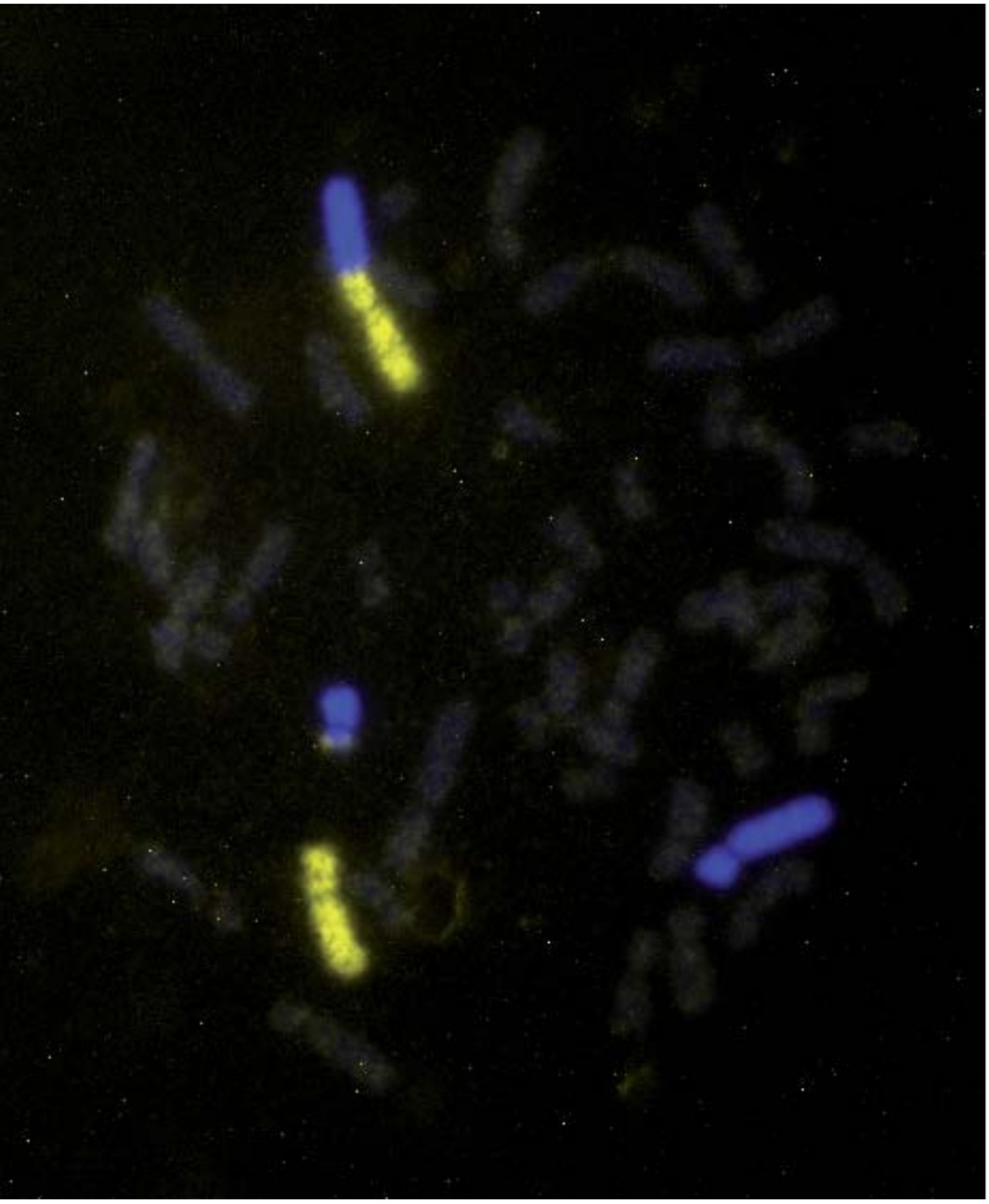
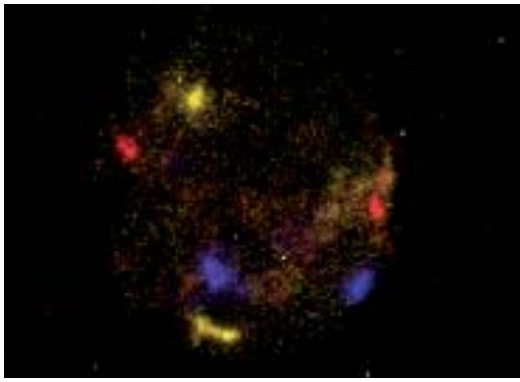
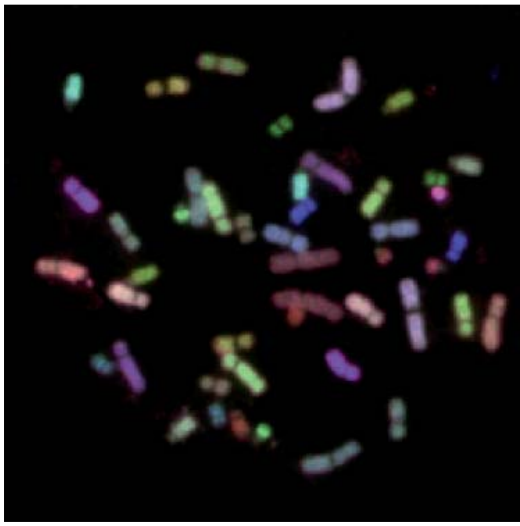
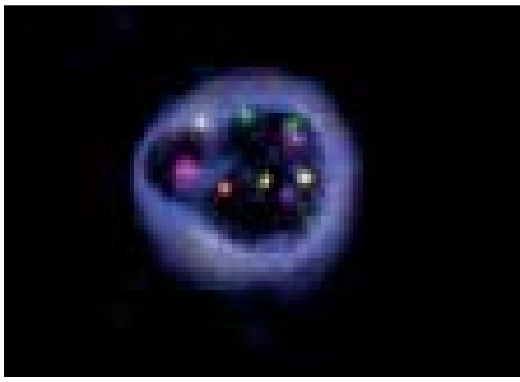
PALM User Protocols

