Drosophila Protocols

Edited by
William Sullivan
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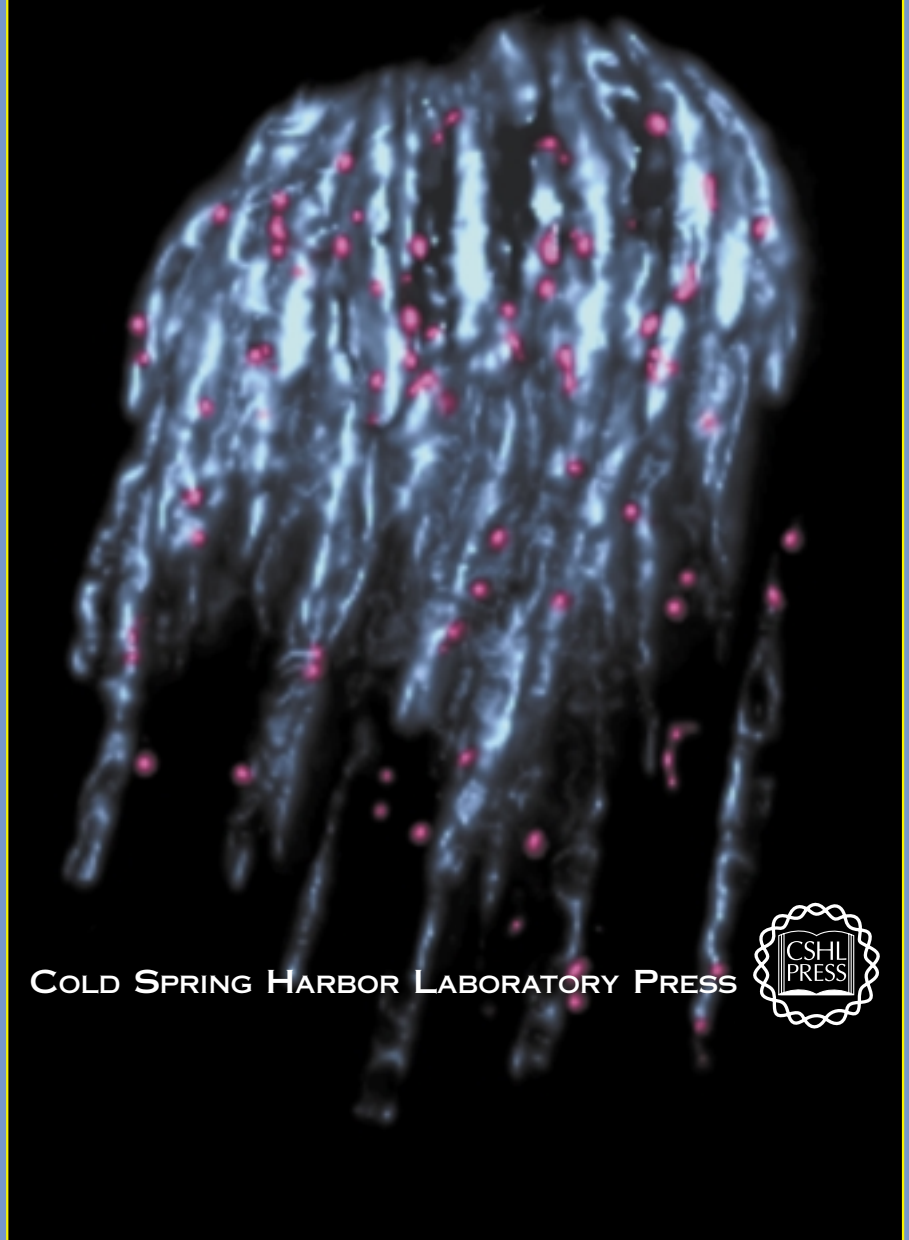

Cold Spring Harbor Laboratory Press

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Cold Spring Harbor Laboratory Press
Cold Spring Harbor, New York

Drosophila Protocols

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Front cover (printed hardcover) In situ hybridization to the histone gene cluster provides a means of identifying compound chromosome-2-bearing sperm during the process of individualization in the testes of CS(2)EN/Omales. (Courtesy of Abby Dernburg from Dernburg et al., Genetics 143: 1629 [1996].)

Back cover (printed hardcover) Individualized sperm bearing a compound chromosome 2 are identified through in situ hybridization to the histone gene cluster in the testes of CS(2)EN/Omales. (Courtesy of Abby Dernburg from Dernburg et al., Genetics 143: 1629 [1996].)

Library of Congress Cataloging-in-Publication Data

Drosophila protocols / [edited by] William Sullivan, Michael Ashburner, R. Scott Hawley.
p. cm.
Includes bibliographical references.
ISBN 0-87969-585-4 (cloth) -- ISBN 0-87969-586-2 (pbk.)
I. Drosophila--Laboratory manuals. I. Sullivan, William, 1957- II. Ashburner, M. III. Hawley, R. Scott.

QL537.D76 D765 2000
595.77Õ4--dc21
00-020100

10 9 8 7 6 5 4 3 2

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Preface

LARRY SANDLER WOULD OFTEN TELL HIS STUDENTS, ÒI can make a living pushing flies, but you canÕt.Ó He was referring to the fact that during his days as a graduate student and po doc, formal genetic analysis yielded many of the most exciting findings in *Drosophila*. However, since that time, *Drosophila* has become an organism of choice for molecular, biochemical, and cellular studies, and many key insights rely on a combination of approaches. For students just beginning to work with *Drosophila*, mastering techniques and approaches to be used in combination with Òfly pushing,Ó Larry correctly concluded would be an essential element of their training.

With new techniques being generated at an ever increasing rate, LarryÕs advice is ev more appropriate. Although the variety of approaches currently available to the fly community makes *Drosophila* research particularly exciting, it is also daunting. It requires all of us to step outside the comfort zone of tried-and-true procedures we learned as graduate students and post-docs. Establishing a new technique in the lab is often a leap of faitl and requires a large commitment of time and resources. Therefore, reducing this activation barrier was foremost in our minds when we developed a strategy for revising the Laboratory Manual that accompanied Michael AshburnerÕs *Drosophila: A Laboratory Handbook* (1989), known to the community as the Grey Book. Unlike the original edition, this book makes no attempt to be comprehensive. We felt liberated from this responsibility because there exists a number of other excellent *Drosophila* protocol manuals, including AshburnerÕs original more comprehensive work (*Drosophila: A Laboratory Manual*, 1989). Instead, we chose to provide in-depth descriptions of a select set of protocols that are most likely to be used by the *Drosophila* community in the next decade. Each protocol includes enough basic information to be useful to the uninitiated, in sufficient detail to serve as a useful reference for the more experienced.

We arrived at a ÒTop 37Ó list by soliciting the advice of the *Drosophila* community and surveying the literature for the most frequently used protocols. Some of the protocols, such as RNA interference, are currently used by only a few laboratories, but are likely soon to become more routine. Some widely used protocols, such as transformation, are not included because there already exist a number of excellent published descriptions of this technique.

The protocols are grouped into six sections: Chromosomes, Cell Biology, Molecular Biology, Genomics, Biochemistry, and The Organism. Several of the protocols assume a knowledge of basic molecular techniques and may require users to refer to one of the

many excellent sources of such methods. Very little genetics is included in this volume, because much of this material is covered in the Grey Book. New advances in Drosophila genetic techniques will be included in a planned second edition of the Grey Book. In addition to an introduction describing the purpose of each procedure, the chapters include many figures, tables, illustrations, and examples of the kinds of data that can be produced. We believe that these features make for a more useful and interesting reference. It is our hope that it will be read between time-points for ideas and new approaches.

The manual also includes six appendices. Reprinted in Appendix 1 is the Table of Contents of the original edition of the protocol manual. This has been included because the revision required the sacrifice of many useful protocols from that book. (Although it was published more than a decade ago, most labs still have a copy and the taped covers attest to its continued value as a reference.) Appendix 2 consists of line drawings of many key aspects of Drosophila biology and development. These have been plucked from a number of sources and slightly modified to serve as templates for figures and transparencies. Appendix 3 includes a number of commonly used solutions, buffers, and recipes used by the Drosophila community. Appendix 4 lists the toxic and dangerous reagents used in these protocols. Also included are Suppliers and Trademarks in Appendices 5 and 6, respectively.

This book is in print only because of the enthusiastic support of both the Drosophila community and the good folk at Cold Spring Harbor Laboratory. To all of the contributing authors, we owe a hearty thanks. Without exception the authors put a great deal of time, effort, and thought into the project. The majority of the chapters required only minor changes, and the authors diligently responded to any of our suggestions. This made our job relatively painless and sometimes even pleasant. (And to those authors who were late returning manuscripts and wondering if they were the very last, rest easy, the person concerned atoned with an excellent chapter and is forgiven.)

The publishing group at Cold Spring Harbor was a pleasure to work with. Throughout the entire project, it was clear that quality was their top priority. John Inglis and his team had an extraordinary ability to focus our rather vague ideas about the book and marshal the resources and personnel to carry them out. We thank Dotty Brown for the wonderful job she did with the copy editing, drawings, figures, appendices, and indexing. We also want to thank Marilu Hoeppner for cheerfully carrying out that most difficult task of ensuring the accuracy and consistency of the book. Finally, we wish to give a special thanks to Mary Cozza for her exceptional organizational skills and equally important her firm, but gentle, prodding.

W. Sullivan
M. Ashburner
R. Scott Hawley

Abbreviations

Acetyl CoA	acetyl coenzyme A
ACh	acetylcholine
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AEL	after egg laying
AL	antennal lobe
ALH	after larval hatching
AP	alkaline phosphatase
BAC	bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate (see X-phosphate)
BDGP	Berkeley *Drosophila* Genome Project
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BFD	Berkeley Fly Database
BLAST	basic local alignment search tool
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
BSS	balanced salt solution
b/w	black/white
BWSV	black widow spider venom
CALI	chromophore-assisted laser inactivation
CAT	chloramphenicol acetyltransferase
CCD	charge-coupled device
CDS	coding sequence
CF	chemical fixation
CHEF	contour-clamped homogeneous electric field
CLSM	confocal laser-scanning microscopy
CMFDG	5-chloromethylfluorescein di-D-galactopyranoside
CNS	central nervous system
ConA	concanavalin A
CPD	criticial point dried
CPITC	coumarin phenylisothiocyanate
CPRG	chlorophenol red-D-galactopyranoside
DAB	3,3-diaminobenzidine
DABCO	1,4-diazabicyclo-(2,2,2)-octane
DAPI	4,6-diamidino-2-phenylindole

DCV	*Drosophila* C virus
DDBJ	DNA Database of Japan
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DIC	differential interference contrast
DIG	digoxigenin
DLM	dorsal longitudinal flight muscle
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DOP-PCR	degenerate oligonucleotide-primed PCR
DRES	*Drosophila*-related EST sequences
DSHB	The Developmental Studies Hybridoma Bank (University of Iowa)
dsRNA	double-stranded RNA
DT-A	diphtheria toxin A subunit
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDGP	European *Drosophila* Genome Project
EGFP	ÒenhancedÓ GFP
EJC	excitatory junctional current
EJP	excitatory junctional potential
EM	electron microscopy
EMBL	European Molecular Biology Laboratory
EMS	ethyl methane sulfonate
ENU	ethyl nitrosourea
EP	enhancer promoter
ERG	electroretinogram
EST	expressed sequence tag
FAQs	frequently asked questions
FCS	fetal calf serum
FETi	fast extensor tibiae motor neuron
FIGE	field inversion gel electrophoresis
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FLP-FRT	site-specific FLP recombinase–FLP recombination target
FRT	FLPase recombination target
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester
FTP	file transfer protocol
GF	giant fiber
GFP	green fluorescent protein
GIFTS	Gene Interaction in the Fly Transworld Server
GST	glutathione-*S*-transferase
GTPase	guanosine triphosphatase
HAME	*p*-hydroxyl benzoic acid methyl ester
HEPES	N-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)
HGT	high gelling temperature
HHMI	Howard Hughes Medical Institute
HL	hemolymph-like

HMDS	hexamethyldisilazane
HMW	high molecular weight
HPF	high-pressure freezing or high-pressure freezer
HPF-FS	high-pressure freezing/freeze-substitution
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
hsp	heat shock promoter
HU	hydroxyurea
iACT	inner antenno-cerebral tract
IdU	5-iodo-2 -deoxyuridine
iEM	immunoEM
ip	intraperitoneal
IPTG	isopropyl- -D-thiogalactopyranoside
IR	infrared
kb	kilobase
KC	Kenyon cell
LB medium	Luria-Bertani medium
LM	light microscopy
LMW	low molecular weight
LocI	local interneuron
MAb	monoclonal antibody
Mb	megabase
MB	mushroom body
mEJCs	miniature EJCs
$[Mg^{++}]$	magnesium ion concentration
MM3 medium	modified M3 medium
MRP	mechanoreceptor potential
MT	microtubule
Mt	metallothionein
$[Na^{+}]$	sodium ion concentration
NA	numerical aperature
ND	neutral density
NBT	nitroblue tetrazolium
NIDA	National Institute on Drug Abuse
NGS	normal goat serum
NMJ	neuromuscular junction
NPG	n-propyl gallate
NOR	nucleolus organizer region
n-syb	neuronal synaptobrevin
NVOC-C1	6-nitroveratryl chloroformate
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDMN	posterior dorsal mesothoracic nerve
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
PI	propidium iodide
PIPES	piperazine-N,N -bis(2-ethanesulfonic acid)
PLT	progressive lowering of temperature

PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PPF	paired-pulse facilitation
ppm	peripodial membrane
PPD	p-phenylenediamine
PTP	post-tetanic potentiation
Rac	ricin A chain
RH	relative humidity
Ril	relay interneurons
RNA	ribonucleic acid
RNA-i	dsRNA genetic interference
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SETi	slow extensor tibiae motor neuron
SIT	silicon intensifier target
SNF	soluble nuclear fraction
STF	short-term facilitation
STS	sequence tagged site
syb	synaptobrevin
TBS	Tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
TEM	transmission electron microscopy
TEMED	N,N,N ,N ,-tetramethylethylenediamine
TEP	transepithelial potential
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
TeTxLC	tetanus toxin light chain
TEVC	two-electrode voltage-clamp
TLC	thin-layer chromatography
TRITC	tetramethylrhodamine isothiocyanate
TSA	tyramide signal amplification system
TTM	tergotrochanteral jump muscle
TTMn	tergotrochanteral motor neuron
TUNEL	terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end-labeling
UV	ultraviolet
UAS	upstream activation sequence
URL	uniform resource locator
UTR	untranslated regions
VNC	ventral nerve cord
WGA	wheat germ agglutinin
WPP	white prepupal stage
WWW	World Wide Web
X-gal	5-bromo-4-chloro-3-indolyl -D-galactopyranoside
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate
YAC	yeast artifical chromsome

Chromosomes

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1

Preparation and Analysis of *Drosophila* Mitotic Chromosomes

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MITOTIC CHROMOSOME CYTOLOGY HAS AN IMPORTANT ROLE in many areas of *Drosophila* research. It is routinely needed for characterization of the mitotic phenotypes elicited by mutations affecting chromosome structure and/or behavior (for review, see Gatti and Goldberg 1991). In addition, mitotic cytology has proven to be essential for cytogenetic analysis of heterochromatin (for review, see Gatti and Pimpinelli 1992). Heterochromatin cannot be cytologically dissected by polytene chromosome analysis, because the bulk of this material is included in the chromocenter. However, heterochromatin breakpoints can be precisely determined in mitotic chromosomes processed with high-resolution banding techniques, such as quinacrine, Hoechst, and N-banding (for review, see Gatti and Pimpinelli 1992). Finally, good mitotic preparations are essential for fine mapping of repetitive DNA sequences along heterochromatin by in situ hybridization (see, e.g., Palumbo et al. 1994; Pimpinelli et al. 1995; Dernburg et al. 1996) and for immunolocalization of chromosomal proteins (see, e.g., Pak et al. 1997; Fanti et al. 1998; Platero et al. 1998).

In this chapter, we present the protocols routinely used in our laboratories for mitotic chromosome preparation, chromosome banding, and fluorescent in situ hybridization (FISH). In addition, we describe our fixation and immunostaining procedures for protein localization along mitotic chromosomes.

PREPARATION AND STAINING OF LARVAL BRAIN MITOTIC CHROMOSOMES

Although mitotic chromosome preparations can be obtained from embryonic and gonial cells of both sexes, the tissue that provides the best mitotic figures is the larval brain. This tissue contains two major types of dividing cells: the neuroblasts and the ganglion mother

cells (Hofbauer and Campos-Ortega 1990). The neuroblasts divide either symmetrically, producing two neuroblast stem cells, or asymmetrically, producing another neuroblast and a smaller cell called the ganglion mother cell. The ganglion mother cell divides only once, producing two daughter cells that differentiate into neurons. Several squashing techniques have been developed for preparation of larval brain mitotic chromosomes (Ashburner 1989; Gonzalez and Glover 1993). Below, we present a series of squashing protocols that a routinely used in our laboratories for a variety of experimental purposes. These procedures are minor modifications of a basic technique developed 25 years ago (Gatti et al. 1974) and can be successfully used for preparing mitotic chromosomes of various *Drosophila* and mosquito species (Gatti et al. 1976; Pimpinelli et al. 1976; Bonaccorsi et al. 1980, 1981). To characterize various aspects of mitotic chromosome morphology and behavior, larval brains can be squashed either in aceto-orcein to obtain orcein-stained chromosomes (Protocol 1.1) or in 45% acetic acid to obtain unstained preparations (Protocol 1.2). Unstained material can be then stained with Giemsa to obtain permanent preparations, processed with a variety of banding techniques, or used for in situ hybridization.

Preparation of Orcein-stained Chromosomes

Depending on the experimental purpose, aceto-orcein squashes can be prepared by three different experimental regimes, which are summarized in Protocol 1.1. First, dissected brains can be squashed in aceto-orcein without colchicine treatment and hypotonic shock (i.e., Protocol 1.1, with the omission of steps 3 and 4). This procedure allows observation of all phases of mitosis and permits evaluation of the mitotic index and the frequency of anaphases (Gatti and Baker 1989). However, chromosome morphology is poorly defined in these preparations.

In the second regime, colchicine treatment is omitted, but brains are incubated in hypotonic solution (i.e., Protocol 1.1, with the omission of step 3). Hypotonic treatment improves metaphase chromosome spreading and causes sister chromatid separation allowing examination of chromosome condensation and detection of hyperploid and polyploid metaphases. However, hypotonic shock disrupts anaphase (Brinkley et al. 1980), and anaphase figures are almost absent in hypotonically treated brains.

In the third regime (Protocol 1.1), brains are incubated in vitro with colchicine, treated with hypotonic solution, fixed, and squashed. This procedure provides a large number of well-spread metaphase figures (200Ð400 per brain) that can be analyzed for chromosome morphology, the presence of chromosome aberrations, and the degree of ploidy. However, because colchicine disrupts spindle microtubules, inducing metaphase arrest followed by chromosome overcontraction, colchicine treatment must be omitted if the degree of chromosome condensation has to be evaluated.

Preparation of Unstained Chromosomes

Unstained brain chromosomes can be prepared according to the same experimental regimes described above for aceto-orcein squashes. The procedure for this type of preparation is given in Protocol 1.2.

Giemsa Staining

Although well-sealed aceto-orcein squashes remain in good condition for 1Ð2 months, there are cases in which permanent chromosome preparations are needed. This can b

done by staining preparations obtained according to Protocol 1.2 with 2% Giemsa in a phosphate buffer at pH 7.0. We routinely use Giemsa from Merck, but other Giemsa brands work just as well. The timing of Giemsa staining varies with the Giemsa brand and should be adjusted to obtain the desired staining. After Giemsa staining, chromosome preparations are differentiated by washing the slides in tap water. Giemsa stain is additive and thus if chromosomes are not sufficiently stained, the slides can be stained again in 2% Giemsa, until the desired stain is obtained. After washing in tap water, the slides are air-dried and then mounted in Euparal (Carolina Biological Supply) or similar medium.

PROTOCOL 1.1*

Aceto-Orcein Squashes of Larval Brains

Materials

Supplies and Equipment

Siliconized slides, used only as a support for drops of either saline or hypotonic solution (for preparation, see Protocol 12.6 [Dernberg] or Protocol 6.1 [Kennison] in this volume.)

Dissecting tools
- dissecting microscope
- forceps (2 pairs; e.g., Dumont #5 Biologie)

Petri dish (35 x 10 mm) with cover

Nonsiliconized slides and coverslips (22 x 40 mm or 22 x 22 mm)

Blotting paper

Depilatory wax (found in most cosmetic shops) or nail polish

Solutions and Reagents

Saline (0.7% NaCl in H_2O)

(Optional) Colchicine (10^{-3} M) in H_2O

(Optional) Hypotonic solution (0.5% sodium citrate $2H_2O$ in H_2O)

Fixative

Acetic acid/methanol/H_2O (ratio 11:11:2)

Use freshly prepared fixative.

Preparation of 2% Aceto-orcein

Boil synthetic orcein powder (Gurr, BDH Laboratory Supplies, Poole, Dorset BH15 1TD England; Phone: +44 1202 660444; Fax: +44 1202 666856; Web site: http://www.bdh.com) in 45% acetic acid for 45 minutes in a reflux condenser. We usually prepare 5% orcein, which is subsequently diluted to 2% with 45% acetic acid.

Before use, remove particulate matter from aceto-orcein either by filtration through blotting paper or by centrifugation in a microcentrifuge.

CAUTION: acetic acid, colchicine, methanol (see Appendix 4)

*In all protocols, H_2O indicates glass distilled and deionized.

Method

1. Dissect larval brains as follows:

   a. Transfer drops of saline (~50 l) onto a siliconized slide. Place one to three actively crawling third-instar larvae in each drop.

   b. Use two pairs of thin forceps (e.g., Dumont #5 Biologie) to grab the larval mouth parts and the larval body at a position approximately two thirds of its length in the posterior part, and then pull apart.

      Note: The brain usually remains attached to the head together with several imaginal disks and the salivary glands.

   c. Use the forceps to remove the brain from the remainder of the head.

      Note: There is no need to completely remove the imaginal discs, which will not interfere with squashing, but the more rigid mouth parts should be completely removed.

2. Wash the dissected brains in saline at room temperature for 1–5 minutes.

3. Transfer the brains to a 35 x 10-mm petri dish containing 2 ml of saline supplemented with a drop (~100 l) of 10^{-3} M colchicine. Cover the petri dish and incubate at 25¼C for 1.5 hours.

4. Transfer the brains into a drop (~50 l) of hypotonic solution placed on a siliconized slide and incubate at room temperature for 10 minutes.

   Note: The incubation time is critical because treatments that exceed 10 minutes may induce sister chromatid separation in metaphase chromosomes.

5. Fix the brains at room temperature in a freshly prepared mixture of acetic acid/methanol/H_2O (11:11:2). Check fixation under the dissecting microscope, and keep the brains in the fixative until they begin to become transparent (10–20 seconds).

   Note: We usually fix the brains in 2 ml of fixative placed in a 35-mm petri dish.

6. Transfer fixed brains individually into small drops (12 l) of 2% aceto-orcein placed on a very clean, dust-free nonsiliconized coverslip (20 x 20 or 22 x 22 mm). Between one and four brains may be transferred to a corresponding number of drops placed on the same coverslip.

7. Leave the brains in the aceto-orcein drops for 1–2 minutes and then lower a very clean, dust-free nonsiliconized slide onto the coverslip; surface tension will cause the coverslip to adhere to the slide. Invert the sandwich and squash between two or three sheets of blotting paper.

   Note: Squashing should be performed in two steps: First exert a gentle pressure to remove the excess of aceto-orcein and then squash very hard. The gentle presquashing prevents coverslip sliding and the consequent damage to the preparation.

8. Seal the edges of the coverslip either with melted depilatory wax or with nail polish.

   Note: Well-sealed slides can be stored at 4¼C for 1–2 months without substantial deterioration.

PROTOCOL 1.2

Unstained Squashes of Larval Brains

Materials

Supplies and Equipment

Sliconized slides, used only as a support for drops of either saline or hypotonic solution (for preparation, see Appendix 3)
Dissecting tools
- dissecting microscope
- forceps (2 pairs; e.g., Dumont #5 Biologie)

Petri dish (35 x 10 mm) with cover
Nonsiliconized slides and siliconized coverslips (20 x 20 mm or 22 x 22 mm)
Blotting paper
Razor blade

Solutions and Reagents

Saline (0.7% NaCl in H_2O)
(Optional) Colchicine (10^{-3} M) in H_2O
(Optional) Hypotonic solution (0.5% sodium citrate ¥ $2H_2O$ in H_2O)
Acetic acid (45%)
Absolute ethanol, chilled at -20¼C
Liquid nitrogen or dry ice

CAUTION: acetic acid, colchicine, dry ice, ethanol, liquid nitrogen (see Appendix 4)

Method

1. Perform steps 1-5 of Protocol 1.1 above.

   Note: Incubation in 10^{-3} M colchicine (step 3) and hypotonic treatment (step 4) may be omitted depending on the desired type of preparation.

2. Transfer fixed brains individually into small drops (2) of 45% acetic acid placed on a very clean, dust-free siliconized coverslip (20 x 20 or 22 x 22 mm). Between one and four brains may be transferred to a corresponding number of drops placed on the same coverslip.

   Note: Siliconized coverslips are used instead of nonsiliconized coverslips (as in step 6, Protocol 1.1).

3. Leave the brains in the 45% acetic acid drops for 1-2 minutes and then lower a very clean, dust-free nonsiliconized slide onto the coverslip; surface tension will cause the coverslip to adhere to the slide. Invert the sandwich and squash between two or three sheets of blotting paper.

