

M E T A  
S Y S T E M S

# XCyte Lab Manual

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XCyte lab manual  
April 2003  
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## What about mFISH...



- simultaneous presentation of all 24 different human chromosomes with one single hybridization
- analysis of hidden or complex chromosome aberrations
- composition of marker chromosomes

## ... or mBAND?



- color banding pattern along one chromosome
- higher level of precision within one chromosome
- detection of intrachromosomal rearrangements
- detection of break points

Dear FISHerman,

Welcome to the XCyte lab manual. This brochure intends to give you an insight into the mFISH/mBand laboratory procedure. You will find the hybridization protocol, of course. And in addition some hints concerning the equipment, the handling and storage of the reagents and, just in case, the troubleshooting. To make it easy to survey, we have illustrated our protocol with ideograms here. Along these lines the lab manual is an extended pack insert (as it is enclosed in each XCyte kit). Beyond that you will find some background information on FISH and fluorescence microscopy as well as a short guide for the analysis with the Isis FISH imaging system.

Enjoy the XCyting colors!

Yours sincerely

MetaSystems GmbH

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# 1 Fluorescence *in situ* Hybridization

This chapter gives a short introduction into the FISH technique, the basics of the hybridization procedure as well as the special features of the multicolor applications mFISH and mBAND, and presents the MetaSystems XCyte mFISH and mBAND kits.

## 1.1 FISH

The technique of fluorescence *in situ* hybridization (FISH) is based on the reassociation of complementary DNA single strands. The probe is made of specific DNA pieces, whose nucleotides are labeled with fluorescent molecules. Denatured DNA from a certain sample forms the target. Complementary sequences of probe and target DNA are then allowed to reanneal. The fluorescence signal corresponding to the specific part of the double stranded DNA is detected by fluorescence microscopy.

Painting probes are used for a special type of FISH application. Chromosome or chromosomal region specific painting probes give prominence to a whole chromosome or a chromosomal region. The chromatin of chromosome preparations forms the target. This technique simplifies the analysis of numerical and structural chromosomal aberrations.

## 1.2 mFISH

The multicolor fluorescence *in situ* hybridization (mFISH) uses various fluorescence dyes to detect different painting probes at the same time. This offers the simultaneous presentation of all 24 different human chromosomes with a single hybridization in particular.

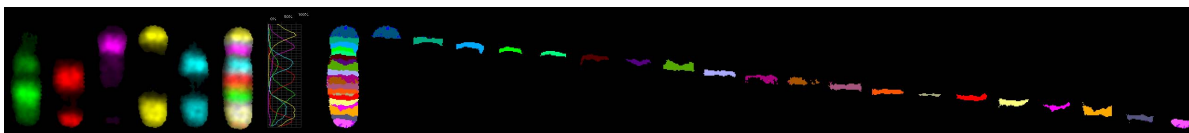
The detection of at least 24 different chromosome painting probes is achieved with five varicolored fluorochromes. Each paint is labeled with one of this five fluorochromes or a unique combination of them (combinatorial labeling). The separation of different excitation and emission spectra is guaranteed by appropriate filter sets (→ Chapter 2).

The resulting unequivocal color signature for each chromosome allows the analysis of numerical aberrations, hidden or complex chromosome aberrations, or to describe the composition of marker chromosomes.

### 1.3 mBAND

mBAND is a proprietary technique, which combines region specific paints to get a color banding pattern over the entire chromosome length. The region specific paints are partially overlapping and labeled with different fluorochromes or combinations of these.

The partial overlap of adjacent banding probes results in a multitude of unique color ratios along the chromosome. Color ratio analysis allows to resolve the chromosome into a selectable number of bands of similar ratios. This quantitative color ratio analysis effectively multiplies the resolution of the region specific probes. The color banding pattern is independent of the chromatin condensation.



mBAND takes multicolor analysis to a higher level of precision: it allows the determination of breakpoints and detects intra-chromosomal rearrangements like interstitial deletions, inversions, insertions, or duplications.

### 1.4 Direct and Indirect Labeled Probes

There are two possibilities to couple the fluorochromes to the DNA. Whereas in the direct method the fluorochrome is build into the DNA directly, the indirect labeling uses a reporter molecule to which a fluorochrome coupled antibody has to be bound following the hybridization procedure. The advantage of the direct labeling is, that no further post hybridization detection steps are necessary. On the other hand in the indirect method allows signal amplification is possible.

The MetaSystems' XCyte mFISH and mBAND uses five different fluorochromes. Four of them are coupled directly to the probes, namely DEAC (Diethylamino-coumarin), FITC, Spectrum Orange™, and Texas Red®. The other labeling is carried out using Biotin as a reporter molecule, which has to be detected by Streptavidin-Cy™5. The signal of this fluorochrome could be amplified by additional detection steps with biotinylated anti-Streptavidin and again with Streptavidin-Cy™5.

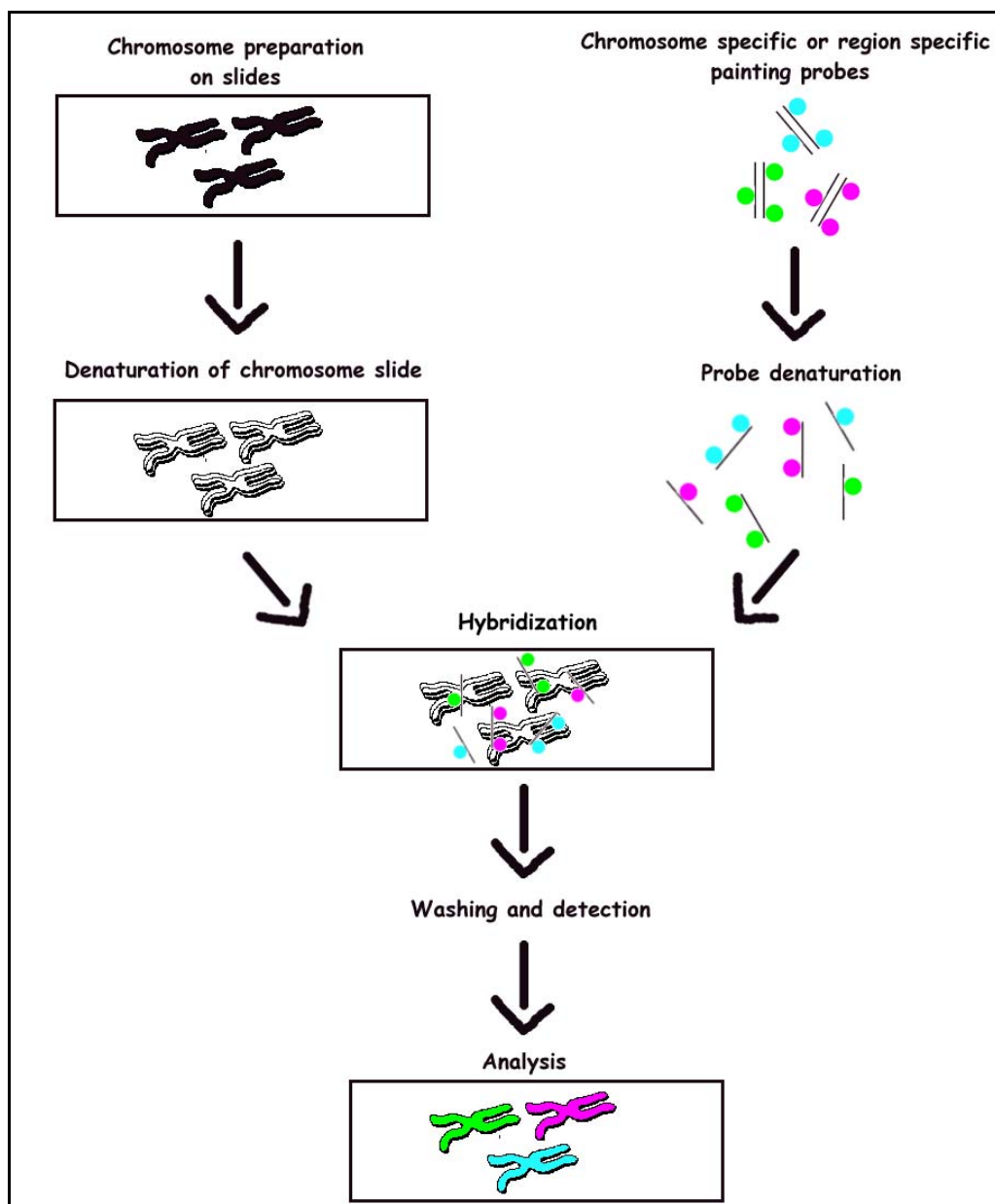
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## 1.5 The Hybridization Procedure

First you need a chromosome preparation from the case of interest and, of course, the painting probe.

The FISH procedure as such is composed of only four steps: denaturation of probe and target, hybridization, washing and detection and then analysis.

In other words: Separate the DNA of the painting probe and the DNA of the chromosomes into their single strands. Put probe and chromosomes together and let them build new double stranded DNA between the chromosomes and their complementary DNA peaces in the probe. Remove probe DNA that has not found an adequate partner. And than enjoy the colored chromosomes (and analyze them...).



The mFISH and mBAND procedures do not differ from that of 'simple' FISH.

- **Denaturation of probe and target:**  
Denaturation of DNA double strands could be induced by increasing temperature or pH of environmental solutions or by organic solvents. A combination of organic solvent and increased temperature is used for the denaturation of the probe and the target in general. This method is used for the probe denaturation here.  
For denaturation of the chromosomes we recommend a treatment with a basic solution to increase the pH, which is carried out in sodium hydroxide (0.07N NaOH) at room temperature.
- **Hybridization:**  
Reannealing of probe and target occurs in the presence of hybridization buffer. The hybridization is carried out in a humidified chamber for 2-4 days at 37°C.
- **Washing and detection:**  
Post hybridization washing is necessary to remove the remaining hybridization buffer and to undo unspecific probe binding. Afterwards the indirect labeled probes are detected with an additional fluorescence dye. Finally, the counterstain is applied.
- **Analysis:**  
Fluorescence signals are detected by fluorescence microscopy. Images have to be captured for every fluorescent dye with different single band pass filter sets. All six color channels (for the five different fluorochromes and the DAPI counter stain) are superposed by the Isis software. Image processing leads to karyotypes which than could be analyzed. Several software features support the analysis.

## 1.6 The MetaSystems' XCyte mFISH and mBAND Kits

The XCyte painting probes are supplied ready-to-use. They are already dissolved in hybridization buffer. The kits have been proven to give good results with several cell types, for example lymphocytes, amniocytes, bone marrow, or various cultured cell lines.