FISH Hybridization









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FISH Hybridization

FISH Hybridization

Introduction

Fluorescence In Situ Hybridization (FISH) is a highly sensitive and specific methodology with broad applications in diagnostics and research. Numerical or structural chromosomal changes can be detected quickly not only in metaphase chromosomes from cultured cells but also in interphase nuclei and tissue sections. FISH-probes can range from single locus or chromosome-specific (e.g., centromere probes) to painting probes highlighting specifically whole chromosomes or chromosome arms.
By combining various different fluorescent dyes for multiple different probes all chromosomes of whole karyograms can be distinguished by color-paints (Multi Color FISH). Even cryptic small translocations may become visible by such methods.
The following FISH related protocols have been optimized for laser microdissection technology from Carl Zeiss by Dr. Sabine Langer (TU Munich, Human Genetics Dept.). They have not been thoroughly tested in the ZEISS Labs but were successfully applied for Post-Doctoral training courses at the TU Munich in cooperation with microdissection specialists from Carl Zeiss. Therefore these methods can be regarded as helpful guidelines for starting FISH experiments in combination with PALM Systems.

Some steps in this protocols have been optimized for using MembraneSlides and can therefore differ from common routine protocols.

1. Chromosome Preparation

(RNase A treatment, Pepsination and Denaturation)

Reagents:

RNase A stock: 10 mg/ml in 10 mM Tris-HCl (pH 7.5) + 15 mM Sodium Chloride
10% **Pepsin** stock in 0.01 M HCl solution (Sigma-Aldrich)
Denaturation solution: 70% Formamide (pure) in 2x SSC
(e.g. 35 ml Formamide + 15 ml 2x SSC, pH 7.0 for 50 ml solution)
PBS buffer
2x **SSC** buffer (pH 7.0)
dd**H_2O** (double distilled or other highly pure water)

