

Module 4

FISH on Medicago truncatula chromosomes and extended DNA fibres

Olga Kulikova, Ton Bisseling

Local organiser : Eva Kondorosi

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1. INTRODUCTION

Fluorescence *in situ* hybridisation (FISH) technique has opened new possibilities for genome mapping. Effective utilization of this technique for the rapid orientation and ordering of clones facilitates physical mapping and positional cloning efforts.

The detection of chromosomal DNA sequences in cytological preparations was initially based on application of radioactively labelled RNA and DNA probes. A major breakthrough came with the introduction of fluorescent labels, (in)directly linked to DNA probes and visualized by fluorescence microscopy. FISH appeared superior to previous *in situ* technologies in providing better spatial resolution along with the potential of simultaneously using different fluorescence systems for multi-probe analysis. The number of targets to be analysed simultaneously, depends on the number of fluorochromes with different excitation and emission wavelengths.

Initially, the FISH technique was applied to metaphase chromosomes to assign clones to specific chromosome regions and to order clones along chromosomes. Chromosomes at this stage are highly condensed, which limits the optical resolution of adjacent FISH targets to Mbs rather than kbs. To improve spatial resolution of FISH mapping and accessibility of chromosomal DNA targets, a more decondensed chromosome preparation should be considered to replace the highly condensed metaphase chromosomes in plants. Pachytene chromosomes, which are much less condensed than metaphase chromosomes, are better material for the development of a cytogenetic map by FISH. However, the production of high-quality pachytene spreads in comparison to metaphase chromosomes is technically more demanding and the success for satisfactory results may differ between related species and even between genotypes of the same species (review by De Jong et al., 1999).

To further improve the mapping resolution for closely linked clones, FISH has been used on extended genomic DNA fibres from interphase nuclei (Parra and Windle, 1993 ; Fransz et al., 1996).

The first FISH mapping on *M. truncatula* chromosomes was done by Gerbah et al. (1999). 5S rDNA and 45S rDNA were used as probes on metaphase chromosomes of the R-108-1 and Jemalong J5 accessions. Both genotypes have a single NOR (nucleolar organizing region), Jemalong J5 has three 5S rDNA loci, whereas R-108-1 has only two.

M. truncatula Jemalong A17, which is the standard line in most current molecular genetic studies, was used for karyotyping of pachytene chromosomes (Kulikova et al., 2001). Pollen mother cells at late pachytene clearly display eight fully paired bivalents with total complement length of 406 μm that is 20 times longer than at mitotic metaphase. DAPI staining of pachytene chromosomes demonstrated striking differences in chromatin density. Brightly fluorescent heterochromatic blocks are detected in the pericentromeric regions of all chromosomes. Distal regions of the chromosome arms generally consist of weakly fluorescing euchromatin. All chromosomes can be identified based on chromosome length, centromere position, heterochromatin patterns and the positions of three repetitive sequences (5S rDNA, 45S rDNA and the *MtR1* tandem repeat), visualised by FISH. The correlation between genetic linkage

groups and chromosomes was determined by FISH mapping of BAC (Bacterial Artificial Chromosome) clones. Most of the linkage group specific BACs gave distinct FISH signals in euchromatic parts of the chromosomes and no background labelling was observed, suggesting that the interstitial segments of *M. truncatula* chromosomes contain a relatively low number of repeated sequences. Furthermore, the degree of chromatin condensation in a euchromatic part of chromosomes was shown to be about 300 kb per μm , similar to that of *Arabidopsis* euchromatin which varies between 150-300 kb/ μm (Fransz et al., 1998 ; Fransz et al., 2000). All these features together make *M. truncatula* an attractive plant model for FISH mapping.

During this part of the course the following experiments are planned :

1. FISH with tandem repeats 5S rDNA, 45S rDNA, *MtR1* and *MtR2* on metaphase chromosomes of two accessions of *M. truncatula* Jemalong A17 and DZA315.16.
2. FISH with the same tandem repeats and three BAC clones on pachytene chromosomes of Jemalong A17 and DZA 45.5.
3. FISH with BAC clones on extended DNA fibres of Jemalong A17.

