Formalin-Fixed and Paraffin-Embedded Tissue Sections

Christine Sommerlad, Yasmin Mehraein, Marina Giersberg, Kersten Marben, Harald Rieder, and Helga Rehder

Introduction

In situ hybridization in tissue sections has primarily been associated with RNA analysis in gene expression studies. More recently, in situ DNA hybridization in tissue sections has also gained increasing importance. The tissue context allows the correlation of specific genetic and morphological characteristics which is not possible by genetic analysis on a single cell level, e.g. in metaphase preparations or isolated interphase nuclei. Different cell types may be distinguished and malignant cells may be differentiated from non-malignant cells by genetic as well as morphological criteria.

The maintenance of the original tissue context may also be of fundamental importance when tissue areas carry different genetic markers – a situation often found in tumors.

A major advantage of this technique is the use of formalin-fixed and paraffin-embedded archival material, allowing the investigation of cases in which vital cells for conventional cell culture and metaphase chromosome

✉ Harald Rieder, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany (*phone* +49-6421-28-62986; *fax* +49-6421-28-63984; *e-mail* riederh@mailer.uni-marburg.de)
Christine Sommerlad, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany
Yasmin Mehraein, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany
Marina Giersberg, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany
Kersten Marben, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany
Helga Rehder, Klinikum der Philipps-Universität, Institut für Klinische Genetik, Zentrum für Humangenetik, Bahnhofstraße 7, Marburg, 35033, Germany

analysis are not, or no more available, thus giving access to a wide pool of material hitherto not considered for genetic studies.

Due to distinctive features of the archival material, in situ hybridization in tissue sections is complicated by a variety of problems which arise either from characteristics of the tissue itself or from preparation procedures. Major difficulties include reduced probe penetration, high level of tissue autofluorescence, cell overlap or truncated nuclei, and last but not least, low hybridization efficiency caused by a long-formalin fixation.

To overcome these problems, different pretreatment procedures have been developed for tissue sections, involving proteolytic digestion (e.g. treatment with pepsin, trypsin, proteinase K), microwave treatment, preincubation in formamide solutions, acid treatment or the so called chaotropic treatment with NaSCN, first described by Hopman and colleagues in 1991.

Principles and applications

Although light microscopy is well established in histology, in situ hybridization with fluorescence detection has the major advantages (compared to bright-field microscopic detection), of a higher sensitivity and the possibility of using various fluorescent dyes for multi-target detection. The application of direct fluorochrome labeled probes greatly facilitates the detection procedure.

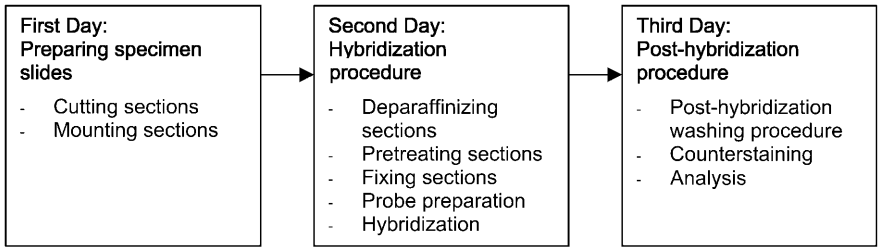
Main diagnostic applications of FISH in tissue sections relate to questions concerning:

- abortion material where fetal and maternal cells may be distinguished, as in the placenta or where the differentiation between tissue types allows correlation between genetic markers and morphological features in cases of genetic mosaicism,
- tumor material where tumor cells can be distinguished from normal cells, and seemingly normal cells can be unmasked to show the presence of genetic abnormalities. Cell clones with different genetic markers can be separated from each other and, because the tissue maintains its original cell formation, can be related directly to the tissue regions in haemotoxylin and eosin(HE)-stained serial sections.

In the following, we outline a protocol which has proved highly efficient for hybridization with direct fluorochrome labeled probes on formalin-fixed paraffin-embedded tissue sections.