1.1 RNase A treatment

- Dip chromosome slide shortly in 2x SSC.
- Prepare a 1:200 RNase dilution in 2x SSC.
  (e.g. for a 200 µl volume/slide: 1 µl RNase A stock + 199 µl 2x SSC)
- Pipet 200 µl RNase solution onto each chromosome slide, cover with a coverslip and incubate for 1/2 hour at 37°C in a humid chamber.
- Wash/shake 3 x 5 minutes in 2x SSC at RT (coverslip swims off)

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1.2 Pepsin digest

- Prepare Pepsin working solution, preheat at 37°C.
  (e.g. using a 50 ml volume: 49.5 ml ddH_2O+ 0.5 ml 1N HCl + 15 µl Pepsin stock)
- Incubate chromosome slides for 5 minutes at 37°C in Pepsin working solution.
- Wash 2 x 5 minutes in PBS at RT.
- Wash in ascending Ethanol series (70%, 90%, 100%) for 3 minutes each.
- Let chromosome slides air-dry.

1.3 Denaturation

- Prepare 70% Formamide denaturation solution, preheat at 73°C.
  (e.g. using a 50 ml volume: 35 ml Formamide + 15 ml 2x SSC, pH 7.0)
- Denature chromosome slides for 2 minutes at 73°C in denaturation solution.
- Wash in ice-cold (-20°C) ascending Ethanol series (70%, 90%, 100%) for 3 minutes each.

2. Preparation of Chromosome Paints

2.1 DOP-PCR Amplification

By using DOP-PCR starting from e.g. flow-sorted chromosomes ("1st generation pool") a higher amount of specific DNA probe ("2nd generation pool") is created.

Reagents:

10x **PCR buffer**
50 mM **$MgCl_2$**
5 mM **dNTP**
Primer 6MW: 100 µM (5' – CCG ACT CGA GNN NNN NAT GTG G – 3')
Taq-Polymerase 5U/µl: (e.g., GIBCO BRL)
dd**H_2O** (double distilled or other highly pure water)

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Pipetting scheme:

Final concentration		1x µl
1x	10x PCR buffer	2.5
2 mM	50 mM $MgCl_2$	1.0
0.2 mM	5 mM dNTP	1.0
2 µM	100 µM 6MW	0.5
	ddH_2O	18.8
1U	Taq-Polymerase	0.2
	DNA 1st generation pool	1.0
		Σ= 25

PCR program:

1 x	94°C, 3 min
35 x	94°C, 1 min ; 56°C, 1 min ; 72°C, 4 min
1 x	72°C, 20 min
	hold 4°C forever

If required: Check PCR products on a 1% agarose gel using 3 µl of each PCR product.

2.2 DOP-PCR Fluorescent Labeling

The "2nd generation pool" (as produced in 2.1) is used for the fluorescent labeling of DNA-probes. This protocol shows examples for both a direct (Cy3) and a linker based (DIG) labeling.

Reagents:

10x **PCR Buffer**
50 mM **$MgCl_2$**
5 mM dNTP (**A,G,C**)
5 mM **dTTP**
Primer 6MW: 100 µM (5' – CCG ACT CGA GNN NNN NAT GTG G – 3')
Taq-Polymerase 5 U/µl
DIG (Digoxigenin)-11-dUTP (125 nmol)
Cy3-dUTP (25 nmol)

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Cy3-PCR Setup:

Final concentration		1x µl
1x	10x PCR Puffer	2.5
2 mM	50 mM $MgCl_2$	1
0.2 mM	5 mM AGC	1
0.15 mM	5 mM dTTP	0.75
0.05 mM	1 mM Cy3-dUTP	1.25
2 µM	100 µM 6MW	0.5
	ddH_2O	16.8
1 U	Taq-Polymerase	0.2
	DNA 2nd generation pool	1
		Σ=25

Digoxigenin-PCR Setup:

Final concentration		1x µl
1x	10x PCR Puffer	2.5
2 mM	50 mM $MgCl_2$	1
0.2 mM	5 mM AGC	1
0.15 mM	5 mM dTTP	0.5
0.05 mM	1 mM DIG-11-dUTP	2.5
2 µM	100 µM 6MW	0.5
	ddH_2O	15.8
1 U	Taq-Polymerase	0.2
	DNA 2nd generation pool	1
		Σ=25

PCR program:

1 x	94°C, 3 min
35 x	94°C, 1 min ; 56°C, 1 min ; 72°C, 4 min
1 x	72°C, 20 min
	hold 4°C forever

FISH Hybridization

Check PCR products on a 1% agarose gel using 3 µl of each PCR product. The products are usually too large showing a "smear" starting in the kbp range. Therefore a subsequent digestion using DNase I is required.