   Note: Squashing should be performed in two steps: First exert a gentle pressure to remove the excess acetic acid and then squash very hard. The gentle presquashing prevents coverslip sliding and the consequent damage to the preparation.

4. Freeze the slides either on dry ice or in liquid nitrogen.

5. Flip off the coverslip with a razor blade and immediately immerse the slides in absolute ethanol at Ð20¼C, and leave for 15 minutes. Air-dry the slides and store them at 4¼C until further processing.

CHROMOSOME-BANDING TECHNIQUES

The classical chromosome-banding techniques developed for mammalian chromosomes do not differentiate the euchromatic arms of *Drosophila* mitotic chromosomes. However, some of these techniques produce a sharp and highly reproducible banding of *Drosophila* heterochromatin (see, e.g., Holmquist 1975; Gatti et al. 1976). For example, the use of quinacrine-, Hoechst- and N-banding differentiates *Drosophila* heterochromatin into 61 cytological entities (Figure 1.1C), allowing precise localization of heterochromatic breakpoints (see, e.g., Gatti and Pimpinelli 1983, 1992; Pimpinelli and Dimitri 1989; Dimitri

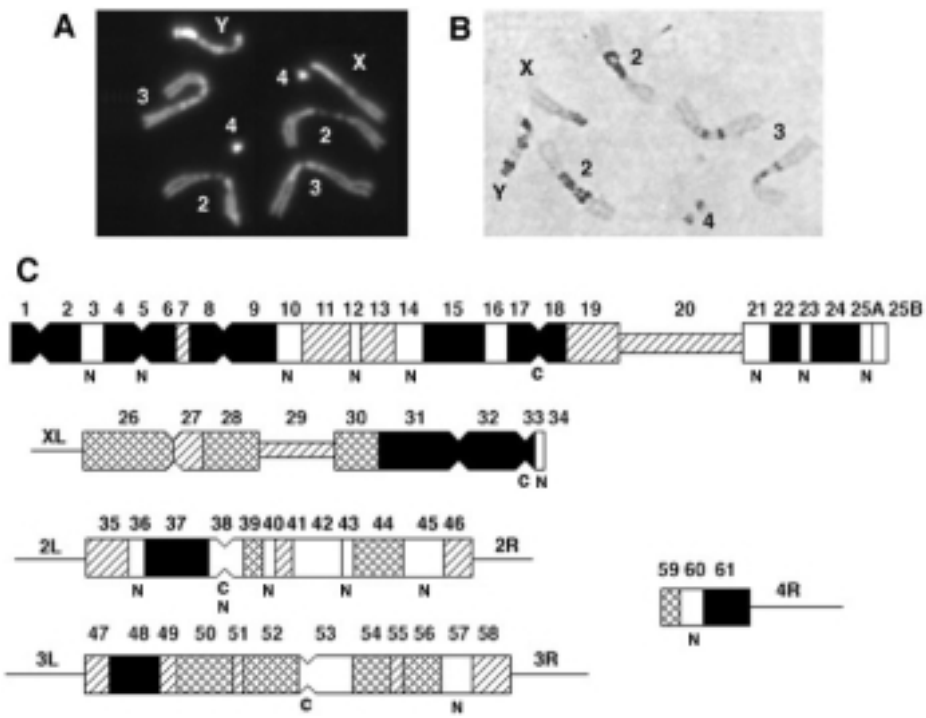

Figure 1.1. Examples of Hoechst-stained (A) and N-banded (B) metaphases. (C) A cytological map of *D. melanogaster* heterochromatin. The diagrams have been constructed by examining prometaphase chromosomes sequentially processed for quinacrine-, Hoechst- and N-banding. The entirely heterochromatic Y chromosome, the X chromosome, and the second, third, and fourth chromosome heterochromatin are schematically represented left to right and from top to bottom. Only the heterochromatic portions of chromosomes are shown; euchromatin is depicted as a straight line. The narrow cross-hatched regions of the X and Y chromosomes represent the nucleolus organizer regions. C indicates the position of the centromere; the location of the fourth chromosome centromere has not been precisely determined. (Solid segments) Bright fluorescence; (cross-hatched segments) moderate fluorescence; (open segments) no fluorescence. The N letters below each diagram indicate the regions that are consistently stained by the N-banding procedure. (Reprinted, with permission, from Pimpinelli et al. 1976.) For example, in B, the third chromosome heterochromatin exhibits an intensely stained block and two less heavily stained regions. The latter are not consistently observed and were not considered as N-bands.

1991). These banding techniques can also be successfully used to differentiate mitotic heterochromatin of various Drosophila and mosquito species (Gatti et al. 1976; Pimpinelli et al. 1976; Bonaccorsi et al. 1980, 1981).

Hoechst 33258 and 4«,6-diamidino-2-phenylindole (DAPI) stainings produce identical banding patterns, differentiating mitotic heterochromatin into blocks that differ for the degree of fluorescence (Figure 1.1A). Both compounds are general indicators of AT-richness along the chromosomes (Verma and Babu 1989; Sumner 1990), and the Hoechst and DAPI-bright regions have been shown to contain large numbers of AT-rich satellite repeats (Bonaccorsi and Lohe 1991; Lohe et al. 1993).

Quinacrine staining produces a banding pattern similar but not identical to the Hoechst banding. The major difference between these two banding patterns is in the bulk of X-chromosome heterochromatin (region h31–h32) which is Hoechst bright and quinacrine dull. This region, which accommodates the relatively AT-rich 359-bp satellite DNA (Hilliker and Appels 1982; Lohe et al. 1993), can therefore be cytologically identified by sequential staining with Hoechst and quinacrine (see below).

The N-banding procedure specifically stains the nucleolus organizer regions (NORs) of several animal and plant species (Matsui and Sasaki 1973; Funaki et al. 1975). In Drosophila melanogaster, N-banding stains 16 major heterochromatic blocks (see Figure 1.1B) that are not fluorescent after Hoechst, DAPI, or quinacrine staining and do not correspond to NORs. N-banded regions are all enriched in the AAGAG repeats cloned from the 1.705 g/cm^3 satellite DNA (Bonaccorsi and Lohe 1991; Lohe et al. 1993). In addition, N-banded heterochromatic blocks are specifically immunostained with antibodies directed to the GAGA factor (see Figure 1.2C).

As shown in Figure 1.1, some heterochromatic regions that are poorly differentiated by Hoechst, DAPI, or quinacrine banding are sharply defined by the N-banding procedure. The cytological map of D. melanogaster heterochromatin (Figure 1.1) indicates which banding procedure should be applied for the best differentiation of each heterochromatic region. The most efficient method in our experience for defining a heterochromatic breakpoint is sequential application of two banding procedures such as Hoechst- (or DAPI-) and N-banding.

The degree of resolution of the banding techniques depends on the degree of elongation of heterochromatic regions. Thus, for all of the banding procedures described below, chromosomes should be prepared according to Protocol 1.2, with the omission of colchicine treatment.

PROTOCOL 1.3

Hoechst Staining

The following procedure (Gatti et al. 1976; Gatti and Pimpinelli 1983) is a modification of a protocol developed by Latt (1973) for mammalian chromosomes. For other Hoechst-staining protocols, see Holmquist (1975) and Hazelrigg et al. (1982).

Materials

Supplies and Equipment

Slides, prepared according to Protocol 1.2, with the omission of colchicine treatment
Rubber cement

Solutions and Reagents

Hoechst 33258 (0.5 g/ml) dissolved in HB

Hoechst Buffer (HB)

150 mM NaCl
30 mM KCl
10 mM Na_2HPO_4
Adjust to pH 7.0.

Mounting Buffer

160 mM Na_2HPO_4
40 mM sodium citrate
Adjust to pH 7.0.

CAUTION: Hoechst 33258, KCl, Na_2HPO_4 (see Appendix 4)

Method

1. Rehydrate air-dried slides in HB at room temperature for 5 minutes.
2. Stain slides in Hoechst 33258 (0.5 g/ml in HB) at room temperature for 10 minutes.
3. Wash briefly (~5 seconds) in HB.
4. Place slides in a vertical position and allow to air-dry.
5. Mount in either HB or Mounting Buffer.
6. Seal around the edges of the coverslip with rubber cement.

Notes: The slides may be stored in the dark at 4¼C for 1Ð2 days before observation; we have found that this treatment reduces fluorescence fading.

Fluorescence fading depends on both the lamp and the set of filters used for epifluorescence. If the degree of fading is too high, the slides may be mounted in Vectashield (H-1000; Vector Laboratories) or in similar media containing glycerol and antifading compounds (see Appendix 3).

PROTOCOL 1.4

DAPI Staining

Materials

Supplies and Equipment

Slides, prepared according to Protocol 1.2, with the omission of colchicine treatment

Solutions and Reagents

2x SSC (see Appendix 3)
DAPI (0.2 g/ml), dissolved in 2x SSC
Vectashield or other mounting media (H-1000; Vector Laboratories)

CAUTION: DAPI (see Appendix 4)

Method

1. Rehydrate air-dried slides in 2x SSC at room temperature for 5 minutes.
2. Stain in DAPI (0.2 g/ml in 2x SSC) at room temperature for 5 minutes.
3. Wash briefly (~5 seconds) in 2x SSC.
4. Place slides in a vertical position and allow to air-dry.
5. Mount in Vectashield or in similar media containing glycerol and antifading compounds.

PROTOCOL 1.5

Quinacrine Staining

The following quinacrine staining protocol was developed by Gatti et al. (1976). For other quinacrine-banding techniques, see Vosa (1970), Ellison and Barr (1971), and Faccio Dolfini (1974).

Materials

Supplies and Equipment

Slides, prepared according to Protocol 1.2, with the omission of colchicine treatment
Rubber cement

Solutions and Reagents

Quinacrine dihydrocloride (0.5%; Gurr), dissolved in absolute ethanol
Absolute ethanol

CAUTION: ethanol, quinacrine dihydrocloride (see Appendix 4)

Method

1. Immerse slides in absolute ethanol for 5 minutes.
2. Stain slides in 0.5% quinacrine dihydrochloride (in absolute ethanol) for 10 minutes.
3. Wash slides twice (5 seconds each) in absolute ethanol.
4. Place slides vertically and allow to air-dry.
5. Mount in H_2O.
6. Seal around the edges of the coverslip with rubber cement. Store the slides in the dark at 4¼C before observation.

   Note: This treatment improves the degree of differentiation and reduces fluorescence fading. Here again (see Protocol 1.3, step 6) if fluorescence fading is too high, mount the slides in Vectashield (H-1000; Vector Laboratories) or similar media.

PROTOCOL 1.6

N-Banding

The N-banding procedure presented below is essentially that of Funaki et al. (1975) with minor modifications (Pimpinelli et al. 1976). Best results are obtained when preparations obtained according to Protocol 1.2 are aged for 2Ð5 days at 4¼C before processing; the preparations give a sharper N-banding compared to either fresh or older slides.

Materials

Supplies and Equipment

Slides, prepared according to Protocol 1.2, with the omission of colchicine treatment

Solutions and Reagents

NaH_2PO_4 (1 M, pH 4.2)
Giemsa (4%; Merck), dissolved in phosphate buffer
Euparal (Carolina Biological Supply) or similar medium for mounting

Phosphate Buffer

8 mM KH_2PO_4
6 mM Na_2HPO_4
Adjust to pH 7.0.

CAUTION: Giemsa, KH_2PO_4, Na_2HPO_4 (see Appendix 4)

Method

1. Immerse slides in 1 M NaH_2PO_4 at 85¼C and incubate for 15 minutes.
2. Rinse slides in H_2O at room temperature.
3. Stain slides in 4% Giemsa (in phosphate buffer at pH 7.0) at room temperature for 20 minutes.
4. Rinse slides in tap water.

   Note: Remember that Giemsa staining is additive and that insufficiently stained preparations can be restained (see above).
5. Let the slides air-dry and mount in Euparal or similar medium.
6. Examine slides using phase-contrast optics.

PROTOCOL 1.7

Sequential Quinacrine-, Hoechst-, and N-banding

The following protocol describes sequential quinacrine-, Hoechst- and N-banding. However, sequential application of Hoechst- and N-banding is sufficient for precise determination of most heterochromatic breakpoints. Quinacrine staining may be used only for

breakpoints falling into regions that are Hoechst-bright and quinacrine-dull (Gatti and Pimpinelli 1983).