Outline

Essential steps working with FISH on formalin-fixed paraffin-embedded tissue sections are shown in the following scheme:



Materials

First day: preparing specimen slides

Equipment

- Microtome (e.g. Leitz Type 530-492)
- Water bath (e.g. GFL Type 1052)

Supplies

- Positively charged slides (e.g. Super Frost/Plus Menzel) or coated slides (poly-l-lysine, silane)

Specimens

- Paraffin blocks of conventionally embedded tissue

Second day: hybridization procedure

Equipment

- Two water bath circulators (for simultaneous use), one heating up to 100°C (e.g. RM 6 Lauda GFL 20 l)
- Heating plate, heating up to 50°C
- Shaker (e.g. Vibrax, IKA Labortechnik)
- Microwave with temperature sensor (can be used facultatively for alternative wash procedure)

Supplies

- Coverslips, 22×22 mm and 22×60 mm
- Rubber Cement (e.g. Fixogum, Marabu)
- Heat resistant Coplin jars

Reagents and solutions

- Xylol 100%
- Ethanol 100%, 85% and 70%, store at 4°C
- 1 M NaSCN, autoclaved
- 0.2 M HCl
- Pepsin, lyophilized 2500-3500 units/mg
- 0.9% NaCl, pH 2.0
- 2×SSC, pH 7.0 (prepare from 20×SSC)
- 70% Formamide in 2×SSC: Deionise formamide using mixed bed resins (e.g. Dowex, mix 70 ml deionised formamide with 10 ml 20×SSC, add distilled water to 100 ml volume, adjust to pH 7.0
- Formalin 3.7% in phosphate buffered saline

Third day: post-hybridization procedure

Reagents and solutions

- 2×SSC/0.3% Nonidet p40
- DAPI staining solution: 0.2 µg DAPI/ml 2×SSC, prepare from stock solution (200 µg DAPI/ml distilled water)
- Mounting medium for fluorescence microscopy (e.g. Vectashield)

Procedure

First day: preparing specimen slides

1. Cut 5 µm thick paraffin sections using a microtome.
2. Float the section on a protein free water bath at 40°C.
3. Mount the section on slides.
4. Air dry the slides.
5. Bake the slides overnight at 56°C.

Second day: hybridization procedure

- Prewarm Coplin jar with NaSCN to 80°C in a water bath
- Prewarm a bottle with 0.9% NaCl and an empty Coplin jar in a 37°C water bath
- Adjust the heating plate to approx. 47°C

It is advisable to place the Coplin jars onto a gently moving shaker during incubation to improve the tissue-solution contact.

Deparaffinization

1. Immerse slides in Xylol: 3×10 min.
2. Dehydrate slides in 100% ethanol: 2×5 min.
3. Dry slides on a heating plate: 2-3 min.

Acid treatment

Treatment with acid (e.g. HCl) pulls the basic histone proteins away from DNA; especially in formalin fixed material, it helps to deproteinize the DNA if signals are weak.

4. Immerse slides in 0.2 N HCl at room temperature: up to 20 min.
5. Rinse in distilled water: 3 min.
6. Rinse in 2×SSC: 3 min.

Chaotrope treatment

Chaotrope treatment by a solvent disrupting molecular structures – which is a compound or group of secondary structures that interfere with the mechanism which stabilizes the complex proteins, nucleic acids and polysaccharides. These agents denature macromolecules primarily through their disruption of the water lattice.

7. Incubate slides in 1 M NaSCN, 80°C: 30 min.
8. Rinse in distilled water: 1 min.
9. Immerse slides in 2×SSC at room temperature (solutions can be kept for step 13): 2×5 min.
10. Turn down water bath to 73°C and place Coplin jar containing formamide into it.

Note: Caution: glass jars may crack if not prewarmed!

Protease treatment

11. Add lyophilized pepsin to the bottle of prewarmed 0.9% NaCl, pH 2.0, to a final concentration of about 1250-1750 units/ml, invert several times to mix and pour into the prewarmed Coplin jar.
12. Remove slides from 2×SSC, blot off excess liquid and incubate in pepsin solution at 37°C: 10 min.

