PALM User Protocols

Chromosome Preparation

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Chromosome preparation

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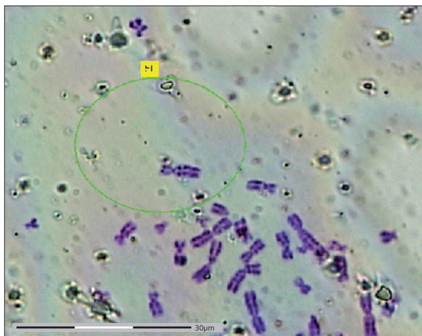
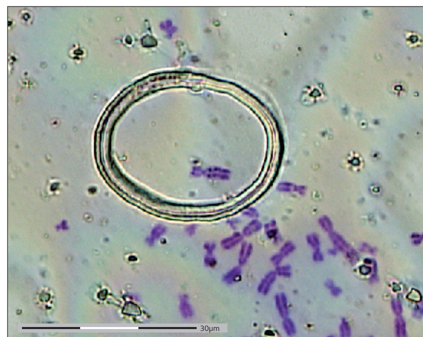
1 Introduction

Laser capture microdissection (LCM) is a process to collect pure cell populations for subsequent DNA and RNA extraction. Although laser microdissection is generally used to isolate specific cells from fixed tissue sections, it has been also effective for isolation of individual chromosomes. Preparation of chromosome paints (Kubičková et al. 2002), FISH hybridization (Langer et al. 2005) and DOP-PCR (Hobza et al. 2004) combined with LCM are already described.
One challenge of microdissecting chromosomes is the minimal diameter of samples collected. Non-contact LCM transfers the dissected pure material directly into a collection device. This enables the fast procurement of a homogeneous specimen of just 0.5 µm in diameter without intrusion into the adjacent area.


I.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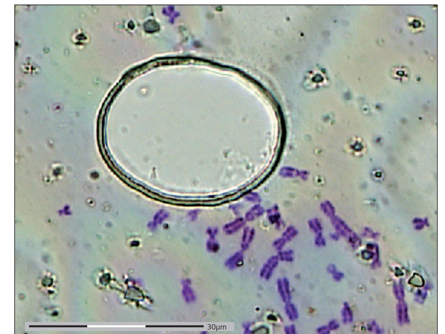
III.

IV.

Non-contact Laser Capture Microdissection of a metaphase chromosome

I. human metaphase chromosomes fixed onto PEN membrane (objective: 100x oil)
II. cutting of selected chromosome (objective: 100x oil)
III. non-contact LCM of selected chromosome by membrane lifting (objective: 100x oil)
IV. isolated region after lifting

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2 Consumables and components to work with chromosomes

2.1 MembraneSlides

MembraneSlide 0.17 PEN	Order No. 415190-9061-000
MembraneSlide 0.17 PET	Order No. 415190-9071-000
MembraneSlide 1.0 PEN	Order No. 415190-9041-000
MembraneSlide 1.0 PET	Order No. 415190-9051-000
FrameSlide PET	Order No. 415190-9101-000

MembraneSlides are glass slides covered with a membrane on one side.
This membrane is easily cut together with the sample and acts as a stabilizing backbone during lifting. Therefore even large areas can be lifted by a single laser pulse without affecting the morphological integrity.
Due to the short working distance of routine 100x (oil) and 63x (oil) magnifying objectives only 0.17 mm thin membrane (PEN, PET) covered glass slides can be used in combination with these objectives!

An alternative strategy is to use long distance objectives for higher magnifications (20xLD, 40xLD or 63xLD, **not available for 100x!**). There you have to adapt the working distance to the different glass slides by moving the correction collar on the objective to the right position (see figure 2).