Materials

Supplies and Equipment

Slides, prepared according to Protocol 1.2, with the omission of colchicine treatment
Coplin jar
Rubber cement

Solutions and Reagents

Quinacrine dihydrocloride (0.5%; Gurr), dissolved in absolute ethanol
Ethanol (70%, 95%, and absolute)
Destain (methanol/acetic acid [3:1])
HB (see p. 10)
Hoechst 33258 (0.5 g/ml), dissolved in HB
Giemsa (4%; Merck), dissolved in phosphate buffer (see p. 12) at pH 7.0
Mounting media

CAUTION: acetic acid, ethanol, methanol, quinacrine dihydrocloride (see Appendix 4)

Method

1. Perform quinacrine staining according to Protocol 1.5.
2. After analysis of quinacrine banding, peel off the rubber cement from the coverslip edges and place the slides into a Coplin jar containing H_2O. Keep the slides in H_2O until the coverslip slides off. To facilitate coverslip sliding, shake the jar and/or gently tap against the bench.
3. Place slides vertically and allow to air-dry.
4. Wash slides in 70% ethanol for 5 minutes.
5. Destain slides by washing three times (30 minutes each) in a 3:1 solution of methanol/acetic acid.
6. Place slides vertically and allow to air-dry.
7. Stain slides with Hoechst 33258 according to Protocol 1.3.
8. After analysis of Hoechst banding, dismount the slides as described in step 2.
9. Wash slides in 95% ethanol for 5 minutes.
10. Air-dry slides and store them at 4¼C for 2–5 days.
11. Process slides with the N-banding technique, according to Protocol 1.6.

IN SITU HYBRIDIZATION

The fluorescent in situ hybridization (FISH) technique permits fine mapping of both middle and highly repetitive DNA sequences along *D. melanogaster* heterochromatin (see, e.g., Abad et al. 1992; Palumbo et al. 1994; Pimpinelli et al. 1995; Berghella and Dimitri

1996; Dernburg et al. 1996). Best results are obtained when this technique is coupled with DAPI staining and digital recording of fluorescent signals. For example, if digital images of the FISH signals and DAPI fluorescence are detected separately using a charge coupled device (CCD camera), they can be then pseudocolored and merged using suitable computer programs. This allows precise overlapping of the DAPI banding (which is identical to the Hoechst 33258 banding) and the FISH signals, facilitating the assignment of the repetitive sequence under study to specific regions of the cytological map of *Drosophila melanogaster* heterochromatin (Figure 1.1). We describe here the FISH procedures that are routinely used in our laboratories. These procedures have been adapted to *Drosophila* chromosomes by modifying the FISH technique of DÕAiuto et al. (1993). For other FISH procedures for *Drosophila* chromosomes, see Gonzalez and Glover (1993).

PROTOCOL 1.8

Fluorescent In Situ Hybridization

Materials

Supplies and Equipment

Slides (2–3 days old) prepared according to Protocol 1.2. The most suitable chromosome preparations for in situ hybridization are those prepared according to Protocol 1.2 without colchicine pretreatment, but with hypotonic shock.

Coplin jars

Coverslips (22 x 22 mm)

Moist chamber at 37¼C

Rubber cement

Solutions and Reagents

Biotin-11-dUTP (Enzo) or digoxigenin-11-dUTP (Boehringer Mannheim)

Nick translation kit (Boehringer Mannheim)

Sonicated salmon sperm DNA (3 g per slide)

Sodium acetate (3 M, pH 4.5)

Ethanol (70%, 90%, and absolute) at room temperature

Ethanol (70%) chilled at –20¼C

Ethanol (90%) chilled at 4¼C or on ice

2x SSC (see Appendix 3)

0.1x SSC

Detection reagents

Labeling with a single probe: Depending on the label, use either:

- Fluorescein isothiocyanate (FITC)-conjugated avidin (DCS grade; Vector Laboratories; 3.3 g/ml, diluted in SBT)
- Rhodamine-conjugated antidigoxigenin sheep IgG, Fab fragments (Boehringer Mannheim; 2 g/ml, diluted in SBT)

Double labeling: Use a mixture of the following two reagents:

- 3.3 g/ml FITC-conjugated avidin (Vector Laboratories)
- 2 g/ml rhodamine-conjugated antidigoxigenin sheep IgG, Fab fragment (Boehringer Mannheim), diluted in SBT

DAPI (0.2 g/ml), dissolved in 2x SSC
Vectashield H-1000 (Vector Laboratories) or other mounting media (see Appendix 3)
Rubber cement

Hybridization Mixture (10 l per slide)

ultrapure formamide (J.T. Baker)	5 l
50% dextran sulfate	2 l
20x SSC	1 l
H_2O	2 l

Denaturation Solution

ultrapure formamide (J.T. Baker)	35 ml
20x SSC	5 ml
H_2O	10 ml

Mix all components and pour into a Coplin jar.

Wash Buffer 1

50% formamide
2x SSC

Blocking Solution

3% bovine serum albumin (BSA)
0.1% Tween-20
4x SSC

SBT Solution

1% BSA
0.1% Tween-20
4x SSC

SSC/Tween-20 Solution

0.1% Tween-20
4x SSC

CAUTION: ethanol, DAPI, digoxigenin, formamide (see Appendix 4)

Method

Probe Preparation and Denaturation

1. Label 1 g of probe by nick translation using either biotin-11-dUTP or digoxigenin-11-dUTP, following the manufacturerÕs instructions.
2. Mix the labeled DNA (40Ð80 ng per slide) with sonicated salmon sperm DNA (3 g per slide). Add 0.1 volume of 3 M sodium acetate (pH 4.5) and 2 volumes of cold absolute ethanol (chilled at Ð20¼C). Place at Ð80¼C for 15 minutes and centrifuge 13,600 g for 15 minutes to pellet the DNA. Aspirate the ethanol. Dry the pellet in a Savant speed vac.

3. Resuspend the DNA pellet in the hybridization mixture by vortexing.
4. Denature the probe mixture at 80¡C for 8 minutes. Place the probe on ice until used.

Slide Denaturation

1. Place the Coplin jar containing the Denaturation Solution in a water bath at 70¼C.
2. Dehydrate 2Ð3-day-old slides (prepared according to Protocol 1.2) by immersion in 70%, 90%, and absolute ethanol at room temperature (3 minutes each). Air-dry slides.
3. Transfer the slides into the Denaturation Solution at 70¼C and leave for exactly 2 minutes. To avoid a temperature drop of this solution, do not immerse more than three to four slides at any given time.
4. Quickly transfer the slides to cold 70% ethanol at Ð20¼C and incubate for 3 minutes. Then dehydrate sequentially through cold 90% and absolute ethanol at 4¼C (3 minutes each). Air-dry slides.

Hybridization

Do not allow slides to dry during the following passages.

1. Apply 10 l of Hybridization Mixture to each of the denatured slides. Cover each slide with a 22x 22-mm coverslip; avoid forming air bubbles. Seal the edges of the coverslip with rubber cement.
2. Incubate slides in a moist chamber at 37¼C overnight.

   Note: The hybridization temperature we routinely use for middle repetitive probes is 37¼C. However, for simple sequence satellite DNAs, other hybridization temperatures should be used, depending on their nucleotide composition and sequence. For each satellite probe, the hybridization temperature is maintained at 8Ð12¡C below its melting temperature value (for details, see Bonaccorsi and Lohe [1991] and Lohe et al. [1993]).
3. Peel off the rubber cement, gently remove the coverslip, and wash slides three times in Wash Buffer 1 at 42¼C for 5 minutes (each wash).
4. Wash slides three times in 0.1SSC at 60¼C for 5 minutes (each wash).
5. Remove excess liquid from around the specimen and apply 100 l Blocking Solution to each slide. Cover with a 22x 22-mm coverslip and incubate at 37¼C for 30 minutes.

Biotin-labeled DNA Detection

1. Gently remove the coverslip and blot excess liquid from around the specimen.
2. To each slide, apply 80 l of 3.3 g/ml FITC-conjugated avidin, diluted in SBT. Cover with a 22x 22-mm coverslip and incubate at 37¼C for 30 minutes in a dark, humid chamber.

3. Remove the coverslips. Wash slides three times in SSC/Tween-20 at 42¼C for 5 minutes (each wash).
4. Stain with 0.2 g/ml DAPI (in 2x SSC) at room temperature for 5 minutes.
5. Rinse in 2x SSC at room temperature.
6. Mount slides in Vectashield H-1000 (Vector Laboratories) or similar antifading mountants. Seal the coverslip edges with either rubber cement or nail polish and store in the dark at 4¼C.

   Note: Slides can be stored for weeks without substantial deterioration.

Digoxigenin-labeled DNA Detection

1. Gently remove the coverslip and blot excess liquid from around the specimen.
2. To each slide, apply 80 l of 2 g/ml rhodamine-conjugated antidigoxigenin sheep IgG, Fab fragments, diluted in SBT. Cover with a 22-mm coverslip and incubate at 37¼C for 30 minutes in a dark, humid chamber.
3. Proceed as described in steps 3Ð6 above in Biotin-labeled DNA Detection.

Double Labeling

For simultaneous in situ hybridizations with biotin- and digoxigenin-labeled probes, perform the following steps:

1. Mix together 40Ð80 ng (per slide) of each labeled probe.
2. Follow the procedure for single probes:
   a. Probe Preparation and Denaturation (steps 2Ð4, omit step 1)
   b. Slide Denaturation (steps 1Ð4)
   c. Hybridization (steps 1Ð5)
3. (Signal detection) Add 80 l of 3.3 g/ml FITC-conjugated avidin, 2 g/ml rhodamine-conjugated antidigoxigenin sheep IgG, Fab fragment, diluted in SBT. Cover with a 22x 22-mm coverslip and incubate at 37¼C for 30 minutes in a dark, humid chamber.
4. Proceed as described in steps 3Ð6 above in Biotin-labeled DNA Detection.

IMMUNOSTAINING PROCEDURES

The methanol/acetic acid fixation techniques described above preserve chromosome morphology very well, but they remove a substantial fraction of chromosomal proteins. Thus, preparations obtained by these techniques are not suitable for immunolocalization of proteinaceous components of metaphase chromosomes. We have therefore developed a series of fixation procedures that result in good chromosomal quality with minimal removal of proteins. We stress here that different fixation protocols result in differential extraction of chromosomal proteins. Thus, for each protein under study, develop a fixation procedure that minimizes its removal from the chromosomes. In addition, we have

found that the timing of hypotonic treatment is also critical. For example, certain proteins cannot be visualized by immunostaining hypotonically treated preparations (e.g., the Modulo protein, Method 4 [Protocol 1.9]), suggesting that the hypotonic shock removes these proteins from metaphase chromosomes. Despite these problems, by playing with the above parameters, we have empirically determined the conditions that allow immunolocalization of several proteins along mitotic chromosomes. These experiments showed that certain fixation procedures result in artifacts. One of the most common artifacts is the absence of immunostaining of condensed chromosomes, which appear embedded into a fluorescent halo (Figure 1.2a). For example, this immunofluorescence pattern is usually observed when chromosomes fixed according to Method 2 (Protocol 1.9) are stained with antibodies directed to HP1. However, if chromosomes are fixed according to Method 1 (Protocol 1.9), the HP1 protein remains associated with the chromosomes and exhibits a preferential localization in the heterochromatic regions (Figure 1.2b). An obvious interpretation of these results is that the lack of immunostaining of mitotic chromosomes reflects the fixative-induced extraction of HP1. Thus, before concluding that a particular protein is not associated with chromosomes, it is highly advisable to examine its behavior using a variety of fixation techniques.

Below are four fixation/immunostaining protocols. Each of these protocols proved to be suitable for the immunolocalization of one or more proteins along mitotic chromosomes.

PROTOCOL 1.9

Fixation and Immunostaining

Materials

Supplies and Equipment

Silconized slides, used only as a support for drops of either saline or hypotonic solution (for preparation, see Appendix 3)
Dissecting tools
- dissecting microscope
- forceps (2 pairs; Dumont #5 Biologie)

Syringe or tungsten needle
Nonsiliconized slides and siliconized coverslips (18 mm)
Blotting paper
Razor blade

Solutions and Reagents

Saline (0.7% NaCl in H_2O)
Hypotonic solution (0.5% sodium citrate $2H_2O$ in H_2O)
Liquid nitrogen
1x PBS (phosphate-buffered saline)
Primary and secondary antibodies, diluted in PBS/BSA (see below)
DAPI (0.05 g/ml), dissolved in 2x SSC
Vectashield H-1000 (Vector Laboratories)