The number of tests depends on the size of the hybridized area. 60µl of probe cocktail are sufficient for 5 hybridizations using a 24x24mm<sup>2</sup> coverslip, or 8 hybridizations using an 18x18mm<sup>2</sup> coverslip.

The hybridization protocol and the corresponding label scheme are enclosed with each kit. The hybridization procedure is the same for all XCyte kits.

For research use only!

**Warning:** *Painting probes contain formamide. Handle carefully. Avoid contact with skin; wear gloves while handling the reagents.*

To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light!

Centrifuge all vials prior to opening to collect the contents at the bottom of the vials!

The XCyte mFISH and mBAND uses five different fluorochromes. Four of them are coupled directly to the probes, namely FITC, DEAC (Diethylamino-coumarin), Spectrum Orange™ and Texas Red®. The other labeling is carried out using Biotin as a reporter molecule, which has to be detected by Streptavidin-Cy™5. The signal of this fluorochrome could be amplified by additional detection steps with biotinylated anti-Streptavidin and again with Streptavidin-Cy™5.

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### 6.1.1 The 24XCyte mFISH Probe Kit

The 24XCyte kit contains 24 different chromosome painting probes specific for the 24 different human chromosomes. Each paint is labeled with one of five different fluorochromes or a unique combination of them.

The 24XCyte mFISH kit is available in three different pack sizes:

	Volume	Reference Number
24XCyte	60µl	D-0125-060-MC
24XCyte	120µl	D-0125-120-MC
24XCyte	600µl	D-0125-600-MC

### 6.1.2 The XCyte mBAND Probe Kits

Each XCyte mBAND kit contains a mix of region-specific partial painting probes specific for one chromosome. The probes are labeled with one of up to five different fluorochromes or a combination of them, respectively.

The XCyte mBAND kits are available in three different pack sizes:

mBAND kit	Volume	Reference Number
XCyte ##	24 $\mu$ l or 60 $\mu$ l or 120 $\mu$ l	D-02##-024-MC
XCyte 1	24 $\mu$ l	D-0201-024-MC
XCyte 1	60 $\mu$ l	D-0201-060-MC
XCyte 1	120 $\mu$ l	D-0201-120-MC
XCyte 2	24 $\mu$ l	D-0202-024-MC
...	...	...
XCyte X	60 $\mu$ l	D-0223-060-MC
XCyte Y	60 $\mu$ l	D-0224-060-MC

### 6.1.3 The 21XMouse mFISH Probe Kit for Mouse



The 21XMouse kit contains 21 different chromosome painting probes specific for the 21 different mouse chromosomes. Each paint is labeled with one of five different fluorochromes or a unique combination of them.

The 21XMouse mFISH kit is available in two different pack sizes:

	Volume	Reference Number
21XMouse	60 $\mu$ l	D-0425-060-MC
21XMouse	120 $\mu$ l	D-0425-120-MC

#### 6.1.4 Stability of MetaSystems' Probes

The MetaSystems XCyte mFISH and mBAND probes are stable for at least half a year from delivery, if stored properly:

- Store at -20°C
- Avoid repeated freeze-thaw-cycles (divide probe cocktail into appropriate aliquots)
- Avoid temperatures above 30°C

Stability tests have shown that the probes still work fine after a two-year-storage in the freeze. Hybridized slides should be stored at -20°C, too.

#### 6.1.5 The B-tect Detection Kit

The B-tect detection kit contains reagents required for the Cy<sup>TM</sup>5 detection of the Biotin labeled painting probes. The kit includes DAPI counter stain and antifade.

□□□□□	blocking reagent	Store at -20°C
■ ■ ■ ■ ■	detection 1+3 (streptavidin-Cy <sup>TM</sup> 5)	Store at -20°C
■ ■ ■ ■ ■	detection 2 (biotinylated $\alpha$ -streptavidin )	Store at -20°C
■ ■ ■ ■ ■	DAPI/antifade	Store at 4°C

The B-tect detection kit is recommended for the 24XCyte mFISH kit and the XCyte mBAND kits containing Biotin labeled probes: (XCyte 1 - XCyte 12, XCyte X)

Some of the XCyte mBand kits are without Biotin labeled probes. For those we offer the DAPI/antifade reagent separately (XCyte 13 - XCyte 22).

The B-tect detection kit is available either for 10 and 20 applications. One application means one slide independent of the hybridized area.

	Volume	Reference Number
B-tect	10 Applications	D-0901-060-IR
B-tect	20 Applications	D-0901-120-IR
DAPI / Antifade	10 Applications	D-0902-060-DA
DAPI / Antifade	20 Applications	D-0902-120-DA

## 1.7 Literature on mFISH and mBAND

### A small selection:

- *M. R. Speicher, D. C. Ward* (1996)  
The coloring of cytogenetics.  
Nat Med 2:1046-1048.
- *E. Schröck, T. Veldman, H. Padilla-Nash, Y. Ning, J. Spurbeck, S. Jalal, J. P. Schaffer, P. Papenhausen, C. Kozma, M. C. Phelan, E. Kijeldsen, S. A. Schonberg, L. Biesecker, S. du Manoir, T. Ried* (1997)  
Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities.  
Hum Genet 101: 255-262.
- *I. Chudoba, A. Plesch, T. Lörch, J. Lemke, U. Claussen, G. Senger* (1999):  
High-resolution multicolor-banding: a new technique for refined FISH analysis of human chromosomes.  
Cytogenet Cell Genet, 84:156-160.
- *C. Johannes, I. Chudoba, G. Obe* (1999):  
Analysis of X-ray-induced aberrations in human chromosome 5 using high-resolution multicolour banding FISH (mBAND).  
Chromosome Res 7: 625-633.
- *V. S. Lestou, R. D. Gascoyne, C. Salski, J. M. Connors, D. E. Horsman* (2002):  
Uncovering novel inter- and intrachromosomal chromosome 1 aberrations in follicular lymphomas by using an innovative multicolor banding technique.  
Genes Chromosomes Cancer, 34: 201-210.
- *K. Michalova, Z. Zemanova, J. Brezinova* (2002):  
Analysis of structural chromosomal aberrations by mFISH and mBAND

techniques.

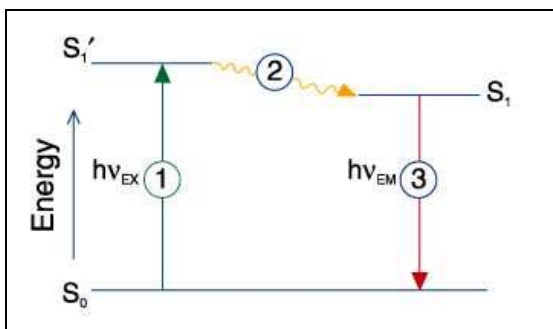
Early prenatal diagnosis, fetal cells and DNA in the mother - present state and perspectives. The Karolinum Press, Prag, pp 284-292, ISBN: 80-246-0397-7.

- *C. Schoch, T. Haferlach, S. Bursch, D. Gerstner, S. Schnittger, M. Dugas, W. Kern, H. Löffler, W. Hiddemann (2002)*  
Loss of genetic material is more common than gain in acute myeloid leukemia with complex aberrant karyotype: a detailed analysis of 125 cases using conventional chromosome analysis and fluorescence in situ hybridization including 24-color FISH.  
*Genes, Chromosomes & Cancer*, 35, 20-29.
- *Heller, H. Starke, V. Trifonov, N. Rubtsov, U. Wedding, I. Loncarevic, C. Bleck, U. Claussen, T. Liehr (2002)*  
A complex translocation event between the two homologues of chromosomes 5 leading to a del(5)(q21q33) as a sole aberration in a case clinically diagnosed as CML: characterization of the aberration by multicolor banding.  
*Int. J. Oncol.*, 20, 1179-1181.
- *M. Van Gele, J.H. Leonard, N. Van Roy, H. Van Limbergen, S. Van Belle, V. Cocquyt, H. Salwen, A. De Paepe, F. Speleman (2002)*  
Combined karyotyping, CGH and m-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma.  
*Int. J. Cancer*, 101, 137-145.
- *H. Van Limbergen, B. Poppe, L. Michaux, C. Herens, J. Brown, L. Noens, Z. Berneman, R. De Bock, A. De Paepe, F. Speleman (2002)*  
Identification of cytogenetic subclasses and recurring chromosomal aberrations in AML and MDS with complex karyotypes using M-FISH.  
*Genes, Chromosomes & Cancer*, 33, 60-72.
- *A. Weise, H. Starke, A. Heller, H. Tönnies, M. Volleth, M. Stumm, G. Senger, A. Nietzel, U. Claussen, T. Liehr (2002)*  
Chromosome 2 aberrations in clinical cases characterized by high resolution multicolour banding and region specific FISH probes.  
*J. Med. Genet.*, 39, 434-439.

## 2 Fluorescence Microscopy and Image Capturing

### 2.1 Fluorescence

Fluorescence is a photochemical process, which takes place in a special type of molecules the so-called 'fluorophores', or 'fluorochromes'. When a fluorochrome absorbs light, it is shifted into an excited state. A part of the absorbed energy is lost by internal structural changes and interactions with other molecules. Returning in its ground state, the fluorochrome emits light. Due to the energy loss during excited-state lifetime the wavelength of the emitted light is longer compared to the absorbed light. This difference in wavelength is called 'Stokes shift'. Each fluorochrome has its own characteristic excitation and emission spectrum.



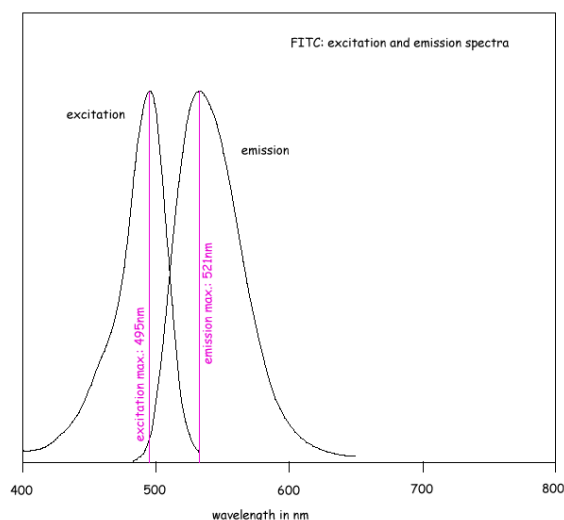
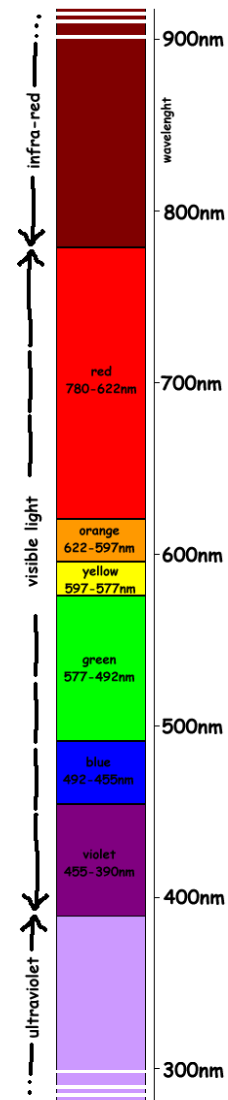
The basic of fluorescence is a three stage process:

- ① excitation
- ② excited-state lifetime (loss of energy)
- ③ fluorescence emission

The fluorochrome is excited repeatedly during illumination. Nevertheless, photochemical reactions may result in the irreversible destruction of the fluorochrome, which then cannot be excited any longer (photo bleaching or fading).

