CHAPTER 3

Painting whole chromosome sets in hybrids using GISH

1. Introduction to the topic

The size of a genome is usually typical of a species and is generally speaking proportional to the complexity of an organism, simply because more complex organisms require greater coding potential for a higher diversity of gene products. However, this is not the whole story, since some phyla of the multicellular eukaryotes have vast ranges of nuclear DNA amounts which cannot be attributed to differences in complexity. This is particularly true of the flowering plants in which, for example, *Fritillaria assyriaca* has 1,200 times more DNA than *Arabidopsis thaliana* (1C DNA = 0.2 pg). Within this group, even very closely related species can have vastly different DNA amounts. For example, *Vicia faba* (bean) has seven times more DNA than *Vicia sativa* (common vetch). Evidently, a large proportion of the genome of higher eukaryotes has no apparent function, and comprises repetitive DNA sequences localised in heterochromatic blocks or dispersed throughout the genome. This repetitive component may be responsible for the structural divergence of genomes, and may be an important component of speciation. Indeed, genome analysis in plant genera such as *Secale* (VERSHININ et al., 1995) and *Lolium* (JENKINS et al., 2000) has recovered repeat families which have evolved independently and are characteristic for a particular species. It is this difference in repetitive DNA in the genomes of related species that is exploited in this experiment.

Genomic *In Situ* Hybridisation (GISH) differs from FISH in that whole genomic DNA, rather than particular clones or PCR products, is used to

detect complementary sequences on chromosomes *in situ*. If discrimination is good, GISH allows the detection of particular chromosome sets in hybrids and polyploids, particular chromosomes in substitution and addition lines, or alien segments in introgression lines (Fig. 3.1). It has, therefore, great utility in cell biology and phylogenetic studies, and can be an important component of introgression mapping.

2. Scientific problem

The scientific problems of this experiment are to:

- highlight the rye genome in a hybrid with wheat in somatic tissue at various stages of the mitotic cell cycle. Triticale was the first successful intergeneric hybrid between wheat (AABB) and rye (RR) and has the genomic formula $2n = 6\times = 42$ (AABBRR).
- detect supernumerary B chromosomes of rye in hexaploid wheat ($2n = 6\times = 42 +$ Bs).

3. The research skills you will acquire

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

- Labelling genomic DNA by nick translation
- Labelling B-specific DNA sequences by PCR-labelling
- Cytological preparation of chromosomes for GISH/FISH
- DNA-DNA hybridisation
- Detection of the probe
- Fluorescence microscopy and photography
- Image processing, analysis, interpretation and presentation.

