CHAPTER 1

Chromosomal localisation by FISH of ribosomal RNA genes in plant mitotic chromosomes

1. Introduction to the topic

1.1. Why are we using *Vicia faba, Crepis capillaris* and *Arabidopsis thaliana?*

Three plant species have been chosen for the present investigation, which are quite different with respect to chromosome number and size. A peculiarity of *Crepis capillaris* is that it contains a variable number of B chromosomes per cell. B chromosomes are accessory chromosomes, which are dispensible and which are known to harbour few if any genes. It will be interesting to see if the ribosomal genes map to the Bs. *Arabidopsis thaliana* is an important genetic model plant with a completely sequenced genome. The genome size is one of the smallest among flowering plants. The 1C nuclear DNA amount is approximately 120 Mbp.

1.2. What does the acronym FISH mean?

FISH stands for Fluorescent *In Situ* nucleic acid Hybridisation. This technique is based on the interaction between a single stranded (denatured)

labeled probe (DNA or RNA) and the single stranded cellular target (DNA or RNA). The method can be used to localise a sequence of interest within the cell and in its components such as cytoplasm, nucleus or chromosomes (see Figs 1.1, 1.2).

2. Scientific problem

The scientific problem in this part of the course is to physically map the numbers and chromosomal positions of both 45S and 5S rRNA gene clusters in plant species, namely *Vicia faba* (2n = 12), *Crepis capillaris* (2n = 6 + Bs) and *Arabidopsis thaliana* (2n = 10).

3. The research skills you will acquire

In conducting this experiment you will acquire research skills in the following:

- Preparing slides for FISH from plant root tip meristems
- Labelling of the DNA probe
- DNA—DNA hybridisation
- Detection of the probe following *in situ* hybridisation
- Fluorescence microscopy and photography
- Image processing, analysis, interpretation and presentation