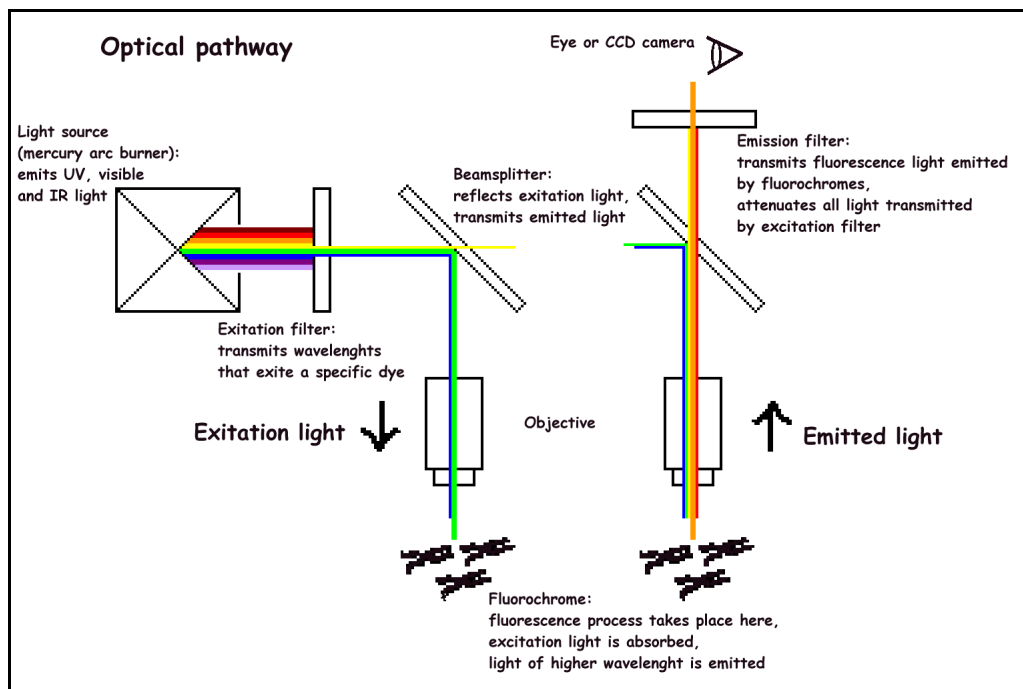
The basic feature for the sensitivity of the fluorescence techniques is the Stokes Shift, it allows discriminating between the emitted light and the absorbed light by a beam splitter.

### 2.2 Fluorescence Microscopy



In fluorescence microscopy the slide preparation is illuminated through the objective. As a light source usually a mercury vapor arc burner is used, which emits ultraviolet, visible and infrared light. The appropriate excitation wavelength for a particular fluorochrome is selected using an excitation filter. The light passes the excitation filter and is focused through the objective onto the slide preparation. The emitted light passes the emission filter, which transmits light within a bandwidth according to the emission spectrum of the fluorochrome.

The dichromatic beam splitter inserted between excitation filter and object reflects the short-waved excitation light onto the slide preparation. The longer-waved fluorescence light emitted by the object passes the beam splitter nearly completely and reaches the emission filter. This is necessary to create a dark background so that the fluorescence signal can be easily seen (surface reflections of the excitation light and backscattered light are reduced).



## 2.3 The UV Light Source

Arc burners are used as a light source for multicolor fluorescence applications in general because they generate enough excitation light intensity to furnish emission capable of detection. They are filled with high-pressure gas. The light source is powered by a power supply furnishing enough start-up power to ignite the burner by ionizing of the gaseous vapor and keep it burning by a minimum of flicker.

If DAPI is used, a mercury arc burner is needed. The spectrum of this light source is far from continuous, most of its light output is concentrated in few narrow lines.

**Note:** The lamp has a limited lifetime (e.g. 200 hours for HBO 100, or 300 hours for HBO 103). It loses efficiency and is more likely to shutter, if used beyond its rated lifetime. The lifetime is reduced by switching it on and off repeatedly.

**Note:** Let the lamp cool down for 30 minutes before switching it on again.

**Note:** It takes about 15 minutes to reach maximal intensity after ignition.



**Note:** Adjust the lamp. An evenly illuminated image field is fundamental in mFISH. The light arc has to be centered and focused. The light arc and its mirror image should be side by side and on the same size (see figure). Refer to the operating instructions of the microscope.

**Warning:** Mercury arc lamps require caution during operation because of the danger of explosion due to the high internal gas pressures and extreme heat during use. Never use a lamp outside of its housing or observe the lamp directly when it is burning, this can cause serious eye damage. The lamp should not be handled with bare fingers in order to avoid inadvertent etching of quartz envelope. Change bulbs only after the lamp has had sufficient time to cool off.

## 2.4 Fluorescence Filter Sets

A filter set including beam splitter, excitation and emission filter has to match to the excitation and emissions characteristics of the given fluorochrome. For multicolor fluorescence applications special small banded filter sets are necessary to separate adjoining colors sufficiently.

In all modern fluorescence microscopes the excitation and emission filters and the beam splitter are incorporated in a filter cube. If the filter turret cannot accommodate 6 filter cubes, one or two dual-band-pass-filter sets are necessary. In this case the change of the excitation filter is carried out by an additional filter wheel or slider inserted between the UV-lamp and the microscope.

The excitation and emission maxima of each fluorescence dye used in the XCyte mFISH and mBAND kits are given in the table overleaf.

Fluorochrome	Emission/ excitation maximum	Color of fluorescence signal	Color of excitation light	Color in Isis software (default settings)
DAPI (counterstain)	455nm/345nm	light-blue	violet	violet (blue of first triplet)
DEAC	480nm/426nm	blue/turquoise	blue	light blue (blue of second triplet)
FITC	521nm/495nm	green	turquoise	green (green of first triplet)
Spectrum Orange™	588nm/559nm	yellow	green	red (red of first triplet)
Texas Red®	615nm/595nm	red	yellow/orange	magenta (red of second triplet)
Cy™5	670nm/649nm	near IR (not visible)	red	yellow (green of second triplet)

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**Note:** Capture the DEAC channel at the end of the sequence to avoid cross talk with the DAPI counterstain (implemented as default setting in Isis software).

**Note:** Close the light pathway after capturing or looking through the microscope. Prevent the filters from heat damage by closing the shutter or by moving the filter turret and/or the filter wheel to a free position.

**Note:** Avoid infrared filters in the optical pathway especially in front of the CCD camera. The IR-filters adsorb part of the excitation light and nearly the complete emission light for Cy™5.

Reading filter specifications:

X: excitation filter

M: emission filter (barrier filter)

BS or D: beamsplitter (dichroic mirror)

B or BP: bandpass filter, with cut-off both to the lower and higher wavelengths

L or LP: longpass filter, open (transmitting) light to the red side of the spectrum (higher wavelengths)

Example:

360/40 X BP (DAPI excitation filter recommended for mFISH)

is a bandpass filter for excitation with a maximum transmission of 360nm and a bandwidth of 40nm

The filters have to be mounted correctly. A small arrow on the edge of the filter indicates the orientation. It should point into the filter cube (in direction of the object). The beamsplitter should be mounted so, that the

reflecting surface is turned in a 45° angle to the light source and the object, respectively. The reflecting surface is marked by an arrow tip or a facet cut.

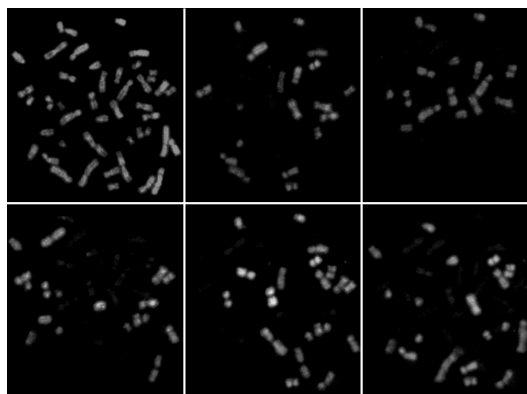
## 2.5 The CCD Camera and Image Capturing

A CCD -charge-coupled device- camera converts light energy into an electronic charge. Photons (of the emitted fluorescence light in our case) interact with the silicon atoms of silicon diode photosensors and generate electrons. The silicon diode photosensors are arranged in a matrix. The generated electronic charges in the pixel wells are read out (emptied) in fixed intervals (50 times per second, European video standard).

For an integrated image the charges from many photons are collected. The video output is stopped and light is allowed to fall on the CCD for a prolonged period.

A standard CCD camera is sensitive in the range of 300nm to 900nm (depending on CCD type). The sensitivity maximum covers the visible light, the CCD is less sensitive in the near infrared.

For the multicolor applications mFISH and mBAND a monochrome camera is used. Each fluorescence color is captured separately. First the DAPI filter set is turned into the light path. The counterstaining makes it easy to find a nice metaphase and to bring it into the focus plane. The first photograph of this metaphase is taken. Then the filters have to be changed to capture the FITC signals, and then the SpectrumOrange™ signals, and so on. This results in six black and white images for each mFISH metaphase. These six pictures are processed and analyzed by the Isis software.



For further reading on technical details we recommend the following websites:

[www.microscopy.fsu.edu](http://www.microscopy.fsu.edu) An excellent online tutorial by Mortimer Abramowitz and Michael W. Davidson.

[www.chroma.com](http://www.chroma.com) Download of the 'Handbook of optical filters for fluorescence microscopy' by Jay Reichman.

[www.probes.com](http://www.probes.com) 'Handbook of Fluorescent Probes and Research Products', Molecular Probes

[www.omegafilter.com](http://www.omegafilter.com) Testing filter characteristics online: 'Curvomativcs'.