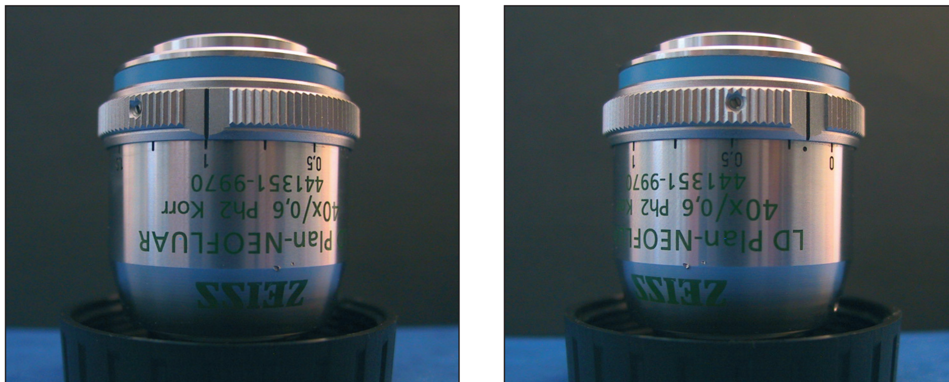

Figure 2

left image:	MembraneSlide	1.0 mm	->	recommended setting	= 1
right image:	MembraneSlide	0.17 mm	->	recommended setting	= dot
without image:	FrameSlide		->	recommended setting	= between dot and 0

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Carl Zeiss MicroImaging (CZMI) offers 1 mm and 0.17 mm slides covered with polyethylene naphthalate (PEN)-membrane (MembraneSlide PEN). This PEN-membrane (2 µm thickness) is highly absorptive in the UV-A range, which facilitates laser cutting. The PEN-membrane can be used for all kind of applications.

In addition to MembraneSlide PEN, CZMI offers polyethylene teraphthalate-membrane covered (MembraneSlide PET) slides for special processes. The PET-membrane is 1.4 µm thick and highly absorptive in the UV-A range, too. The PET-membrane does not exhibit any autofluorescence itself and is therefore dedicated excellently for fluorescence applications.
It is also suitable to perform laser ablation of unwanted specimen. Any downstream applications are feasible.

Alternatively the PET-membrane is available attached to a metal frame (FrameSlide PET).
In fluorescence applications (FISH) even weak signals can be detected due to low signal to noise ratio. The frame structure of FrameSlide PET enables immunohistological staining with minimal volume (< 1 ml) and is resistant to microwave treatment. The special bonding is inert and adapted to heat treatment (up to 95°C under wet conditions or up to 180°C in dry heat) so that the membrane does not ruffle during the heating process.

2.2 Collectors

2.2.1 CapMover

CapMover is designed to be equipped with a

SingleTube Collector 200/500 or
SingleCap Collector 200/500 or
TubeCollector 2x200/2x500

for 200 µl and 500 µl tubes/caps.

2.2.2 RoboMover

RoboMover is a device for automated harvesting and sorting of different kinds of microdissected specimen in a higher throughput process. The non-contact LCM process can be operated automatically or manually. The RoboMover can be reconfigured with different collectors, e.g. SingleTube Collector, TubeCollector 8x500, CapturePlate Collector 96 or SlideCollector 48.

Chromosome preparation

2.3 Collection devices

2.3.1 AdhesiveCap

AdhesiveCap 500 clear	Order No. 415190-9211-000
AdhesiveCap 200 clear	Order No. 415190-9191-000

The intention of AdhesiveCaps is to allow non-contact LCM without applying any capturing liquid into the caps.
Beside the quick relocation of the lifted samples in the cap due to instant immobilization there is no risk of evaporation and crystal forming of buffers during extended specimen harvesting.
For more details and handling, please see also AdhesiveCap product information.

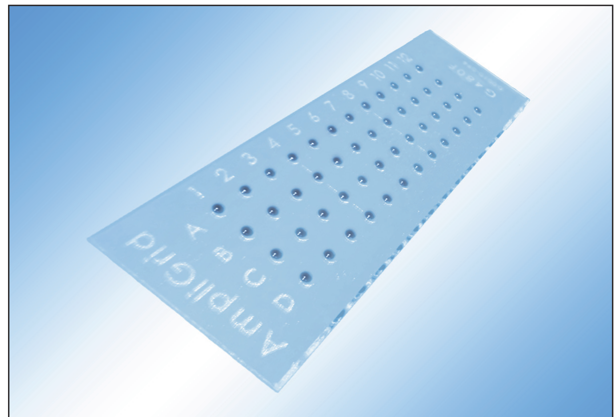
Note: Carl Zeiss recommends AdhesiveCap as collection device for most experiments.