2.3 DNase I Digest

For best results in hybridization the labeled DNA should be digested to sizes of about 300-700 bp.

Reagents:

10x **Nick Translation Buffer**: 0.5 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 0.5 mg/ml BSA
DNase I-Stock (3 mg/ml)
0.5 M **EDTA**
dd**H_2O**

Digestion reaction:

To 25 µl of labeled amplification product add:
5 µl of 10x Nick Translation Buffer
5 µl of diluted DNase I (~1:1000; e.g., 1 µl DNase in 1 ml ddH_2O)
15 µl of ddH_2O

- Mix and incubate for 30-45 minutes at 15°C.

Check probe size again on a 1% agarose gel. Ideal size should be in the range of 300-700 bp. Comment: Optimal DNase I concentration and digestion times may vary depending on size of original DNA.

3 Hybridization of Painting Probes (e.g., Chr. 1, 2, 3, 4)

3.1 Ethanol Co-Precipitation

Reagents:

Cot-1 DNA: (Roche)
Salmon testis DNA: (Sigma)
3M **Sodium Actetate**
100% **Ethanol**

FISH Hybridization

Mixture for Ethanol Co-Precipitation:

DNA-Pool	Volume in µl	Cot-1 DNA in µl	Salmon testis DNA in µl	Σ	3M NaOAc in µl (1/10 Vol.)	EtOH 100% in µl (2.5 Vol.)
Chr. 1-Cy3 Chr. 2-Cy3 Chr. 3-Dig Chr. 4-Dig	8 8 6 6	30	5	63	6.3	174

- Mix by flipping the tube
- Store precipitation mix at –20°C over night.
- Next day: Spin down for 30 minutes with 13000 rpm at 4°C
- Discard supernatant
- Wash pellet with 400 µl of 70% Ethanol and spin down for 10 minutes at 13000 rpm
- Air-dry pellet about 10-20 minutes

3.2 Hybridization-Mix und Hybridization

Reagents:

deionized **Formamide**: (Sigma)
Mastermix: 30% Dextransulfate in 4x SSC
Fixogum rubber cement (Marabu)

- After air-drying: dissolve pellet from above in 6 µl deionized formamide by shaking at 42°C for at least 30 minutes.
- Add same volume (6 µl) of Mastermix (30% Dextransulfate in 4x SSC) and mix carefully.
- Denaturate mix at 78°C for 7 minutes.
- Pre-hybridize Mix for 30 minutes at 42°C.
- Pipet hybridization mix onto chromosome slide, put a coverslip on top and seal it with Fixogum. (Size of coverslip: 12x12 mm or 15x15 mm).
- Hybridize slide over night in a hybridization box at 37°C (option: using a waterbath).

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3.3 Post-Hybridization

Reagents:

4x **SSC** / 0.2% **Tween-20**
3% **BSA** solution (in 4x SSC/0.2% Tween-20)
DAPI-Stock: (4`,6-Diamidino-2-phenylindole, 0.2 mg/ml in ddH_2O)
Sheep **anti-Dig**oxigenin FITC (Roche), 1:100 diluted in: 4x SSC/Tween-20 with 1% BSA

