FISH for prokaryotes

General comments: Once cells have been fixed, they can be used for FISH. The procedure is quite straightforward:

- Simply pipette your sample on suitable slides (coated or non-coated, depending on your cells*), let dry;
- Dehydrate your cells on the slide in a series of different EtOH solutions;
- Prepare a hybridization solution containing the nucleic acid probe, apply it to the cells on the slide;
- Hybridize at appropriate temperature (normally 46 °C, for about 90 min.),
- Wash your 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.

* See page 9 on slide and coating procedures.

Procedure:

1) Prepare specimen on a teflon coated slide: spot fixed cells (2-15 µl (or even more, amount depending on the cell concentration, and/or if the sample is to be quantified) and let air dry over night, or dry at ~46°C, for about ~20 min.

2) In the meantime, prepare the hybridization buffer and keep at room temperature.

3) Dehydrate specimen on the slide in an increasing ethanol series (3 min each in 50, 80 and 100 % ethanol).

4) In the meantime, thaw the oligonucleotide probes (diluted to appropriate working solutions, ~ 30 ng/µl for Cy3 and Cy5 labeled probes, or ~50 ng/µl for FLUOS labeled probes). Protect them from light. The probes do not necessarily have to be stored in an ice-box (only if they are kept longer than 1 h at room temperature).

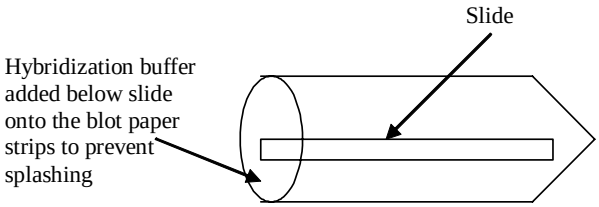
5) Drop 8-10 µl of hybridization buffer onto the wells, without scratching the teflon surface.

6) Add 1 µl of each probe, without scratching the teflon surface.

   Comment to 5-6): 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 cap, and then apply the master mix (about 10 µl) to each well on the slide.

7) Prepare a hybridization tube (50 ml Falcon tube) by folding a piece of tissue, putting it into the tube and pouring the rest of the hybridization buffer onto the tissue.

8) Immediately transfer the slide into the hybridization tube and place the tube on a (e.g. styrofoam rack, and then place the rack in the oven.



9) Incubate the hybridization chamber with the slide in the hybridization oven (46°C) for about 1.5 hours.

10) In the meantime, prepare the washing buffer and preheat this buffer at 48°C (water bath).

11) Rinse the hybridization buffer with the washing buffer from the slide and incubate the 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 equal for all processed samples.

12) Remove the washing buffer with cold, distilled water (do not detach the cells!) and dry the slide quickly (compressed air).

13) Before microscopy, embed the slide with an embedding agent and put a cover slip onto the slide. 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.

Comments: For further notes on storing, handling and microscopy of slides after FISH, see page 9.

Hybridization Buffer for *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 formamide and MQ (ultra pure water), depending on the applied stringency:

% 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

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

Washing buffer for *in situ* hybridization at 46°C (washing step 20 min at 48°C)

Mix in a 50 ml Falcon tube:

- 1 ml 1 M Tris/HCl pH 8,0
- 5 M NaCl and 0,5 M EDTA pH 8, 0 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 50 µl 10 % (w/v) SDS,
- Preheat the washing buffer at 48°C prior to use,
- Check that you have a bottle of cool sterile distilled water (for the final washing step).

Labelling and storage of nucleic acid based probes

Oligonucleotide probes:

Oligonucleotides can be labelled in at least three different ways:

1) With radioactive compounds (e.g. 35S, Giovannoni et al., 1988);
2) Non-fluorescent compounds such as biotin and digoxigenin (Schönhuber et al, 1997);
3) Fluorescent compounds such as Cy-3, Cy-5, FLUOS or Alexa derivates (see table on the following page).

Of these three alternatives, fluorescent labelling presents the easiest alternative. Radioactive labelling was practiced only in the early history of the FISH development. Non-fluorescent labelling is useful for samples with a strong autofluorescing background. Labelling should be done at the 5'- terminus of the oligonucleotide. Labelling can be done by onself in the lab (see for example Amann, 1998). However, this is quite tedious and time-consuming (costs about 1-2 days or longer, depending on expected yields/efficiencies, etc). Nowadays, fluorescently labelled oligonucleotide probes can be easily and quickly (within a few days or a week) obtained from several biotechnological companies (e.g. Molecular Probes/Invitrogen, MWG Biotech, Operon, Sigma, Thermo Hybaid). Expected costs: approximately 100-150 € per probe, depending on type of fluorescent dye, length of probe and desired probe concentration. The stock solution normally lasts for at least 100-1000 (depending on applied concentrations) hybridization experiments. The stock solution should be separately stored (at -20 ºC, in dark). To avoid too much re-thawing of the stock solution (and thus degradation of the nucleic acid probes), aliquots of working solutions should be prepared (e.g. 50 µl of 30 or more ng/µl, in several aliquots).

