FISH of human clinical samples (Swidsinski 2011)

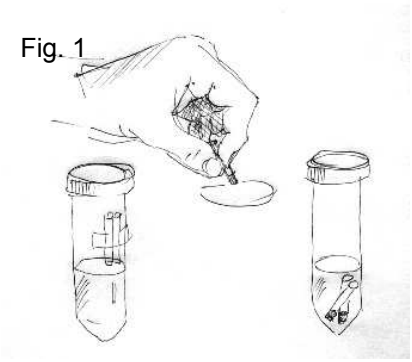
Generally:
1. Do not use formalin/paraformaldehyd fixation or any technique of freezing
2. Use water free Carnoy solution for fixation
3. Shorten the exposure to water containing solutions for the absolute necessary minimum.

1. Human stool samples

1.1. Collecting human stool samples

The 4 to 10 mm long stool cylinders were punched-out from the stool (Fig. 1) using plastic drinking straws with an inside diameter of 3 mm (Schlecker, Germany). The drinking straws were pre-cut to 4 cm in length and handed out to participants of the study together with 50 ml Falcon tubes filled with 20 ml of Carnoy solution (6/6/1 vol.ethanol/glacial acetic acid/chloroform). The pieces of drinking straw with the stool inside were put into the 50 ml Falcon tube, fixated in Carnoy for 24 hours at room temperature. Sample should not be kept longer than 3 months in the Carnoy solution. The stool cylinders were then removed from the straws with a plastic rod, embedded into paraffin using standard techniques, cut longitudinally into 4 µm sections, and placed on SuperFrost plus slides (R. Langenbrinck, Emmendingen, Germany). The straws do not need to be sterilised. The bacterial number introduced with hand is negligible´.[3]

Fig. 1



1.2. Preparation of human stool samples for FISH-Method

Take stool sample and put it directly in a poly propylene tube containing modified Carnoy solution prepared according to the following proportions. Do not flatten the sample and do not use a shaker.

6 part of ethanol absolute
6 part of acetic acid glacial
1 part of chloroform extra pure

Incubate at room temperature for 12 h, better 24 h. Sample can be kept in Carnoy solution for up to 3 months.

In case no cylinder is available:
You can take soft stool sediments directly from Carnoy`s solution and tup it on on a slide.[5] First, make a 1 cm circle with a pap pen on the slide , let dry , then pipette ca 50 µl (you can take a plastic transfer pipette) of the stirred sample on the slide and let dry at 50°C for 30 min. Then you can hybridize directly.

Embedding

Do not use an automated Paraffin station in order to avoid contamination.

Decant Carnoy and take out the stool sample carefully (don't squeeze). Put it on a paper towel to let dry 30 sec up to 1 min. Test with a toothpick if its solid. If stool is in the straw yet, remove it carefully using a glass or plastic rod.

Remove the stool sample in the tube carefully and fill in ca. 5 ml of xylene (p.a.).

Let sample incubate overnight.

Decant the xylene and let the stool sample roll out of the tube on a paper towel. Leave it there for 10 min. to evaporate xylene.
Then take the sample carefully (use fingers or a pinzette without squeezing) and put in a pre-warmed embedding mold with paraffin. Incubate for 2 hour at 75 °C.

Take the mold out of the oven and it on ice. Use a toothpick to place the sample in the middle of the mold. Put pre-identified embedding cassette on top of mold, and fill with paraffin.

Store at 4°C for 15 min, then store at -20°C for at least 15 min before cutting.

2. Preparation of human endoscopic biopsies, bigger tissue samples, stool or colon samples from mice and rats for FISH-Method

Take sample and put it directly in a tube containing modified Carnoy solution, prepared according to the following proportions. Cut big samples in smaller pieces, not bigger then 20 mm. Do not open and do not wash colon samples, leave intestinal wall and the contents as intact as possible. Cutting pressing washing introduces biases, which can not be evaluated. Use a poly propylene tube (2 ml Eppendorf tube, 15 ml -50 ml tube).
Use enough solution in each step (amount depending on the sample -Table 1).