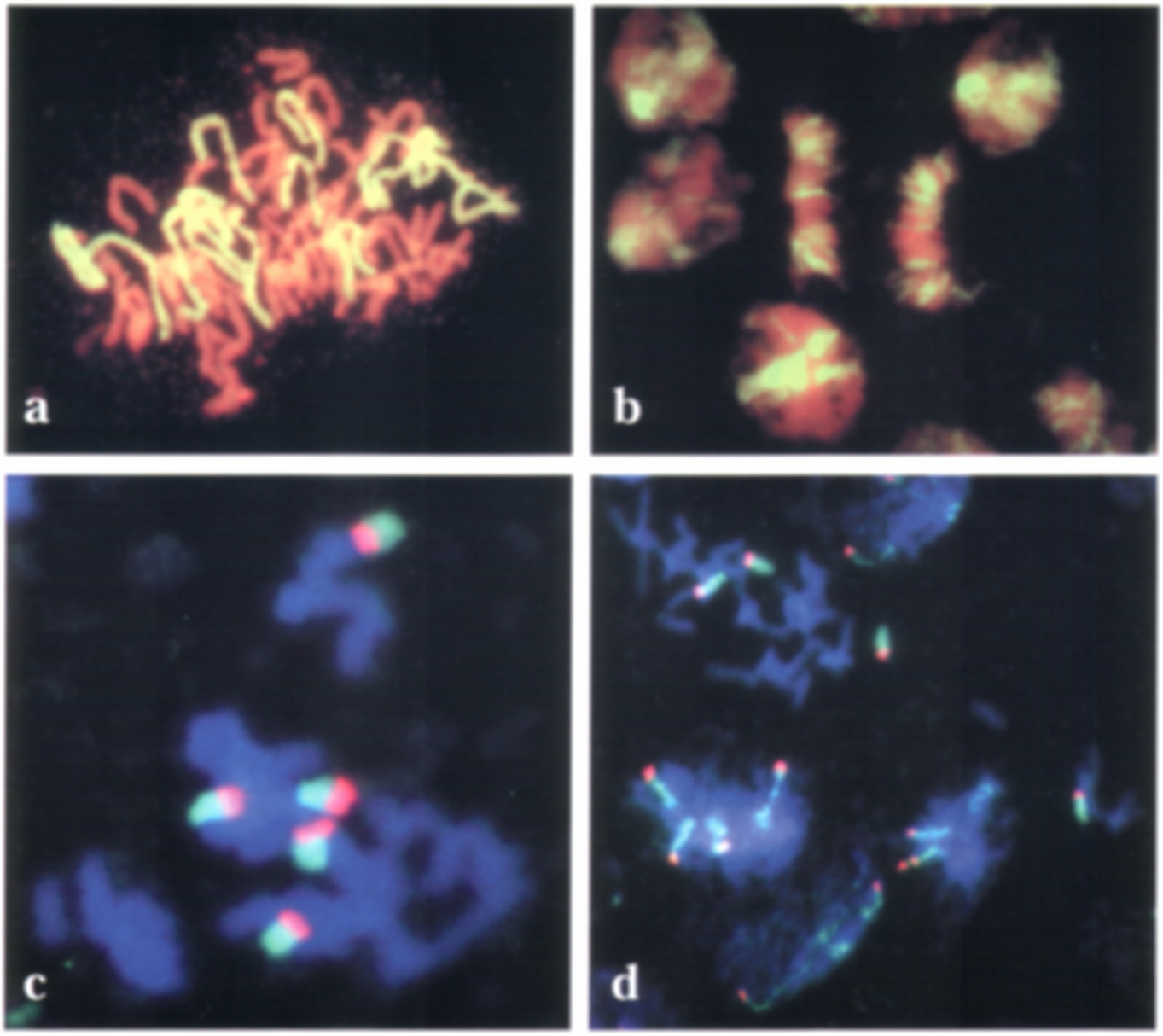

Fig. 3.1. (a, b): *Triticum durum* x *Secale cereale* hybrid (Triticale; 2n = 6x = 42; AABBRR). Green/yellow fluorescence — genomic *in situ* hybridisation to rye chromatin, orange/red — counter staining with propidium iodide. (c, d): *Triticum aestivum* Lindström (2n = 6x = 42; AABBDD + 0—6 B chromosomes from *Secale cereale*). Green fluorescence — genomic *in situ* hybridisation to rye chromatin, red — fluorescence *in situ* hybridisation with rye B-specific DNA sequence as a probe, blue — counter staining with DAPI

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

- What is the chromosomal constitution of Triticale?
- What is the main difference between GISH and FISH?
- Why do we use wheat genomic DNA as a blocking agent in GISH with Triticale?
- Are there any DNA sequences specific for rye B chromosomes? If so — where do they map on Bs?
- What is the advantage of simultaneous *in situ* hybridisation of rye-specific clonal and genomic DNA probes fto hexaploid wheat carrying rye B chromosomes?

5. Report

You will be expected to present your results in the form of an illustrated written report or as a research poster.

6. Methods

6.1. Chromosome and slide preparation

The following procedure is designed to produce large numbers of highly condensed C-metaphases which are suitable as substrates for GISH.

(1) Germinate seeds at 22±1°C in a dark incubator on filter paper moistened with tap water in 14 cm Petri dishes.