    Note: Make sure that temperature has reached 37°C after adding all slides (at most 6/jar) and check temperature during the whole procedure.
13. Rinse in 2×SSC at room temperature: 2×5 min.
14. Place on the slide warmer to dry: 2 min.

Postfixation

15. Immerse slides in 3.7% buffered formalin solution at room temperature: 10 min.

    Note: Do not shake Coplin jars during this step.
16. Rinse in 2×SSC at room temperature: 2 x 5 min.
17. Place on the heating plate to dry: 2 min.

Denaturation

18. Place slides into 70% formamide/2×SSC at 73°C: 5 min.

    Note: Make sure that temperature has reached 71-73°C after adding all slides (at most 6/jar) and check temperature during the whole procedure.
19. Dehydrate slides in icecold 70%, 85% and 100% ethanol: 1-2 min each.
20. Dry slides at room temperature.

    Note: If working with direct fluorochrome labeled probes, all following steps should be performed protected from the light (in subdued light or in a dark room if possible).

Hybridization

21. Prepare 10-15 µl hybridization mixture containing labeled probe, blocking DNA, 50% formamide and 10% dextran sulfate. If commercially available probes are used, prepare according to the manufacturers recommendations (e.g. in the cases demonstrated in Figs. 3A,B a mixture of centromere and locus specific probes was used.
22. Cap tightly and denature at 73°C in a water bath: 5 min.
23. Apply denaturated hybridization mixture onto the slide.

24. Place a 22×22 mm coverslip on the hybridization area, seal with rubber cement, dry on heating plate .
25. Place slides in a humidified box and incubate in a 37°C incubator overnight (at least 14-18 h).

Third day: post-hybridization procedure

Prewarm Coplin jar containing 2×SSC/0.3% NP 40 in a 73°C water bath.

Washing

1. Carefully remove rubber cement with forceps.
2. Immerse slides in 2×SSC/0.3% NP 40 at room temperature and float off coverslips.
3. Place slides into 2×SSC/0.3% NP 40 at 73°C: 2 min.

   Note: Make sure that the temperature has reached 71-73°C after adding all slides, at most 6 slides/jar.

More rapidly, the solution can be prewarmed to 73°C by a microwave if the temperature in the Coplin jar is controlled by a sensor. When proceeding immediately, the washing procedure may even be performed without using a water bath because the decrease in the temperature during incubation is negligible.

4. Place slides into 2×SSC/0.3% NP40 at room temperature.
5. Immerse slides in 2×SSC: 5 min.
6. Immerse slides in DAPI/2×SSC: 5 min.
7. Immerse slides in 2×SSC: 5 min.
8. Remove excess liquid by wicking off the edge of the slide, add one drop of mounting medium (e.g.Vectashield)/slide and cover with a 22×60 mm coverslip.
9. Analyze hybridization signals using an epifluorescence microscope equipped with filter combinations adequate for the fluorochromes used.

Analysis (basic remarks for signal evaluation in tissue sections): To provide reliable results, the counting of overlapping and ruptured nuclei should be avoided. Out of focus signals should be considered and focus-

sing the whole depth of the section is therefore necessary. It has to be kept in mind that part of the nuclei is truncated by the preceding tissue sectioning, leading to a loss of genetic material and reduced numbers of hybridization signals. Thus diagnostic results in tissue sections, although based on information from a single cell, require the analysis of large numbers of nuclei and statistical evaluation.

Results

We performed this method on tissue sections of various thicknesses (2, 5, 10, 15 and 20 μm) of archived, formalin-fixed and paraffin-embedded tissue specimens derived from placenta, fetal organs, lymphomas and breast cancers. Centromeric probes as well as single copy probes were successfully employed. Mainly centromeric probes used in a large variety of samples of different tissue origin were the basis for our statistical evaluation of FISH results.