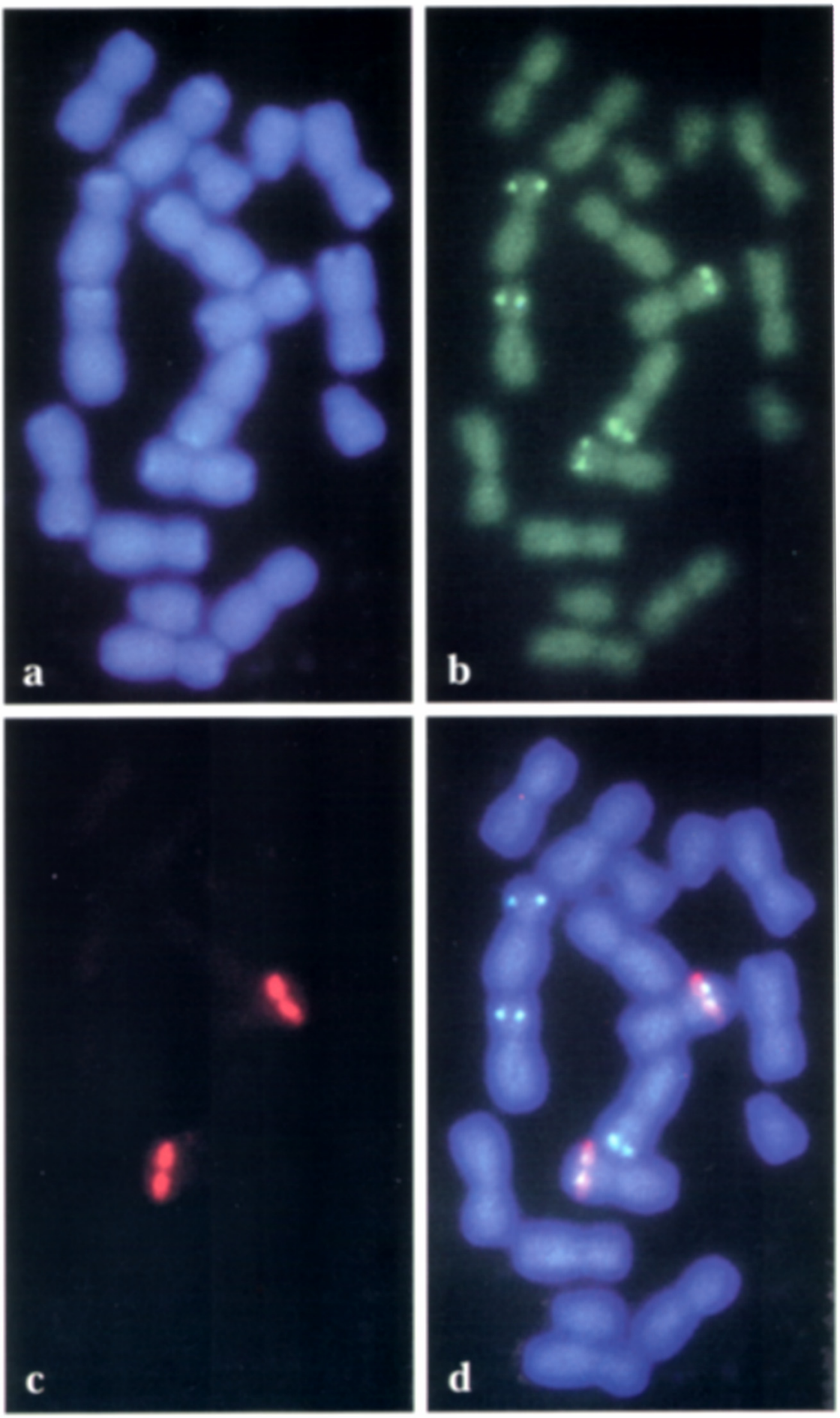

Fig. 1.1. *Secale cereale* (2n = 2x = 14 + 4B; genome RR); double target *in situ* hybridisation with rDNA. (a) blue fluorescence — counterstaining with DAPI, (b) green — 5 loci of 5S rDNA, (c) red — 2 loci of 25S rDNA, (d) — overlay of a-c images