2. CHROMOSOME PREPARATIONS

FISH technique has been applied to mitotic and meiotic cell spreads in a large number of animal and plant species. In general, much attention must be paid to the quality of the chromosome preparation. This goes even more for preparation of plant chromosomes where the presence of thick cell walls and limited numbers of cells at appropriate stage hamper good chromosome preparation with many cell spreads.

Mitotic chromosome preparations

Most cytogenetic studies involve the morphological analysis of mitotic metaphase chromosomes of fast growing meristematic cells. Root tips are in general used as source of mitotic metaphase chromosomes. To arrest cells at metaphase stage *M. truncatula* roots are treated with β -bromonaphtalene. The effects of the treatment are :

- Inhibition of formation/breakdown of cell division spindles
- Better spreading of chromosomes at metaphase
- Stronger chromosome condensation of metaphase chromosomes resulting in more distinct centromeres and secondary constrictions.

The commonly used fixative for plant chromosomes is acetic acid-ethanol 1:3, also known as Carnoy's Fluid. This mixture is prepared just before use to minimize ester formation. It penetrates the tissue very rapidly and a fixation time of 3 h will normally be sufficient.

The digestion of plant cell walls by pectolytic enzymes is an essential step in the procedure for making high quality chromosome preparations. The chromosomes and nuclei should be well separated and free of cytoplasm, debris and dirt. Non-specific signals mostly occur on cytoplasm

and cell debris. Furthermore, a thick layer of cytoplasm can mask the chromosomes and hinder the access of probe and detection reagents.

Pectolytic enzymes are available at different grades of purity and most combinations of cellulases and pectinases will be effective in breaking down plant cell walls in a short time. We routinely work with mixtures of cellulase RS and pectolyase Y23, which work at low concentration while their pronase activity is negligible.

There are several techniques for making chromosome preparations. The cell spreading technique was originally developed by Pijnacker & Ferwerda (1984) for potato chromosomes. It consists of dissecting a piece of root (or other material) in a drop of 60% acetic acid with fine needles, thus producing a suspension of single cell and small cell aggregated.

Once a series of chromosome preparations have been obtained, all slides should be checked under the phase contrast microscope. Selection criteria are :

- “dark appearance of the chromosomes”
- number of complete metaphase spreads
- spreading of chromosomes
- absence of cytoplasm and cell walls, debris and dirt.

Only the best preparations should be selected for the *in situ* hybridization experiments ! Store them in a tightly closed box on a dry and cool place (+4°C). For longer storage wrap the box in aluminium foil and keep at -80°C.

Cell spreading technique for root tips of M. truncatula

Material

5 day-old seedlings of *M. truncatula* Jemalong A17 and DZA 315.16 were grown on wet filter paper.

Reagents

- Freshly prepared ice-cold Carnoy`s fixative; ethanol - glacial acetic acid (3:1)
- 10mM citrate buffer pH 4.5
- pectolytic enzyme mixture : 0,15% (w/v) cellulase RS, 0.15% (w/v) pectolyase Y23 in citrate buffer
- 60% glacial acetic acid

Procedure

1. Treat root tips for 4 h with a saturated aqueous solution of β -bromonaphtalene at 14°C to accumulate metaphase cells.
2. Fix root tips in freshly prepared fixative for 2-3 h.
3. Place fixed root tips in a small watch glass with water and wash for 2x5 min.
4. Replace the water by citrate buffer and wash 2x5 min.
5. Incubate the material in enzyme mixture for 1¹/₄ h in a moist chamber at 37°C.