The hybridization efficiency was dependent on the tissue type, the conservation of the tissues with respect to autolysis as well as necrosis, and the period of formalin fixation rather than of paraffin embedding. Regarding centromeric probes, diagnostic results could be achieved in approximately 60% of placental tissues, 20-90% of fetal tissues (depending on the different tissue types), 90% of lymphoma and 60-70% of breast cancer samples.

Characteristic signal distribution patterns were found after successfully performed hybridization. They clearly differentiated between chromosome and/or gene copy numbers, but not between different levels of ploidies. Exclusion of polyploidy required the application of several chromosome probes in single, double or multiple color FISH. The combined use of differently labeled gene specific and centromeric probes of the same chromosomes helped to distinguish between changes in the gene copy number, due to complete chromosome gains or losses and due to structural chromosome rearrangements (Figs. 1, 2). Double in situ hybridization using human DNA and viral RNA probes was performed for the identification of Ebstein Barr virus positive tumor cells. By the combination of using chromosome-specific FISH with immunhistochemistry to identify tumor cell-specific antigens, a correlation of cytogenetic, immunological and morphological criteria was possible.

The section thickness as well as the chromosome copy numbers directly influenced the FISH results with respect to the detection of the true karyotype. The highest number of spots per nucleus present in at least

5% of all nuclei corresponded well with the true copy number of the respective chromosome and was independent of section thickness and also of chromosome count. The fraction of the nuclei which represented the true chromosome number decreased with the copy number of this chromosome and increased with the section thickness. In placental tissues, for instance, it was highest in monosomy and lowest in tetrasomy at a section thickness of 20 μm. Except for monosomies, the detection of low grade mosaicism usually required sections thicker than 5 μm and the evaluation of an increased number of cells.

The average number of spots per nucleus was also used to identify monosomies, disomies and trisomies in thin tissue sections, while detection of tetrasomies required sections of more than 15 μm. The influence of section thickness on signal distribution patterns was dependent on the density of nuclei within the tissue formation. The placenta, for instance,

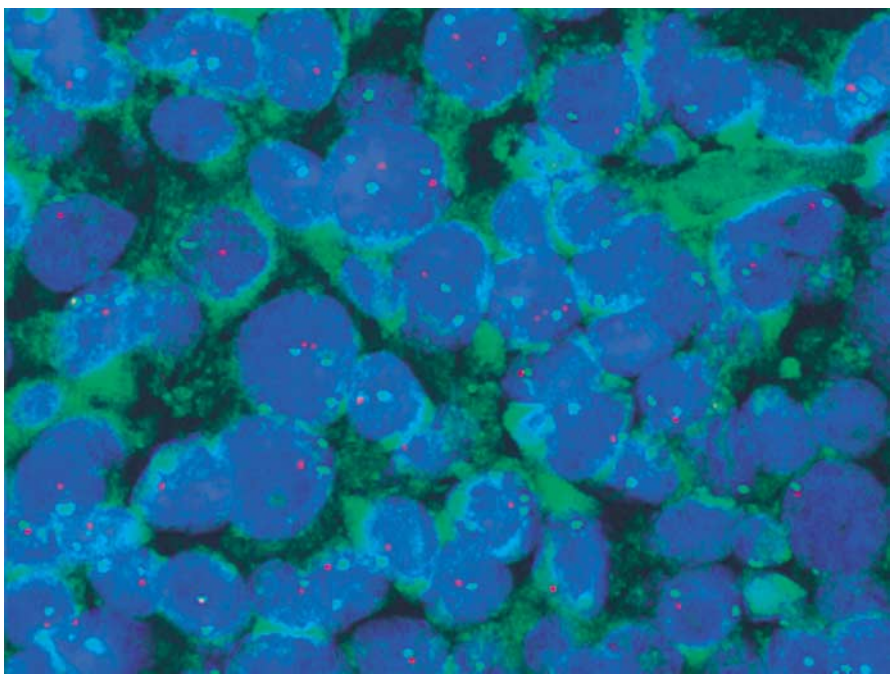


Fig. 1. Dual color FISH with differently labeled DNA probes for the p53 gene (red), localized on the short arm of chromosome 17, and for centromeric alpha satellite DNA (green) of chromosome 17, performed on sections of formalin-fixed archival breast cancer tissue. Most nuclei show two chromosome 17 centromeric together with only one p53 signal pointing to a loss of p53