2.3.2 Other microfuge tubes

In case you use commercially available plasticware (e.g. ABgene #AB-0350; 0.5 ml tubes) without adhesive filling, a capturing liquid is necessary to attach the microdissected samples.

2.3.3 AmpliGrid AG480F

Using the SlideCollector 48 in conjunction with AmpliGrid technology from Advalytix enables working in a higher throughput LCM.
The AmpliGrid technology allows DNA amplification and sequencing in an extremely low volume (1 µl) directly on chip.



(Please see brochure: On-chip Single Cell Analysis for MicroBeam; to order at ZEISS with brochure no. 60-3-0002/e)

3 Chromosome Preparation

3.1 Chromosome Preparation from peripheral blood cells

Reagents:

RPMI Medium	(Biochrom # FG1385)
Fetal Bovine Serum	(Biochrom # S0113)
Penicillin/Streptomycin: 10000µg/ml	(Biochrom # A2213)
Phytohemagglutinin	(Biochrom # M5030)
Colcemid	(Biochrom # L6231)

Hypotonic Solution: 0.075 M KCl
Fixation Mix: Methanol/Glacial Acetic Acid (3:1)

Labware:

Falcon tubes (15 ml)
Glass pipettes (230 mm) and sucker
MembraneSlides (0.17 mm or 1 mm, PET or PEN; FrameSlide PET)
Hettich Centrifuge Universal 32R

Prearrangements:

preheat 0.075 M KCl at 37°C
freshly prepare Methanol/Glacial Acetic Acid (3:1) and store at -20°C
put on ice shortly before use

Protocol:

- set up blood cultures (sterile flask):

RPMI Medium	8 ml
Fetal Bovine Serum	2 ml
Phytohemagglutinin	0.2 ml
Penicillin	0.05 ml
heparinized blood	8 drops ~ 0.3 ml (adult)
	5-6 drops ~ 0.2 ml (newborn)

- incubate at 37°C for 72 hours

Chromosome preparation

Colcemid treatment:

sway the flask	
add Colcemid	100 µl

• incubate at 37°C for 30 min

Note: Prolonged Colcemid treatment may result in shrunken chromosomes.

• after colcemid incubation transfer the suspension into a 10-15 ml tube
• centrifuge at 1000 rpm for 10 min

Metaphase preparation:

remove supernatant
resuspend pellet in 6 ml KCl (0.075M), by adding drop by drop during careful mixing at room temperature

• incubate at 37°C for 10-20 min
• spin down at 1000 rpm for 10 min
• discard supernatant (Caution: pellet is somewhat wobbly)
• resuspend pellet cautiously in some drops of ice-cold Fixation Mix (see Reagents) (stored on ice during the next steps)
• slowly fill up with Fixation Mix to 6 ml

Note: It is very important to resuspend the pellet carefully. This means to pipette the suspension carefully up and down or to mix it by gently knocking at the tube.

• repeat the centrifugation step and resuspend till supernatant gets clear after the last centrifugation step
• resuspend the pellet in about 2-5 ml Fixation Mix

Note: Adjust volume for pellet resuspension to the wanted density of cells and metaphase spreads on the slides. If a high amount of cells is present in the suspension use more Fixation Mix to thin it out. More slides can be prepared this way.

Chromosome preparation

3.2 Chromosome Preparation from cell lines (EJ28, HepG2, HeLa)

Depending on the cell type, adequate media, antibiotics etc. have to be used for the cultivation.

Please, see protocols:
"Cell Culture, non-contact Laser Capture Microdissection and recultivation" or
"Live Cells and Molecular Analysis"

Reagents:

Hanks' medium (with Ca++, Mg++) (Biochrom # L 2035)
Trypsin / EDTA (Biochrom # L 2143)
Colcemid (Biochrom # L 6231)
Hypotonic Solution: Hanks'/Aqua dest. (1:3)
Fixation Mix: Methanol/Glacial Acetic Acid (3:1)

Labware:

Falcon tubes (15 ml)
Glass pipettes (230 mm) and sucker
MembraneSlides (0.17 mm or 1 mm; PET or PEN)
Hettich Centrifuge Universal 32R