6. Select a root tip and put it on a clean slide in 2 μ l water. Tap the root tip with a blunt dissection needle and examine whether a fine suspension has formed. (*If there are still large tissue fragments then extend the enzymatic incubation time for further digestion. Once a fine suspension has been obtained, replace the enzyme mix by citrate buffer or water. The digested material should be kept on ice.*)
7. Add about 15 μ l of 60 % acetic acid to clear the fine suspension and make the cells sticky. (*The cells move in the droplet and settle down at the periphery of the droplet, forming a ring of cells. To prevent disposition of too many cells at the same ring position the droplet should be stirred now and then with the needle, without touching the glass surface.*)
8. Place the slide on a hot block at 45°C, add another 15 μ l of 60 % acetic acid and keep on stirring for 30-60 s. (*In this step acid-soluble proteins and various cytoplasmic components will dissolve in the acetic acid and clear up the spread preparation. Omitting this step will result in chromosome spreads that are still surrounded by cytoplasm. Too little acetic acid or a too high density of cells will result in an undesirable layer of cytoplasmic debris. Monitor the results under the phase contrast microscope. Add more acetic acid, if needed. More cells will adhere to the slide at the periphery of the droplet forming a ring that varies in shape due to the stirring.*)
9. Precipitate cells by carefully pipetting drops of fixative around the droplet with cleared cell suspension and wait until the fixative covered the whole glass slide. Discard excessive solution by tilting the slide and pipette more fixative on the preparation.
10. Dry the preparation using a hairdryer or air-dry the preparations.
11. Examine the preparation under the phase contrast microscope at 40X magnification and select the best ones for *in situ* hybridisation.

Meiotic chromosome preparations

The major drawback of metaphase chromosomes, despite all technical progress in FISH technology, remains its highly condensed nature that hampers to distinguish DNA targets separated by less than 1-2 Mb. As the spatial resolution is inherent to the higher order organization of the chromosome, less condensed chromosome structures are required for resolving physically closely linked targets.

Another approach has been applied in plants, involving meiotic prophase chromosomes. Pachytene homologous chromosomes are fully paired to form long well differentiated bivalents, 10-50 times longer than mitotic metaphase counterparts. For example, the pachytene and metaphase chromosomes in *M. truncatula* differ by a factor 20. The spatial resolution of pachytene chromosomes is about 50 kb in euchromatin and more than 1 Mb in heterochromatin.

Cell spreading technique for pachytene chromosomes of M. truncatula

The spreading technique for meiotic material is essentially the same as for mitotic chromosomes with the following exceptions :

- Meiotic chromosomes are isolated from pollen mother cells. Finding the right stage is a matter of experience. Collect young, unopened flower buds at different stages and the anthers should still be green.
- Digestion of the thick callose wall of pollen mother cells at meiosis requires β -1, 3 glucanase, callase or the snail gut enzyme cytohelicase.

Material

Inflorescences of *M. truncatula* genotypes of Jemalong A17 and DZA 45.5. Since the anthers containing pollen mother cells at pachytene are small and meiosis are not synchronized use entire flower buds for the preparation of pachytene chromosome spreads. Immature flower buds smaller than 2.0 mm contain the appropriate meiotic stages.

Reagents

- Freshly prepared ice-cold Carnoy`s fixative ; ethanol-glacial acetic acid (3:1)
- 10 mM citrate buffer pH 4.5
- Pectolytic enzyme mixture: 0,3 % (w/v) cellulase RS, 0.3 % (w/v) pectolyase Y23, 0.3 % (w/v) cytohelicase in citrate buffer
- 60 % glacial acetic acid

Procedure

1. Fix whole inflorescences for 3-4 h. The fixative is refreshed several times until the flower buds are yellow-white and the solution remains clear.
2. Select flower buds of appropriate size.
3. Place fixed flower buds in a small watch glass with water and wash for 2x5 min at 20°C (room temperature).
4. Replace the water by citrate buffer and wash 2x5 min.
5. Incubate the material in enzyme mixture for 2¹/₄ h in a moist chamber at 37°C.
6. Select a flower bud and put it on a clean slide in 2 μ l water. Remove connective tissue by using two needles.
7. Transfer flower bud to another clean slide in 2 μ l water.
8. Tap the flower bud with the tip of a blunt dissection needle and examine if a fine suspension has formed.
9. Add about 15 μ l of 60 % acetic acid to clear the fine suspension and make the cells sticky. Place the slide on a hot block at 45°C, add another 15 μ l of 60 % acetic acid and keep on stirring for 30-60 s.

10. Precipitate cells by carefully pipetting drops of ethanol-acetic acid fixative around the droplet with cleared cell suspension and wait until the fixative covers the whole glass slide. Discard excessive solution by tilting the slide and pipette more fixative on the preparation.
11. Dry the preparation using a hairdryer or air-dry the preparations.
12. Examine the preparation under phase-contrast microscope without cover glass. Only the best preparations without cell walls and negligible cytoplasm are good enough for your FISH work !

