# Evaluation of Polymicrobial Involvement Using Fluorescence In Situ Hybridization (FISH) in Clinical Practice

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## Abstract

The involvement of microorganisms in infection is often deduced from their presence during disease and absence in healthy humans or animals, which is an oversimplification. The proof of direct involvement is decisive. Fluorescence in situ hybridization (FISH) combines the specific identification of microorganisms and the morphological aspect of the host tissues and is as a consequence especially helpful for these purposes.

The present manuscript describes FISH methods which we use in ambulatory patients for Polymicrobial Infections and Bacterial Biofilms of the Charité Hospital to visualize pathogens (pathogenic consortia) in clinical samples.

**Keywords** Polymicrobial infections, Pathogenic consortia, FISH

## 1 Introduction

The contemporary understanding of infections is based on identifying microorganisms in diseased persons that are absent in healthy persons. However, the presence of a bacterium (or bacteria) in health does not mean that it is healthy or at least harmless (chapter by Benedetta Bottari et al. "FISHing for Food Microorganisms").

*Neisseria meningitidis* is part of the normal nonpathogenic flora in the nasopharynx of up to 5–15 % of adults. Its causative involvement in meningitis is however beyond doubt, since it is the only bacterium found in the inflamed cerebrospinal fluid.

Detecting bacteria at the site of an infection is more appropriate for identifying the infectious agent than its absence within normal colonization. Difficulties arise when multiple organisms are present at the infection site. In this case, the criminological experiment is decisive. A transfection of the suspected bacteria to healthy animals helps to uncover potential pathogens.

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However, when none of the involved microorganism causes infection, does this exclude the harmful potential of a group? No.

A well-known example is the induction of Vincent's angina by Rosebury, who transferred plaque-infected material holding different components [1]. While single microorganisms were innocuous and incapable to initiate infection, it was possible to cause disease with the combination of different species. The required consortium was called the "Pathogenic Quartet" and included the following species that were isolated from a patient diagnosed with Vincent's angina: a spirochete, a fusiform *Bacillus*, a *Vibrio*, and an anaerobic *Streptococcus*. Rosebury's conclusion was that each of these species is a member of healthy indigenous flora, but they may cooperate and form an unmanageable complex structure.

In nature, microorganisms build diverse consortia in which single participants complement each other and display specific properties, which cannot be discovered in one of the participants or in other associations. Can some of these consortia be pathogenic? Yes.

We should await the presence of such consortia on surfaces which contact the outer world such as the skin, mouth, intestine, vagina, etc.

Can the role of these consortia be proved in transfection experiments? Presently, no.

Rosebury transfected not really a consortia but a mix of isolated cultured single strains. This should be only in exceptional cases successful. The problem is that until now, we are unable to cultivate polymicrobials. When more than three bacterial strains are incubated in the same culture, their growth is getting unpredictable, and one of the strains suppresses and overgrows the others. Polymicrobial culture is a challenge for future research.

In the absence of polymicrobial cultures, a link between the consortium of distinct species and their involvement in disease can be established directly by visualizing pathogenic consortia within biofilms and microbial infiltrates in host tissues via fluorescence in situ hybridization (FISH).

We have successfully used this approach in case of colonic cancer [2], inflammatory bowel disease [3], gallstones [4], tonsillitis [5], appendicitis [6], bacterial vaginosis [7], candidiasis [8], and urethritis [9].