[www.amershambiosciences.com](http://www.amershambiosciences.com) Download of Handbook of 'Fluorescence Imaging Principles and Methods' (Homepage ⇒ Literature ⇒ literature search: Fluorescence, Handbook)

## 3 Equipment and Reagents

### 3.1 Lab Equipment

- Water bath at 37°C, 70°C and 75°C
 

37°C ≈	70°C ≈	75°C ≈
-----------	-----------	-----------
- Refrigerator (4°C)
 

4°C ❄
----------
- Freezer (-20°C)
 

❄❄❄
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- Incubator 37°C
 

37°C
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- Microcentrifuge
 

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- Tubes: 0,5ml
 

🧴
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- Coplin jars for 50ml or 100ml
 

🧴
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- Humidified chamber
 

37°C ≈
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- Variable micropipettes: e.g. 1µl - 20µl, 50µl - 100µl, 500µl - 1ml
- pH meter or pH indicator sticks
- Thermometer
- Timer
- Gloves
- Coverslips: 18x18mm<sup>2</sup> or 22x22mm<sup>2</sup> or 24x24mm<sup>2</sup> (depending on the area to hybridize) and 24x60mm<sup>2</sup> (covering the whole slide)
- Rubber Cement, e.g. Fixogum (Marabu, Germany)
- Chromosome preparation on slides (→ Chapter 4.1)

### 3.2 Reagents Required

The following reagents are required for the hybridization procedure. They are **not** included either in the 24Xyte mFISH kit or the Xyte mBAND kit or the B-tect detection kit.

- **Water, deionized or distilled (Aqua dest.)**  
We recommend doubled distilled water.
- **100% Ethanol, denatured**
- **1N NaOH** 1N Sodium Hydroxide:  
Dissolve 40g Sodium hydroxide pellets ( $M=40.00\text{g/mol}$ ) per liter of final volume in distilled water.
- **1xPBS** Phosphate Buffered Saline Solution:  
120mM NaCl, 7mM  $\text{Na}_2\text{HPO}_4$ , 3mM  $\text{NaH}_2\text{PO}_4$  and 2,7mM KCl:  
Add 7,01g NaCl (Sodium chloride,  $M=58,44\text{g/mol}$ ),  
0,99g  $\text{Na}_2\text{HPO}_4$  (di-Sodium hydrogen phosphate,  $M=141,96\text{g/mol}$ ),  
0,41g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (Sodium dihydrogen phosphate Monohydrate,  $M=137,99\text{g/mol}$ )  
and 0,20g KCl (Potassium chloride,  $M=74,56\text{g/mol}$ )  
per liter of final volume to distilled water.  
We recommend to take ready to use solutions available from several manufacturers.
- **Tween20** Polyoxyethylenesorbitan-monolaurate  
Syrup (e.g. Sigma P-1379)  
Tween™ is a trademark of ICI America, Inc.
- **20xSSC** Saline-sodium Citrate Buffer:  
3.0M NaCl and 0.3M  $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ :  
Add 175.2g NaCl (Sodium chloride,  $M=58,44\text{g/mol}$ )  
and 88.3g  $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$  (Citric Acid Trisodium Salt Dihydrate resp. tri-Sodium citrate Dihydrate,  $M=294,10\text{g/mol}$ )  
per liter of final volume to distilled water.  
We recommend to take ready to use solutions available from several manufacturers.
- **Pepsin** Pepsin stock solution: Dissolve 1g pepsin (Sigma, P-7012) in 50ml sterile distilled  $\text{H}_2\text{O}$ , store in 500 $\mu\text{l}$  aliquots at  $-20^\circ\text{C}$ .
- **RNase A:** RNase stock solution: Dissolve 25mg RNase A (Roche, 109142, 25mg) in 2,5ml 2xSSC. Incubate for 10min at  $100^\circ\text{C}$ . Store in 100 $\mu\text{l}$  aliquots at  $-20^\circ\text{C}$ .
- **1N HCl** 1N Hydrochloric Acid
- **37% Formaldehyde (Formalin)**



- **1M MgCl<sub>2</sub>** 1M Magnesium chloride:  
9,52g MgCl<sub>2</sub> (M=95,21g/mol)  
or 20,33g MgCl<sub>2</sub>\*H<sub>2</sub>O (Magnesium chloride Hexahydrate,  
M=203,30g/mol)  
add water up to a final volume of 100ml.

## 4 Preparation

This chapter gives a step-by-step protocol for the hybridization procedure. Each unit of the protocol starts with a short introduction followed by a list of the reagents required for the next steps and a list of preparations which should be done before getting started (Chapter 4.4 to 4.8). There are some additional hints (Notes) making your work easier.

This hybridization protocol differs from most known FISH protocols using formamide for chromosome denaturation. We recommend this protocol because the procedure is less aggressive and preserves the structure and morphology of the chromosomes. Furthermore, we achieve better results in comparison to the formamide protocol.

**Note:** Processes, which change the DNA structure of chromosomes or probe, are critical. They occur at high temperature and/or exposure to organic solvents or changes of the pH value (denaturation of chromosomes, denaturation of probe, first post hybridization washing). Under these circumstances take great care for environmental conditions such as concentration, temperature and pH of solution and time of incubation.

### 4.1 Chromosome Preparation on Slides

The essential basis for an optimal hybridization is a high-grade chromosome slide preparation. The care and accuracy you invest in the preparation will pay off in the analysis: the nicer your chromosomes, the easier their analysis.

Metaphase spreads are prepared according to the conventional cytogenetically methods. Check slides for spreading and morphology under phase contrast before hybridization.

We recommend to use frosted slides and to mark them with a pencil. Note that other markers (special lab markers also) and stickers may not be resistant to the hybridization procedure. Be careful with slides, which are marked with diamond marker: if the slides are wet, it is difficult to distinguish the right from the wrong side.

Slides should not be older than two weeks. We recommend preparing the slides one day prior to hybridization. For long-term storage keep slides at  $-20^{\circ}\text{C}$ .

Remaining cytoplasmic proteins may impair with the hybridization. Hence, we recommend a protein digesting pretreatment prior to hybridization (→ Chapter 4.4).

RNase pretreatment is less effective for the hybridization procedure of **human** chromosomes according to our experience.



For **mouse** chromosomes we recommend an RNase pretreatment prior to the pepsin pretreatment.

**Note:** Further fixation or 'aging' processes may inhibit the hybridization. This is valid especially for slides or cell suspensions, which are stored for several month or years.

## 4.2 Stock Solutions

We recommend to prepare stock solutions for the pre- and post-hybridization washing steps. They can be used up to three month. Store in a dark place at room temperature. For long term storage use sterilized or fresh distilled water.

200ml **0.07N NaOH**

14ml 1N NaOH

186ml Aqua dest.

500ml **2xSSC**, pH7.0-7.5

450ml Aqua dest.

50ml 2xSSC

200ml **1xSSC**, pH7.0-7.5

190ml Aqua dest.

10ml 20xSSC

500ml **0.1xSSC**, pH7.0-7.5

497.5ml Aqua dest.      **or**      450ml Aqua dest.

2.5ml 20xSSC                      50ml 1xSSC

500ml **4xSSCT** (4xSSC containing 0.05% Tween), pH7.0-7.5

400ml Aqua dest.

100ml 20xSSC

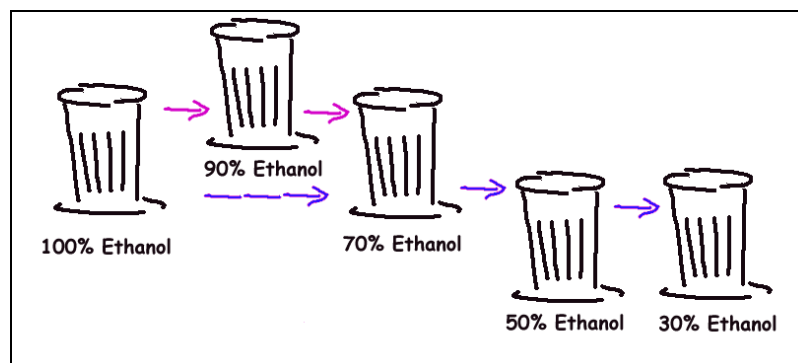
250µl Tween20

## 4.3 Ethanol Series

The ethanol series are necessary for re- and dehydration of the slide preparation. The rehydration procedure ensures that the chromosomes are prepared well for a following washing or incubation step. This kind of 'soaking' improves the effectiveness of agents solved in aqueous solution. On the other hand reactions of the preparation with e.g. buffer substances are stopped by the dehydration procedure. This is especially important if strong solvents are used or in case that the slide preparations will be stored for a longer time.

Two ethanol series are needed. One with 100%, 90% and 70% ethanol for the protein digestion pretreatment procedure (→ Chapter 4.4). And another one with 100%, 70%, 50% and 30% ethanol for the pretreatment and denaturation procedure of the chromosome slides (→ Chapter 4.6).

Prepare five Coplin jars, one for each ethanol concentration. The 100% and the 70% ethanol are used in both series. These solutions are useable for four to six weeks. They should be stored at room temperature. Use lids to protect the solutions from evaporation.



	100ml Coplin jar	50ml Coplin jar
<b>100% Ethanol</b>	100ml Ethanol (100%)	50ml Ethanol (100%)
<b>90% Ethanol</b>	90ml Ethanol (100%) 10ml Aqua dest.	45ml Ethanol (100%) 5ml Aqua dest.
<b>70% Ethanol</b>	70ml Ethanol (100%) 30ml Aqua dest.	35ml Ethanol (100%) 15ml Aqua dest.
<b>50% Ethanol</b>	50ml Ethanol (100%) 50ml Aqua dest.	25ml Ethanol (100%) 25ml Aqua dest.
<b>30% Ethanol</b>	30ml Ethanol (100%) 70ml Aqua dest.	15ml Ethanol (100%) 35ml Aqua dest.

## 4.4 RNase Pre-Treatment for Mouse Chromosomes



For mouse chromosomes we recommend an RNase pre-treatment. The RNase pre-treatment is followed by the protein digesting pre-treatment immediately (→ Chapter 4.5). There is no need to do this RNase treatment for human chromosomes, in general.

### Solutions required:

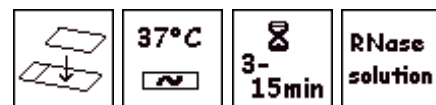
- 2xSSC
- **RNase stock solution:**  
Dissolve 25mg RNase A (Roche, 109142, 25mg) in 2,5ml 2xSSC.  
Incubate for 10min at 100°C. Store in 100µl aliquots at -20°C.