Prearrangements:

preheat cell culture medium, Hanks' and Trypsin/EDTA at least at room temperature
preheat hypotonic solution at 37°C
freshly prepare Methanol/Glacial Acetic Acid (3:1) and store at -20°C;
put on ice shortly before use

Protocol:

- set up cell cultures (sterile flask):

RPMI Medium	9 ml
Fetal Bovine Serum	0.9 ml
Penicillin	0.09 ml
cell suspension	20 µl (depending on the cell type)

- incubate at 37°C for 72 hours (depending on the cell type)

Chromosome preparation

Colcemid treatment:

4-16 hours before culture stop	
add Colcemid	100-200 µl

Note: Prolonged Colcemid treatment may result in shrunken chromosomes

Trypsinization:

remove medium	
rinse with Hanks' solution	5 ml
remove the solution	
add Trypsin/EDTA	3 ml

- incubate at 37°C for 5 minutes

Note: If cells are hard to trypsinize, knock gently at the flask

to stop trypsinization:	
add medium	8 ml
transfer the suspension into a Falcon tube	

- spin down at 1000 rpm for 10 minutes

Metaphase preparation:

remove supernatant
resuspend pellet in 8 ml KCl (0.075M),
containing Hanks'/Aqua dest. (1:3) by adding it drop by drop

- incubate at 37°C for 30-40 min
- spin down at 800-1000 rpm for 10 min
- discard supernatant (Caution: pellet is somewhat wobbly)
- resuspend pellet carefully in some drops of ice-cold Fixation Mix (stored on ice during the next steps)
- slowly fill up with Fixation Mix to 4 ml

Note: It is very important to resuspend the pellet carefully. This means to pipette the suspension carefully up and down or to mix it by gently knocking at the tube.

Chromosome preparation

- repeat the centrifugation step and resuspend till supernatant gets clear after the last centrifugation step
- resuspend the pellet in about 2-5 ml of Fixation Mix

Note: *Adjust volume for pellet resuspension on the wanted density of cells and metaphase spreads on the slides. If a high amount of cells is present in the suspension use more Fixation Mix to thin it out. More slides can be prepared this way.*

3.3 "Chromosome Dropping"

- dip a plain slide into 100% ethanol, rinse in distilled water and let air dry
- store the so prepared slides at -20°C until starting to drop the chromosome suspension
- take out a slide immediately before dropping the chromosomes (Defrosting causes a wet slide and makes spreading much easier.)

Note: *When using a routine 1 mm glass slide underneath the 0.17 mm MembraneSlide for "stabilization", the chromosomes will not bounce during dropping, thus improving chromosome spreading.*

- put about 2-4 drops of fixed cell suspension from a distance of about 20 cm onto an ice-cold and wet MembraneSlide
- cover a heating block with wet tissues
- let the slides dry on the heating block for 1 hour at 37°C
  This so called 'chromosome aging' has direct influence on the quality of the metaphases.

Chromosome preparation

4 Chromosome staining: GTG staining (Giemsa staining)

Reagents:

Giemsa Stain Solution	(Sigma Aldrich # GS 500)
Di-sodium hydrogen phosphate solution (1/15 mol/l)	(Merck # 1.065871)
Potassium dihydrogen phosphate solution (1/15 mol/l)	(Merck # 1.04875)
Trypsin (1:250)	(PAA # L11-002)
70% Ethanol	

Prearrangements (prepare all solutions freshly):

Giemsa Stain:

Di-sodium hydrogen phosphate solution	50 ml
Potassium dihydrogen phosphate solution	50 ml
Giemsa Stain Solution	8 ml

Trypsin solution:

PBS (1x)	100 ml
Trypsin (1x)	3 ml

Note: *Use only freshly prepared trypsin!*

Protocol:

- soak slide into Trypsin 5 seconds
- dip slide into 96 % Ethanol
- dip slide into Aqua dest
- soak slide into Giemsa stain for 4 minutes
- dip slide into Aqua dest
- let the slide air dry

Note: *The quality of chromosome spreads is highly depending on the time period of trypsinization and the time period of previous chromosome aging.*