FISH combines the specific identification of microorganisms and the morphological aspect and is especially helpful for identification of polymicrobial consortia involved in local infection. Each single bacterium possesses $10^3$–$10^5$ ribosomes of which each ribosome owns the same copy of ribosomal RNA. Some of the regions of the rRNA are strain-specific; others are universal for species, families, or even kingdoms. Oligonucleotides synthesized complimentary to rRNA sequences and labeled with fluorescent dye are called FISH probes. When added to samples containing bacteria,

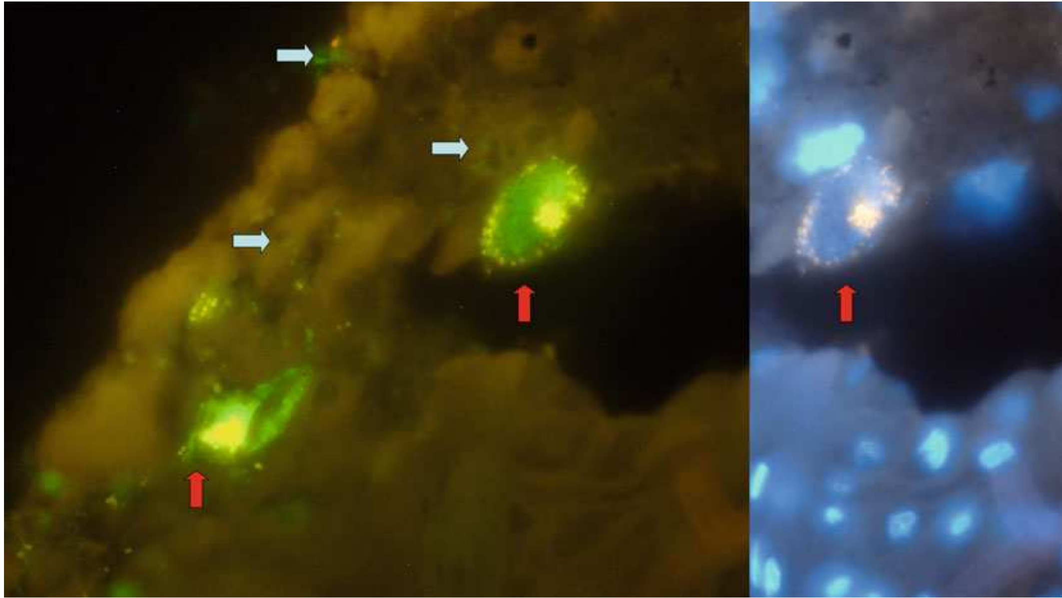

**Fig. 1** Isolated islands of bacteria attached to desquamated epithelial cells ×1,000: mouth and surgically removed material; universal bacterial probes (Eub338 FITC, green fluorescence), and *Burkholderia* (Burkho-Cy3, yellow fluorescence) on the *left*. Unspecific DAPI stain of the DNA is overlaid with *Burkholderia* fluorescence on the *right*

FISH probes hybridize with the rRNA of the bacterial ribosomes. No additional enhancement of fluorescence is necessary and bacteria can be visualized directly with a fluorescence microscope due to the large number of ribosomes in each bacterium.

Multicolor FISH enables the identification of potentially all bacterial groups in spatial relation to each other and in relation to histological structures of the host. Any biological material can be studied for in situ presence of bacteria and bacterial biofilms, including smears from tonsils or vagina, desquamated epithelial cells in the urine, tissue biopsies, surgically removed tissues, saliva, perspiration, exudation, sperm samples, and medical devices removed from the body (Figs. 1, 2, 3).

FISH protocols described here are standard protocols, which are used for ambulatory patients in the Laboratory for Molecular Genetics, Polymicrobial Infections, and Bacterial Biofilms at the Charité Hospital in Berlin, Germany.