- Remove Fixogum CAREFULLY
- Wash 3x 5 minutes at 42°C in 4x SSC/0.2% Tween-20.
- Wash 3x 5 minutes at 60°C in 1x SSC.
- Dip in 4x SSC/0.2% Tween-20.
- Pipet 1 ml of 3% BSA solution onto chromosome slide and incubate for 30 minutes at 37°C in a humid chamber
- Dip in 4x SSC/0.2% Tween-20.
- Incubate with 150 µl sheep anti-DIG FITC (diluted 1:100) for 45 minutes at 37°C in a humid chamber
- Wash 3x 5 minutes at 42°C in 4x SSC.
- Incubate with 1 ml DAPI (1:10000 dilution of stock in ddH_2O) for 3 minutes at RT.
- Rinse in water and let air-dry

4 Preparation of Centromeric Probes (e.g., Chr. 7, 17, 8, 11)

4.1 pUC-Insert PCR

Necessary reagents:

10x **PCR Buffer**
50 mM **$MgCl_2$**
Detergent **W1** (Polyoxyethylene ether W1; 1%; Sigma-Aldrich)
5 mM **dTTP**
Primer: 10 µM pUC forward
10 µM pUC reverse
Taq-Polymerase 5U/µl

- Take 2 µl of a 1:10 diluted pUC-plasmid preparation containing the respective centromer region (Chr. 7, 17, 8, or 11) and use it for pUC-Insert PCR.

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Pipetting Scheme:

Final concentration		1x µl
1x	10x PCR Puffer	2
1.5 mM	50 mM $MgCl_2$	0.6
0.05%	W1 (1%)	1
250 µM	5 mM dNTP	1
0.2 µM	pUC forward (10 µM)	0.4
0.2 µM	pUC reverse (10 µM)	0.4
	ddH_2O	12.4
0.05 U/µl	Taq-Polymerase	0.2
	pUC-DNA (e.g., Chr. 7)	2
		Σ=20

PCR program:

1 x	95°C, 5 min
30 x	95°C, 45 sec ; 66°C, 45 sec ; 72°C, 1 min
1 x	72°C, 5 min
	hold 4°C forever

Check PCR products on a 1% agarose gel using 3 µl of each PCR product. Depending on the used probes you will get defined fragments on the gel.

4.2 Nick-Translation with DNase I Digest

For labeling the centromeric probes use the entire pUC-Insert PCR product from 4.1 (~20µl).

Necessary reagents:

10x **Nick-Translation Buffer**: 0.5 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 0.5 mg/ml BSA
0.1 M **Mercaptoethanol**
0.5 mM **A,G,C**: from 100 mM stocks of dATP, dGTP and dCTP
Cy3-dUTP (25 nmol)
DIG (Digoxigenin)-11-dUTP (125 nmol)
DNA-Polymerase I (5 U/µl)
DNase I (3 mg/ml)

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Pipetting scheme :

Final concentration	Stock	µl
1x	10x NT Puffer	5
0.01 M	0.1 M Mercaptoethanol	5
0.05 mM	0.5 mM AGC	5
0.05 mM	1 mM DIG or Cy3	2.5
	DNA-Polymerase I	2
	1:5000 DNase I	8
	DNA	20
	ddH_2O	2.5
		Σ=50

- Incubate for about 2 hours at 15°C.

Check probe size again on an agarose gel. Ideal probe size should be in the range of ~500 bp. If the product is too big, redo the digest by simply adding another 5µl of DNase I (1:5000) into the probes and incubate another 2 hours at 15 °C.

4.3 Evaporation and Hybridization

Necessary reagents:

Cot-1 DNA (GIBCO BRL)
Hybridization Mix (7 µl): 4,55 µl deionized formamide (Sigma)
0.7 µl 20x SSC
0.7 µl 5% Dextransulfate in 4x SSC (Sigma)
1.05 µl ddH_2O
Fixogum rubber cement (Marabu)

Important: Dilute fluorescent probes from the Nick-Translation at 1:10 !!!