(2) Select seedlings with a root of 1—2 cm and immerse immediately in 0.05% aqueous colchicine (Appendix-1) for 5 h at room temperature in the dark.

(3) Rinse seedlings in tap water and detach roots. Fix immediately in a fresh mixture of ethanol: acetic acid (Appendix-2) and store at −20°C until required.

(4) Wash material for 3× 5 min in citrate buffer (Appendix-3) to remove fixative.

(5) Replace citrate buffer with 1—2× vol enzyme solution (1 ml; Appendix-4) and incubate for about 1.5—2 h at 37°C.

(6) Replace carefully the enzyme with citrate buffer, place the material on ice and wash for 15—30 min.

(7) Remove the root-tips from the rest of the root.

(8) Transfer carefully one root-tip and to a dish of 45% acetic acid. If possible remove the cap and extrude the meristem under a dissecting microscope. When the material becomes transparent transfer it in 3—4 µl of 45% acetic acid with a micropipette to the centre of a clean slide (Appendix-5).

(9) Apply carefully a 22 × 22 mm coverslip and squash the preparation between folded filter paper.

(10) Freeze the preparation in a deep freeze, flick off the coverslip with a blade and flood with pre-chilled ethanol: acetic acid fixative.

(11) Immerse the slide in absolute ethanol for 30 min and air-dry.

(12) Scan under ×20 and ×40 phase contrast to identify well-spread metaphases.

(13) Slides may be stored at room temperature for several days or in the freezer for several weeks.

6.2. Slide pre-treatment

The slides are pre-treated with RNAse in order to remove RNA from the preparations which could otherwise bind to the DNA probe and cause excessive background noise.

(1) Add 200 µl RNase A solution (Appendix-6) to each slide, cover with plastic coverslip (made from autoclavable plastic bags) and incubate for 1 h at 37°C.

(2) Remove coverslips and wash slides for 3× 5 min in 2× SSC at RT.

(3) Place slides in freshly prepared 1% (v/v) formaldehyde in 1× PBS (Appendices-7, 8) for 10 min at RT.

(4) Wash for 3× 5 min in 2× SSC at RT.

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

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

6.3. Labelling of DNA

This section describes the labelling of genomic DNA for use as a GISH probe. Probe DNA is labelled with digoxigenin-11-dUTP (1 mM; Roche, 1093 088) or rhodamine-4-dUTP (1 mM Fluoro Red; Amersham, RNP 2122; avoid intensive light) by nick translation according to manufacturer's instructions (Roche, kit 976 776).

(1) Prepare on ice the standard labelling mix containing:

reagent	volume (µl)
dATP (0.4 mM; vial 2)	2.50
dCTP (0.4 mM; vial 3)	2.50
dGTP (0.4 mM; vial 4)	2.50
dTTP (0.4 mM; vial 5)	1.67
10x buffer (vial 6)	2.00
DNA for labelling (0.1—2.0 µg in SDW)	max. 6.00
digoxigenin-11-dUTP (1 mM) or rhodamine-4-dUTP (1 mM)	0.83
enzyme mix	2.00
if necessary, make up final volume to 20 µl with SDW	**20.00**

(2) Incubate for 90 min at 15°C.

(3) Stop reaction by heating at 65°C for 10 min.

(4) 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 37°C for 20 min.

(5) Resuspend pellet in an appropriate volume of sterile distilled water (SDW) or 1× TE buffer and store at −20°C.

This section describes the labelling of D1100 rye B-specific DNA sequence for use as a FISH probe. Probe DNA is labelled with digoxigenin-11-dUTP (1 mM; Roche, 1093 088) or rhodamine-4-dUTP (1 mM Fluoro Red; Amersham, RNP 2122; avoid intensive light) by PCR-labelling according to the following instructions:

(1) Prepare on ice the standard labelling mix containing:

reagent	volume (µl)
SDW	27.50
10× reaction buffer with $MgCl_2$	5.00
dATP (2.5 µM)	2.00
dCTP (2.5 µM)	2.00
dGTP (2.5 µM)	2.00
dTTP (2.5 µM)	3.25
DNA for labelling (1: 100 diluted plasmid prep)	2.00
M13 universal forward sequencing primer (5 pmol/ml)	2.00
M13 universal reverse sequencing primer (5 pmol/ml)	2.00
digoxigenin-11-dUTP (1 mM) or rhodamine-4-dUTP (1 mM)	1.75
Taq polymerase (5 u/µl, Promega buffer B)	0.50
total volume	**50.00**

(2) Run PCR programme as follows:
94× 1 min, 35 cycles of 94× 40 sec, 55× 40 sec, 72× 1 min, 1 cycle of 72× 5 min.

(3) Non-incorporated nucleotides are removed from solution by ethanol precipitation. Add 6.25 µl 4M LiCl and 125 µl 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 37°C for 20 min.

(4) Resuspend pellet in an appropriate volume of SDW or 1 × TE buffer and store at −20°C.

6.4. Denaturation and hybridisation

During this part of the procedure the probe and substrate DNA is made single stranded and allowed to reanneal.

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

solution	µl/slide	final conc.
100% formamide (Appendix-9)	20	50%
50% w/v dextran sulphate (Appendix-10)	8	10%
20× SSC (Appendix-11)	4	2×
10% w/v aq. SDS (Appendix-12)	4	1%
probe DNA	X	25—100 ng/slide
blocking DNA (sheared genomic DNA of rye or wheat)	Y	25—100× probe
SDW	Z	–
total volume	**40**	–

Use an appropriate combination of X, Y and Z to make up the final vol to 40 µl.

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

(3) Add 38 µl denatured hybridisation mix to each slide and cover with plastic coverslip. Load slides into Hybaid humidity chamber and set the machine to 70—80°C for 5—10 min.

(4) Cool slides to 37°C and hold overnight.

6.5. Stringent washing

These steps remove the probe bound non-homologously to the substrate leaving DNA—DNA hybrids with a sequence identity of 85—90% under these conditions.

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

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

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

6.6. Detection of the probe

This part of the procedure detects the digoxygenated probe with fluorescent antibodies. The probe labelled with Fluoro Red can be visualised directly and requires no further treatment.

(1) Wash slides briefly in Tween/4× SSC at RT (Appendix-13).

(2) Drain slides and add 1 ml of blocking reagent (milk solution or BSA; Appendix-14) to each slide. Apply coverslips and incubate for 15—30 min at RT.

(3) Remove coverslips, drain slides and add 45 µl of FITC-conjugated anti-dig (Appendix-15) to each slide. Replace coverslips and incubate for 1 h at 37°C.

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

(5) Drain slides and add 1 ml of blocking agent (milk solution or RSA; Appendix-14) to each slide. Apply coverslips and incubate for 15—30 min at RT.

(6) Remove coverslips, drain slides and add 30 µl of FITC-conjugated anti-sheep antibodies (Appendix-16) to each slide. Replace coverslips and incubate slides for 1 h at 37°C.

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

6.7. Visualisation and photography

(1) Dehydrate 1 min each in 70%, 90% and 100% ethanol, and air dry at RT in dark for 15 min.

(2) Apply one drop of mounting solution A (**for slides labelled with digoxigenin and detected with FITC conjugated antibodies only**; Appendix-17) or B (**for slides labelled with Fluoro Red**; Appendix-18).

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

(4) 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.

(5) Locate the top left corner of the preparation under the FITC/PI or rhodamine filter (Appendix-19) using a ×10 objective. Systematically scan under ×25, ×40 or ×100 oil immersion to locate useful cells. Photograph onto either Kodak 400 Gold Ultra colour negative film or Fujicolor Super G 400 colour negative film.

(6) Change to the DAPI filter (Appendix-18) and photograph again. Avoid extended examination, particularly at the UV wavelength, which will fade the probe signal.

7. Appendix

(1) 1.25 mM (0.05%) aqueous solution of colchicine

9.51 mg colchicine/100 ml distilled water

The solution can be stored for several days in a refrigerator in the dark.