## 2 Materials

Apart from standard cell biological and molecular cytogenetic equipment, including standard solutions (e.g., ethanol, methanol, formamide, formaldehyde, xylene, etc.), no more specialized items

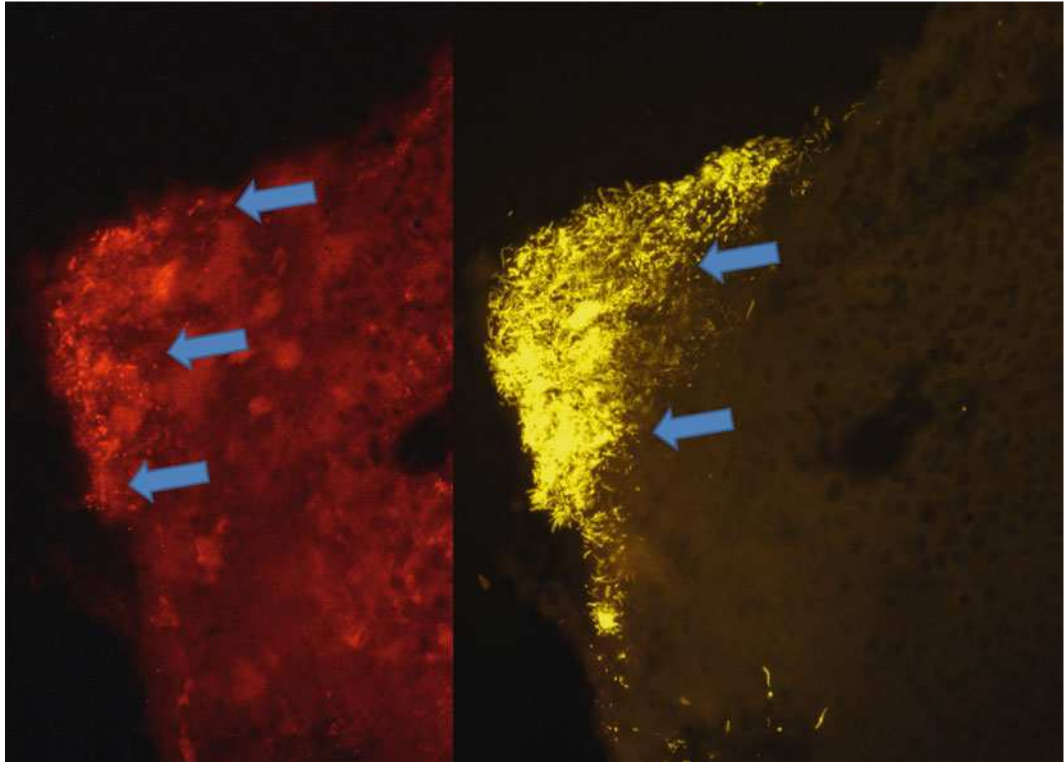

**Fig. 2** Multicolor FISH of superficial tonsils infiltrates ×400. Gamma proteobacteria as a part of superficial infiltrate (Gam42-Cy5, red fluorescence). The main group involved in infiltration is a *Fusobacterium nucleatum* (Fnuc Cy3, yellow fluorescence)

are required. The equipment needed for FISH is listed in the chapter by Thomas Liehr et al. "The Standard FISH Procedure".

However, skilled laboratory staff with experience in FISH microscopy is necessary for performing this protocol. Although the techniques applied here do basically not differ from those used by pathologists, they cannot be delegated to the staff of the routine pathology department, because the preparation of tissue sections is performed with no regard to possible microbiological cross contaminations, and bacteria are massively present in the environment. This contamination is easily avoided when materials in which single steps are performed are renewed after each sample, and instruments are kept clean and are often changed.

In addition, a routine pathologic laboratory uses automatic equipment, vessels, and containers in which large parts of sterile and highly contaminated samples are processed simultaneously, leading to enrichment of bacteria in solutions, massive microbial cross contamination, and diagnostic biases.