represents a tissue with a low density of nuclei. In tumors which were characterized by a high tumor cell density, e.g. lymphomas or ductal breast carcinomas, 5 µm sections were sufficient for the evaluation of the signal numbers. The investigations aimed at identifying high level gene amplifications such as the proto-oncogenes HER-2/neu or c-myc in ductal breast cancer were even hampered by thick sections, so the use of 5 µm sections was preferred.

Troubleshooting

Hybridization efficiency and tissue morphology are usually negatively correlated. Aggressive pretreatment procedures improving hybridization efficiency (e.g. long incubation times of NaSCN or proteolytic digestion)

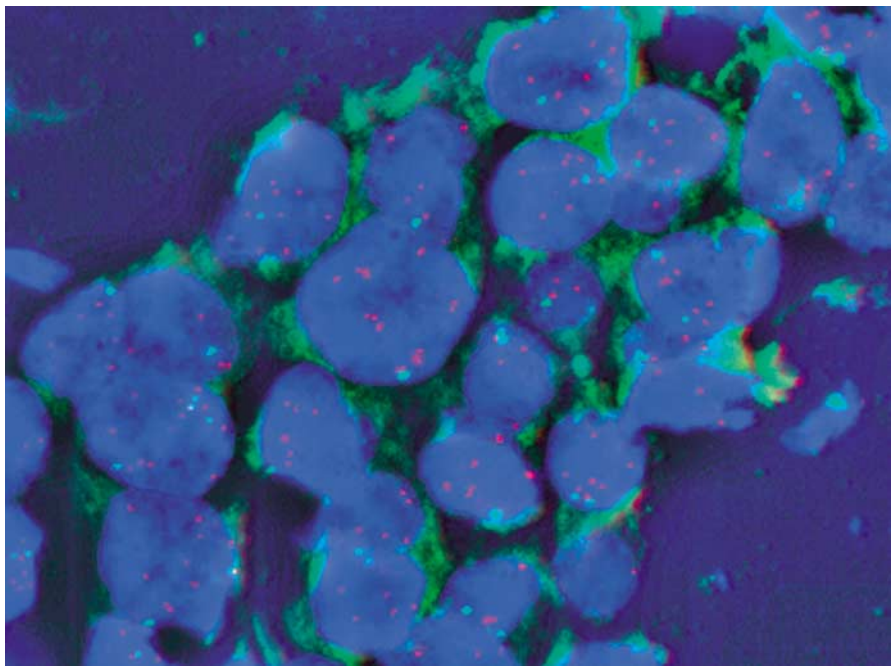
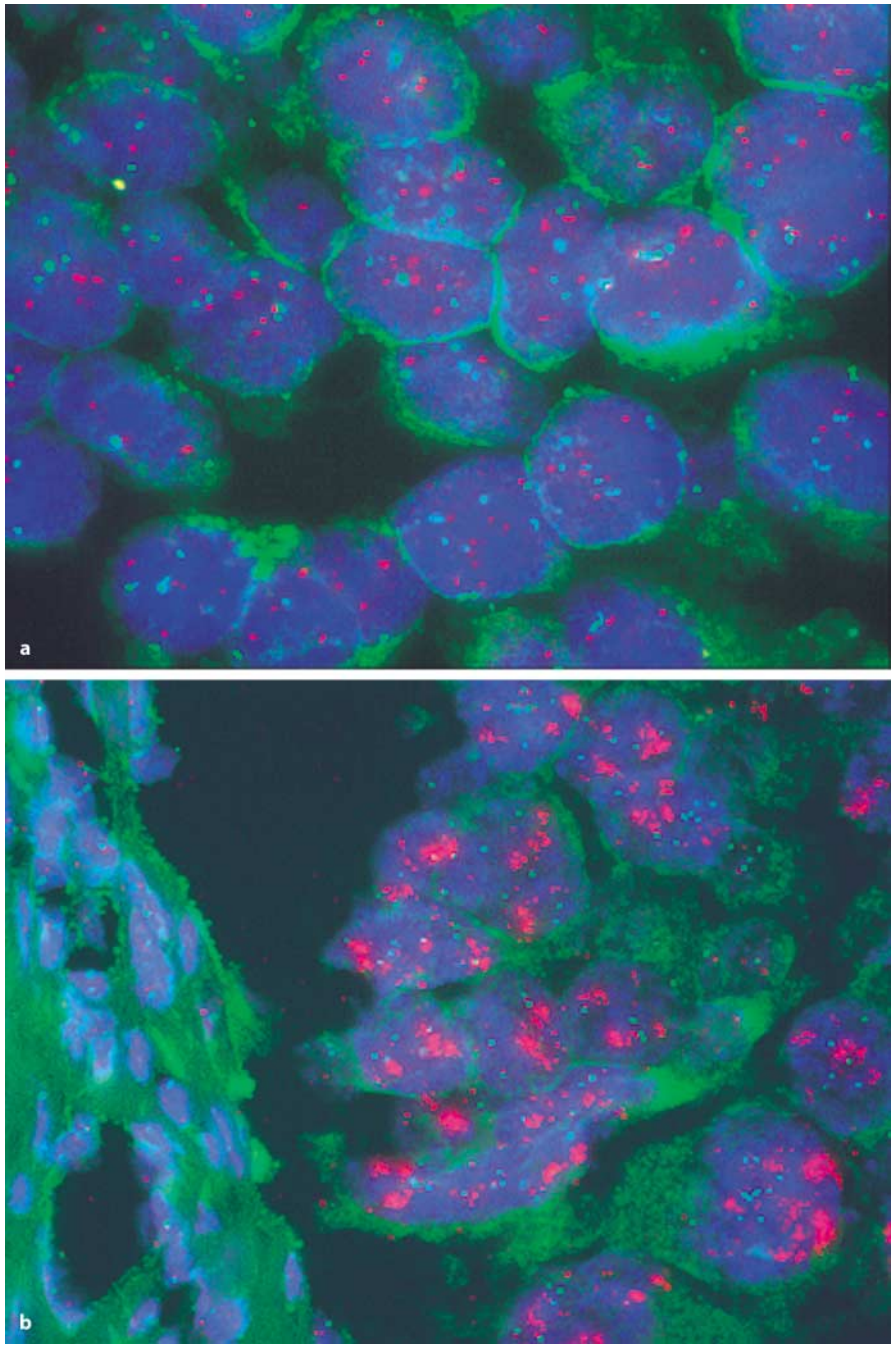