### Preparation:

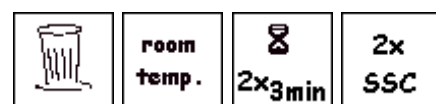
- Preheat incubator (37°C).
- Prepare RNase solution (100µl for each slide):  
Add 1µl RNase stock solution to 99µl 2xSSC.
- Prepare for the protein digesting pretreatment (→ Chapter 4.5):  
Preheat water bath to 37°C.  
Preheat 0,01N HCl: Add 1ml 1N HCl to 99ml Aqua dest.  
Thaw one pepsin aliquot during the SSC washing step.

### Procedure:

- Apply 100µl of the RNase solution to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and **incubate** for 3min for lymphocytes or up 10-15min amniocytes at 37°C.



- **Wash** slides in 2xSSC for 2x 3min



- Start with protein digesting pretreatment immediately (→ Chapter 4.5).

## 4.5 Protein Digesting Pre-Treatment prior to Hybridization

Remaining cytoplasmic proteins of the cells may obstruct the denaturation of the chromosomal DNA and, thus, impair with the hybridization. This may result in high fluorescent slide background and weak or inhomogeneous fluorescence signals. Check slides under phase contrast and pre-treat the slides, if necessary.

The pepsin solution digests the cytoplasmic proteins, enabling a better probe penetration. The following postfixation step binds the chromosome onto the slide again and stabilizes their structure.

### Solutions required:

- 1N HCl
- Aqua dest.
- 1xPBS
- 70%, 90%, 100% ethanol
- **Pepsin stock solution:**  
Dissolve 1g Pepsin (Sigma, P-7012) in 50ml sterile distilled H<sub>2</sub>O, store in 500µl aliquots at -20°C.
- **Postfixation solution, 1% formaldehyde in 1xPBS + 50mM MgCl<sub>2</sub>:**  
60µl 37% Formaldehyde  
2ml 1xPBS  
100µl 1N MgCl<sub>2</sub>

This solution can be used for three to five days. Store at 4°C.

**Note:** The pepsin concentration refers to the given pepsin type. For different types of pepsin the concentration and the incubation time have to be determined.

**Note:** It is necessary to preheat the hydrochloric acid solution without the pepsin. Add the pepsin to the preheated solution shortly before immersing the slides in order to take advantage of the whole activity of the pepsin. Otherwise, longer incubation time may be required to get the same degree of digestion.

**Note:** If the protein digestion pretreatment is done immediately prior to pre hybridization washing, the ethanol series for dehydration and rehydration are not necessary. Transfer slide from 1xPBS into preheated 2xSSC directly (→ Chapter 4.6).

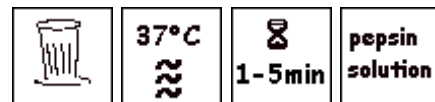
Preparation:

- Preheat water bath to 37°C.
- Preheat 0,01N HCl:  
Add 1ml 1N HCl to 99ml Aqua dest.
- Thaw one pepsin aliquot (500µl)

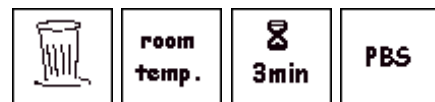
Procedure:

- Add the thawed pepsin aliquot to the preheated 0,01N HCl immediately, mix well

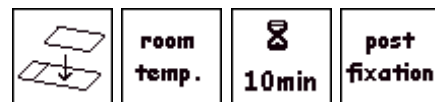
- Immediately immerse the slides into the pepsin solution and incubate 1-2min for amniocytes and lymphocytes or up to 5min for bone marrow



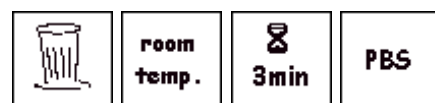
- Wash slides in 1xPBS for 3min



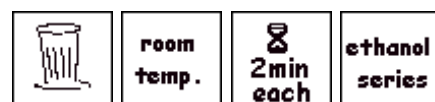
- Apply 100µl of the postfixation to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and incubate for 10min at room temperature



- Wash slides in 1xPBS for 3min



- Dehydrate slides in 70%, 90%, 100% ethanol for 2min each



- Let air dry

- For longer storage (> 1 day) keep slides at -20°C

## 4.6 Pretreatment and Denaturation of Chromosome Slides

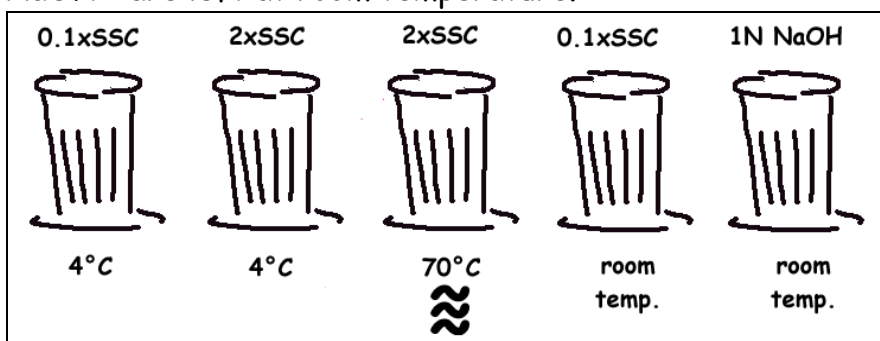
For denaturation the chromosomes are treated with a basic solution to increase the pH (0.07N NaOH). The chromosomes are stabilized in SSC buffer prior to the denaturation. Afterwards, the chromosomes are rinsed in SSC buffer again to stop the denaturation process and to stabilize their structure.

### Solutions required:

- 0.1xSSC, pH7.0-7.5
- 2xSSC, pH7.0-7.5
- NaOH 0.07mol/l
- Ethanol series: 100%, 70%, 50%, 30%

### Preparation

- Prepare five Coplin jars for the prehybridization washing steps: one with 0.07N NaOH, two with 0.1xSSC and another two with 2xSSC.
- Check the pH value (at room temperature) of each solution before using it.
- Put one Coplin jar with 0.1xSSC and one with 2xSSC into the refrigerator. Preheat a Coplin jar with 2xSSC to 70°C in a water bath. The remaining two Coplin jars - one with 0.1xSSC and one with 1N NaOH - are left at room temperature.



- These solutions may be used for three to five days, except the preheated 2xSSC, which has to be prepared fresh prior to each denaturation procedure.
- Check the temperature of the preheated solution. Notice that the given temperature in the protocol specifies the temperature of the


solution, which may be different from the temperature indication of the water bath.


**Note:** Preheat Coplin jar together with waterbath to avoid cracking. Check pH at room temperature before heating up.


**Note:** Start the probe denaturation during pretreatment and denaturation of the metaphase spreads. Time the procedure so that the prepared slide has just dried as the probe prehybridization is completed (→ Chapter 4.7, start with probe denaturation process after removing the slide / Coplin jar from the water bath).


Procedure:


- Rehydrate slide: 100%, 70%, 50%, 30% ethanol, 1min each


	room temp.	1min each	ethanol series
---	------------	-----------	----------------
- Transfer slide into 0.1xSSC at room temperature for 1min


	room temp.	1min	0.1x SSC
---	------------	------	----------
- Incubate slide in 2xSSC at 70°C for 30min


	70°C	30min	2x SSC
---	------	-------	--------
- Remove Coplin jar from water bath\*, let cool down to 37°C (takes about 20min)


	room temp.	approx. 20min	2x SSC
---	------------	---------------	--------
- Transfer slide to 0.1xSSC at room temperature for 1min

	room temp.	1min	0.1x SSC
--	------------	------	----------
- Denature slide in 0.07N NaOH at room temperature for 1min

	room temp.	1min	0.07N NaOH
---	------------	------	------------
- Put slide into 0.1xSSC at 4°C for 1min

	4°C	1min	0.1x SSC
---	-----	------	----------
- Put slide into 2xSSC at 4°C for 1min

	4°C	1min	2x SSC
---	-----	------	--------
- Dehydrate slide: 30%, 50%, 70%, 100% ethanol for 1min each

	room temp.	1min each	ethanol series
---	------------	-----------	----------------
- Let air dry

Denaturation procedure adapted from: Fritz et al, Hum Genet (1998)103:441-449; Rieder et al, Leukemia (1998)9:1473-1481

\* start probe denaturation here

## 4.7 Probe Denaturation and Hybridization

The denaturation of the probe is induced by formamide solution at increased temperature. The probe is already dissolved in a hybridization mixture containing formamide and buffer solutions. For denaturation it only has to be heated up. Allow the probe to prehybridize for half an hour to reduce unspecific binding of short or repetitive DNA pieces.

### Solutions required:

- Probe cocktail (■ ■ ■ ■ ■)
- Probe cocktail per hybridization:  
Use
- 4 $\mu$ l for  $\varnothing=12$ mm coverslip,
  - 7 $\mu$ l for 18x18mm<sup>2</sup> coverslip,
  - 10 $\mu$ l for 22x22mm<sup>2</sup> coverslip,
  - 12 $\mu$ l for 24x24mm<sup>2</sup> coverslip, or
  - 24 $\mu$ l for 24x50mm<sup>2</sup> coverslip (for whole slide).

We recommend dividing the probe cocktail into appropriate aliquots to avoid repeated freeze thaw cycles.

### Preparation

- Preheat water bath and incubator.
- Prepare and preheat humidified chamber.

**Note:** Start the probe denaturation during pretreatment and denaturation of the metaphase spreads ( $\rightarrow$  Chapter 4.6). Time the procedure so that the prepared slide has just dried as the probe prehybridization is completed. Pipette the denatured and prehybridized probe onto the denatured chromosome preparation immediately.

**Note:** If you have a thermocycler in your lab, you could use it for the probe denaturation and prehybridization. (program: 75°C for 5min, 10°C for 30s, 37°C for 30min)

For research use only!

**Warning:** *Painting probes contain formamide. Handle carefully. Avoid contact with skin; wear gloves while handling the reagents.*

To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light!

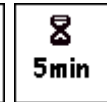
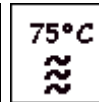
Centrifuge all tubes prior to opening to assemble the contents at the bottom of the tube!

Procedure:

- Pipette the required volume of probe cocktail into a tube.



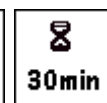
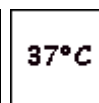
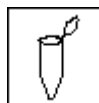
- Denature the probe by incubating at 75°C for 5min



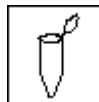
- Put on ice briefly



- Incubate at 37°C for 30min



- Spin briefly to collect probe cocktail

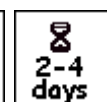


- Pipette the denatured and prehybridized probe cocktail onto the **denatured** chromosome preparation (→ Chapter 4.6)

- Overlay with coverslip

- Seal with rubber cement

- Incubate  
2-4 days for mFISH or  
1-2 days for mBAND  
in a humidified chamber at 37°C



## 4.8 Post Hybridization Washing Steps and Detection of the Biotin-Labeled Probes with Cy<sup>TM</sup>5

The post hybridization washing is necessary to remove the remaining hybridization buffer and to undo unspecific probe binding. The indirect labeled probes have to be detected with Cy<sup>TM</sup>5. Amplify their fluorescence signal, if necessary (→ Chapter 2).