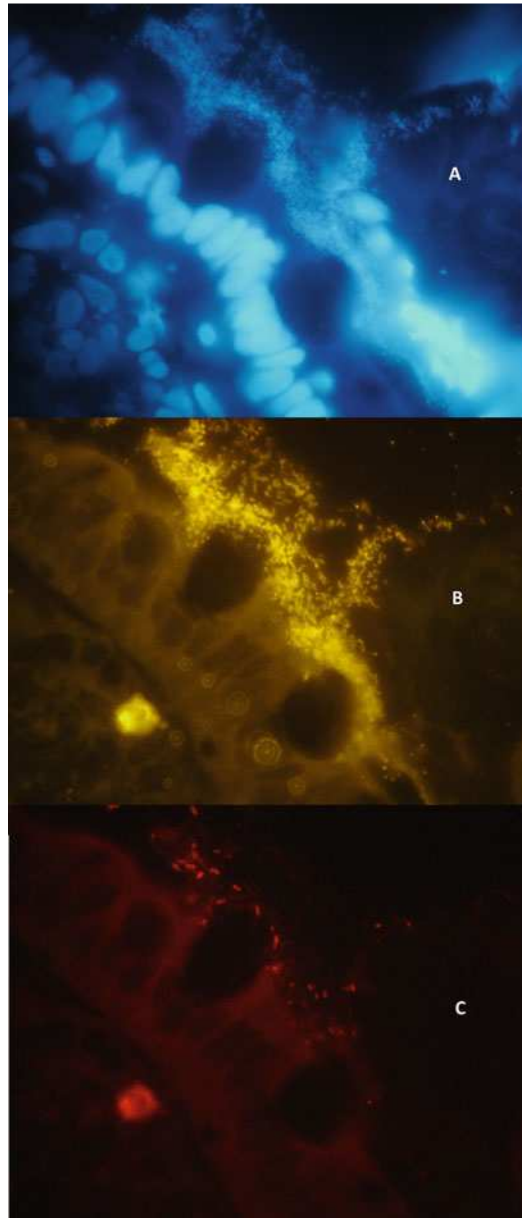


**Fig. 3** Prolific bacterial biofilm covers the colonic mucosa in a patient with Crohn's disease ×1000 multicolor FISH: (**a**) DAPI stain of DNA structures; (**b**) Bac303 Cy3; Bacteroides; orange fluorescence; (**c**) EREC Cy5; *Eubacterium rectale*; and red fluorescence

## 3 Methods

Bacteria-specific FISH probes are inexpensive and can be purchased from many oligonucleotide manufacturers (such as MWG Biotech, Ebersberg, Germany). A probe purchased for 50 € is sufficient for

### 3.1 Ribosomal RNA-Based FISH for Evaluation of Polymicrobial Consortia in Clinical Settings

at least 5,000 hybridizations. Over 200 FISH probes targeting the bacterial rRNA at a domain, group, and species level are described in the literature and can be freely accessed online over resources like www.microbial-ecology.net/probebase and www.arb-silva.de/fish-probes. New probes can be developed in case of specific clinical questions.

#### 3.1.1 Bacteria-Specific FISH Probes

Probes routinely used for evaluation of intestinal samples in clinical settings are Bac 303, EREC, Fprau, Bif 153, and EBAC representing *Bacteroides/Prevotella* and *Enterobacteriaceae*.

Probes routinely used for evaluation of urogenital samples in clinical settings are GardV, Lab, Ato, Cor, EBAC, and EUB338 probes representing *Gardnerella*, *Lactobacillus*, *Atopobium*, *Coriobacterium*, *Enterobacteriaceae*, and *Eubacteria*, respectively (*see* **Notes 1** and **2**).

The choice of FISH probes must be adjusted to the specific requirements of the biotope and the aims of the research. Probes which deliver unsure results in the microbiome should be avoided (*see* **Notes 1–3**).

FISH is an excellent tool for the assessment of spatial structure. However, results must be interpreted carefully. In case FISH probes seem to detect bacterial groups that were never described in the specific biotope or anatomical location even in case of an apparent high specificity of the probes, the presence of these groups must be confirmed using alternative methods such as culturing, polymerase chain reaction with subsequent cloning, and sequencing.