3. EXTENDED DNA FIBRES

A dramatic improvement of mapping resolution has recently been achieved with the development of extended DNA fibres technology for FISH mapping. This technique allows spatial resolution of 1 kb that surpasses those in all other FISH techniques and approaches the physical distances estimates in molecular restriction mapping protocols. DNA fibre technology is based on the release from interphase nuclei of protein depleted chromatin fibres and subsequent fixation on a microscope slide. The stretched chromatin fibres are largely free of proteins and decondensed to length values approaching native DNA. By hybridising labelled DNA probes to the DNA fibres hybridisation, targets become visible as linear fluorescent tracks, the microscopical length of which corresponds to the molecular size of the DNA sequences. Additionally, the order of signals directly reflects the physical order of sequences. The method is referred to as direct visualization hybridisation (DIRVISH) or DNA fibre FISH.

The DIRVISH method is based on the total disruption of nuclei and chromatin structure using detergent and EDTA, followed by stretching of the DNA fibres along the glass surface by tilting the microscopic slide. The stretching degree is close to 2.94 kb/ μm , which agrees with the expected length for the duplex B-DNA.

As the microscopical view often reveals non uniform, complex patterns of beaded fluorescent signals, the question arises as to how hybridisation signals should be distinguished from the background and how the it should be interpreted ? Like molecular markers in gel electrophoresis, proper bench markers and controls should be included during FISH. The nature of these matters depends on the intended purposes. For ordering closely linked DNA sequences no specific markers are required as the sequences themselves serve as markers for each other. However, the DNA targets should not be too far from each other, otherwise a clear-cut linkage remains elusive. To distinguish a hybridisation signal from background fluorescent dots a minimum target size of about 10 kb is recommended, which generates a 3 μm linear fluorescent signal. Smaller targets can only be identified by simultaneous detection of flanking markers with the other fluorochrome. The presence of two different fluorescent signals in close approximately denotes a positive hybridisation signal. More conveniently, if a larger DNA sequence containing target is available, for example a cosmid, than the whole cosmid should be hybridised and detected with another fluorochrome. This will reveal size and position of the small target.

Isolation of plant nuclei

This section describes a protocol for isolating nuclei as adapted from Liu and Whittier (1994). The final concentration of nuclei should be approximately 5×10^5 per ml, although this is more guideline than a prerequisite.

Material

Young leaves of *M. truncatula* Jemalong (~1g) are collected and frozen in liquid nitrogen.

Reagents

- Nuclei isolation buffer (NIB), freshly prepared and kept on ice (10 mM Tris-HCl, pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1.0 mM spermine, 0.1 % (v/v β -mercaptoethanol)
- Liquid nitrogen
- 10 % Triton X-100 in NIB
- Mortar and pestle
- Set of nylon mesh filters
- 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) in Vectashield antifade mounting medium (Vector Laboratories)

Procedure

1. Grind the tissue in a mortar to a fine powder with liquid nitrogen.
2. Transfer the powder to a 50 ml jar containing 20 ml ice-cold NIB and incubate for 5 min.
3. Filter the homogenate through consecutive nylon mesh filters (170, 120, 50 and 22.4 μ m).
4. Add 1/20 volume of 10 (v/v) Triton X-100 in NIB to the filtrate.
5. Centrifuge the suspension at 2000 g for 10 min at 4°C.
6. Resuspend the pellet in 200 μ l NIB.
7. Mix 1 or 2 μ l suspension with 5 μ l of 1 μ g/ml DAPI in Vectashield, using a UV-filter set for DAPI.
8. Nuclei should be more or less intact. Long extended DAPI fluorescent threads indicate premature lyses of nuclei, which may effect the preparation of extended DNA fibres later in the protocol.

Preparation of extended DNA fibres

The protocol for releasing DNA fibres from nuclei is adapted from Parra and Windle (1993) using SDS and EDTA. Lysis of the nuclei occurs by means of the detergent, while EDTA disrupts chromatin integrity. At a high EDTA concentration (50 mM) the distortion of the chromatin structure is rather abrupt and results in a criss-cross pattern of free extended DNA fibres. A 10-x lower concentration (5 mM) will release DNA fibres more gradually and generates long parallel strands (Fransz et al., 1996).