Chromosome preparation

5 Non-contact Laser Capture Microdissection (LCM)

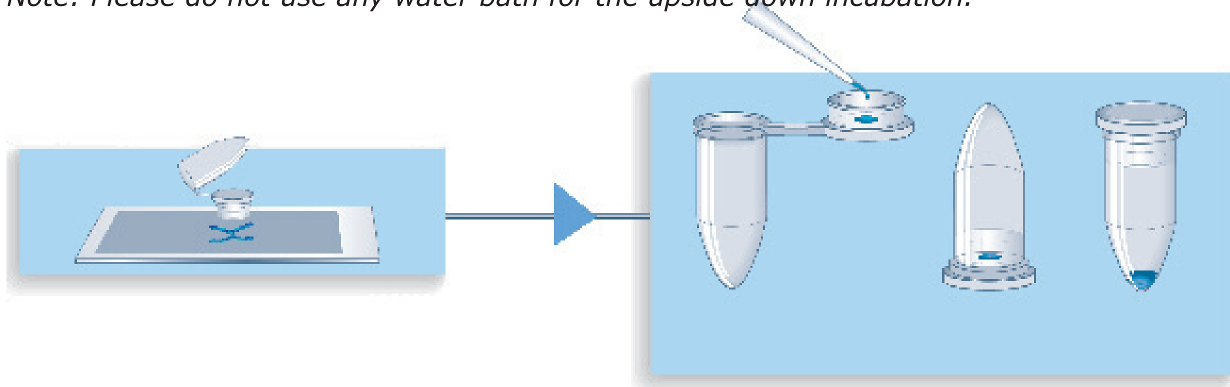
5.1 Collection procedures

Please, additionally have a look into the MicroBeam user manual.

5.1.1 “Dry” collection into AdhesiveCap

- after LCM into AdhesiveCap add a buffer of your own choice and incubate “upside down” for 2-18 hours depending on chromosome preparation

  Note: Please do not use any water bath for the upside down incubation.



- subsequently centrifuge the liquid into the tip of the tube and then apply the routine procedure

5.1.2 “Wet” collection into other microfuge tubes

When using “unfilled” routine microfuge tubes it is necessary to fill a liquid into the cap prior to LCM. This improves the adhesion of the captured chromosomes. Igepal CA-630 in the Capturing Buffer (see next page) allows to smear out a small amount of liquid in the whole cap area. Alternatively the chromosomes can be lifted directly and precisely in a 3 µl droplet of buffer of your own choice.

Note: All kinds of aqueous solutions will run dry after a while.

Chromosome preparation

Prearrangements:

Capturing Buffer:

0.5 M EDTA pH 8.0	20 µl
1 M Tris pH 8.0	200 µl
Igepal CA-630 (SIGMA #I-3021)	50 µl
Proteinase K 20 mg/ml (Qiagen #19131)	100 µl
ddH2O	fill up to 10 ml

Note: *Always prepare a fresh mixture of Capturing Buffer and Proteinase K.*

Protocol:

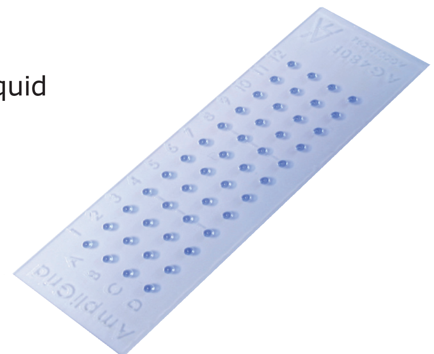
- take an autoclaved cap or tube
- pipette 3-15 µl of Capturing Buffer or DNase-free water in the middle of the cap
- check the right position of the correction collar (see page 4)
- put the cap/tube into the collector and position it above the selected chromosome
- perform non-contact LCM of selected chromosome or chromosome parts
- put the cap onto the tube
- centrifuge the tube at full speed for 5 minutes (Hettich Centrifuge, Universal 32R)
- add 10-15 µl Capturing Buffer containing Proteinase K onto the chromosomes (which are now located in the tip of the tube)
- vortex gently, digest for 2 - 18 hours at 55 °C followed by a final heating step at 90 °C for 10 min to inactivate Proteinase K
- if not going on immediately, store the samples in a refrigerator at 4 °C

Note: *The time necessary for complete digestion depends on the type and the number of isolated chromosomes.*

- after the Proteinase K digest and the inactivation step you may start with your routine downstream application.