When using multicolor FISH, a large variation in practicable fluorochromes exists, but only four of them can be error-free discriminated by the human eye regardless of all possible nuances of color shades. The four colors used on a regular basis are orange, dark red, green, and blue represented by carbocyanine (Cy) 3, Cy5, fluorescein isothiocyanate (FITC), and 4′,6-diamidino-2-phenylindole (DAPI) as counterstain, respectively. The advantages of these appropriate fluorochromes are the slow bleaching, demonstrating little autofluorescent background and allowing high-quality micrographs. Cy3 is most resilient to bleaching, followed by Cy5 and FITC. Alexa fluorochromes corresponding to Cy3, Cy5, FITC, and DAPI are likewise practicable but about ten times as expensive as Cy fluorochromes (chapter by Michael Sommerauer et al. "Optical Filters and Light Sources for FISH").

In our experience with other than the abovementioned fluorochromes, there was a massive increase of signals which could not be definitively assigned to bacteria.

### 3.2 Optimally Fixated Material

Paraffin embedding and preparation of histological sections is the only time-consuming and elaborate step leading to additional costs for personal. Optimal materials to perform FISH are biopsies; eluates from swabs; urine sediments; surgically removed tissues; sections of nylon-membrane strips placed into the oral cavity or

prepuce/vagina or attached to skin overnight; and sections of adhesive tape attached to the anal region and removed after 60 s. Smears on glass slides are less appropriate since the arrangement of cells on each slide is artificial and unique. Hybridizations of the same sample performed under the exact same conditions using different bacterial FISH probes on different glass slides are not quantitatively comparable.

#### 3.2.1 General Rules to Avoid Biases

In aqueous solutions, the DNA is unstable, leading to a decreased intensity of specific hybridization signals and increased background fluorescence. To achieve optimal relation between high-specific bacterial and low-background fluorescence, we recommend the following:

- Do not use formalin/paraformaldehyde fixation, but rather use water-free modified Carnoy's solution.
- Do not use any technique of freezing while preserving samples. Freezing and thawing are deleterious for many microorganisms and especially for biofilm structures.
- Shorten the exposure to water containing solutions to the absolute minimum; drop all rehydration steps which are often used in histology.
- Hold hybridization time as short as necessary (30–90 min depending on material). With longer exposure, bacteria may detach from their original place and spread over the surface of histological cuts, leading to contaminations of sterile locations.
- Use polypropylene tubes (e.g., 2 ml Eppendorf tube, 15–50 ml Falcon tube) because polypropylene is resistant to Carnoy.

#### 3.2.2 Collecting, Fixating, Transporting, and Embedding of Samples

Different fixatives were tested, and the best results were achieved with modified nonaqueous Carnoy's solution composed of 6/6/1 vol. ethanol/glacial acetic acid/chloroform.

For Tissue Biopsies and Surgically Removed Material

1. Samples of human tissue can be directly placed in a modified Carnoy fixative and stored or shipped at room temperature (RT) to the laboratory. All mechanic pressure (flattening the sample or the use of a shaker) should be avoided because it may injure the biofilm structure. The amount of modified Carnoy's solution should exceed the volume of tissue sample by a factor of 20 (Table 1; *see* **Note 4**).
2. The minimal incubation time for biopsies in Carnoy fixative is 12 h but preferably at least 24 h. In case of larger tissue pieces, prolong the incubation time for 4 h for each additional millimeter of the tissue size. If the added Carnoy fixative is less than ten times the tissue/sample volume, the storage should not

**Table 1**
**Amount of Carnoy depending on the sample size**

| Sample size ($mm^3$) | Volume of Carnoy (ml) |
|---|---|
| 1 | 0.5 |
| 5 | 5 |
| 6–20 | 15 |

exceed 2 weeks. In case of excess of Carnoy (more than 20-fold), excellent results could still be obtained after storage for 6 months and probably longer.