Reagents

- PBS solution (10 mM sodium phosphate, pH 7.0, 140 mM NaCl)
- STE buffer (0.5 % (w/v) SDS, 5 mM EDTA, 100 mM Tris-HCl, pH 7.0)
- Ethanol/acetic acid (3:1)

Procedure

1. Spin down 50 µl of the nuclei suspension at 3600 for 5 min.
2. Resuspend the pellet in 50 µl PBS.
3. Pipette droplet of 2µl of the suspension onto one end of a clean microscope slide and air dry for 2-3 min.
4. Lyse the nuclei by adding 30 µl of STE to the slide and incubate for 6-7 min.
5. Tilt the slide at an angle of 30 degrees to let the buffer float downwards. The DNA fibres, which are partly attached to the glass surface, will move downwards and stretch.
6. Air dry slides and fix the DNA fibres by soaking in ethanol/acetic acid (3: 1) for 2 min.
7. Dry the slides.

4. PROBES

The quality and properties of the probes are critical in FISH experiments. It is crucial to use pure DNA samples as probes or as blocking material.

DNA isolation

Plasmid DNA isolation was done by using High Pure Plasmid Isolation Kit according manufacture protocol (Roche).

BAC DNA was isolated according to the alkaline lysate method.

DNA labelling

FISH is based on the use of non-radioactive labels that are incorporated into the probe DNA. Two types of FISH methods can be discerned : the **direct** and **indirect** methods. In the **direct** method the label that has been incorporated into a probe that can be examined by fluorescence microscope immediately after the hybridisation has been finished. The fluorochromes that are currently used for direct *in situ* hybridisation include Fluorescein isothiocyanate (FITC), Rhodamine (TRITC), Texas Red, 7-amino-4-methyl-coumarin-3-acetic acid (AMCA), Cy3 (indocarbocyanine) and Cy5 (indodicarbocyanine). The advantage of using direct *in situ* hybridisation is the ease of visualization of hybridisation signals. A drawback is that the sensitivity may not be high enough for the detection of low copy or dispersed sequences. In this

case the availability of fluorescein-conjugated antibodies raised against Fluorescein enables amplification of the signal.

In the **indirect** method a non-fluorescent (either biotin or digoxigenin) is incorporated into the DNA. After *in situ* hybridisation the label has to be detected by incubation with either avidin or streptavidin (in the case of biotin) or antibodies raised against digoxigenin. The (strept)avidin and anti-digoxigenin molecules are linked with one of the fluorochromes mentioned above, and can be visualized by fluorescent microscopy.

Uniformly labelled probes can be obtained in various ways. Different probes will be used in this course. BAC clones are labelled by nick translation by using Biotin-Nick Translation kit and Digoxigenin-Nick Translation kit according manufacture's protocol (Roche). Clone pCT4.2, which contains a 5S ribosomal DNA repeat unit (~500 bp) of *Arabidopsis thaliana* in pBS (Campbell et al., 1992), is PCR-labelled with biotin-16-dUTP (Roche) according to the instructions of the manufacturer. Clone pTA71, which contains a 9.1 kb fragment of 18S-5.8S-26S rDNA of common wheat (Gerlach and Bedbrook, 1979), is labelled with digoxigenin-11-dUTP using the High Primed Labelling Kit (Roche). Clones pMtR1 and pMtR2 contain two pericentromeric repeats in pGEM-T (Promega). They are labelled by PCR with either digoxigenin-11-dUTP or biotin-16-dUTP (Roche).

Checking the labelling

It is important to check if the DNA to be used for *in situ* hybridisation is sufficiently labelled because this may limit the detection. The labelling can be detected using a colour reaction based on alkaline phosphatase (AP) conjugate and nitro blue tetrazolium (NBT) on a Southern dot blot (standard Roche protocol).