All chromosomes have to be counterstained with DAPI. To reduce photobleaching an anti-fading reagent is applied.

The last washing step with PBS avoids the formation of salt crystals on the slide while drying. Salt crystals may impair the fluorescence microscopy.

### Solutions required:

- 1xSSC, pH7.0-7.5, 75°C
- 4xSSCT = 4xSSC, pH7.0-7.5 containing 0.05% Tween<sup>TM</sup>20, room temperature
- 1xPBS, room temperature
- *blocking reagent* (□□□□□)
- *detection 1+3* (■ ■ ■ ■ ■)
- *detection 2* (■ ■ ■ ■ ■)
- *DAPI/antifade* (■ ■ ■ ■ ■)

### Preparation

- Prepare two Coplin jars for the post hybridization washing steps: one with 1xSSC and one with 4xSSCT.
- Thaw the *blocking reagent* (□□□□□).
- Check the pH value (at room temperature) of each solution before using it.
- Preheat the 1xSSC to 75°C in a water bath.
- Check the temperature of the preheated solution. Notice that the given temperature in the protocol specifies the temperature of the solution, which may be different from the temperature indication of the water bath.

During blocking step and detection slides should be incubated at 37°C in the humidified chamber.

Dispose solutions after each washing step.

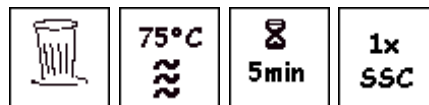
**Note:** Preheat Coplin jar together with waterbath to avoid going to pieces. Check pH at room temperature before heating up.

Tween™ is a trademark of ICI America, Inc.; Cy™ is a trademark of Amersham Pharmacia Biotech Limited, Inc.

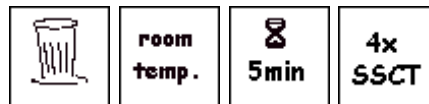
Procedure:

- Remove rubber cement and coverslips carefully

- Wash** slides in preheated (75°C) 1xSSC for 5min



- Incubate** slides in 4xSSCT for 5min



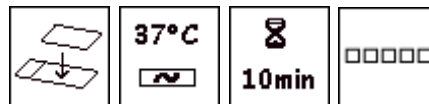
**Note:** The XCyte mBAND kits for chromosomes #13 to #22

(XCyte13, XCyte14, XCyte15, XCyte16, XCyte17, XCyte18, XCyte19, XCyte20, XCyte21, XCyte22)

do **not** contain biotin labeled probes. They do **not** have to be detected with Cy5. Continue with counterstaining.

**Blocking Step:**

- Apply 50µl of *blocking reagent* (□□□□) to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and **incubate** at 37°C for 10min

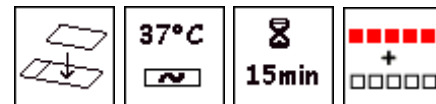


- Flip off coverslips, put slides into 4xSSCT and continue with the next step

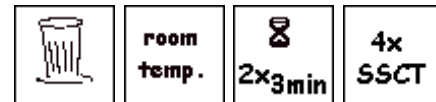


### Detection Step 1:

- For each slide mix 50 $\mu$ l *blocking reagent* (□□□□□) with 1 $\mu$ l *detection 1+3* (■ ■ ■ ■ ■) reagent. Apply 50 $\mu$ l to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and **incubate** at 37°C for 15min



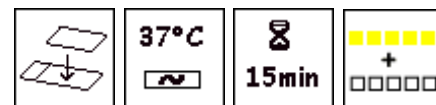
- Wash** slide 2x 3min in 4xSSCT



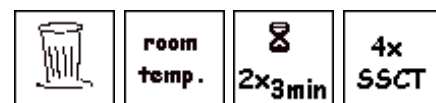
**Note:** Detection steps 2 and 3 are optional. They are only necessary if the Cy<sup>TM</sup>5 fluorescence signal is weak and you want to amplify the signal. This depends on the microscope, the filter set and the UV lamp (→ Chapter 2) and on the quality of the hybridization. Otherwise continue with counterstaining.

### Detection Step 2:

- For signal amplification mix 50 $\mu$ l *blocking reagent* (□□□□□) with 1 $\mu$ l *detection 2* (■ ■ ■ ■ ■) reagent. Apply 50 $\mu$ l to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and **incubate** at 37°C for 15min

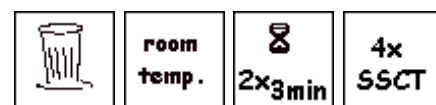
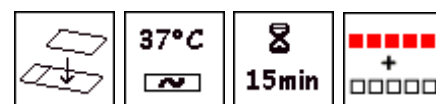


- Wash** slide in 4xSSCT for 2x 3min



### Detection Step 3:

- See detection step 1
- Wash** slide in 4xSSCT for 2x 3min



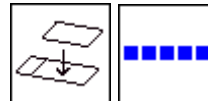
**Counterstaining:**

- **Wash** slide in 1xPBS for 3min



- Drain fluid off and blow dry with a rubber ball or let air dry

- Apply 20µl of *DAPI/antifade* (■■■■■)



- Overlay with a 24x60mm<sup>2</sup> coverslip

Store slides at -20°C. Hybridization signals are fine for at least six month.

## 5 Analysis Procedure

This chapter deals with the analysis of the hybridized slide. It does not intend to give the technical instructions how to use the microscope or the software. For the details of the software features or the operating of the microscope refer to the Isis manual or the operating instructions of your microscope. And this chapter cannot tell you how to interpret your results. But it may help you to find your way from the image acquisition to the correct analysis of aberrant chromosomes.

### 5.1 Image Capturing and Pre-Processing

The first and most important step of the analysis procedure is the correct image acquisition. The fluorescence illumination of the microscope should be carefully adjusted to achieve a uniformly illuminated field. Each pictures for the different color channels has to be sharply defined. Use the automatic integration control. Be careful while changing the fluorescence filters. Avoid shaking the microscope while capturing. Use the highest grade of automation your system permits.

#### Image capturing:

- Select the experiment type for mFISH, mouse-mFISH or the mBAND's (→ Chapter 5.4)
- Capture all six color channels (or all five or four or three for some mBANDs → Chapter 5.4)

#### Image processing:

- Apply background correction
- Define region (if necessary)
- Apply automatic upper and lower threshold
- Correct pixel shift (if necessary).  
Do not use the automatic register color function for mBAND.
- Maximize the metaphase

To include all color channels make sure that the spectrum symbol is selected before you apply these commands. This is most important in order to preserve the correct fluorescent ratio of the raw image.

### Karyotype chromosomes:

- Adjust the object threshold
- Separate the chromosomes
- Enter karyotype view
- Apply automatic classification (for mFISH)

### Analysis

- Single color gallery
- Binary display
- False colors

## 5.2 The mFISH Analysis for Human Chromosomes

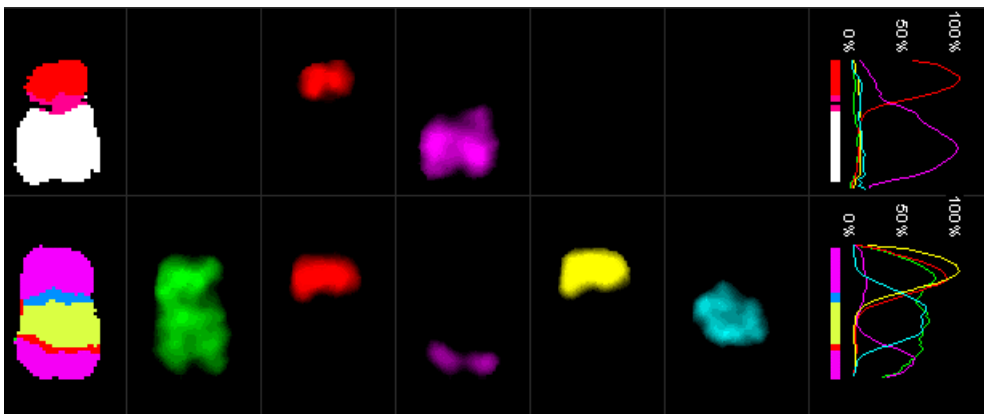
The classification of the chromosomes in mFISH experiments is based on the specific fluorochrome combination of each single chromosome according to the 24XCyte labeling scheme. For the false color representation each chromosome class is related to a defined color. The information for the classification and the matching 'false' color are combined in a classifier. This means that you have to select the right classifier to classify the chromosomes. The standard classifier for the mFISH experiments is called *LABEL*.

Essential for a successful classification are

- a careful slide preparation to minimize the signal background and to avoid disturbing fluorescent dirt,
- a regular hybridization to receive homogenous fluorescence signals and
- a correct image acquisition.

**Important Notice:** The karyotype established by the Isis program should be considered as a suggestion only. This suggestion must be verified or corrected by an experienced cytogeneticist. The program itself is not capable of making diagnostic decision at any time.

The software makes several sophisticated features available for the analysis of the aberrant chromosomes. An important tool is the single color gallery, which provides the whole data information on each chromosome.



### 24XCyte - Labeling Scheme

#	DEAC	FITC	Spectrum Orange™	Texas Red®	Cy™5
1					Yellow
2	Blue				
3				Magenta	
4		Green			
5			Red		
6		Green			Yellow
7	Blue				
8				Magenta	Yellow
9			Red		Yellow
10	Blue	Green			
11		Green		Magenta	
12		Green	Red		
13	Blue			Magenta	
14	Blue		Red		
15			Red	Magenta	
16	Blue	Green			Yellow
17		Green		Magenta	
18		Green	Red		Yellow
19	Blue			Magenta	Yellow
20	Blue		Red		Yellow
21			Red	Magenta	Yellow
22	Blue	Green		Magenta	
X	Blue	Green	Red		
Y	Blue			Magenta	

Depending on lab conditions it may be necessary to modify the classifier. With the color classifier training you define a new correlation between the fluorochrome combination for each chromosome class and its assigned false color. This proceeding adapts the software to your lab conditions actually, so you could neglect some unspecific background for instance. Before the classifier training is started, a correct karyotyp has to be generated. This means that the chromosomes have to be assigned to their correct

chromosome classes. Use the information from the label scheme to check or correct the karyotype. Smaller rearrangements on the assigned chromosomes have no influence on the classifier training.