3. A change of Carnoy by longer storage time is preferable.
4. After decanting the modified Carnoy solution, the same volume of ethanol (absolute) is added, and the sample is incubated at 4 °C for a time period based on the size of the sample. Biopsy samples up to 3 mm are incubated for 15 min and big tissue samples (4–20 mm) for 2 h (*see* **Notes 5–6**).
5. After cold incubation, the ethanol is poured, and the tissue is put very carefully—without squeezing—into a new tube with ca. 5 ml of xylene (p.a.) and incubated overnight at RT.
6. When the xylene is decanted the following day, the sample is transferred carefully in an embedding mold with pre-warmed, melted paraffin. When the samples are larger than 5 mm, they are first put on a paper towel for 10 min to achieve the evaporation of the xylene before putting the sample into the paraffin. Small samples (<3 mm) are incubated for 1 h at 75 °C, samples up to 10 mm for 2 h at 75 °C, and large samples (10–20 mm) overnight at 65 °C.
7. After this first incubation, the paraffin is disposed and fresh paraffin is added and incubated for 2 h. This step is not necessary for small samples. After this, the mold is taken out of the incubator and placed on ice. The sample is positioned in the middle of the mold by using a toothpick. While the paraffin is coagulating, a pre-identified embedding cassette is put on top of the mold. Some warm paraffin is poured on this cassette to connect with the mold. These molds are first stored at 4 °C during 15 min followed by storage at −20 °C for at least 15 min. After complete coagulation of the paraffin, 4 μm cut sections can be made and put on a glass slide (Superfrost Plus slide) used for histological research. These slides are incubated for 1 h at 50 °C to assure the connection between the cut section and the slide.
8. The deparaffinization of the slides is obtained by putting them four times for 2–3 min each time at RT first in successive xylene

(p.a.) baths and next in successive ethanol (absolute) baths. The slides are incubated for 25 min at 50 °C. The sections are encircled with a thin line with the pap pen and dried.

9. A lysozyme step (Carl Roth, Germany) is only necessary for some strains of mainly gram-positive bacteria (depending on the probe). The entire section is covered by lysozyme solution (1 mg $ml^{-1}$) and incubated at 37 °C for 15–90 min in a humid pre-warmed chamber (depending on the sample). The optimal time has to be evaluated for each kind of sample. The lysozyme can introduce biases in polymicrobial communities by destroying, for example, *Proteobacteria* completely. It is important to obtain the best results in regard to the specific target species and other bacterial groups.
10. After incubation, the slides are flushed with distilled water and dried for 5 min at 50 °C.

For Fluid Secretions, Lavage, and Urine

1. Fluid secretions and lavages are done by MDs. The collection and fixation of urine samples is performed by the patient. Women are asked not to wash the urogenital region in the evening before sample collection and to use the first part of the morning urine, which increases the number of desquamated epithelial cells in urine sediments. Men need to pull the foreskin back over the glans penis before urine collection.
2. Two milliliters of the liquid sample are mixed with 8 ml Carnoy fixative in 15 ml Falcon tubes. These samples are not stable due to the high water amount. Therefore, the time for delivery to the laboratory should not exceed 4 weeks. Shorter periods are preferable. At arrival in the laboratory, fixated fluids and secretions should be centrifuged and the fixative solution decanted. Then Carnoy fixative is added to the sediment in a proportion of at least 1:20. Such prepared sediments can be stored until 6 months at RT.
3. Then there is a 1 cm circle drawn with a pap pen on the Superfrost Plus glass slide. Then 5 μl of the stirred sample is transferred to the slide with a plastic pipette within the area of hybridization and dried at 50 °C for 30 min. 5 μl of the final aliquot are convenient to use for single hybridizations. In case of urine sediments fixated as described above, this volume represents 30 μl of the initial urine volume.

### 3.3 The Hybridization

The hybridization of slides from tissues and fluids follows the same protocol.