Reagents

- Anti-dig-AP conjugate or streptavidin-AP conjugate
- Buffer 1 (100 mM maleic acid, 150 mM NaCl, adjust pH to 7.5 with NaOH)
- Buffer 2 (1 % blocking reagent in buffer 1)
- Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5)

Procedure

1. Spot 2, 20, 200 and 2000 pg of labelled DNA on a small piece of Hybond N+ (Amersham) hybridisation membrane.
2. Cross-link the DNA to the membrane by UV light using a UV cross-linker
3. Wash filters briefly (1 min) in 10 ml of buffer 1 + 0.3 % Tween 20.
4. Incubate for 30 min in 5 ml buffer 2.
5. Dilute anti-dig-AP or streptavidin-AP conjugate 1:5000 in buffer 2 and incubate the filter 30 min.
6. Wash twice 15 min with 10 ml buffer 1 to remove the unbound antibody conjugates.

7. Incubate the filter in 5 ml freshly prepared colour substrate in the dark, the colour should develop now (100 µl NBT/BCIP in 5 ml buffer³).
8. Check the labelling by comparing the label with control DNA.

5. FLUORESCENCE *IN SITU* HYBRIDISATION

FISH involves the following steps :

1. The procedure starts with a three-step pre-treatment of slides. RNase incubation is performed to reduce non-specific probe hybridisation to non-target nucleic acids. The pepsin treatment reduces proteinaceous compounds and so clears up non-specific signals in background. Pachytene preparations of *M. truncatula* do not require pepsin treatment. The incubation in formaldehyde is needed to cross-link chromosome proteins and so enhances their stability during subsequent hybridisation steps.
2. The hybridisation mixture is now applied on the chromosome preparations. After a combined denaturation of probe and chromosome DNA, the slides are incubated overnight.
3. Upon hybridisation, un-hybridised and non-specifically bound probe DNA is removed by washing under more stringent conditions than during hybridisation.
4. For biotin and digoxigenin labelled probes, additional detection steps are required to visualize the hybridisation sites.
5. Extended DNA fibre preparations do not need any pre-treatment to improve DNA accessibility, so no pepsin and formaldehyde treatment.

Reagents

- Hybridisation buffer (HB50), containing 50 % deionized formamide, 2xSSC and 50 mM sodium phosphate pH 7.0
- 3 mM NaAc (pH 5.5)
- Ice-cold ethanol
- 20 % dextran sulphate in HB50
- 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0)
- 100 µg/mL of RNase in 2x SSC
- PBS (10 mM sodium phosphate pH7.0, 140 mM NaCl)
- 1% formaldehyde in PBS
- Ethanol (70 %, 90 %, 100 %)
- SF50 buffer (50 % formamide, 2xSSC pH 7.0)

DNA probe mixture

1. Take the required amount of each DNA probe (200-250 ng).
2. Add 50 µg fragmented Salmon Sperm DNA.
3. Add 0.1 volume 3M sodium acetate (pH 5.5) and 2.5 volume 100 % ethanol (-20°C), mix well.

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4. Place the solution for 30 minutes on ice.
5. Spin down for 30 minutes at 4°C.
6. Remove supernatant and wash pellet in 70 % ethanol.
7. Spin down for 15 min at room temperature.
8. Remove supernatant and resuspend the pellet in 10 µl HB.
9. Dissolve the DNA for 15 minutes in a 37°C water bath.
10. Add 10 µm of 20 % dextran sulphate in HB (pre-heated to 37°C).
11. Denature the DNA by placing it for 5 minutes in a boiling water.
12. Place the DNA for 3 minute on ice and spin down briefly.

Pre-treatment of slides

1. Dry the slides at 60°C for 30 min.
2. Pipette 100 µl of RNase solution and cover with a 24x50 mm cover slip.
3. Incubate at 37°C for 60 min.
4. Rinse in 2x SSC at RT for 3x5 min.
5. Incubate the slides in 0.01M HCl for 2 min.
6. Add 100 µm of 5 µg/ml (20 units/ml) pepsin in 0.01M HCl on each slide, cover with a cover slip and incubate at 37°C for 10 min.
7. Wash slides in water for 2 min and in 2x SSC two times for 5 min.
8. Rinse in PBS for 5 min.
9. Fix in 1 % formaldehyde at RT for 10 min.
10. Rinse in PBS for 2x5 min.
11. Dehydrate the slides through an ethanol series (70 %, 90 %, 100 %). Each step 2 min
12. Air-dry the preparations.
13. Drop hybridisation mix on target area of the slide.
14. Cover the solution with 24x24 mm cover slip.
15. Denature chromosomes and DNA at 80°C (hot plate) for 2 min.
16. Put the slides in a moist chamber and incubate overnight at 37°C.