5.1.3 "Wet" collection onto AmpliGrid AG480F

A preloading of the 48 ReactionSites of the AmpliGrid with 1 µl liquid (e.g., 1% Glycerol in water) enables prolongation of the working time and increases adhesion of the captured samples.
The LCM process of 48 ReactionSites can be operated automatically and is controlled by RoboSoftware.



Chromosome preparation

6 Literature

PALM Systems

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Höfler H and Werner M
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Diagn Mol Pathol, 2000, 9,1: 14-19

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Chromosoma, 2006, 116,2: 135-145

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Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma In Situ of human urinary bladder.
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Chromosome preparation

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Lab Invest, 2000, 80,5: 709-718

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Lab Invest, 2005, 85,4: 582-592

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Laser pressure catapulting followed by B actin gene identification in Japanese quail macrochromosomes and microchrosomosomes using Teflon-coated coverslip slides.
J Microsc, 2005, 218: 219-224

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J Pathol, 2003, 199: 50-57

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DOP-PCR

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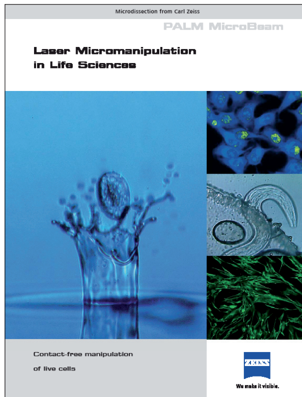
Genomics, 1992, 13: 718-72

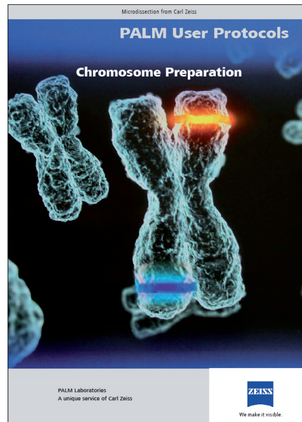
Chromosome preparation

Brochures and protocols

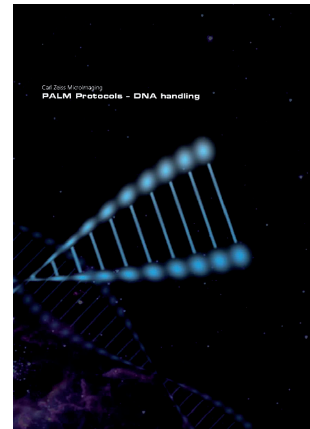
Live cells



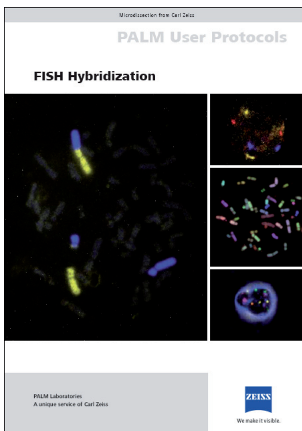
Chromosomes



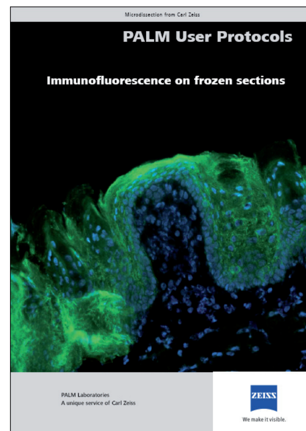
DNA



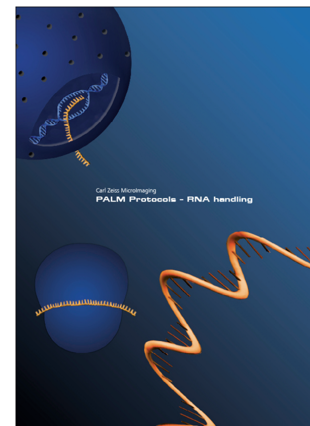
FISH



Immunofluorescence



RNA



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PALM Protocol - Chromosome Preparation

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