**Note (Isis 3 and 4):** Do not modify the classifier *LABEL*. Create a new classifier based on the initial settings of the classifier *LABEL*.

Once you have generated a classifier it can be used for the analysis of all normal or aberrant metaphases, which have been hybridized under comparable conditions.

### 5.3 The mFISH Analysis for Mouse Chromosomes

The experiment type for mouse mFISH is called *mF-mouse*.

21XMouse - Labeling Scheme



	DEAC	FITC	Spectrum Orange™	Texas Red®	Cy™5
1	Blue		Red	Purple	
2	Blue		Red		Yellow
3		Green			
4			Red		
5				Purple	
6	Blue	Green			Yellow
7	Blue				Yellow
8		Green			Yellow
9			Red		Yellow
10				Purple	Yellow
11		Green	Red		Yellow
12		Green	Red	Purple	
13		Green		Purple	Yellow
14	Blue				
15	Blue	Green			
16	Blue		Red		
17	Blue			Purple	
18					Yellow
19		Green	Red		
X		Green		Purple	
Y			Red	Purple	

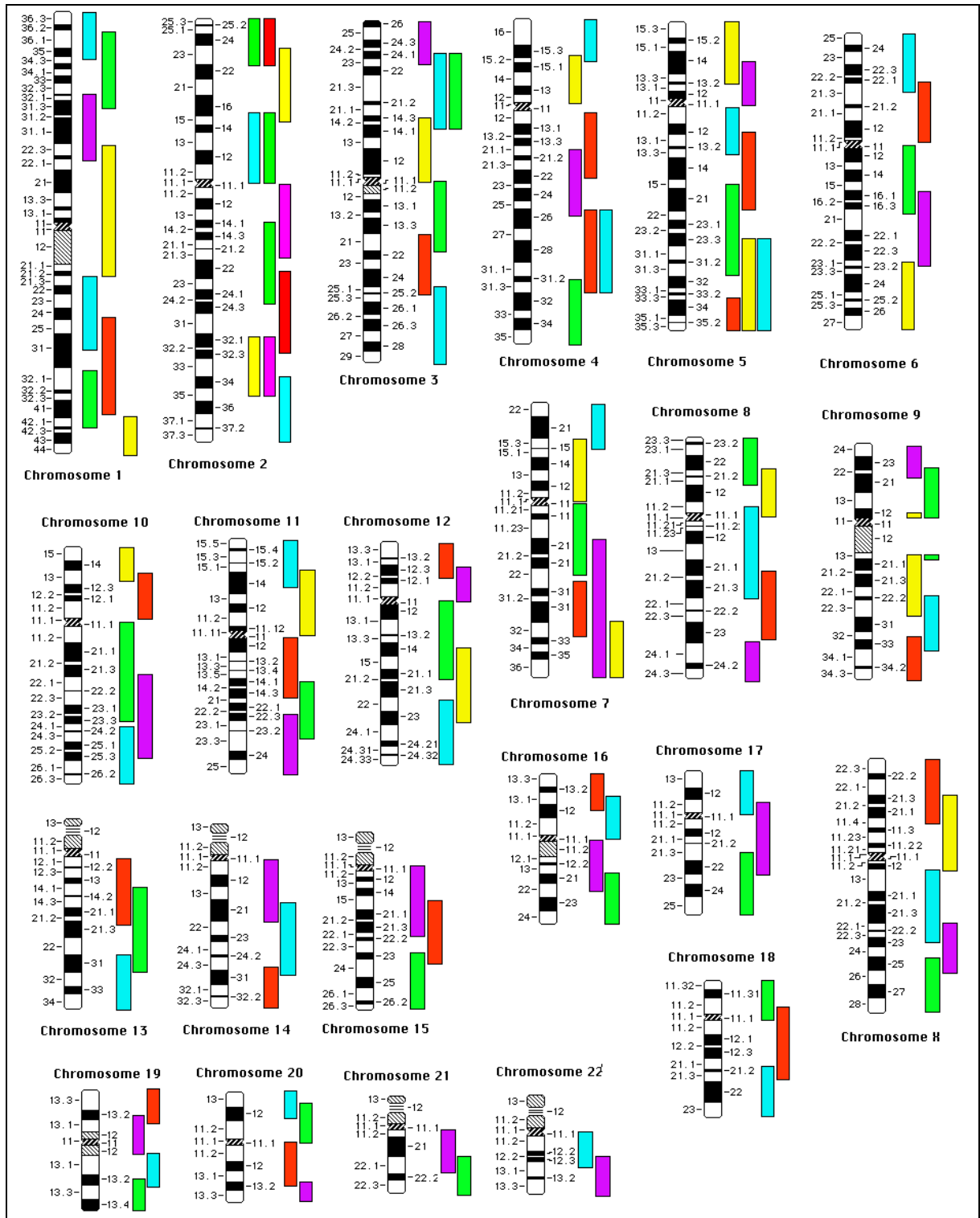
### 5.4 The mBAND Analysis

The mBAND probes comprise region specific probes labeled with different fluorochromes or fluorochrome combinations. The single labeling schemes for all different chromosomes are given below.

These particular partial chromosome paints exhibit a gradual intensity decrease from their center towards their ends. The overlaps of the intensity profile of adjacent probes result in color ratio variations along the chromosome that are quantitated by the Isis software. Pseudo-color

assignment yields a definable number of color bands per chromosome. This quantitative color ratio analysis effectively multiplies the resolution of the region specific probes.

mBAND probe kit	Number of region specific probes	Used fluorochromes					Detection of biotin labeled probes	Exerimenttyp	mBAND classifier
		DEAC	FITC	Spectrum Orange™	Texas Red®	Biotin/Cy™5			
XCyte 1	8	+	+	+	+	+	Yes	mB1-12	Xcyte01
XCyte 2	8	+	+	+	+	+	Yes	mB1-12	Xcyte02
XCyte 3	6	+	+	+	+	+	Yes	mB1-12	Xcyte03
XCyte 4	6	+	+	+	+	+	Yes	mB1-12	Xcyte04
XCyte 5	7	+	+	+	+	+	Yes	mB1-12	Xcyte05
XCyte 6	5	+	+	+	+	+	Yes	mB1-12	Xcyte06
XCyte 7	6	+	+	+	+	+	Yes	mB1-12	Xcyte07
XCyte 8	5	+	+	+	+	+	Yes	mB1-12	Xcyte08
XCyte 9	5	+	+	+	+	+	Yes	mB1-12	Xcyte09
XCyte 10	5	+	+	+	+	+	Yes	mB1-12	Xcyte10
XCyte 11	5	+	+	+	+	+	Yes	mB1-12	Xcyte11
XCyte 12	5	+	+	+	+	+	Yes	mB1-12	Xcyte12
XCyte 13	3	+	+	+			No	mB13	Xcyte13
XCyte 14	3	+		+	+		No	mB14	Xcyte14
XCyte 15	3		+	+	+		No	mB15	Xcyte15
XCyte 16	4	+	+	+	+		No	mB16	Xcyte16
XCyte 17	3	+	+		+		No	mB17	Xcyte17
XCyte 18	3	+	+	+			No	mB18	Xcyte18
XCyte 19	4	+	+	+	+		No	mB19	Xcyte19
XCyte 20	4	+	+	+	+		No	mB10	Xcyte20
XCyte 21	2		+		+		No	mB21	Xcyte21
XCyte 22	2	+			+		No	mB22	Xcyte22
XCyte X	5	+	+	+	+	+	Yes	mBX	XcyteX



## 6 Troubleshooting

If the result is not as nice as you expected...

The FISH procedure consists of a lot of different preparation steps, so there are several factors, which may influence the result. It can be difficult to find out which of these factors interfered with your hybridization. The following hints are based on our own experience.

- If you have carried a lot of successful hybridizations and, suddenly, one does not work, the problem could be solved by doing the whole procedure again. It might be that a little mistake creeps in you routine. (In general this mistakes are not reproducible, so don't worry, this could happen to everybody :-) )
- Poor hybridization signals could be caused by different factors. Low FITC signals, for example,
  - could be a hint for a low pH in one of the washing solutions or
  - could indicate some trouble with the fluorescence filters or
  - (after image capturing) some high-fluorescent artifacts influence the integration time.
- So do not focus on only one potential for the source of trouble, reflect on the whole process: hybridization procedure, microscopy, and image processing.
- Before you are going to change something in the lab-procedure, check your analysis procedure and the optical pathway first.

### 6.1 Preparation

#### Weak or no hybridization signals:

- *Chromosome slide is not adequately denatured:*  
Ensure that washing solutions were made according to the protocol.  
Check the temperature of the preheated solutions.  
Check the pH value of each solution. It has to be between pH7.0 and pH7.5 at room temperature.  
Ensure that denaturation time for slide in 0.07N NaOH is exactly one minute.  
Ensure that washing solutions are not stored too long: Prepare fresh.  
Store in a dark place at room temperature.

- *Probe cocktail is not adequately denatured:*  
Ensure that the denature temperature is correct.
- *Incubation time for hybridization is too short:*  
Incubate 2-4 days for hybridization in humidified chamber: Prevent from drying up. Check the incubation temperature.
- *Metaphase spreads contain cytoplasmic proteins:*  
Apply protein digesting pretreatment prior to hybridization.
- *Chromosome slide is too old:*  
Slides should not be older than two weeks. We recommend preparing slides one day before hybridization. For long-term storage keep slides at -20°C.

#### Low Cy5 signals:

- Apply detection steps 2 and 3.  
Avoid air bubbles under coverslip during incubation.  
In case that DAPI/antifade is applied already onto the slide: Remove coverslip carefully. Rinse slide with 70% ethanol. Dehydrate slide in 70%, 90% and 100% ethanol for 2min each. Let air-dry. Apply detection steps 2 and 3.

#### Low FITC signals:

- *The FITC fluorophores are very sensible to low ph (pH>7.0).*  
Check the pH value of each solution. It has to be between pH7.0 and pH7.5 at room temperature.
- Apply protein digesting pretreatment prior to hybridization.

#### High slide background:

- *Metaphase spreads contain cytoplasmic proteins:*  
Apply protein digesting pretreatment prior to hybridization.
- *Post hybridization washing not adequate:*  
Ensure that washing solutions were made according to the protocol.  
Check the temperature of the preheated solutions.  
Check the pH value (at room temperature) of each solution before using it.  
Ensure that washing solutions are not stored too long: Prepare fresh.  
Store in a dark place at room temperature.