Washing

1. Wash the slides in coplin jars containing SF50 at 42° for 3x5 min.
2. Wash the slides in 2x SSC.

Immunodetection of biotin- and digoxigenin- labelled probes

Reagents

- Non-fat dry milk (from local store)
- Blocking reagent (Roche)
- 4T buffer (4xSSC pH 7.0, 0.05 % (v/v) Tween-20)
- 4M buffer (4xSSC, containing 5 % non-fat dry milk)
- TNT buffer (100 mM Tris-HCL pH 7.5, 150 mM NaCL, 0.05%(v/v) Tween-20)

- TNB (100 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5 % Roche blocking reagent)
- Avidin~Texas Red (Vector Laboratories)
- Goat-anti-avidin~biotin (Vector Laboratories)
- Sheep-anti-dig~FITC
- Rabbit-anti-sheep~FITC
- 2-5 µg/ml DAPI in Vectashield (Vector Laboratories)

Procedure

1. Rinse the slides briefly in 4T.
2. Incubate in 4M at 37°C for 30 min to prevent non-specific binding of antibodies to the glass surface.
3. Rinse briefly with 4T.
4. Add 100 µl Avidin~Texas Red (2.5 µg/ml) in 4M , cover with 25x50 mm cover slip and incubate in a moist chamber at 37°C for 30- 60 min.
5. Rinse in 4T for 2x5 min.
6. Rinse in TNT for 5 min.
7. Add 100 µl of goat-anti-avidin~biotin (10 µg/ml) and sheep-anti-digoxigenin (10 µg/ml) in TNB, cover with 25x50 mm cover slip and incubate in a moist chamber at 37°C for 30-60 min.
8. Rinse in TNT for 3x5 min.
9. Add 100 µl of 2.5 µg/ml Avidin~Texas Red and 10 µg/ml rabbit-anti-sheep~FITC in TNB, cover with 25x50 mm cover slip and incubate in a moist chamber at 37°C for 30-60 min.
10. Rinse in TNT for 3x5 min.
11. Dehydrate the slides in an ethanol series of 70 %, 90 % and 100 %, 1 min each and air dry.
12. Mount with DAPI (4',6-diamidino-2-phenylindole) in Vectashield anti-fade solution (Vector Laboratories) (2-5 µg/ml).

6. FLUORESCENCE MICROSCOPY

In the FISH experiments we will make use of green (FITC) and red (TRITC or Texas Red) fluorochromes in different combination with avidin and antibodies like anti-digoxigenin.

Fluorescence microscopy combines a number of special features not shared by other types of microscopes. It enables observation of very small objects undetectable by any other type of conventional light microscope and provides the possibility to visualise specified substances or objects selectively. The principle behind fluorescence microscopy is that a photon of a particular wavelength (excitation wavelength) excites an electron in the fluorochromes, making it jump into an outer electron shell. The excited electron is unstable and on returning to its ground (stable) state loses energy, which is emitted as light (fluorescence). According to Stoke's Law the wavelength of emitted light is always longer than the excitation wavelength. In our experiments we will excite DAPI with ultraviolet light resulting in blue fluorescence, FITC - with blue light giving green fluorescence and TRITC - with green light resulting in red fluorescence.

The light source for fluorescence microscopy is usually an ultrahigh pressure mercury vapour lamp, which emits ultraviolet, visible and infrared light. The microscope has excitation filters to select the correct wavelengths of light for the particular fluorochromes. The selected wavelengths are focused on the specimen using the objective lens. Most fluorescing compounds and fluorochromes gradually decrease in intensity upon exposure to light. This, so-called, "fading effect" can be diminished by anti-fading agents like Vectashield®.

Chromosome preparations will be analysed under a Zeiss Axioskop fluorescence microscope equipped with CCD camera. The captured digital images will be cropped and optimised for contrast and brightness using Adobe Photoshop (Adobe Inc.). Cytological distances between FISH signals will be measured with "MicroMeasure", a freeware software programme from Colorado State University (<http://www.colostate.edu/Depts/Biology/MicroMeasure>).

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