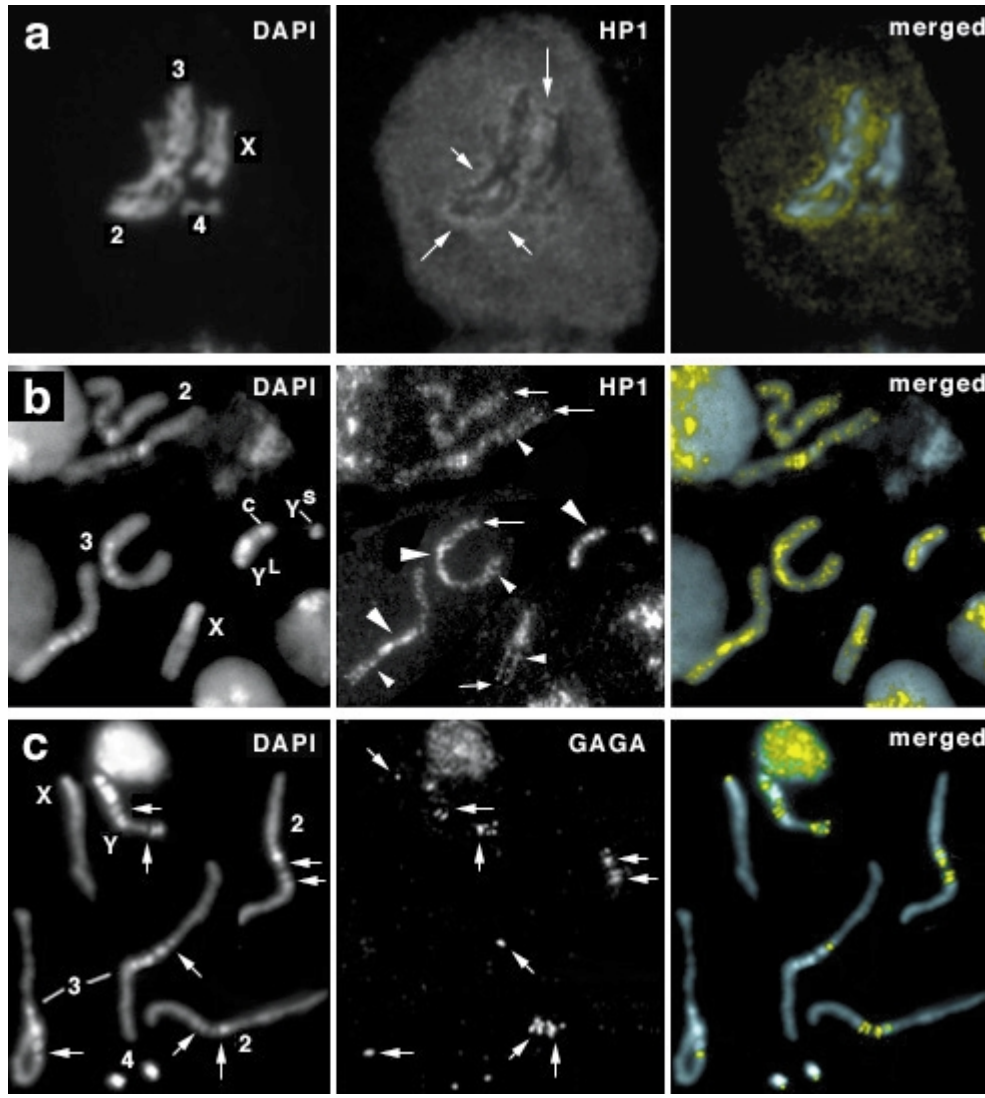

Figure 1.2 Immunostaining of larval brain cells with antibodies directed to the HP1 and GAGA proteins. (a) A prometaphase figure fixed and immunostained for HP1 according to Method 2 (Protocol 1.9). Note that the chromosomes are unstained and surrounded by a fluorescent halo (arrows). (b) A prometaphase figure fixed according to Method 1 (Protocol 1.9) and immunostained for HP1. HP1 is clearly associated with the chromosomes and enriched at the heterochromatic (arrowheads), telomeric (arrows), and several euchromatic (small arrowheads) regions. (Reprinted, with permission, from Fanti et al. 1998.) (c) A prometaphase figure fixed according to Method 2 (Protocol 1.9) and immunostained for the GAGA factor. Note the accumulation of this protein in discrete heterochromatic regions, which correspond to the N bands (arrows).

Fixative 1

Methanol/acetic acid/H_2O (ratio 10:2:1)
Use freshly prepared solution.

Fixative 2

Methanol/acetic acid/H_2O (ratio 5:2:3)
Use freshly prepared solution.

Fixative 3

45% acetic acid containing 2% formaldehyde
Use freshly prepared solution.

PTX Solution

1x PBS
1% Triton X-100

PBS/BSA

1x PBS
1% BSA
Alternatively, substitute 1% nonfat dry milk for BSA.

CAUTION: acetic acid, DAPI, formaldehyde, liquid nitrogen, methanol (see Appendix 4)

Method 1

The following procedure has been successfully applied for immunostaining with anti-HP1 antibodies (Fanti et al. 1998; the antibodies used were produced by James et al. 1989). For another protocol for HP1 immunostaining, see Pak et al. (1997).

1. Dissect brains in saline as described in Protocol 1.1, step 1.
2. Transfer brains into a drop of hypotonic solution (~50 μl) placed on a siliconized slide and leave at room temperature for 2 minutes.
3. Transfer brains into a 5-μl drop of freshly prepared Fixative 1 placed on a clean, dust-free 18x 18-mm siliconized coverslip. During fixation, which should not exceed 2 minutes, use either a syringe or a tungsten needle to fragment the brains.

   Note: Brains fixed in solutions containing a low proportion of acetic acid are rather hard and need to be fragmented before squashing.
4. Lower a clean, dust-free nonsiliconized slide on the coverslip, invert the sandwich, and squash between two to three sheets of blotting paper.
5. Freeze the slides in liquid nitrogen and flip off the coverslip with a razor blade.
6. Immediately immerse the slides in PBS at room temperature and leave for 5 minutes.
7. Transfer the slides into PTX and leave at room temperature for 10 minutes.
8. Transfer the slides into PBS/BSA and incubate at room temperature for 30 minutes.
9. Remove excess of PBS/BSA from around the specimen and cover it with 15 μl primary antibody diluted in PBS/BSA. Place a clean 18x 18-mm nonsiliconized coverslip over the preparation and incubate at 4¼C overnight in a humid chamber.
10. Gently remove the coverslip and wash the slides three times in PBS at room temperature (5 minutes each wash).
11. Remove excess of PBS/BSA from around the specimen and cover it with 15 μl secondary antibody diluted in PBS/BSA. Place a clean 18x 18-mm nonsiliconized coverslip over the preparation and incubate at room temperature for 1 hour in a dark, humid chamber.

12. Gently remove the coverslip and wash the slides three times in PBS at room temperature (5 minutes each wash).
13. Stain the slides with 0.05 g/ml DAPI (in 2x SSC) at room temperature for 5 minutes.
14. Wash slides in PBS at room temperature for 5 minutes.
15. Mount in Vectashield H-1000 (Vector Laboratories) or similar antifading media; seal the edges of the coverslip with either rubber cement or nail polish.

Method 2

Method 2 has been used for immunolocalization of the GAGA (Huang et al. 1998), E(var)3-93D, and Psc proteins (L. Fanti and S. Pimpinelli, unpubl.); the antibodies used were produced by Tsukiyama et al. (1994), Dorn et al. (1993), and Rastelli et al. (1993), respectively. Method 2 is identical to Method 1, with the exception of steps 2 and 3.

For Method 2, note the following changes to the procedure given for Method 1:

In step 2, brains are hypotonically swollen for 10 minutes.

In step 3, brains are immersed in Fixative 2 for approximately 3 minutes and then fragmented using either a syringe or a tungsten needle.

Method 3

This procedure has proven to be suitable for immunolocalization of the Ph protein (the antibody used was produced by DeCamillis et al. [1992]; the immunolocalization experiments are unpublished results of L. Fanti and S. Pimpinelli). Method 3 differs from Method 1 only in steps 2 and 3.

For Method 3, note the following changes to the procedure given for Method 1:

In step 2, brains are hypotonically treated for 10 minutes.

In step 3, brains are immersed in Fixative 3 for 8 minutes and then transferred to a 5- l drop of the same fixative placed on a 18 x 18-mm siliconized coverslip (in this method, brains do not need to be fragmented).

Method 4

This procedure is identical to Method 3 with the omission of the hypotonic treatment (step 2). It has been used by Perrin et al. (1998) to immunolocalize the Modulo protein using antibodies generated by Garzino et al. (1987).

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2

In Situ Hybridization to Somatic Chromosomes

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PARDUE AND GALL (1969; GALL AND PURDUE 1969) PIONEERED the technique of in situ hybridization as a means to visualize and physically map specific sequences on *Drosophila* polytene chromosomes. Hybridization techniques can also be used to localize sequences on smaller, diploid chromosomes, such as condensed mitotic chromosomes (see Pimpinelli et al., this volume). Variations of the method also allow the hybridization of probes to chromosomes within intact cells and tissues, rather than to chromosomes isolated from their cellular context and flattened on slides. This chapter presents methods for hybridizing fluorescent probes to chromosomes in whole-mount *Drosophila* tissues. These methods allow the investigation of nuclear organization even at stages where chromosomes are decondensed (as in interphase) or, for other reasons, cannot be discriminated in the light microscope. Consequently, they are useful for addressing a variety of cell biological questions.

In addition to enhancing our understanding of somatic chromosome organization, this experimental approach has also revealed interactions among meiotic chromosomes in *Drosophila* females, which spend much of meiosis in a compact ball called the karyosome (Dernburg et al. 1996a). Fluorescent in situ hybridization (FISH) methods can also be used to karyotype individual nuclei using chromosome-specific markers (Dernburg et al. 1996b). With appropriate fixation conditions, hybridization to chromosomal DNA can be performed in conjunction with immunostaining, allowing the colocalization of cellular or chromosomal proteins (see, e.g., Franke et al. 1996; Marshall et al. 1996, 1997).

CHOICE OF TISSUE FOR INVESTIGATION OF CHROMOSOMES

The *Drosophila* embryo has provided an excellent model system for a variety of questions in chromosome biology, including investigations of somatic chromosome architecture and chromosome-associated proteins. Its advantages include the ease of obtaining populations of staged specimens (see Sisson, this volume), as well as a variety of techniques for experimental manipulation, fixation, and staining of specific cellular components. The

organization of the late blastoderm embryo, with a monolayer of nearly synchronous nuclei at the surface, greatly facilitates microscopic imaging and analysis. For many reasons, embryos are often the tissue of choice for cell biological studies in Drosophila.

Significant differences, however, have been documented when chromosome architecture in embryos has been compared to that of more ÒtypicalÓ somatic nuclei. In the rapidly dividing blastoderm embryo, chromosomes never have enough time between mitoses to relax from the polarized configuration induced by anaphase chromosome movement. Consequently, they retain a ÒRablÓ configuration throughout interphase (Rabl 1885): In early embryonic nuclei, centromeric regions are clustered at the nuclear pole closest to the embryo surface, with the chromosome arms extending down along the long axis of the nucleus, resulting in a constrained position along this axis for any given locus (Hiraoka et al. 1993; Dernburg et al. 1996c; Marshall et al. 1996). Conversely, nuclei that experience longer interphases, such as those in larval neuroblasts or imaginal tissues, reveal a marked reduction in this polarity, probably as a consequence of chromosome diffusion (Dernburg et al. 1996c; Cenci et al. 1997; Marshall et al. 1997). Somatic pairing between homologous chromosomes only becomes extensive at the end of blastoderm development (Hiraoka et al. 1993; Fung et al. 1998), probably as another consequence of the rapid pace of early cell cycles. Even in nuclei that divide much less frequently, the extent of somatic pairing can be increased by lengthening the cell cycle (Golic and Golic 1996), implying that chromosomes retain the capacity to diffuse within the nucleus and that homologous regions can associate throughout interphase.

These established differences between nuclei at different developmental stages, as well as other stage- or tissue-specific variations yet to be discovered, warrant careful consideration of the experimental approach. Embryo tissues are the most easily isolated, fixed, hybridized, and imaged. They also tend to produce superior ratios of signal to background in hybridization experiments compared to other whole-mount somatic tissues. However, if answers to the questions being addressed may be influenced by developmental stage, then chromosome organization must be examined within the appropriate context.

METHODS FOR PROBE SYNTHESIS AND LABELING

Either single-copy or repetitive DNA elements can be targeted in FISH experiments, but the more abundant the target, the greater the likelihood of a detectable signal. Nevertheless, good detection equipment (see p. 54 [Microscopy and Image Analysis]) and some of the better fluorescent labeling reagents have made it possible to detect probes covering as little as a few kilobases of single-copy genomic DNA. Further improvements in labeling technology may push the sensitivity still further.

Why Use Fluorescence-based Detection?

Compared with other histochemical methods, fluorescent probe detection provides several major advantages for in situ localization of DNA sequences. Fluorescent probes remain physically bound to the site of hybridization, providing optimal spatial localization. The signal-to-background provided is also superior, because the tissue itself does not usually fluoresce strongly. Multiple fluorescent probes can be discriminated in the same sample through the use of specific excitation and emission filters. Fluorescent reagents are stable for long periods if stored frozen and protected from light, and detection is simple and rapid. Microscopy techniques that reduce out-of-focus information allow fluorescent signals to also be localized with great precision within a three-dimensional volume.

FISH probes may be labeled directly with fluorophores, usually by incorporation of fluorescently conjugated nucleotides, or with haptens such as biotin or digoxigenin, which are then detected with fluorescent-binding reagents. Direct labeling is very convenient and tends to produce optimal signal/background staining. In particular cases, fluorescent probes (especially short oligos) will penetrate tissues better than secondary detection reagents such as antibodies or streptavidin.