1. A hybridization solution (Table 2) is prepared, in which the amount of formamide varies depending on the FISH probe.

**Table 2**
**Composition of hybridization solutions**

| % of formamide | Formamide (μl) | $H_2O$ (μl) | NaCl 5M (μl) | Tris-HCl 1M (pH 7.4) (μl) | SDS 10 % (μl) |
|---|---|---|---|---|---|
| 0 | 0 | 1,600 | 360 | 40 | 10 |
| 1 | 20 | 1,580 | 360 | 40 | 10 |
| 5 | 100 | 1,500 | 360 | 40 | 10 |
| 10 | 200 | 1,400 | 360 | 40 | 10 |
| 15 | 300 | 1,300 | 360 | 40 | 10 |
| 20 | 400 | 1,200 | 360 | 40 | 10 |
| 25 | 500 | 1,100 | 360 | 40 | 10 |
| 30 | 600 | 1,000 | 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 |

2. This solution is pre-warmed at 46 °C (or the probe-specific hybridization temperature; *see* **Note** 7), and 0.5 μl of probe (50 ng $\mu l^{-1}$) is added to 50 μl of hybridization buffer (per sample section). This amount of hybridization buffer is enough to cover the section completely.
3. The section is incubated for 45 min to 3 h at 46 °C in a humid pre-warmed chamber and in the dark. The incubation time should be optimized for different bacterial groups.
4. Wash buffer (Table 3) is prepared and warmed at 48 °C during this incubation period. The wash buffer composition depends on the formamide concentration used for hybridization.
5. After incubation, the slides are flushed with distilled water, and two slides are put back-to-back in a 50 ml Falcon tube completely filled with the pre-warmed wash buffer.
6. The tubes are incubated in a 48 °C water bath for 5 min; then they are flushed with distilled water.
7. The slides are dried in an upright position in an oven at 50 °C for 5 min in the dark.
8. Sections are placed in a cardboard slide folder, covered with 50 μl DAPI solution (1 μg $ml^{-1}$), and incubated for 5–10 min at RT in the dark.

**Table 3**
**Composition of wash buffer**

| % of formamide 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 | 9,000 | 40.0 | 1,000 | 30 | 0 |
| 1 | 900 | 9,000 | 40.0 | 1,000 | 30 | 0 |
| 5 | 636 | 6,300 | 42.7 | 1,000 | 30 | 0 |
| 10 | 450 | 4,500 | 44.5 | 1,000 | 30 | 0 |
| 15 | 318 | 3,180 | 45.8 | 1,000 | 30 | 0 |
| 20 | 225 | 2,150 | 46.4 | 1,000 | 30 | 500 |
| 25 | 159 | 1,490 | 47.0 | 1,000 | 30 | 500 |
| 30 | 112 | 1,020 | 47.5 | 1,000 | 30 | 500 |
| 35 | 80 | 700 | 47.8 | 1,000 | 30 | 500 |
| 40 | 56 | 460 | 48.0 | 1,000 | 30 | 500 |
| 45 | 40 | 300 | 48.2 | 1,000 | 30 | 500 |
| 50 | 28 | 180 | 48.3 | 1,000 | 30 | 500 |
| 55 | 20 | 100 | 48.4 | 1,000 | 30 | 500 |

Materials necessary:
- Lysozyme (Carl Roth, Germany)

9. Then they are flushed with distilled water and dried for 5 min in the dark in an oven at 50 °C in an upright position.
10. The slides can be kept dry for about 6 weeks in cardboard folders at RT in the dark.

### *3.4 Evaluation*

This is the most expensive part. The microscope can however be shared at the beginning with other research groups. In our laboratory, we use a Nikon E600 fluorescence microscope (Nikon; Tokyo, Japan; 40,000 €). We also use a Digital Microscope Camera ProgRes® CFcool (7,000 €) and accompanying software (Jenoptik, Jena, Germany). The color camera is necessary for documentation and performance of multicolor FISH pictures to demonstrate the spatial relationship between single microbial groups and to exclude cross hybridizations of unrelated FISH probes.