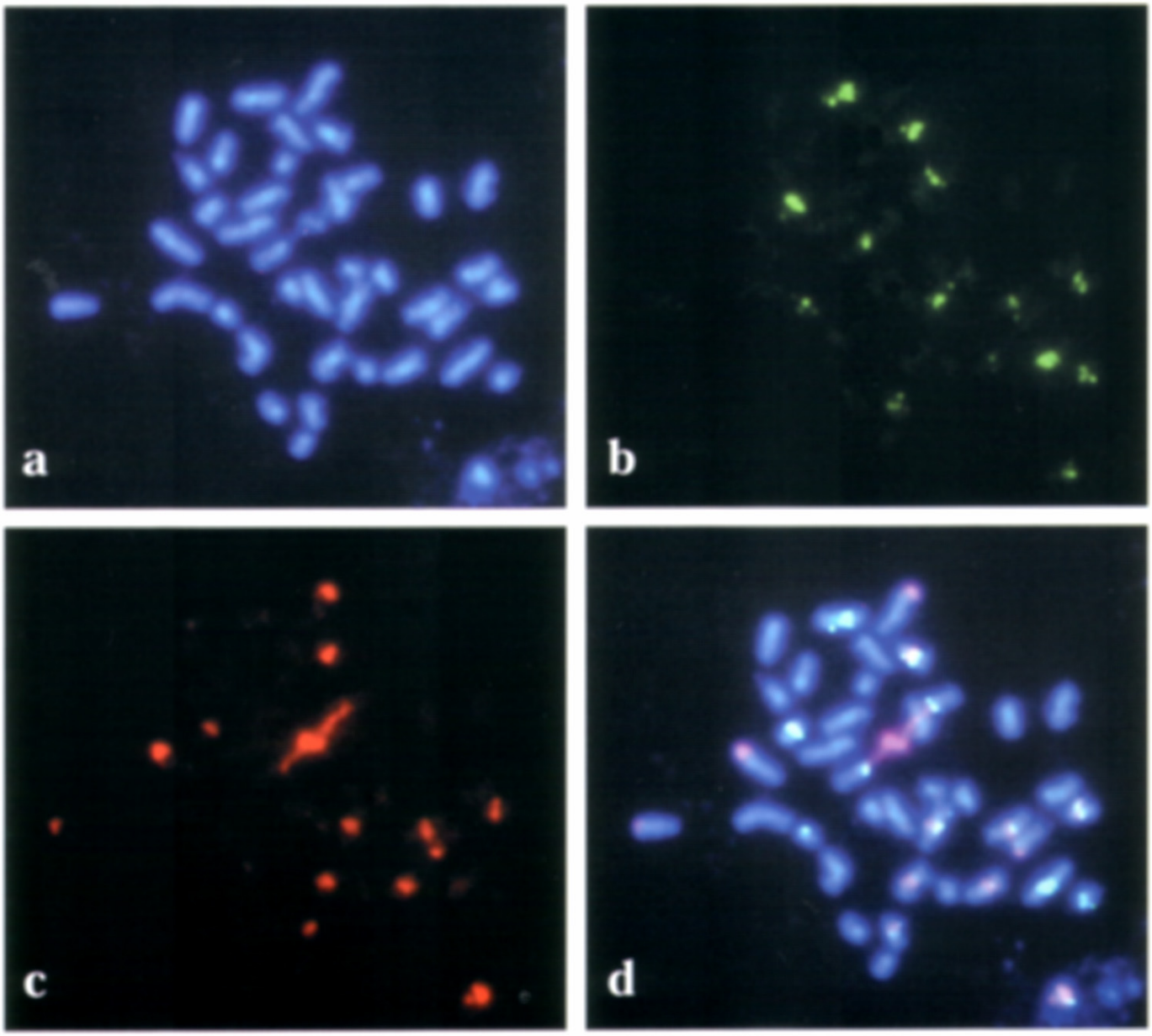

Fig. 1.2. *Brassica napus* (2n = 4x = 38; genomes AACC); double target *in situ* hybridisation with rDNA. (a) blue fluorescence — counterstaining with DAPI, (b) green — 12 loci of 5S rDNA, (c) red — 14 loci of 25S rDNA, (d) — overlay of a-c images

4. Scientific goals and questions you should be able to answer

4.1. Chromosomal location of rRNA genes

- On which chromosome type(s) in the respective species are the FISH signal(s)?
- Can you draw a karyotype for these species on which you have mapped the 45S ribosomal genes?
- Where do the 5S rRNA genes map?
- Are the 5S rRNA genes on the same chromosome as the 45S rDNA?
- If the 5S loci are in the same linkage group as 45S rDNA, are the 5S rRNA genes proximal or distal to the NORs?

4.2. B-Chromosomes of *C. capillaris*

- Can you detect FISH signals (45S and/or 5S) on the B-chromosomes?
- If ribosomal genes also map to B-chromosomes, what does this mean with respect to the origin and evolution of B-chromosomes?

5. Report

You will be expected to conduct the experiment alone or in a small team. You will present your results in the form of an illustrated protocol. In addition you might be selected to present your results in the form of a poster or short oral presentation.

6. Methods

6.1. Chromosome and slide preparation

(1) Germinate seeds at 20°C in the dark on filter paper moistened with tap water in Petri dishes.

Vicia faba: the seeds are soaked first up to two days in running tap water. Before germination on filter paper, the seed coat can be removed with the use of fine needles. Use primary roots about 1—3 cm long for further colchicine treatment.

Crepis capillaris and *Arabidopsis thaliana*: seeds can be sown directly onto moist filter paper. Check germination daily under a stereo microscope. Alternatively, meristems from *in vitro* culture material can be used.

(2) Optional step: For synchronisation of mitosis in apical root meristems of *Vicia faba* the following treatment is recommended. Select seedlings with a root of 1—3 cm and briefly immerse in 1.25 mM aqueous hydroxyurea (Appendix-1). Transfer to Petri dishes containing filter paper moistened with the same solution and incubate for about 24 h at room temperature (RT) in the dark. Rinse 3× with tap water, transfer to Petri dishes containing filter paper moistened with tap water, and allow to recover for ~24 h at RT in dark.

(3) Treatment for metaphase accumulation:

Vicia faba — 1—2 cm root tips are cut off by means of the razor blade and immediately immersed in 0.05% aqueous colchicine (Appendix-2) for 3—4 h at RT in dark.

Crepis capillaris — small seedlings immersed in 0.02 M 8-hydroxyquinoline (Appendix-3), for 2 h at RT and 2 h at 4°C.

Arabidopsis thaliana — small seedlings immersed 0.02 M 8-hydroxyquinoline, for 0.5 h at RT and 0.5 h at 4°C.