## 6.2 Microscopy

### No signals at all:

Check the optical pathway:

- *UV lamp:*  
Check the power switch. (→ Chapter 2.3).  
Check the change over shift between microscopy-light-path and the adjusting-aid (if your microscope has one).
- *Shutter:*  
Make sure that the shutter is open.
- *Diaphragm:*  
Make sure that the diaphragm is completely open.
- *Objective:*  
Swing the objective into the light path correctly.
- *Fluorescence filters:*  
Rotate the suitable filter cube into the light path. If your microscope has an excitation filter wheel, make sure that the right filters are combined.
- *Ocular or camera:*  
Check the change over shift.

### Low signals and longer integration time as usual in only one color channel:

- Check, whether the filter cube is in the right position and mounted correctly.

### Inhomogeneous illumination:

- Adjust the UV light properly. (→ Chapter 2.3)
- Check the lifetime of the UV lamp.

### Diffuse signals:

- *Immersion oil:*  
Use enough immersion oil.  
Do not mix up different immersion oils.  
Clean up the objective lens.  
Use immersion oil for fluorescence.

- *DAPI/antifade:*  
Do not use too much DAPI/antifade.  
15 to 20  $\mu$ l per slide (24mmx60mm coverslip) are enough.
- If your microscope has an excitation filter wheel, make sure that the right filters are combined.
- Clean up the objective lens.

### 6.3 Analysis

#### Processed image looks very strange:

- Make sure that the spectrum symbol (to include all color channels) is selected before you apply any commands for image processing.

#### Chromosomes show small edges in false color view:

- *Images are shifted against each other:*  
Correct pixel shift.  
Do not use the automatic register colors function for mBAND.

#### Automatic mFISH classification does not work properly:

- *Wrong classifier:*  
Make sure, that you have selected the right classifier.
- *The Texas Red<sup>®</sup> signals are highlighted in the Cy<sup>TM</sup>5 channel as well:*  
Amplify the Cy<sup>TM</sup>5 signals by applying detection steps 2 and 3.
- *The chromosomes do not fit into the karyotype form:*  
Reduce the size of the chromosomes.

## 6.4 Frequently Asked Questions

- *Can I combine two or more mBANDs in one hybridization?*  
We do not recommend it, because the analysis becomes very difficult. For complex rearrangements with different chromosomes it is easier to hybridize each XCyte mBAND for the chromosomes in question separately. If there is only one slide available for the hybridization, you could use small cover slips and arrange them close together (e.g.  $\varnothing=12\text{mm}$ , use  $4\mu\text{l}$  probe cocktail each).
- *Can I dilute the 24XCyte probe?*  
No. We cannot guarantee proper results.
- *Cool packs were nearly defrosted when package arrived. Could it cause any problem?*  
No. Cool packs are used to avoid high temperatures during shipment. Probes are stable for several days at room temperature.
- *Is it necessary to verify the mFISH results with conventional chromosome paints?*  
No. The filter based mFISH approach allows a direct inspection of the original hybridization signals. There is no need for additional control experiments.
- *Is it possible to use my own detection reagents?*  
Yes. If you have already established the detection of Biotin labeled FISH probes with Cy<sup>TM</sup>5 in your lab, you could apply your own procedure (→ Chapter 1.4).
- *Can I use my own DAPI or antifading reagent?*  
Yes, but be careful: First, DAPI has to be applied at a low concentration to avoid crosstalking to the DEAC signals (DAPI/Antifade: 250ng/ml). Second, some antifading reagents do not work well for all fluorochromes.
- *Can I use a slide, which is already stained with Giemsa (G-banding) for FISH?*  
Yes, but note, that this procedure does not work in all cases. Please refer to chapter 6.5.
- *Can I hybridize a slide again, which already had been hybridized before?*  
Yes, but it might be, that the old hybridization signal is not removed completely. Please keep that in mind, while you are doing your analysis. For details refer to chapter 6.5.

## 6.5 Tricks for Delicate Cases -for Advanced FISHerman only-

In this chapter we want to give some hints or ideas for doing FISH under difficult conditions: It might be that you have not the possibility to prepare cell suspensions fresh, and you are depending on very old suspension or slides. Or there is only one slide available and you have to get a result in any case.

The following ideas came up from our experiences with difficult preparations from our customers over the last couple of years. We were not successful in all cases, but in most we obtained good results. So you may profit from our experiences.

The following ideas or recipes are for advanced FISHerman only. Knowledge of the theoretical backgrounds and a lot of practice with the hybridization and analysis procedures are fundamental for this kind of experimental FISHing. Please note that these things depend on lab conditions also, and may differ from case to case.

### Remove DAPI/antifade and coverslips from slides:

Imagine that the coverslip is broken, or got out of place, or is covered with dirt, or you want apply additional detections steps because of weak Cy5 signal, or you are not satisfied with the quality of the hybridization and want to do it again (perhaps to apply pepsin digesting pretreatment?), or...

In all this cases you have to remove the old coverslip and to get rid of the antifade:

So remove coverslip carefully. Rinse slide with 70% ethanol. Dehydrate slide in 70%, 90% and 100% ethanol for 2min each. Let air-dry.

Slides are now prepared for

- a new coverslip (apply DAPI/antifade again), or
- additional detection steps (start with washing step: 4xSSCT for 3min),  
or
- a Pepsin digesting pretreatment and a new hybridization, or
- a new hybridization.

### mFISH on G-banded slides:

The basic problem is to get rid of the immersion oil:

Wash the slide in fresh 100% Xylol for 10-30min.

Wash slide in Carnoy fixative (methanol and glacial acetic acid 3:1) for 15 min. Dehydrate slide in 70%, 90% and 100% ethanol for 2min each. Let air-dry.

Pepsin pre-treatment is not necessary, the Trypsin has already done this job during the G-banding procedure.

Chromosome preparation:

The aging and fixation of chromosome preparation determine the degree of denaturation. That is effective for the stability of the chromosomal structure, and for the 'binding' of the chromosomes onto the slide as well.

- The older the slides the more difficult the denaturation, in general. Thus, we recommend preparing the slides one day prior to hybridization. The slides should be stored in no case at room temperature for more than one week. Store slides at  $-20^{\circ}\text{C}$  in a freezer for long term storage (more than a few days). Be careful with additional fixation or 'aging' processes, they may inhibit the hybridization.
- The NaOH denaturation procedure is less aggressive and preserves the structure and morphology of the chromosomes in comparison to a formamide denaturation procedure. If the NaOH denaturation was not successful, you may try a formamide denaturation of slides (according to our protocol for the XCP painting probes). For very resistant chromosomes increase the denaturation temperature to  $75^{\circ}\text{C}$  or up to  $80^{\circ}\text{C}$ . But keep in mind that you risk to over-denature the chromosomes! ( $\rightarrow$  Appendix)
- Check the quality of the denatured slide under phase contrast prior to the hybridization:  
If the chromosomes appear in light gray and look a little bit blown out, the denaturation is ok.  
If the chromosomes appear in dark gray or black, the denaturation was not successful.  
If the chromosomes appear in very light gray and their structure is lost, the chromosomes are over-denatured.  
If you insert this quality check in daily routine, you may get an idea of the interaction between treatment and chromosomes. These experiences allow you to handle delicate cases.
- In our experience an RNase pretreatment has no affect on the hybridization procedure of human chromosomes.
- The only situation in which we recommend a further fixation of your chromosomal preparation is, when the fresh prepared slide has to be hybridized at the same day. Allow the slide to 'age' at a hot plate at  $45^{\circ}\text{C}$  for 3 hours.

*To hybridize a slide again, which already had been hybridized before:*

You could use a slide, which was already hybridized, for a new hybridization

- to improve the quality of the hybridization by applying a pepsin digesting pretreatment, or a more aggressive denaturation, or
- to apply another painting probe.

For the second slide denaturation we recommend a formamide denaturation to remove the old signals more effectively (→ Appendix).

But it might be, that the old hybridization signal is not removed properly. Please keep that in mind, while you are doing your analysis.

## Appendix

### Formamide Protocol for Chromosome Painting Probes XCP

#### Denaturation of Chromosome Slides

##### Solutions required:

- Denaturation solution: 70% formamide in 2xSSC, pH7.0
- 70% ethanol, -20°C
- 90%, 100% ethanol, room temperature

##### Procedure:

- Prewarm the denaturation solution to 70°C
- Immerse 2 slides into the denaturation solution, incubate for 3min
- Transfer slides to icecold 70% ethanol, incubate for 3min
- Transfer to 90% and 100% ethanol, incubate for 3min each
- Let air dry

**Note:** If you want to denature more than two slides simultaneously, the denaturation solution has to be preheated even higher. Increase denaturation temperature by 1°C for each slide.

If you have a hot plate, apply the following procedure:

##### Procedure:

- Preheat the hot plate to 70°C
- Apply 100µl of the denaturation solution to each slide
- Overlay with a 24 x 60mm<sup>2</sup> coverslip
- Incubate for 3min
- Flip off the coverslip and
- Immediately transfer to a coplin jar with 70% ethanol for 2min
- Subsequently, transfer to a coplin jar with 90% and 100% ethanol, incubate for 2min each
- Let air dry

## **Probe Denaturation and Hybridization**

### **Procedure:**

- Use 10 $\mu$ l of the XCP probe mix per hybridization (22x22mm<sup>2</sup> coverslip); or use 7 $\mu$ l of the probe mix per hybridization (18x18mm<sup>2</sup> coverslip)
- Denature the probe by incubating at 75°C for 5min
- Put on ice briefly
- Incubate at 37°C for 30min
- Spin briefly to collect probe mix
- Pipette the denatured and prehybridized probe mix onto the denatured chromosome preparation
- Overlay with a coverslip
- Seal with rubber cement
- Incubate in a humidified chamber at 37°C overnight

## **Posthybridization Treatment**

### **Solutions required:**

- 1xSSC, pH7.0-7.5, 75°C
- 2xSSCT = 2xSSC, 0.01% Tween20, pH7.0-7.5, room temperature
- 2xSSCT/DAPI = 2xSSCT, 0,2 $\mu$ g/ml DAPI, room temperature
- 1xPBS, room temperature
- mounting medium

### **Procedure:**

- Remove rubber cement and coverslips carefully
- Place the slides in the preheated (75°C) 1xSSC, incubate for 2min
- Transfer slides to 2xSSCT, incubate for 1 min, RT
- Apply 50 $\mu$ l of 2xSSCT/DAPI to each slide, overlay with a 24x60mm<sup>2</sup> coverslip and incubate 2min at room temperature
- Flip off coverslips, put slides into 1xPBS for 2min
- Drain fluid off and blow dry with a rubber ball or let air dry
- 20 $\mu$ l mounting medium
- Overlay with a 24x60mm<sup>2</sup> coverslip