True color micrographs are preferred because they approach reality the most. However, evaluation of fluorescence signals based on micrographs only should be discouraged. In contrast to material composed of bacteria only, human samples contain complex DNA-bearing structures, which may non-specifically bind the oligonucleotides of FISH probes and make it difficult to distinguish them from the bacteria-specific signals. While the human eye can easily

differentiate between real signal and biases, multiple irrelevant signals may appear genuine in micrographs, especially when contrast and intensity are manipulated by software. The nuances are imperceptible on micrographs taken with black and white fluorescence camera. The often used subsequent coloring of the signals is deceptive.

#### 3.4.1 Application of FISH: The Enumeration of Bacteria

Only hybridization signals which are clear and morphologically distinguishable as bacterial cells with at least a triple color identification with universal and group-specific FISH probes and DAPI stain, in the absence of cross hybridization with taxonomically unrelated probes, can be enumerated.

We enumerate bacterial concentrations of homogeneous populations visually in one of the square fields of the ocular raster corresponding to $10 \times 10$ μm of the section surface at $\times 1{,}000$ magnification or $10^9$ bacteria $ml^{-1}$ (a 10 μl sample with a concentration of $10^7$ cells $ml^{-1}$ has on average 40 cells per microscopic field at a $\times 1{,}000$ magnification). In case of uneven distribution of bacteria over the microscopic field, the amount of positive signals is counted in ten fields of the ocular raster along the gradient of distribution and divided by ten.

For microbial populations taking smaller surfaces than $10 \times 10$ μm, the above equation is adopted to a closer $1 \times 1$ μm raster.

In case of urine sediments, bacteria and epithelial cells must be referred to the urine volume and each other. Concentrations of epithelial cells within the $5 \times 5$ mm area of hybridization (corresponding to the initial sample volume) are calculated and converted to numbers of epithelial cells per milliliter of urine. Since adherence is not even, it is recommended to determine the maximal and mean numbers of adherent bacteria per epithelial cell. The overall concentration of adherent bacteria in the urine results from multiplication of the mean number of bacteria per epithelial cell and the concentration of epithelial cells per ml of urine.

For possibilities of evaluation of ISH experiments in microbiology by electron microscope, see chapter by Hannes Schmidt, Thilo Eickhorst "Gold-FISH: In Situ Hybridization of Microbial Cells for Combined Fluorescence and Scanning Electron Microscopy".

## 4 Notes

1. Contrary to the expectations, none of the published FISH probes that were tested in our lab proved to be absolutely specific. Depending on the microbial community investigated (e.g., human or animal intestines, pancreatic duct, gallstones, biliary stents), all FISH probes demonstrated some cross-hybridization when conditions of optimal stringency were applied.

2. Some FISH probes that delivered highly specific results in human samples were cross hybridizing with unrelated probes in murine material. This indicates that the global diversity of bacteria is much higher than we presently accept.
3. When FISH probes for unrelated bacterial groups identify bacteria of similar morphology and equal numbers and at similar locations, the specificity of signals should be evaluated by performing multicolor FISH with probes stained with different fluorochromes. When both micrographs are overlaid, the signals detected by both should not be the same [10].
4. The use of modified Carnoy in a higher ratio does not reveal disadvantages, while a smaller volume increases the proportion of water in the solution, resulting in a decreased quality of hybridization.
5. After paraffin embedding, the fluorescence intensity declines over time (10 % during each year of storage). The reduction of 30 % after 3 years can be critical for less numerous and metabolically active microbial groups. Therefore, it is optimal to perform comparative studies within the first 2 years.
6. The fixation step is followed by embedding in paraffin which is time consuming. Microbiological cross contamination between samples should be avoided by preparing each sample on its own and by averting the use of an automated paraffin station.
7. Any hybridization oven can be used. When lacking such an oven, a microbial incubator that is able to maintain the temperature between 46 and 50 °C is an option.

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