(4) Optional step: Rinse seedlings 3× in tap water and place in ice-cold water for 24 h in dark.

(5) Fix immediately in fresh 3:1 ethanol: acetic acid and store at −20°C.

(6) Wash material for 2× 10 min in citrate buffer (Appendix-4) to remove fixative.

(7) Replace citrate buffer with 1—2× vol enzyme solution (1—2 ml; Appendix-5) and incubate for 1—1,5 h for *Vicia faba*, 50—60 min for *Crepis capillaris* and 30 min for *Arabidopsis thaliana* at 37°C.

(8) Wash material 1× 15 min in enzyme buffer. Place root in the centre of a clean (Appendix-6) slide.

(9) Add a drop of 45% acetic acid, remove the cap and extrude meristem under a dissecting microscope. Apply a 22 × 22 mm coverslip, tap gently with a matchstick and squash between folded filter paper.

(10) Scan under ×10 phase contrast for well-spread metaphases with highly condensed chromosomes.

(11) Freeze preparation in −70°C, flick off coverslip with blade, and air dry immediately at RT.

(12) Slides may be stored dessicated over silica gel in fridge or freezer for several weeks.

6.2. Slide pre-treatment

(1) Place slides in oven at 30—60°C overnight to dry, if just made.

(2) Add 200 µl RNase A solution (Appendix-7) to each slide, cover with plastic coverslip (made from autoclavable plastic bags) and incubate for 1 h at 37°C in a humid chamber.

(3) Remove coverslips, load into rack and wash slides 3× 5 min in 2× SSC at RT.

(4) Place slides in 4% (w/v) aqueous freshly depolymerised paraformaldehyde (Appendix-8) for 10 min at RT.

(5) Wash 3× 5 min in 2× SSC at RT.

(6) Dehydrate 3 min each in 70%, 90% and 100% ethanol, and air dry at RT.

(7) Store slides over silica gel at 4°C.

6.3. Probe DNA labelling

Probe DNA is labelled with digoxygenin-11-dUTP (0.4 mmol/l; Roche, 1093 088) by nick translation according to manufacturer's instructions (Roche, Kit 976776).

(1) A stock dNTP mixture is prepared to avoid repeated thawing and refreezing of kit contents:

3 vol dATP (solution 2)
3 vol dCTP (solution 3)
3 vol dGTP (solution 4)
2 vol dTTP (solution 5)
1 vol digoxygenin-11-dUTP (dig)

Concentration of dNTPs in mixture is 0.1 mmol/l. The stock is stored at −20°C in the dark prior to removing 10 µl aliquots.

(2) Assemble the following reaction mix in a 0.5 ml microfuge tube on ice:

0.1−2.0 µg DNA in SDW
10 µl dNTP mixture
2 µl 10× buffer (solution 6)
make up to 18 µl with SDW
2 µl enzyme mix (solution 7)

N. B. If amount of DNA to be labelled is at the lower end of the range, reduce all volumes by half.

(3) Incubate for 90 min at 15°C.

(4) Add 2 µl 0.2 M EDTA (pH 8.0, Appendix-9) to stop reaction.

(5) Non-incorporated nucleotides are removed from solution by ethanol precipitation. Add 2.5 µl 4 M LiCl and 2.5× vol 100% ethanol from freezer. Mix and leave for at least 30 min at −70°C or 2 h at −20°C. Spin at 13 krpm for 15 min, pipette off supernatant using a drawn-out Pasteur pipette, and dry pellet at RT under vacuum.

(6) Resuspend pellet in an appropriate volume of SDW and store at −20°C.

6.4. Denaturation, hybridisation and washes

(1) Prepare the hybridisation mix as follows and mix thoroughly:

solution	µl/slide	final conc.
100% formamide (Appendix-10)	20	50%
50% w/v dextran sulphate (Appendix-11)	8	10%
20× SSC (Appendix-12)	4	2×
10% w/v aq. SDS (Appendix-13)	4	1%
probe	X	25—100 ng/slide
blocking DNA (sheared salmon sperm DNA, Appendix-14)	Y	25—100× probe
SDW	Z	–
total volume	**40**	–

Use any combination of X, Y and Z to make final volume 40 µl.

(2) Denature hybridisation mix at 70°C for 10 min and plunge into ice for 5 min.

