. Allow to dry.

15) If a counter stain such as a universal DNA stain is to be used, apply 10-50 µl (depending on the size of your sample and hybridization well) DNA stain on your sample. Choose between e.g.:

a. DAPI solution with a concentration between 0,5-1 µg/ml (dissolve in Aqua dest., or dimethylformamide (DMF). Store dark at ° +4 C.

b. SYBR Green I diluted with Aqua dest 1:10 000), incubate in dark for ~10-20 min, at room temperature. Store dark at ° +4 C.

c. Depending on your experimental conditions, other DNA stains may also be useful (e.g. SYBR Gold, SYTOstains etc), please check up appropriate literature.

Incubate at dark, at RT, for 10-30 min.

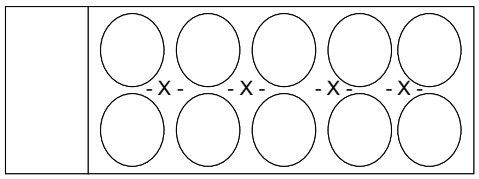
Wash the microscope slide with cold, distilled water and dry the microscope slide as soon as possible carefully with compressed air.

Note, DNA stains are toxic compounds so use a protected space (e.g. a safety hood) clearly notified by safety labels. Collect all waste in a special tray. If you are doing FISH in solutions in tubes, pipette the DNA stain into the tube, incubate, remove and wash via centrifugation.

16) Before microscopy, drop single drops of embedding solution on the microscope slide (see graph below with –X-) and place a cover slip on the microscope slide.

If you are doing FISH in solutions in tubes, pipette your cells onto a microscope slide and drop single drops of embedding solution on the microscope slide (see graph below with –X-X-) and place a cover slip on the microscope slide.

Press carefully on the cover slip to allow the mounting medium get soaked up fully by all wells with samples. Keep the slides at dark prior to microscopy.



Note: Too much mounting medium may lead to detachment of cells.

Comment: If you do not have time to microscope the sample directly after the washing procedure, you may store it in a falcon tube (wrapped in alu-foil) in the fridge, or for longer periods (days) at – 20° C.

17) Keep embedded microscope slides in a dark box until you can microscope them. For evaluation, you can use evaluation protocols like those suggested on www.microbial-systems-ecology.de/lab_issues.html#fish (select "Tables for FISH experiments).

18) After the microscopy:
   a. If you do not wish to save the slides for further microscopy or record, then throw away teflon coated slides in a special dedicated waste tray.
   b. If you wish to reuse your slides for further microscopy or record, remove the cover slip, rinse the slide carefully with distilled water, dry carefully with pressurized air. Store at dark at -20° C.

Comments: For further notes on storing, handling and microscopy of slides after FISH or other FISH protocols for e.g. eukaryotes, consult other literature or see labscript of FISH course www.microbial-systems-ecology.de/fish_course_new.html, or www.arb-silva.de/fish-probes/fish-protocols/ .

References:

Amann, R. 1995. In situ identification of microorganisms by whole cell hybridization with rRNA targeted nucleic acid probes. Methods Molecular Ecology Manual. 3.3.6, 1.

Stoecker, K., Dorninger, C., Daims, H., Wagner, M. 2010. Double Labeling of Oligonucleotide Probes for Fluorescence In Situ Hybridization (DOPE-FISH) Improves Signal Intensity and Increases rRNA Accessibility. Applied and Environmental Microbiology, 76(3), 922.

Hybridization Buffer for standard *in situ* hybridization at 46°C

Pipette into a 2 ml Eppendorf reaction tube:

- 5 M NaCl 360 µl
- 1 M Tris/HCl pH 8,0 40 µl
- Add sterile ddH_2O or MQ (ultra pure water) and formamide as listed below, depending on the stringency of the probes used:

% Formamide (v/v)	Formamide [µl]	MQ [µl]
0	0	1.600
5	100	1.500
10	200	1.400
15	300	1.300
20	400	1.200
25	500	1.100
30	600	1.000
35	700	900
40	800	800
45	900	700
50	1.000	600
60	1.100	500
65	1.200	400
70	1.300	300

- Add 10 % (w/v) SDS 4 µl (SDS is important because it denatures native ribosome structures by removing ribosomal proteins, thus increasing the accessibility of the target sites).

This should be used within the next hours. The buffer should always be freshly prepared for a FISH experiment.

The formamide solution should be of a high quality and stored in dark (wrapped in alu foil) in the fridge. When you add the formamide, use the safety hood and gloves.

If you purchase a larger volume (for example 1 Liter, from Merck, # 1.09684.1000) it is recommended that you aliquot this into e.g. 15-50 ml falcon tubes, and store these at -20º C prior to use. Note: formamide is sensitive for degradation, degraded formamide can lead to unspecific FISH signals!

Important comment:
If probes with different formamide concentrations for optimal stringencies are to be used, then FISH must be performed in several subsequent steps, starting with the probe with the highest formamide concentration.

Washing buffer standard *in situ* hybridization at 46°C (washing at 48°C)

Mix in a 50 ml Falcon tube:

- 1 ml 1 M Tris/HCl pH 8,0
- 5 M NaCl
- 0,5 M EDTA pH 8, 0 (note, only from 20 % formamide)

Corresponding to the following table:

% Formamide in hybridization buffer	[NaCl] in mol/l	NaCl [µl] (from 20% Formamide on, add 500 µl 0.5 M EDTA)
0	0,900	9.000
5	0,636	6.300
10	0,450	4.500
15	0,318	3.180
20	0,225	2.150
25	0,159	1.490
30	0,112	1.020
35	0,080	700
40	0,056	460
45	0,040	300
50	0,028	180
55	0,020	100
60	0,008	40
70	0,000	no NaCl, only 350 µl EDTA

- Add sterile ddH_2O or MQ (ultra pure water) up to 50 ml.
- Add 50 µl 10 % (w/v) SDS.
- Preheat the washing buffer at 48°C prior to use.

Comment: Check that you have in addition a bottle of cooled (+4° C) sterile ddH_2O or MQ (ultra pure water), for the final washing step.