(2) ethanol-acetic acid fixative

3 vols 100% ethanol or methanol
1 vol glacial acetic acid

Mix well. Prepare just before use.

(3) 10 mM citrate buffer (pH 4.8)

A. 2.1 g citric acid monohydrate ($C_6H_8O_7 \times H_2O$)/100 ml distilled water (0.1 M)
B. 2.94 g trisodium citrate dihydrate ($C_6H_5O_7Na_3 \times 2H_2O$)/100 ml distilled water (0.1 M)

Mix 40 ml A and 60 ml B; dilute 10×.

(4) enzyme solution

1.0% w/v cellulase (Calbiochem 21947)
1.0% w/v cellulase (Onozuka RS)
20% v/v pectinase (Sigma P4802)

Dissolve enzymes in 10 mM citrate buffer, divide into 1.5 ml aliquots and store at −20°C.

(5) microscope slides

Place new microscope slides and coverslips into 70% ethanol. Remove and dry with tissue immediately prior to use.

(6) 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).

(7) 1% acid free formaldehyde in 1× PBS (for 100 ml)

37% acid free formaldehyde	3 ml
10× PBS	10 ml
distilled water	87 ml

The solution is unstable. Prepare directly prior to use.

(8) 10× PBS buffer pH 7.0 (for 0.5 litre)

(0.1 M sodium phosphate pH 7.0, 1.4 M NaCl)

Prepare (a) 0.1 M Na_2HPO_4, 1.4 M NaCl and (b) 0.1 M NaH_2PO_4, 1.4 M NaCl

(a) dissolve in 450 ml distilled water:	$Na_2HPO_4 \times 2H_20$	8.01 g
	NaCl	36.82 g

(b) dissolve in 450 ml distilled water: $NaH_2PO_4 \times 2H_2O$ 1.56 g
NaCl 8.18 g

Adjust solution (a) to pH 7.0 with solution (b) ending up with a final volume of 500 ml. Sterilise by autoclaving for 20 minutes at 15 lb/sq in. on liquid cycle.

1× PBS: 10 mM sodium phosphate pH 7.0, 140 mM NaCl

(9) 100% formamide

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

Deionise by adding resin, stirring for 1 h and filtering. Store at −20°C.

(10) 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.

(11) 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).

(12) 10% SDS (sodium dodecyl sulphate)

10 g SDS
100 ml SDW

Dissolve SDS in SDW and filter sterilise.

(13) Tween/4× SSC

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

(14) blocking agents

20× SSC	20 ml
non-fat dry milk	5 g
SDW	80 ml

Dissolve, stir well and divide into 2.0 ml aliquots. Store at −20°C.

Alternatively you can use bovine serum albumin (BSA) as a blocking agent for primary detection:

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.

And normal rabbit serum (RSA) for secondary detection:
1 ml normal rabbit serum (RSA) (5%) (Dako — X902)
19 ml Tween/4× SSC

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

(15) FITC-conjugated anti-dig (200 µg anti-dig-fluorescein fab fragments; Roche — 1207741)

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

(16) FITC-conjugated anti-sheep

3 µl FITC-conjugated anti-sheep antibody (Dako — F135)
297 µl blocking reagent

Mix before use. Avoid intensive, direct light. This represents a dilution of 1:100 to 4 µg/ml.

(17) mounting solution A

DAPI: stock 100 µg/ml in water
PI: stock 100 µg/ml in water

DAPI stock (1:50)	5.0 µl
PI stock (1:250)	1.0 µl
Vectashield	244.0 µl

Store at +4°C in dark. Avoid contamination.

(18) mounting solution B

DAPI: stock 100 µg/ml in water

DAPI stock (1:50)	5.0 µl
Vectashield	245.0 µl

Store at +4°C in dark. Avoid contamination.

(19) 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

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Contents

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ADVANCED MOLECULAR CYTOGENETICS

A PRACTICAL COURSE MANUAL

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