(3) Load slides into the Hybaid hybridisation system and add 38 µl denatured hybridisation mix to each slide and cover with plastic coverslip. Set the thermal cycler to reach and hold a temperature at 80°C for 10 min.

(4) Set the machine to cool slides to 37°C and hold overnight.

(5) Float coverslips off in 2× SSC at 42°C in Coplin jar.

(6) Give slides a stringent wash for 2× 5 min in 20% v/v formamide in 0.1× SSC at 42°C.

(7) Wash slides 3× 3 min in 2× SSC at 42°C, and 3× 3 min in 2× SSC at RT.

6.5. Detection of probe

(1) Wash slides briefly in Tween/4× SSC at RT (Appendix-15).

(2) Drain slides and add 200 µl of BSA block (Appendix-16) to each slide. Apply coverslips and incubate for 5 min at RT.

(3) Remove coverslips, drain slides and add 30 µl of FITC-conjugated anti-dig (Appendix-17) to each slide. Replace coverslips and incubate for 1 h at 37°C.

(4) Wash slides 3× 8 min in wash module in Tween/4× SSC at 37°C.

(5) Drain slides and add 200 µl of rabbit serum block (Appendix-18) to each slide. Apply coverslips and incubate for 5 min in dark at RT.

(6) Remove coverslips, drain slides and add 30 µl of FITC-conjugated anti-sheep antibodies (Appendix-19) to each slide. Replace coverslips and incubate slides for 1 h at 37°C.

(7) Wash slides 3× 8 min in wash module in Tween/4× SSC at 37°C.

6.6. Fluorescent counterstaining and microscopic analysis

(1) Drain and add 100 µl of DAPI (Appendix-20—21) to each slide, apply coverslip, cover and stain for 10 min at RT.

(2) Optional step: Remove coverslip, drain, add 100 µl of propidium iodide (Appendix-22), replace coverslip, cover and stain for 10 min at RT.

(3) Remove coverslip, wash briefly in distilled water, drain and apply one drop of Citifluor or Vectashield (Vector Laboratories).

(4) Place a thin glass coverslip over specimen on slide. Press between filter paper to squeeze out excess antifade.

(5) Store slides for at least 24 h in dark at 4°C to dry antifade and stabilise signal. Slides remain stable for 6 months or more.

(6) Locate the top left corner of the preparation using a ×10 objective and FITC/PI filter (Appendix-23). Systematically scan under ×40 or ×100 oil immersion to locate useful squashes. Photograph onto either Kodak Gold Ultra 400 colour film or Fujicolor Super G 400 colour film, with −2 exposure adjustment and record.

(7) Change to DAPI filter (Appendix-23) and photograph again. Avoid extended examination, particularly at UV wavelength, which will fade probe signal.

6.7. Some questions regarding the method

(1) What are the features of a good preparation for FISH?

(2) What is the reason for applying RNase treatment prior to hybridisation?

(3) Why do the probes for double FISH have to be labelled differently?

(4) What does stringency mean?

(5) How can the strength of a signal be increased?

6.8. Image processing

(1) Image capturing

Digital processing of *in situ* hybridisation signals is the most effective way of data visualisation and presentation. Images can be captured either directly from the fluorescence microscope using a CCD camera or by scanning with a confocal laser scanning microscope, or indirectly from photographs or films by using flatbed or film scanners.

(2) Data saving and storage

Once the image is captured the graphic data need to be saved as a file. There are different formats of graphic files. The most common one is **TIFF** (Tagged-Image File Format), which offers the best quality but usually has no data compression option. It means that one full colour scan of a postcard size photograph at photo quality takes about 6 megabytes (Mb) of storage. Another possibility is **JPEG** (Joint Photographic Experts Group) format, which allows very efficient data compression (1: 10 or more) but with slight and irreversible loss of the original image quality. For serious presentation and publishing purposes **TIFF** format should be used as a standard.

Extensive work with graphics may create enormous amounts of data which soon fill even the largest currently available hard discs. However, there are other ways of data backup and storage. In general, internal network (Intranet) can be used to transfer parts of the large quantities of data from one computer to another. If there is no possibility to use the network then different kinds of high capacity removable discs like ZIP or magneto-optic drives can be

useful. Currently, the best option for permanent storage of huge amounts of data are recordable or rewritable CDs or DVDs, which are easy and convenient to use and offer from 650 Mb (CD) to several gigabytes (Gb, DVD) of disc space.