Summary of physical properties of different fluorescent dyes

For more information on spectral traces see further:
http://www.zeiss.de/C12567BE0045ACF1/?Open
http://www.mcb.arizona.edu/IPC/spectra_page.htm)

Fluorescent Dye	Formula Weight (g/mol)	Absorbance (nm)	Emission (nm)	Extinction Coefficient	Fluorescent Color
TAMRA-dT	870.9	544	576		Yellow-Orange
5-Fluorescein (FITC)	389.4	495	520	73000	Yellow-Green
5-Carboxyfluorescein (FAM)	358.0	495	520	83000	Yellow-Green
6-Carboxyfluorescein (FAM)	537.5	495	520	83000	Yellow-Green
6-Carboxyfluorescein (FAM) CPG	569.5	495	520	83000	Yellow-Green
Fluorescein-dT	815.7	495	520		Yellow-Green
6-Carboxyfluorescein-DMT (FAM-X)	537.5	495	520	83000	Yellow-Green
5(6)-Carboxyfluorescein (FAM)	537.5	495	520	83000	Yellow-Green
6-Hexachlorofluorescein (HEX)	744.1	535	556	73000	Yellow
6-Tetrachlorofluorescein (TET)	675.2	521	536	73000	Yellow-Green
JOE	487.0	520	548	73000	Yellow
LightCycler Red 640	758.0	625	640	0	Red
LightCycler Red 705	753.0	685	705		Red
FAR-Fuchsia (5'-Amidite)	776.0	567	597	150000	Yellow-Orange
FAR-Fuchsia (SE)	776.0	567	597	150000	Yellow-Orange
FAR-Blue (5'-Amidite)	824.0	660	678	150000	Red
FAR-Blue (SE)	824.0	660	678	150000	Red
FAR-Green One (SE)	976.0	800	820	130000	Near-IR
FAR-Green Two (SE)	960.0	772	788	150000	Near-IR
Oregon Green 488	394.0	496	516	76000	Yellow-Green
Oregon Green 500	431.0	499	519	84000	Yellow-Green
Oregon Green 514	494.0	506	526	85000	Yellow-Green
BODIPY FL-X	387.0	504	510	70000	Green
BODIPY FL	273.8	504	510	70000	Green
BODIPY-TMR-X	493.0	544	570	56000	Yellow
BODIPY R6G	322.0	528	547	70000	Yellow
BODIPY 650/665	529.5	650	665	101000	Red
BODIPY 564/570	348.0	563	569	142000	Yellow
BODIPY 581/591	374.0	581	591	136000	Yellow-Orange
BODIPY TR-X	519.0	588	616	68000	Red-Orange
BODIPY 630/650	545.5	625	640	101000	Red
BODIPY 493/503	302.0	500	509	79000	Green
Carboxyrhodamine 6G	441.0	524	557	102000	Yellow
MAX	441.0	525	555	102000	Yellow
5(6)-Carboxytetramethylrhodamine (TAMRA)	412.5	546	576	90000	Yellow-Orange
6-Carboxytetramethylrhodamine (TAMRA)	413.0	544	576	90000	Yellow-Orange
5(6)-Carboxy-X-Rhodamine (ROX)	516.7	576	601	82000	Orange
6-Carboxy-X-Rhodamine (ROX)	516.7	575	602	82000	Orange
AMCA-X (Coumarin)	328.0	353	442	19000	Blue
Texas Red-X	702.0	583	603	116000	Orange
Rhodamine Red-X	654.0	560	580	129000	Yellow-Orange
Marina Blue	252.3	362	459	19000	Blue

Fluorescent Dye	Formula Weight (g/mol)	Absorbance (nm)	Emission (nm)	Extinction Coefficient	Fluorescent Color
Pacific Blue	224.2	416	451	37000	Blue
Rhodamine Green-X	394.0	503	528	74000	Yellow-Green
7-diethylaminocoumarin-3-carboxylic acid	243.0	432	472	56000	Blue-Green
7-methoxycoumarin-3-carboxylic acid	202.0	358	410	26000	Violet
Cy3	508.6	552	570	150000	Yellow
Cy3B	543.0	558	573	130000	Yellow-Orange
Cy5	534.6	643	667	250000	Red
Cy5.5	634.8	675	694	250000	Red
DY-505	0.0	505	530	85000	Yellow-Green
DY-550	667.8	553	578	122000	Yellow
DY-555	636.2	555	580	100000	Yellow-Orange
DY-610	667.8	606	636	140000	Red
DY-630	634.8	630	655	120000	Red
DY-633	751.9	630	659	120000	Red
DY-636	760.9	645	671	120000	Red
DY-650	686.9	653	674	77000	Red
DY-675	706.9	674	699	110000	Red
DY-676	808.0	674	699	84000	Red
DY-681	736.9	691	708	125000	Red
DY-700	668.9	702	723	96000	Red
DY-701	770.9	706	731	115000	Red
DY-730	660.9	734	750	113000	Red
DY-750	713.0	747	776	45700	Near-IR
DY-751	796.8	751	779	220000	Near-IR
DY-782	660.9	782	800	102000	Near-IR
Cy3.5	576.7	581	596	150000	Yellow-Orange
EDANS	307.1	336	490	5700	Blue-Green
WellRED D2-PA	611.0	750	770	170000	Red
WellRED D3-PA	645.0	685	706	224000	Red
WellRED D4-PA	544.8	650	670	203000	Red
Pyrene	535.6	341	377	43000	Violet
Cascade Blue	580.0	399	423	30000	Violet
Cascade Yellow	448.5	409	558	24000	Yellow
PyMPO	467.4	415	570	26000	Yellow
Lucifer Yellow	605.5	428	532	11000	Yellow-Green
NBD-X	276.3	466	535	22000	Yellow-Green
Carboxynapthofluorescein	458.5	598	668	42000	Red
Alexa Fluor 350	295.4	346	442	19000	Blue
Alexa Fluor 405	912.9	401	421	35000	Violet
Alexa Fluor 430	586.8	434	541	16000	Yellow
Alexa Fluor 488	528.4	495	519	71000	Yellow-Green
Alexa Fluor 532	608.8	532	554	81000	Yellow
Alexa Fluor 546	964.4	556	573	104000	Yellow-Orange
Alexa Fluor 555	850.0	555	565	150000	Yellow
Alexa Fluor 568	676.8	578	603	91300	Orange
Alexa Fluor 594	704.9	590	617	73000	Red-Orange
Alexa Fluor 633	1085.0	632	647	100000	Red
Alexa Fluor 647	850.0	650	665	239000	Red