Centromer Mix:

DNA-Pool	µl	Cot-1 DNA in µl	Σ
Chr. 7-Cy3 (1:10)	9		
Chr. 17-Cy3 (1:10)	9	12	44
Chr. 8-Dig (1:10)	7		
Chr. 11-Dig (1:10)	7		

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- Evaporate hybridization mix.
- Dissolve pellet in 7 µl hybridization mix shaking at 42°C for at least 30 minutes.
- Denaturate mix at 78°C for 7 minutes .
- Pipet hybridization mix onto chromosome slide, put a coverslip on top and seal with Fixogum. (Size of coverslip: 12x12 mm or 15x15 mm).
- Hybridize slide over night in a hybridization box at 37°C (option: using a waterbath).

4.4 Post-Hybridization

Reagents:

4x **SSC**/0.2% **Tween-20**
3% **BSA** solution in 4x SSC/0.2% Tween-20
DAPI-stock: 4`,6-Diamidino-2-phenylindole, 0.2 mg/ml, in ddH_2O
Sheep **anti-Dig**oxigenin FITC (Roche); 1:100 diluted in 4x SSC/Tween-20 with 1% BSA

- Remove Fixogum CAREFULLY.
- Wash 3x 5 minutes at 42°C in 4x SSC/0.2% Tween-20.
- Wash 3x 5 Minuten at 60°C in 1x SSC.
- Dip in 4x SSC/0.2% Tween-20.
- Pipet 1 ml of 3% BSA solution onto chromosome slide and incubate for 30 minutes at 37°C in a humid chamber.
- Dip in 4x SSC/0.2% Tween-20.
- Incubate with 150 µl diluted sheep anti-DIG FITC for 45 minutes at 37°C in a humid chamber.
- Wash 3x 5 minutes at 42°C in 4x SSC.
- Incubate with 1 ml DAPI (diluted 1:10000 from stock) for 3 minutes at RT.
- Rinse in water and let air-dry.

FISH Hybridization

5 Literature

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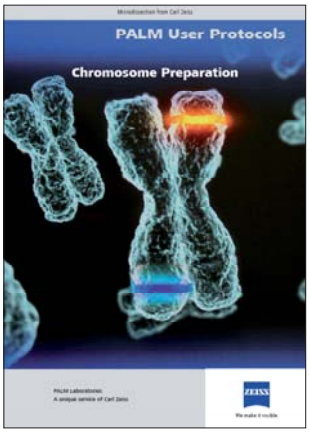
Brochures and protocols

Live cells

Chromosomes



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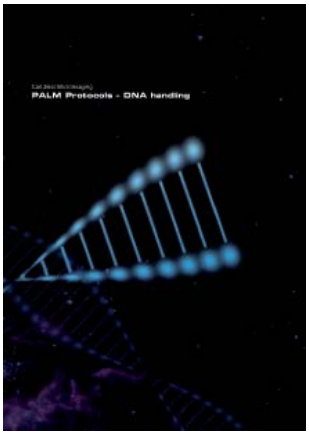
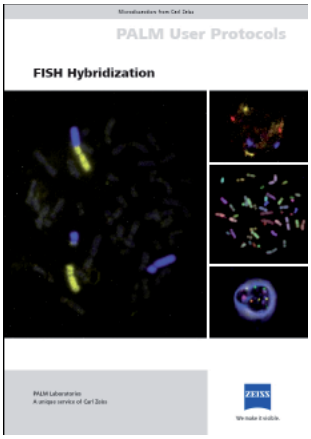
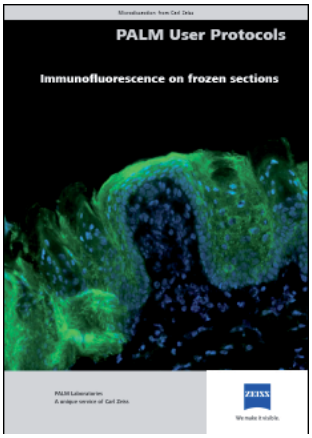