(3) Digital image processing

Graphic data grabbed by the CCD-device or scanned by scanner frequently need to be manipulated by image processing software. Graphics software dedicated to particular image capturing systems and some general graphic applications (i.e. Adobe Photoshop, Micrographx Picture Publisher) offer many useful options like for example: contrast and brightness balancing, gamma correction, cropping and rotating of the images, colour channel separation, superimposition of different images of the same specimen obtained with different filters, quantitative measurements of different parts of the image and so on.

(4) Printing of the images

The printing of the images is the final stage of computer-based graphics processing. The quality of prints obtained largely depends upon the kind of printer and/or media type used. The best print quality is afforded by dye-sublimation printers, which are specially designed for digital photography. However, the cost of printing, especially in full-page format (A4) is still very high. Modern colour laser printers are a good choice, offering good print quality with reasonable costs of consumables, but the printers themselves are still expensive. Recent generations of cheap colour ink-jet printers from leading manufacturers (Epson, Hewlett-Packard, Lexmark, Canon) offer very high quality full-page (A4, A3) colour prints. When special photo paper is used, prints of near photographic quality can be obtained.

7. Appendix

(1) hydroxyurea (Sigma H-8627)

9.51 mg/distilled water (1.25 mM)

(2) colchicine — 0.05%

25 mg/50 ml distilled water (1.25 mM)

(3) 8-hydroxyquinoline — 0.02 M

0.28 g of 8-hydroxyquinoline dissolve in 1000 ml distilled water at 60°C

(4) 10× enzyme buffer — 10 mM citrate buffer (pH 6.0)

0.84 g citric acid monohydrate/40 ml distilled water (0.1 M)
1.76 g trisodium citrate dihydrate/60 ml distilled water (0.1 M)

Mix 2 solutions to make 10× stock. Dilute 10× with distilled water before use.

(5) enzyme solution

3% v/v pectinase (Sigma P4802)
2% w/v cellulase (Calbiochem 21947)
0.2% w/v cellulase (Onozuka RS)

Dissolve enzymes in 10 mM citrate buffer, divide into 1 ml aliquots and store at −20°C.

(6) microscope slides

Place new microscope slides into chromium trioxide solution in 80% w/v sulphuric acid (Merck 33205 5K) for at least 3 h at RT. Wash slides in running water for 5 min, rinse thoroughly in distilled water and air dry. Place into 100% ethanol, remove and dry with tissue immediately prior to use.

(7) 100 µg/ml w/v RNase solution

10 mg RNAse A (Sigma-R-5503)
1 ml 10 mM Tris. HCl + 15 mM NaCl

Boil for 15 min and allow to cool. Store in 20 µl aliquots in 2 ml Eppendorfs at −20°C. When required, add 1980 µl 2× SSC (1:100 dilution).

(8) 4% paraformaldehyde

8 g paraformaldehyde
200 ml distilled water

Add several drops 1 M NaOH to mixture and heat gently over a Bunsen flame to 70°C in fume hood. Stir vigorously until solution is clear. Filter, cool and use immediately.

(9) 0.2 M EDTA

7.44 g EDTA
10 M NaOH

Dissolve EDTA in 50 ml distilled water and adjust to pH 8.0 with 10 M NaOH. Make up to 100 ml with distilled water and autoclave.

(10) 100% formamide

100 ml formamide (Life Technologies)
5 g "Amberlite" IRN-150L monobed mixed resin (Merck 55179 4K)

Store formamide at −20°C. Just before use, deionise by adding resin, stirring for 1 h and filtering.

(11) 50% dextran sulphate

50 g dextran sulphate
100 ml SDW

Dissolve dextran sulphate in SDW. Filter sterilise (0.22 μm) and store at −20°C.

(12) 20× SSC

175.3 g NaCl (3 M)
88.3 g trisodium citrate dihydrate (0.3 M)

Dissolve in 800 ml distilled water and adjust pH to 7.0 with 6 M HCl. Bring to 1 l with distilled water, aliquot and sterilise. Dilute as appropriate before use (0.1× SSC = 5 ml 20× SSC/995 ml water).

(13) 10% SDS

10 g SDS
100 ml SDW

Dissolve SDS in SDW and filter sterilise.