Hapten-labeled probes coupled with secondary detection, on the other hand, are more economical, given the costliness of some of the better fluorescent dNTPs (deoxynucleoside triphosphates). Hapten labeling also provides versatility, because the same probe ma be detected with different secondary reagents in different experiments. Another small advantage is that the fluorescent reagents are only added toward the end of a hybridiza tion procedure, minimizing photodamage during sample handling. Perhaps most importantly, secondary detection allows for signal amplification, because an antibody or avidin molecule that binds to a single hapten molecule can itself carry multiple fluorophores. This may be particularly helpful when laser illumination is used to excite the sample, as in confocal microscopy, or when the signal will be detected by eye. However, the use of multiple layers of antibodies and/or biotin-avidin reagents to enhance the signal also tends to produce high fluorescent background in whole-mount tissues. For this reason, a single application of secondary detection reagent is usually optimal.

Choice of Fluorophores

A wide spectrum of probe labeling and detection reagents is available through severa commercial vendors. These reagents can be very expensive, and the abundance of optior makes the choice confusing. Outlined in this section are criteria for selecting reagents, anc specific recommendations are presented.

FISH probes may be labeled and detected using a variety of fluorophores. The cyanine dyes Cy2 and Cy3 are more photostable and brighter than their spectral counterparts, fluorescein and rhodamine. Indodicarbocyanine, or Cy5, absorbs red light and emits in the far red, providing an additional region of the spectrum for probe detection. With either laser or mercury arc lamp illumination, Cy3 is the best readily available fluorophore, in terms of brightness and photostability (Wessendorf and Brelje 1992; Wiegant et al. 1996). However, it is not useful for FISH if a red-emitting dye such as propidium iodide is used as a counterstain.

With conventional fluorescence technology, up to three FISH probes plus a counterstain can be distinguished in the same sample, using blue (4«,6-diamidino-2-phenylindole [DAPI]), green (fluorescein/Cy2), red (rhodamine/Cy3/Texas Red), and infrared (Cy5) emission. Combinatorial labeling schemes or other tricks can be used to increase the number of probes that can be used together. For experiments involving multiple probes, some consideration should be given to the relative sensitivities of different reagents, fluors, and detection strategies (see below), so that the smallest target is probed in the most easily detectable way.

Fluorescent nucleotides are available from several commercial suppliers. Fluorescein- and other green-labeled dNTPs have not shown dramatic differences in performance. Cy2 is not yet available directly conjugated to nucleotides. Cy3 is available conjugated to 2-deoxyuridine-5-triphosphate (UTP) and 2-deoxycytidine-5-triphosphate (dCTP) (Amersham). Cy5 is currently the sole option for far red emission, and it is also available as nucleotide conjugates (Amersham). At this time, no blue-emitting fluorophore works reliably for FISH probes. Accordingly, this wavelength range is conveniently reserved for a

counterstain such as DAPI or Hoechst dyes 33258 or 33342. With laser illumination, propidium iodide is frequently used as a counterstain.

Digoxigenin-labeled probes may be detected using fluorescein- and rhodamine-conjugated antidigoxigenin F(ab) fragments (Boehringer Mannheim). Commercially available antibodies that recognize digoxin (Jackson ImmunoResearch), although not marketed for FISH, can also detect digoxigenin-labeled probes and yield excellent signal/background. The latter are available conjugated with Cy2, Cy3, and Cy5, which may provide superior performance in terms of brightness, photostability, and versatility.

Biotin can be detected using fluorescent avidin, modified avidins such as UltraAvidin (Leinco Technologies) or NeutraLite Avidin (Molecular Probes), streptavidin, or antibiotin antibodies. Different secondary reagents may work better in different tissues; unmodified avidin tends to result in higher background than the other choices.

Table 2.1 lists some of the many choices available for labeling and detecting probes in the four most useful wavelength ranges. This is not meant to be a comprehensive list, but to point to some basic reagents and commercial suppliers. It is impossible to provide a recommendation of labeling and detection reagents that are optimal for every situation, because success depends on many parameters, including the labeling method used, autofluorescence spectra of different samples, and microscope illumination sources and fluorescence filters. Moreover, new reagents are constantly becoming available.

Fluorescent nucleotides are the most costly component of in situ hybridization experiments. Unfortunately, only a fraction of the dNTP added to the terminal transferase-labeling reaction is incorporated into the probe; the unincorporated nucleotides are generally discarded after ethanol precipitation. For laboratories carrying out FISH on a large scale, it may be worth the effort to reclaim unincorporated nucleotides by high-performance liquid chromatography (HPLC) purification. Protocols for repurifying Cy3- and Cy5-labeled dNTPs have been developed in Pat BrownÕs lab at Stanford and are available on their Web Site at: http://cmgm.stanford.edu/pbrown/mguide/hplc.html

Probe Synthesis

Good probes for whole-mount FISH must meet two criteria: The DNA fragments must be very small, and they must be highly labeled. A labeling scheme (see Protocols 2.1 and 2.2) that has proven reliable involves fragmenting the probe DNA and then adding a mixture of labeled and unlabeled nucleotides to the 3« ends using the enzyme terminal deoxynucleotidyl transferase (TdT). This method can be used to label a variety of DNA probes, regardless of their initial size (e.g., plasmid, cosmid, or P1 clones, PCR products, or total genomic DNA). Short oligonucleotides may also be labeled in this way without digestion, because their small size allows them to diffuse through thick tissues. A potential advantage of end-labeling is that the modified nucleotides are not incorporated into the complementary probe sequence itself and may thus interfere less with hybridization. The free 3« tail may also make haptens more accessible to detection reagents.

Oligonucleotide Probes to Repetitive Sequences from *Drosophila*

The *Drosophila* genome contains a large number of satellite sequences comprising short, regular, or nearly regular repeats, 5Ð12 bases in length. Many of these satellites have been mapped by hybridization to mitotic chromosomes (Dimitri 1991; Abad et al. 1992; Lohe et al. 1993). Some of these sequences are extremely useful for FISH experiments, e.g., several are specific to particular chromosomes. They also provide excellent FISH targets because of their great abundance and simplicity. Probes to such sequences can be generated as synthetic oligonucleotides, 25Ð35 bases in length, and labeled according to th

Table 2.1. A Useful Subset of Available Labeling and Detection Reagents

	Emission			
Type of label or probe	blue[a]	green	red	infrared
Direct label	Cascade Blue-dUTP (M)	FITC-dUTP (A,B,M,N)	Cy3-dCTP/dUTP (A)	Cy5-dCTP/dUTP (A)
Biotinylated probe (B,G,N)	AMCA-antibiotin (J) AMCA-streptavidin (J) AMCA-NeutraLite avidin (M)	Cy2-antibiotin (J) Cy2-streptavidin (J,A) FITC-UltraAvidin (L)	Cy3-antibiotin (J) Cy3-streptavidin (J,A)	Cy5-antibiotin (J) Cy5-streptavidin (J,A)
Digoxigenin-labeled probe (B)	AMCA-antidigoxigenin (B) AMCA-antidigoxin (J)	FITC-antidigoxigenin (B) Cy2-antidigoxin (J)	Rhod-antidigoxigenin (B) Cy3-antidigoxin (J)	Cy5-antidigoxin (J)
Counterstain	DAPI (S) Hoechst 33242/58 (S)	OliGreen (M)[b]	propidium iodide (S)	

(A) Amersham; (B) Boehringer Mannheim; (G) GIBCO-BRL/Life Technologies; (J) Jackson ImmunoResearch; (L) Leinco Technologies; (M) Molecular Probes; (N) New Life Science Products; (S) Sigma.

[a]UV-excitable, blue-emitting dyes are less easily detected than others and are recommended only for detection of very abundant sequences in experiments involving multiple probes.

[b]There is not currently a well-characterized green fluorescent DNA counterstain that is as generally useful as DAPI, Hoechst, or propidium iodide. OliGreen is marketed as a single-stranded nucleic acid detection reagent, but it has been useful as a DNA counterstain in *Drosophila* tissues. It should be tested at a dilution of 1:1000–1:10,000 from the solution provided. YOYO-1 is marketed as a green DNA counterstain, but it binds to other cellular components in whole-mount tissues.

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Table 2.2. Oligonucleotide Probes to Useful Satellite Sequences

Oligonucleotide sequence	Chromosome(s) arm	Total amount of target	Potential uses	Aliases
$(AATAC)_6$	YL	3.5 Mb	sexing/karyotyping	
$(AATAG)_6$	2L (and Y)	0.2 Mb	2-specific if no Y is present	
$(AACAC)_6$	2R	unknown	2-specific (also C(2)EN)	
$(AATAACATAG)_3$	2L and 3L	1.5–2 Mb per chromosome	autosomal centromeres	1.686 g/cm³ satellite
5«-CCCGTACTGGTCCCGTACTGGTCCCGTACTCGGTCCCGTACTCGGT-3«	3R	unknown	3-specific	dodeca satellite
$\{(AAGAG)_6$ $(AAGAGAG)_5$	all chromosomes predominantly 2, 3, Y	genome contains >10 Mb	bulk heterochromatin, bwD insertion	1.705 g/cm³ satellite
$(AATAT)_6$	4 (and Y)	3.5 Mb on 4 (dispersed)	major signal on 4 if no Y is present	1.672 g/cm³ satellite

method described in Protocol 2.2. Some of the most useful sequences are listed in Table 2.2 and are shown in graphical form in Figure 2.1.

The 359-bp Repeat on the X Chromosome

The proximal heterochromatin of the *Drosophila melanogaster* X chromosome contains a huge number of copies of a 359-base sequence originally known as the 1.688 g/cm³ satellite. This sequence provides an excellent FISH target that is specific to the X chromosome (see Figure 2.4). The repeated sequence can be amplified by polymerase chain reaction (PCR) using genomic DNA as a template with the following primers, which were based on the published sequence (Hsieh and Brutlag 1979): 5«-CGGTCATCAAATAATCATT-TATTTTGC-3« (27-mer) and 5«-CGAAATTTGGAAAAACAGACTCTGC-3« (25-mer). Using *Taq* polymerase and standard PCR buffer containing 2 mM Mg^{++}, 25 cycles with an annealing temperature of 55¼C, and a 30-second extension time at 72¼C should produce a single product of 361 bases. Higher-molecular-weight multimers may also be detected. This PCR product can be effectively digested into fragments suitable for labeling using a single enzyme, either *Alu*I or (slightly better) *Tsp*509I.

Probe Template Amplification by DOP-PCR

It may be useful in some cases to amplify DNA before labeling it. DOP-PCR (degenerate oligonucleotide primed-PCR; Telenius et al. 1992) allows amplification of any complex DNA, regardless of sequence. One example of the technique's value for chromosome hybridization is to generate a probe covering hundreds of kilobases of single-copy sequence. A pool of P1 or YAC (yeast artificial chromosome) clones would be difficult to prepare on a large enough scale, but DNA minipreparations of several P1s or gel slices of one or more YACs could each be amplified and then pooled to make a probe. A more extreme version of this technique is chromosome painting, in which a large regional probe, or even a probe covering an entire chromosome arm, is generated from a minute quantity of DNA microdissected from a polytene chromosome squash. A procedure for microdissection of polytene chromosomes is given in Protocol 2.3.

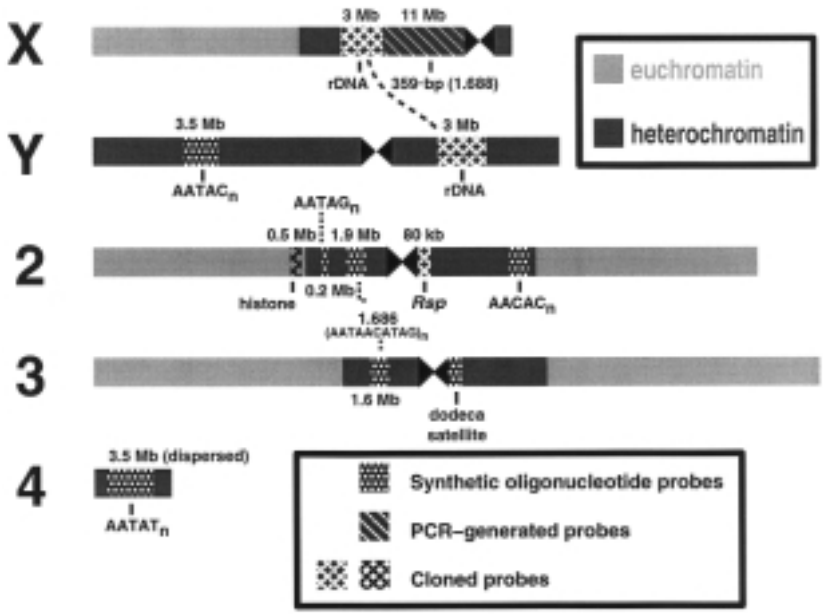

Figure 2.1. Probes to chromosome-specific repetitive sequences in the *Drosophila melanogaster* genome. The five chromosomes are shown schematically, with heterochromatic regions shaded gray and euchromatin in light gray. Probes that localize to only one or two chromosomes are useful tools for cytological studies. For many short, simple satellite repeats within the pericentric heterochromatin, oligonucleotide probes of 30–50 bases in length can be used to mark specific chromosomal targets (see Table 2.2). For the 359-bp repeat on the X chromosome (Hsieh and Brutlag 1979), a single repeat unit can be synthesized by PCR and labeled to make a probe, as described in the text. For some complex sequences such as the 4.8-kb histone gene repeat (Lifton et al. 1978), the 240-bp *Rsp* repeat (Wu et al. 1988), and the ribosomal DNA (28S and 18S subunits), cloned sequences have been used. The positional data in the diagram are adapted primarily from Lohe et al. (1993). The dodeca satellite was discovered and mapped by Villasante and coworkers (Abad et al. 1992; Carmena et al. 1993). The *Rsp* locus was mapped genetically by Brittnacher and Ganetzky (1989) and cytologically by Pimpinelli and Dimitri (1989). The AACAC repeat in 2R heterochromatin was discovered by Zhimulev and coworkers (Makunin et al. 1995). Estimates of the amount of each repeat on the particular chromosome are indicated if available from published work. Certain satellite repeats, including the AATAT and AATAG repeats, are abundant on the Y chromosome, in addition to their unique autosomal positions (Lohe et al. 1993), and are thus primarily useful for studying female tissues. Their distribution on the Y chromosome is not shown here. (Reprinted, with permission, from Dernburg and Sedat 1998.)