Carnoy solution:
6 part of ethanol absolute
6 part of acetic acid glacial
1 part of chloroform extra pure

Table 1

Sample size mm^3	Carnoy ml
1-3	0,5
5	5
6-20	15

Incubate at room temperature for at least 12 h, better 24 h. Tissue can be kept in Carnoy solution for up to 3 months.

Embedding

Don't use an automated Paraffin station in order to avoid contamination. Prepare each sample on its own.

Decant Carnoy solution, add ethanol (absolute) and incubate:
- biopsy samples up to 3 mm size for 15 min at 4°C
- big Tissue samples (4-20 mm) for 2 hours at 4°C

Decant supernatant and repeat washing in ethanol (absolute):
- biopsy samples up to 3 mm size for 15 min at 4°C.
- samples up to 10 mm size for 2 hours at 4°C.
- samples 10-20 mm size over night at 4°C.

Decant supernatant.

Add Xylene (p.a.) and incubate at room temperature.:
- small biopsy for 15 min
- bigger samples for 2 hours

Dispose supernatant and repeat Xylene (p.a.) incubation at room temperature.:
- Small biopsy for 15 min
- Bigger samples over night

Put small samples directly in paraffin (use a toothpick to handle them easily).
Leave big samples on a paper towel for 5 min at room temperature before putting in paraffin and incubate:
- biopsy samples up to 3 mm size for 1 hour at 75°C
- samples up to 10 mm size for 2 hours at 75°C
- samples 10-20 mm size over night at 65°C

Dispose paraffin and add fresh paraffin (not necessary for small biopsies).
Incubate for 2 more hours (not necessary for small biopsies).

Position samples in the embedding mold using a toothpick and place the mold on ice.

Put pre-identified embedding cassette on top of the mold, and fill with paraffin.

Store at 4°C for 15 min, then store at -20°C for at least 15 min before cutting.

3. Steps after paraffin embedment of Carnoy fixates samples

Cutting sections

Cut sections of 4 µm size.

Put section on a glass slide (SuperFrost Plus Slide).
Incubate the sections for 1h at 50°C.

Deparaffinization

Put slides in a rack and do 4 incubations of 2-3 minutes at RT in successive Xylene (p.a.) baths.

Do 4 incubations of 2-3 minutes at RT in successive ethanol (absolute) baths.

Incubate 25 min at 50°C.

Encircle samples with a thin coat of pap pen and let dry.

Lysozyme step

Necessary only for some strains of bacteria (varies depending on the probe).

Add enough Lysozyme solution (1mg/ml) to cover the section.
Incubate at 37°C for 15 min up to 90 min in a humid pre-warmed chamber (varies depending on the sample). The optimal time has to be evaluated in each case. In polymicrobial comunities Lysozyme introduces biases. It is important to find the best result with regard to the specific target and to all other bacterial groups.
Wash by flushing with distilled water.
Dry 5 min at 50°C.

4. Hybridization (Manz , Amann [1,2]):

Prepare hybridization solution. The amount of formamid varies depending on the probe used (Table 1).

Table 1, Hybridization Solutions

% of Formamid required	Formamid (µl)	H_2O (µl)	NaCl 5M (µl)	Tris .HCl 1M (pH 7.4) (µl)	SDS 10% (µl)
0	0	1600	360	40	10
1	20	1580	360	40	10
5	100	1500	360	40	10
10	200	1400	360	40	10
15	300	1300	360	40	10
20	400	1200	360	40	10
25	500	1100	360	40	10
30	600	1000	360	40	10
35	700	900	360	40	10
40	800	800	360	40	10
45	900	700	360	40	10
50	1000	600	360	40	10
55	1100	500	360	40	10

60	1200	400	360	40	10
65	1300	300	360	40	10

Pre-warm solution at 46°C (hybridization temperature required for the probe has been used), then add 0,5µl of probe (at 50ηg/µl) to 50 µl of hybridization buffer (for each section; take enough hybridization buffer to cover the section). Mix it well and immediately put on section and spread to cover it completely.