(14) sonicated salmon sperm DNA

5 mg salmon sperm DNA
1 ml SDW

Dissolve DNA in SDW and sonicate to reduce viscosity. Store at −20°C in 100 μl aliquots.

(15) Tween/4× SSC

2 ml Tween 20 (0.2%) (Merck — 66368 4B)
200 ml 20× SSC (4× SSC)
798 ml distilled water

(16) BSA block

1 g BSA (5%) (Sigma Fraction V, globulin free, A7638)
20 ml Tween/4× SSC

Dissolve BSA in Tween/4× SSC, aliquot into 2 ml and store at −20°C.

(17) FITC-conjugated anti-dig

200 µg anti-dig-fluorescein fab fragments (Roche — 1207741) 1 ml SDW
270 µl BSA block

Dissolve lyophilisate in SDW. Aliquot to 30 µl in 0.5 ml Eppendorfs and store in dark at −20°C. Add BSA block (1: 10 dilution to 20 µg/ml) before use.

(18) rabbit serum block

1 ml normal rabbit serum (5%) (Dako — X902)
19 ml Tween/4× SSC

Mix serum and Tween/4× SSC, aliquot to 2 ml and store at −20°C.

(19) FITC-conjugated anti-sheep

3 µl FITC-conjugated anti-sheep antibody (Dako — F135)
297 µl rabbit serum block

Mix before use. This represents a dilution of 1: 100 to 4 µg/ml.

(20) DAPI

1 mg DAPI (4',6-diamidino-2-phenylindole; Sigma — D9542)
10 ml distilled water

Dissolve DAPI in water and store stock solution (100 µg/ml) at −20°C. Dilute to 2 µg/ml with McIlvaine's buffer, aliquot to 1 ml and store at −20°C.

(21) McIlvaine's buffer (pH 7.0)

solution A: 0.1 M citric acid (2.94 g/100 ml)
solution B: 0.2 M Na_2HPO_4 (3.56 g/100 ml)

Mix 18 ml solution A with 82 ml solution B to make 100 ml buffer.

(22) propidium iodide

10 mg propidium iodide (Sigma — P4170)
100 ml distilled water

Dissolve propidium iodide in water and store stock solution (100 µg/ml) at −20°C. Dilute to 1 µg/ml with 2× SSC, aliquot to 1 ml and store at −20°C.

(23) fluorochrome properties

fluorochrome	excitation	emission	fluorescence	filter	
	wavelength (nm)	wavelength (nm)	colour	Leica	Olympus
DAPI	355	450	blue	A	B-FIL 02
FITC	495	515	green	I3	B-LO 910
rhodamine	545	575	red	N2.1	B-FIL 14
PI	340/530	615	orange/red	I3	B-LO 910

References

Theoretical

MALUSZYNSKA J. and SCHWEIZER D., 1989. Ribosomal RNA genes in B chromosomes of *Crepis capillaris* detected by non-radioactive *in situ* hybridization. Heredity 62: 59—65.

MALUSZYNSKA J. and HESLOP-HARRISON J. S., 1991. Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant Journal 1: 159—166.

FUKUI K. and NAKAYAMA S. (eds), 1996. Plant Chromosome Laboratory Methods. BocaRaton: CRC Press.

SCHWARZACHER T. and HESLOP-HARRISON P., 2000. Practical *in situ* Hybridization. BIOS, Oxford.

WEISS H., PASIERBEK P., MALUSZYNSKA J., 2000. An improved nonfluorescent system for *in situ* hybridization in plants. Biotechnic & Histochemistry 75: 49—53.

Contents

Skrypty Uniwersytetu Śląskiego nr 573

Editor of the Series: Biology
Iwona Szarejko

Reviewer
Maria Olszewska

Editors
Glyn Jenkins
Jolanta Maluszynska
Dieter Schweizer

Contributors
Robert Hasterok
Glyn Jenkins
Jolanta Maluszynska
Wolfgang Miller
Pawel Pasierbek
Dieter Schweizer

Developed under the project
TEMPUS JEP-12037-97

ADVANCED MOLECULAR CYTOGENETICS

A PRACTICAL COURSE MANUAL

Wydawnictwo
Uniwersytetu Śląskiego

Katowice 2001