DOP-PCR is performed using a single primer that has a fixed sequence at its 5« end and several degenerate bases near its 3« end that allow it to anneal at low stringency at many sites in complex DNA. Following several PCR cycles with an annealing temperature of only 30¼C, the annealing temperature is raised so that only products with the primer sequence at each end are amplified in the reaction. An oligonucleotide that has worked well as a primer for *Drosophila* DNA has a recognition site for the enzyme *Bsm*I, which cuts 3« to its recognition sequence, so that it can be removed following amplification: 5«-CCCAACGATG<u>CGATGC</u>NNNNNCAGG-3« (*Bsm*I recognition sequence is underlined; N indicates an equal mixture of all four bases). This primer has resulted in better probes than the most widely used primer for DOP-PCR (5«-CCCGACTCG-GNNNNNNATGTGG-3«) in direct comparisons.

For DOP-PCR amplification of microdissected templates, extreme caution must be taken to avoid any DNA contamination of reagents or tubes, because only a few picograms

of template will be available. Gloves and barrier tips, along with UV irradiation of the solutions (other than those containing template or nucleotides), may help to ensure specific amplification of the desired template. For primary and secondary DOP-PCR procedures, see Protocols 2.4 and 2.5.

PROTOCOL 2.1*

Restriction Enzyme Fragmentation of Probe DNA

Materials

Supplies and Equipment

Polypropylene microcentrifuge tubes (1.5 ml)
Equipment for agarose gel electrophoresis

Solutions and Reagents

Template DNA
BSA (bovine serum albumin) (10 mg/ml; molecular biology grade; New England Biolabs or other supplier)
DTT (dithiothreitol) (100 mM) aqueous solution, stored frozen in aliquots
Restriction enzymes: AluI, HaeIII, MseI, MspI, RsaI, and Sau3AI (New England Biolabs; order at high concentration where available)
Sodium acetate (3 M, pH 5.2)
Ethanol (cold 70% and absolute)
Buffers and reagents for agarose gel electrophoresis

5x 4BC buffer

40 mM Tris-Cl (pH 7.5)
250 mM NaCl
40 mM $MgCl_2$

TE«

10 mM Tris-Cl (pH 7.5)
0.1 mM EDTA

CAUTION: DTT, ethanol, $MgCl_2$ (see Appendix 4)

Method

1. Set up the following reaction in a 1.5-ml polypropylene microcentrifuge tube:

Probe DNA	25 g
5x 4BC buffer	50 l
10 mg/ml BSA	2.5 l
100 mM DTT	2.5 l
H_2O (see Note below)	to ~225 l

Note: The final reaction volume should be 250 l. Adjust volume of H_2O depending on the volume of enzymes required (step 2, below). This reaction is for 25 g of DNA and may be scaled up or down, as desired.

*For all protocols, H_2O indicates glass distilled and deionized.

2. Add 25 units of each restriction enzyme (add all six enzymes as in a multiple digest). Mix and incubate at 37¼C from 2 hours to overnight.
3. Precipitate the digested products as follows:
   a. Add 1/10 volume of 3 M sodium acetate and then 2.5 volumes of cold absolute ethanol. Optional: Chill briefly at Ð20¼C.
   b. Centrifuge in a microcentrifuge at 12,000 g for 15 minutes to pellet the DNA.
   c. Wash DNA pellet with cold 70% ethanol, allow to dry briefly, and then resuspend DNA in TE« to a final concentration of 1 g/ l.
4. Analyze 0.5 g (2%) of the products by electrophoresis through a 2% agarose gel.

   Note: Depending on the complexity of the DNA, either a discrete set or a smear of fragments with an average size of 100Ð150 bp should be detected.
5. Measure the concentration accurately by UV absorption.

PROTOCOL 2.2

Labeling with Terminal Deoxynucleotidyl Transferase

This procedure labels 10 g of probe DNA, enough for 20Ð500 FISH samples, depending on the length and complexity of the target sequence. It can easily be scaled down and carried out in a smaller volume.

Materials

Supplies and Equipment

Microcentrifuge tubes (1.5 ml) (amber microcentrifuge tubes are useful for labeling and storage of fluorescent probes)
(Optional) Sephadex G-25 spin column
Water bath at 95¼C

Solutions and Reagents

5x TdT reaction buffer (Promega)
Labeled and unlabeled dNTPs
TdT (Promega)
Glycogen (20 mg/ml) (molecular biology grade; Boehringer Mannheim or other supplier)
Ammonium acetate (4 M)
Ethanol (cold 75% and absolute)
TE (pH 7.5; see Appendix 3)

CAUTION: ammonium acetate, ethanol (see Appendix 4)

Method

1. Place a 1.5-ml microcentrifuge tube on ice. Add 10 g of DNA fragments in TE« (from step 3c in Protocol 2.1). Heat the tube in a 95¼C water bath for 2 minutes to denature the DNA. Chill rapidly on ice and spin briefly to collect condensate.

2. Add the following to the denatured DNA:

5x TdT labeling buffer	20 l
unlabeled dNTP (whichever the labeled nucleotide analog is derived from, i.e., use dTTP if incorporating a modified dUTP)	13.5 nmoles
labeled dNTP (usually supplied as a 1 m stock solution)	6.75 nmoles (6 l 75 of a 1 m stock solution)
H_2O	adjust volume to 97 l

3. Add 60 units of TdT and incubate at 37¼C for 1 hour.
4. Add 2 l of 20 mg/ml glycogen to act as a carrier.
5. (Optional) Transfer the probe to a 1-ml Sephadex G-25 spin column (or a commercial version) and centrifuge to remove unincorporated nucleotides.

   Note: This may help to reduce background staining (reports vary) but will also result in loss of some precious probe. Spin column purification should not be used when labeling oligonucleotide probes.

6. Precipitate the probe as follows:
   a. Add ammonium acetate to 2 followed by 2.5 volumes of absolute ethanol. Chill at 4¼C.

      Note: Sodium acetate is not used here to minimize the coprecipitation of unincorporated nucleotides.

   b. Centrifuge in a microcentrifuge at 12,000 for 15 minutes to pellet the DNA.

      Note: Probes labeled with fluorescent nucleotides should give a visibly colored pellet.

   c. Aspirate the ethanol carefully, wash the pellet with cold 75% ethanol, and dry briefly.
7. Resuspend in TE. Store at Ð20¼C.

   Note: It is convenient to store probes at a concentration of 50Ð200 ng/l. Probes are stored in TE, but made up during the hybridization procedure in hybridization buffer (see p. 47).

PROTOCOL 2.3

Microdissection of Polytene Chromosomes for DOP-PCR

Materials

Supplies and Equipment

Razor blade

Dissection needles (1-mm glass capillary tubes; one needle will be required per chromosome) (World Precision Instruments 1B100F-4)

Needle puller

Plastic dish containing a strip of double-sided tape or modeling clay for holding needles

Stratalinker or other short-wave UV source

PCR tubes (0.5 ml)

Specialized equipment for microdissection:

- an inverted microscope (preferably equipped with a freely rotating stage)
- microinjection/micromanipulation apparatus
- good-quality phase lens (10–20×) with a long working-distance, to observe chromosomes through the glass slide

Water bath at 95¼C

Solutions and Reagents

Template DNA
Liquid nitrogen
TE (see Appendix 3)
Ethanol (95%)

CAUTION: ethanol, liquid nitrogen, UV radiation (see Appendix 4)

Method

1. Prepare squashes of salivary glands from female third-instar larvae (raised on a rich diet under uncrowded conditions). Use lactic-acetic acid without orcein or other DNA dyes (see Pimpinelli et al., this volume [Protocol 1.2]).

   Note: The ideal squash will have chromosome arms extended with long straight regions that are well separated from each other. This can be achieved by using a slightly greater volume of liquid and/or more pressure than normal in the spreading step of the squash. One good slide can provide the material needed for a large number of dissections.

2. Freeze the slides in liquid nitrogen. Immediately crack the coverslips using a new razor blade as a wedge at one corner and transfer to 95% ethanol. After at least 10 minutes, remove slides and allow to air dry.

   Notes: This step minimizes exposure to acid, which can depurinate the template.
   Slides dipped in liquid nitrogen can crack or splinter. Wear appropriate eye protection.

3. Prepare dissection needles. Wear gloves to avoid contaminating the glass needles. Pull each capillary tube into two needles using any program for microinjection needles on a needle puller (the ends are broken off later, so the exact profile of the needle is not critical).

4. Break off the tip of each needle under a dissecting microscope. Make a clean, even break by drawing the tip of one needle along a broader portion of another needle held perpendicularly, with light and even pressure. With practice, most needles break cleanly to leave a smooth circular edge. Discard any needles with jagged edges. Place the needles in the plastic dish containing a strip of double-sided tape or modeling clay. Irradiate needles by placing the dish in a Stratalinker (or other short-wave UV source) for 10 minutes at full power.

5. Pipette 20 l of TE into each of several clean PCR tubes. Irradiate the tubes for 10 minutes with UV light.

6. Get ready to dissect! Turn on the microdissection equipment.

7. Place a clean needle into the needle holder of the microdissection apparatus and mount over the stage. Adjust the apparatus to give maximum range of motion with-

in the visual field of the microscope. Turn the back pressure on the microinjector to a positive but low value (4Ð6 psi). Mount a slide on the stage, chromosome-side up, and use clips or tape to hold it to the stage securely.

8. Focus the objective and move the stage until a target chromosome is within view (Figure 2.2A). Choosing a specific target requires the ability to recognize the banded pattern of the polytene chromosomes under phase optics without stains such as orcein. To test the procedure, an arbitrary chromosome arm may be chosen.

9. Focus on a plane above the slide, and slowly lower the needle and move it laterally until its tip is in focus. Lower the focus again to see the chromosomes (Figure 2.2B).

10. At a low-speed setting on the micromanipulator, lower the tip of the needle carefully onto the chromosome. Then scrape along the chromosome arm, essentially using the edge of the needle as a bulldozer, moving it laterally while holding it against the slide (Figure 2.2C). As the needle pushes against the chromosome, it will lift off the slide and bunch up into a ball of material. When the end of the chromosome (or the desired region) is reached, continue to move the needle laterally very slowly while lifting it with the micromanipulator; the microdissected material will usually stick to it. Lift the needle well away from the stage.

11. Very carefully, move a tube containing 20 of UV-irradiated TE (from step 5, above) over the needle so that the tip of the needle is immersed in the liquid. Raise the back-pressure on the injector to about 30Ð50 psi. Still holding the plastic tube over the needle with one hand, remove the needle holder from its mount with the other hand. Break off the tip of the needle against the wall of the tube while still holding it immersed in the solution. Gas should start to bubble through the liquid. Withdraw the rest of the needle, trying to avoid removing any liquid from the tube. Set the needle down, cap the tube, label it appropriately, and discard the needle. Turn the injector back pressure down again when this is completed. Repeat the above procedure (steps 7Ð11) for as many chromosomes as desired. Transfer only one dissected chromosome/region per tube; successfully amplified samples may be pooled after the PCR.

12. Heat the tubes at 95¼C in a water bath for 5 minutes.

PROTOCOL 2.3 NOTES

If the air in the microdissection room is dry, dissected chromosome pieces may fly away instead of sticking to the glass dissection needles. The environment may be humidified by heating an open water bath in the room to 80Ð90¼C.

PROTOCOL 2.4

Primary DOP-PCR Amplification

The following amplification procedure may be performed on microdissected material, small volumes of melted gel slice (1Ð5), or small quantities of other DNA templates. Always perform a negative control with no added template.