Fig. 2. Dual color FISH with differently labeled DNA probes for the c-myc gene (red), localized on the long arm of chromosome 8, and for centromeric alpha satellite DNA (green) of chromosome 8, performed on sections of formalin-fixed archival breast cancer tissue. Most nuclei show two chromosome 8 centromeric together with 8-10 c-myc signals indicating an intermediate c-myc amplification


a
b

tend to destroy cell structure and tissue morphology. However, mild pretreatment saving tissue structures, may not be sufficient for probe penetration and successful FISH results. Especially locus specific probes with a single copy target may show dim or a reduced number of signals.

Generally: If the experiment did not work – before changing any of the parameters, try it again!

Problem	Presumed Cause	Solution
Porous paraffin block	– Prolonged storage	– Embed anew
Tissue loss, complete or partial	– Insufficient slide coating – Tissue gets lost during the NaSCN procedure – The tissue was not fixed thoroughly	– Use positively charged (e.g. Super Frost Menzel) or coated slides (e.g. poly-l-lysine, silane) and bake the slides at 56 °C overnight – Check temperature of NaSCN buffer (should not exceed 80 °C) – Decrease the incubation time of NaSCN – Increase the incubation time of buffered formalin (some specimens do not stick to slides at all!)
Poor tissue morphology	– The tissue was digested too extensively – The tissue was extensively fixed (specimen probably exceeded fixation times of 24–48 h) – The tissue was insufficiently fixed or autolytic	– Reduce the incubation time in protease solution – Check temperature of the protease solution – Eliminate or reduce the fixation step with formalin – Choose new specimen – Choose new specimen
High tissue autofluorescence		– Choose new specimen, problem cannot be eliminated

Fig. 3. Dual color FISH with differently labeled DNA probes for the HER-2/neu gene (red), localized on the long arm of chromosome 17, and for centromeric alpha satellite DNA (green) of chromosome 17, performed on sections of formalin-fixed archival breast cancer tissue. **a** Multiple distinct signals of HER-2/neu as well as of centromeric DNA of chromosome 17 point to polysomies of chromosome 17. **b** On the *right*, red signals appear as clusters demonstrating high amplifications of HER-2/neu in tumor cells. Distinct green signals indicate multiple copies of chromosome 17. The HER-2/neu amplifications are estimated at 10-40 signals per nucleus. On the *left*, normal connective tissue with vascular structures is visible showing normal signal patterns.

Problem	Presumed Cause	Solution
Dim or no signal	– The tissue was poorly digested (the target is not sufficiently accessible) – The probe concentration was too low – The hybridization conditions were poor – Loss of DNA (may be indicated by weak DAPI staining)	– Prepare new protease solution directly before use – Check temperature of the water bath (exactly 37+/-1 °C) – Check pH of the protease buffer (exactly 2.0) – Ensure the correct amount of probe. – If working with dual color FISH, only increase the concentration of the probe which shows weaker signals. – Check probe hybridization time (at least 14–16 h) – Check temperature of the hybridization incubator (37+/-1 °C) – Increase fixation time
Probe signal intensity varies within the tissue	– Regional loss of DNA – The hybridization mixture was unevenly distributed on the section	– Do not score the slide if DAPI staining is weak, choose new section – Score the slide if DAPI staining is strong – Ensure that there are no air bubbles in the hybridization mixture during incubation
Signals are speckled over the whole slide	– The stringency conditions were too low	– Check the concentration of the wash buffers – Check temperature of the washing buffer (73+/-1 °C) – Gently agitate the Coplin jar containing the slides to improve the washing – Increase the washing time (5 min)

References

Hopman AHN, van Hooren E, van de Kaa ChA, Vooijs PGP, Ramaekers FCS (1991). Detection of numerical chromosome aberrations using in situ hybridization in paraffin sections of routinely processed bladder cancers. Modern Path. 4, 503-513

Hopman AHN, Classen S, Speel EM (1997). Multi-color brightfield in situ hybridization on tissue sections. Histochemistry Cell Biology. 108, 291-298

Hyytinen E, Visakorpi T, Kallioniemi A, Kallioniemi OP, Isola JJ (1994). Improved technique for analysis of formalin fixed, paraffin embedded tumors by fluorescence in situ hybridization. Cytometry 16: 93-9

Kopf I, Hanson C, Delle U, Verbiene I, Weimark A (1996). A rapid and simplified technique for analysis of archival formalin fixed, paraffin embedded tissue by fluorescence in situ hybridization (FISH). Anticancer-Res. 16: 2533-6

Marben K, Mehraein Y, Jensen K, Rehder H (1997). FISH-derived diagnostic criteria for aneuploidies in paraffin embedded tissue sections. Medizinische Genetik 1/1997.

Marben K, Mehraein Y, Jensen K, Fritz B, Rehder H (submitted) Assessment of aneuploidies in paraffin-embedded placental tissue sections by FISH: Statistical evaluation of characteristic signal distribution patterns

Suppliers

Gesellschaft für Labortechnik mbH, Burgwedel, Germany

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