Fluorescent Dye	Formula Weight (g/mol)	Absorbance (nm)	Emission (nm)	Extinction Coefficient	Fluorescent Color
Alexa Fluor 660	985.0	663	690	132000	Red
Alexa Fluor 680	1035.0	679	702	184000	Red
Alexa Fluor 700	1285.0	702	723	192000	Red
Alexa Fluor 750	1185.0	749	775	240000	Near-IR
Oyster 556	850.0	556	570	155000	Yellow
Oyster 645	1000.0	645	666	250000	Red
Oyster 656	900.0	656	674	220000	Red
5(6)-Carboxyeosin	689.0	521	544	95000	Yellow
Erythrosin	814.0	529	544	90000	Yellow

Comments on handling of probes and microscope slides after FISH, before, during and after microscopy

Handling of nucleic acid base probes and FISH samples:

1) Use 30 ng/µl working solution for Cy3 and Cy5 labelled probes and 50 ng/µl working solution for fluoresceine labelled probes. Comment: sometimes a higher concentration of the probes may be necessary.
2) Always keep probes protected from light; store at -20°C (however, storage at room temperature is ok for a short while (a few h).
3) After hybridisation add two drops of mounting medium (e.g. Citifluor; UK; VectaShield; Moviol (Sigma); different mounting media available at Molecular Probes (Invitrogen)) to slide and cover with cover glass.
4) Do not expose your sample to unnecessary light or laser to avoid bleaching of dye.
5) Best results can be achieved with immediate microscopic observation, otherwise keep sample cold until needed or, for longer storage, freeze at -20°C (without mounting medium!).
6) After microscopic observation and for longer storage of FISH slides, remove mounting medium with 2x washing with sterile destilled water (preferentially cold) dry it under compressed air.
7) Freeze samples at -20°C in the dark; storage time different from sample to sample (from a few days to several weeks/months – however, then these samples should NOT be used for any kind of quantitative assessments).

Microscope slides suitable for FISH

Any types of microscopic slides may be used. However, we recommend teflon-coated microscope slides, with several wells (normally used for immuno fluorescence microscopy), so that several samples or probe combinations can be simultaneously investigated. The slides may be uncoated or coated. Coating of slides may increase adhesion of cells to the slide. There are several different coating protocols. Below are listed two of the most commonly used protocols. For information on microscope slides especially useful for eukaryotes see page 15 and forwards.

Gelatine coating (from Amann, 1998)

1) Clean the slides in a solution of ethanolic KOH (10 % KOH in 95 % EtOH) for 1 hour.
2) Air dry.
3) Dip microscopic slides into a gelatine solution (0,075 % gelatine, 0,01 % chromium potassium sulphate dodecahydrate) at 70 C.
4) Air dry in a vertical position.

Coating of slides with poly-L-Lysin (Reference SIGMA, product P8920)

1) Clean slides in acid alcohol (1 % HCl in 70 % EtOH). Air dry over night at room temperature, or at 60º C for 1-2 h. For cleaner slides, flush with air in order to remove drops.
2) Allow diluted poly-L-lysin (0.01 %, diluted in sterile deionized water) to come to room temperature (100 ml) in coplin jars).
3) Place slides in the coplin jar for 5 min.
4) Drain slides and dry for about 1 h at 60º C or over night at room temperature.

Comment:

- Poly-L-lysin is obtained from Sigma as a 0.1 % (w/v) solution.
- Stock solution may be stored at room temperature until expiration date.
- Diluted solutions should be stored at 4º C and used within 3 months.
- Turbid solutions should of course be discarded.
- The maximum number of slides that can be coated is 900/L of diluted solution.