Incubate for 45 min to 3 hours at 46°C (for optimizing, different bacterial groups should be tested in different times, to obtain the best required time) in a humid pre-warmed chamber and in the dark.

During this time, prepare and pre-warm wash buffer (Table 2). The wash buffer composition depends on formamid concentration used for hybridization. Pre-warm it at 48°C (or temperature required) after starting hybridization.

Take slides out and wash by briefly with distilled water.

Put 2 slides back to back in a 50 ml Falcon tube filled with pre-warmed wash buffer. Incubate in a 48°C (or temperature required) water bath for 5 min.

Table 2. Washing Solutions

% of Formamid used for hybridization	Final NaCl concentration (mM)	NaCl 5M (µl)	H_2O (ml)	Tris.HCl 1M (pH 7.4) (µl)	SDS 10% (µl)	EDTA 0.5M (µl)
0	900	9000	40.0	1000	30	0
1	900	9000	40.0	1000	30	0
5	636	6300	42.7	1000	30	0
10	450	4500	44.5	1000	30	0
15	318	3180	45.8	1000	30	0
20	225	2150	46.4	1000	30	500
25	159	1490	47.0	1000	30	500
30	112	1020	47.5	1000	30	500
35	80	700	47.8	1000	30	500
40	56	460	48.0	1000	30	500
45	40	300	48.2	1000	30	500
50	28	180	48.3	1000	30	500
55	20	100	48.4	1000	30	500

Take slides out and flush with ddH_2O.

Dry in an oven at 50°C for 5 min in the dark (slides upright).

Transfer slides in a cardboard slide folder. Add enough DAPI solution (1µg/ml) to cover the section.

Incubate for 5-10 min at room temperature in the dark.

Wash with ddH_2O.

Dry for 5 min in the dark in an oven at 50°C (slides upright).

The slides can be kept dry for about 6 weeks in cardboard folders at room temperature in the dark.

5. Solutions

NaCl 5M
Tris pH 7,4
EDTA 0,5M pH 8
SDS 10%

DAPI 1µg/ml
Lysozyme 1mg/ml
Formamid p.a.
Carnoy solution modified
Xylene p.a.
Probes at a stock concentration of 200 µM
Paraffin

6. References

1. Amann, R., Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 1990; 172:762-770.
2. Amann, R I., Ludwig W, Schleifer K-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial. Reviews. 1995;59:143-169.
3. Swidsinski A, Loening-Baucke V, Kirsch S, Doerffel Y. [Functional biostructure of colonic microbiota (central fermenting area, germinal stock area and separating mucus layer) in healthy subjects and patients with diarrhea treated with Saccharomyces boulardii]. Gastroenterol Clin Biol. 2010 Sep;34 Suppl 1:S79-92. French. PubMed PMID: 20889010.
4. Swidsinski A, Loening-Baucke V, Herber A.. Mucosal flora in Crohn's disease and ulcerative colitis - an overview. J Physiol Pharmacol. 2009 Dec;60 Suppl 6:61-71.PubMed PMID: 20224153.
5. Swidsinski A, Dörffel Y, Loening-Baucke V, Mendling W, Verstraelen H, DieterleS, Schilling J. Desquamated epithelial cells covered with a polymicrobial biofilm typical for bacterial vaginosis are present in randomly selected cryopreserved donor semen. FEMS Immunol Med Microbiol. 2010 Aug;59(3):399-404. Epub 2010 Apr 20. PubMed PMID: 20497224.

Further Informations:

www.charite.de/arbmkl
www.microbial-ecology.net/probebase
www.arb-silva.de/fish-probes