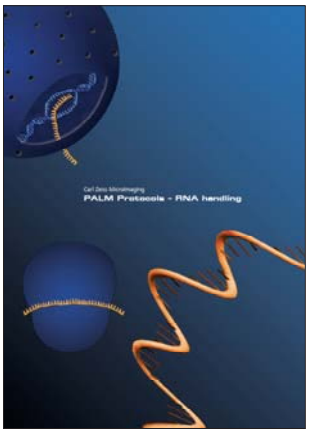
FISH



Immunofluorescence



RNA



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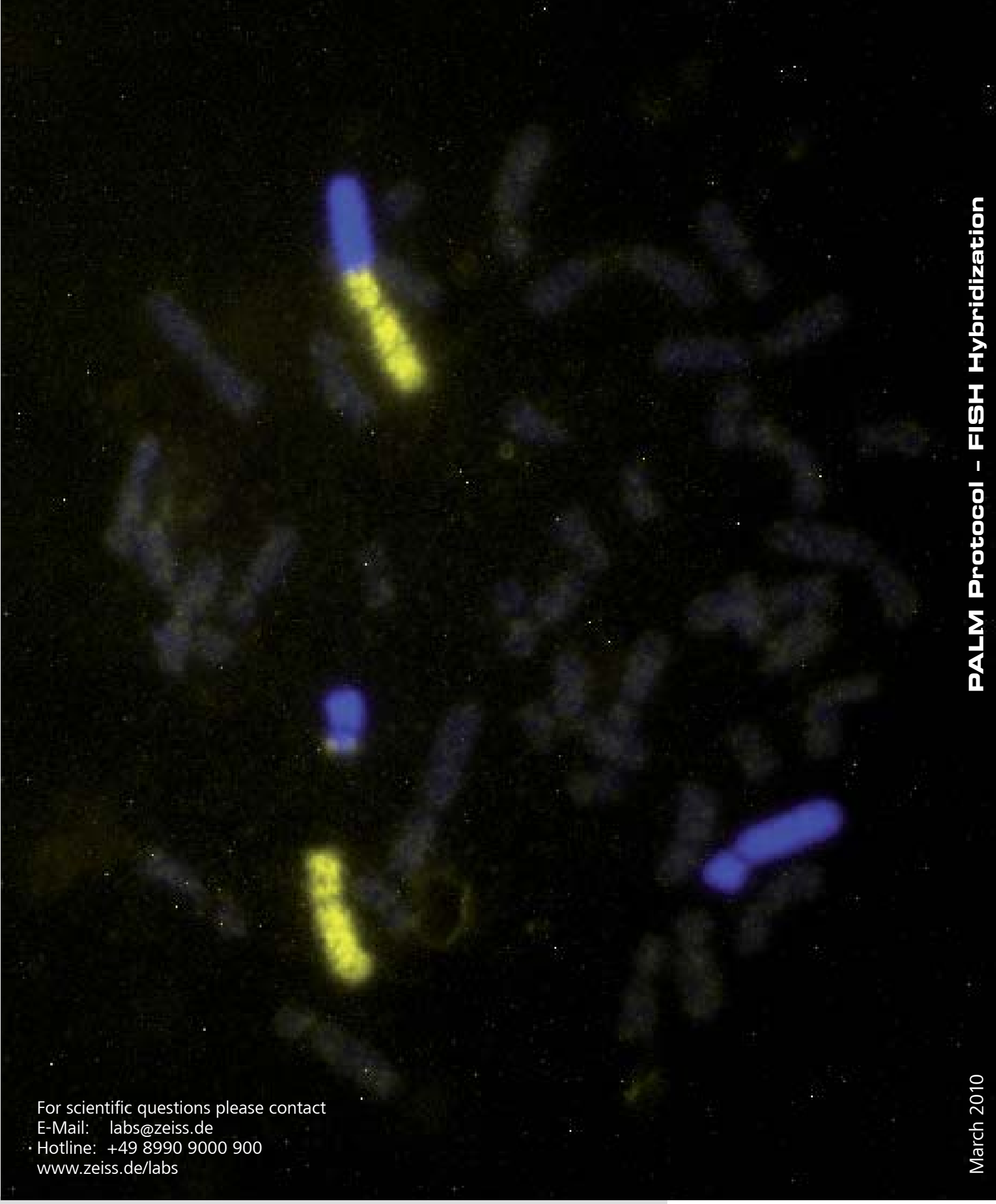

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