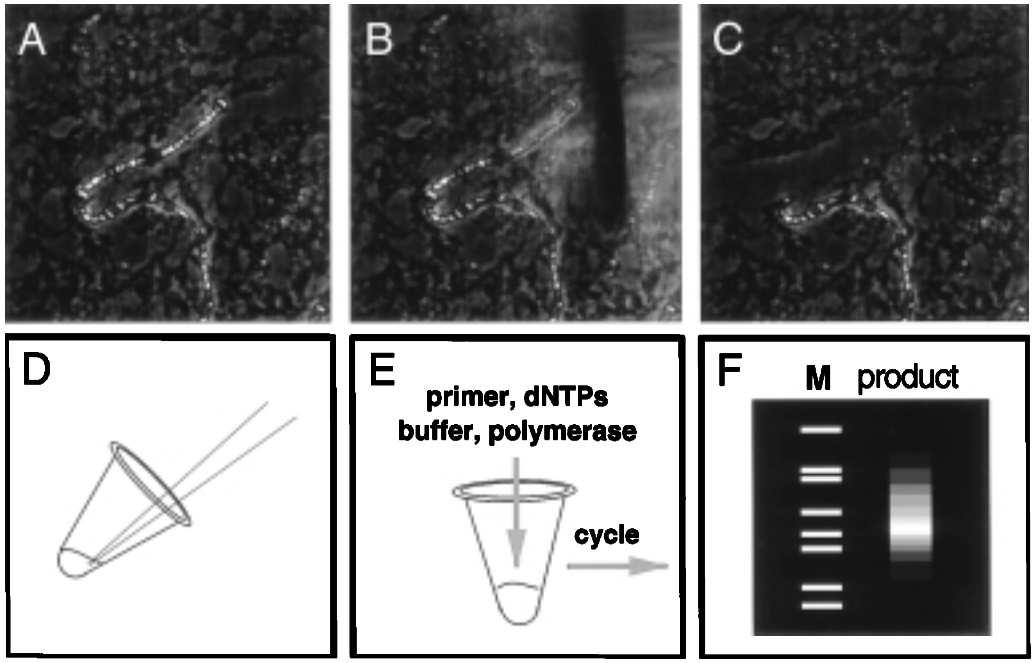

Figure 2.2. Synthesis of chromosome painting probes by microdissection and DOP-PCR. This figure illustrates the steps described in the text for polytene chromosome microdissection and DOP-PCR amplification to generate large regional probes. (A) A portion of a squashed, unstained polytene chromosome arm 2R, visualized with phase optics. (B) The broad tip of a microdissection needle is brought down onto the slide at a position near the end of the chromosome arm. (C) Microdissection has been carried out by dragging the needle from right to left across the chromosome, then lifting it off the slide; the missing distal region of the chromosome arm is now stuck to the tip of the needle. This tiny quantity of DNA (on the order of 1–10 pg) is then transferred to a small volume of liquid in a 0.5-ml microcentrifuge tube. (D) Other components required for DOP-PCR are added, (E) and the amplification is carried out as described in the text. Following a successful amplification, the products can be visualized by gel electrophoresis (F) and will normally comprise a distribution of fragment sizes.

Materials

Supplies and Equipment

PCR tubes (0.5 ml)
Thermal cycler
Equipment for agarose-gel electrophoresis

Solutions and Reagents

Template DNA
$MgCl_2$ (50 mM)
BsmI primer (10 μM stock): 5-CCCAACGATGCGAATGCNNNNNCAGG-3
dNTPs (2 mM each dATP, dCTP, dGTP, and dTTP)
Taq DNA polymerase (10 units/ml)
(Optional) Mineral oil
DNase 1 (1 μg/ml)
Buffers and reagents for agarose-gel electrophoresis

10x DOP-PCR Buffer (Meltzer et al. 1992)

100 mM Tris-Cl (pH 8.3)
500 mM KCl
1 mg/ml gelatin (molecular biology grade)

10x DNase Buffer

0.5 M Tris-Cl (pH 7.5)
0.1 M $MgSO_4$
1 mM dithiothreitol (DTT)
500 g/ml bovine serum albumin (BSA)
Store in aliquots at Ð20¼C.

CAUTION: DTT, KCl, $MgCl_2$, $MgSO_4$ (see Appendix 4)

Method

1. To each tube containing microdissected material (from step 12, Protocol 2.3) or 10 pg to 5 ng DNA template, or no template, add:

10x DOP-PCR buffer	5 l
50 mM $MgCl_2$	2.5 l
BsmI primer (10 M stock)	10 l
2 mM dNTPs	5 l
H_2O	to 48 l
2.5 units Taq DNA polymerase (DNase treated, see Notes below)	l 2

Notes: Overlay PCRs with mineral oil or use a thermal cycler with a heated lid.

DNase treatment is essential to degrade DNA that may contaminate commercial preparations of Taq DNA polymerase. It is convenient to perform this reaction in a PCR tube and the incubations in a thermal cycler. This recipe provides enough enzyme for five PCR samples; scale up or down as needed.

Place a 0.5-ml PCR tube on ice. Add the following to the tube:

H_2O	6.6 l
Taq DNA polymerase (5 units/l)	3 l
10x DNase buffer	1.2 l
1 g/ml DNase	1.2 l

Incubate at 37¼C for 15 minutes. Heat at 90¼C for 10 minutes to inactivate the DNase

2. Perform thermal cycling to amplify the DNA using the following program:

93¼C for 4 minutes

3 cycles:
94¼C for 30 seconds
30¼C for 1 minute

Ramp to 72¼C over 5 minutes (ramp = 7.2 seconds/¼C or 84¼C/minute) and hold second.

3 cycles:
94¼C for 30 seconds
30¼C for 1 minute
72¼C (no ramp) for 2 minutes

36 cycles:
94¼C for 20 seconds
56¼C for 1 minute
72¼C for 2 minutes

Extension cycle at 72¼C for 10 minutes.

3. Electrophorese 2 l of each reaction in DNA sample buffer through a 1.4% agarose/TBE gel. Compare samples with pBR322 digested with Msp I or other appropriate size markers.

Notes: There should be a smear of fragments in each lane from reactions including template DNA. Do not be alarmed if there is also a significant background smear in the control lane (minus template), particularly if the lanes with template look brighter or the fragment size distribution is different (usually longer). If the template is relatively small (e.g., a P1 or a YAC), a discrete banding pattern may be seen, but from microdissected chromosome templates, the mixture of products is usually too complex to see discrete products. In fact, a banding pattern could be an indication of contamination, particularly if the bands are consistent among different lanes.

No amplification at all indicates a problem. If the negative control lane looks identical to the samples with template, pick just one or two of the samples to test as probes, because this may indicate contamination with extraneous DNA in a solution or in the handling procedure.

4. Store the reaction mixture at Ð20¼C.

Notes: It is not necessary to remove oil.

A successful primary amplification provides a nearly inexhaustible source of probe. All of the reaction products now have the primer sequence at each end, so they are simply amplified by a normal, high-stringency PCR using the same primer. In this procedure, contamination is less of a concern, but it is always a good idea to be scrupulously clean when performing the PCR. To generate a probe, simply reamplify a tiny amount (0.5) using Protocol 2.5.

PROTOCOL 2.5

Secondary DOP-PCR Amplification

Materials

Supplies and Equipment

PCR tubes (0.5 ml)
Thermal cycler
Microcon-30 columns (500-l capacity; Amicon)

Solutions and Reagents

10x DOP-PCR buffer (see p. 38)
$MgCl_2$ (50 mM)
dNTPs (1.25 mM each dATP, dCTP, dGTP, and dTTP)
Bsm I primer (10 M stock)
Taq DNA polymerase (10 units/)
(Optional) mineral oil
(Optional) NEB Buffer 2
(Optional) Bsm I enzyme (New England Biolabs, 5 units/)
(Optional) BSA (bovine serum albumin; 10 mg/ml; molecular biology grade; New England Biolabs or other supplier)

(Optional) EDTA (500 mM)
TE (see Appendix 3)

CAUTION: $MgCl_2$ (see Appendix 4)

Method

1. To a PCR tube, add the following:

H_2O	22.5 l
10x DOP-PCR buffer	5 l
50 mM $MgCl_2$	2 l
1.25 mM dNTPs	8 l
BsmI primer	11.5 l
template (primary amplification mixture from step 4, Protocol 4)	0.5 l
Taq DNA polymerase (DNase treatment not necessary)	0.5 l

2. Perform thermal cycling using the following program:

   93¼C for 4 minutes

   16 cycles:
   - 94¼C for 30 seconds
   - 56¼C for 1 minute
   - 72¼C for 2 minutes

   Extension cycle at 72¼C for 10 minutes.

3. (Optional) Perform enzymatic digestion with BsmI enzyme to cleave the ends as follows:

   Note: If the BsmI primer was used for DOP-PCR, the primer sequences can be removed by digestion of the products with BsmI restriction enzyme. This may be useful because it removes the non-specific sequences that do not contribute to probe specificity, but no experimental comparison has been performed.

   a. Without removing the oil, add:

NEB Buffer 2	8 l
10 mg/ml BSA	1 l
H_2O	40 l
BsmI enzyme (5 units/l; New England Biolabs)	3 l

   b. If oil was used in the PCR, centrifuge the tube briefly to mix the aqueous phases. Incubate at 65¼C for 90 minutes.

   c. Add 1 l of 500 mM EDTA to stop the reaction.

4. Purify products as follows:

   a. If an oil overlay was used, remove the oil.

   b. Remove primers, cleaved ends, and other small molecules by centrifuging the sample through a Microcon-30 filter: Pipette the oil-free PCR into the upper chamber of a Microcon-30 filter. Add TE to 450 l. Centrifuge according to the manufacturerÕs instructions (14,000 g for ~12 minutes). Add 450 l of TE and centrifuge again. Collect retentate. If residual volume is very small (<10 l), pipette 10Ð20 l of TE onto the filter, mix by tapping, invert, and place into collection tube. Centrifuge again to pool this rinse with the retentate.

5. Determine the DNA concentration by measuring UV absorbance before proceeding with probe synthesis.
6. Cut and label the PCR products as described in Protocols 2.1 and 2.2 (above) to generate a probe.

FIXATION METHODS

The fixation step is absolutely critical for success with whole-mount in situ hybridization, as defined by two criteria: (1) reliable detection of a specific signal and (2) preservation of the nuclear and tissue structure to the degree required by the experiment. Probe molecules must be able to diffuse through the tissue, so the tissue must be somewhat permeable. At the same time, the sample must be able to withstand the high salt, heat, and formamide required for hybridization without undergoing large-scale structural changes. How this is accomplished is the least generalizable facet of FISH experiments, because various tissues respond differently to identical fixation conditions.

In general, fixation of tissues with moderate concentrations (1.5Ð5%) of formaldehyde provides the best combination of structural preservation and accessibility to hybridization. Ideally, fixation is carried out in an aqueous medium, preferably in an isotonic buffer (e.g., RingerÕs solution or other balanced salts). However, the signal/background can often be increased at the expense of morphology by fixing with a mixture of acetic acid and alcohol, or alcohol alone. In special cases, formaldehyde fixation may not be compatible with other experimental goals (e.g., preservation of cytoskeletal elements) and other fixatives may be substituted. Postfixation with formaldehyde may help to preserve a sample fixed initially with alcohol or acid/alcohol. Glutaraldehyde is an unsuitable fixative as it increases the background fluorescence and prohibits hybridization.

With certain tissues, additional steps will be required for permeability. Such treatments might include incubation with nonionic detergents or dissection to remove physical barriers. Modifications that have proven to be useful with particular tissues include (1) warming the fixative solution to 30Ð37¼C before addition to the sample; (2) fixing at low temperature, typically 0Ð4¼C for 1 hour or more; (3) reducing the formaldehyde concentration to 1Ð2%; and (4) including both 0.1% deoxycholate and 0.1% Triton X-100 in the dissection buffer. Any of these may affect chromosome preservation, however.

Fixation conditions that are compatible with immunodetection are often a good starting point for FISH. Both procedures may even be performed sequentially on the same sample. By combining immunofluorescence and FISH, interactions between chromosomal regions and other components of nuclear architecture may be examined. Immunostaining with antibodies to the nuclear lamina or to nuclear pore proteins is also a good way to delineate the nuclear periphery (Dernburg et al. 1996c; Gotta et al. 1996; Marshall et al. 1996). In general, it is simplest to perform immunostaining steps following FISH. Surprisingly, however, antibodies bound prior to the hybridization can also be detected. Most antibodies show good reactivity after hybridization, although the epitopes recognized by certain monoclonal antibodies may be altered. Soluble proteins may also be extracted to some degree during the hybridization procedure.

Embryos

For *Drosophila* embryos, the best method to use is also the most common fixation procedure for immunostaining, as described by Rothwell and Sullivan (this volume; see Protocol 9.3). Briefly, after bleach dechorionation, transfer embryos to a test tube or vial

containing 3.7% formaldehyde (freshly prepared from paraformaldehyde) in buffer under an equal volume of heptane. Agitate for 10 minutes to keep the phases mixed, then transfer embryos to methanol/EGTA (ethyleneglycol-bis-a[minoethylether]N,N,N ,N - tetraacetic acid) equilibrated on dry ice. After several minutes on dry ice, heat-shock to remove the vitelline membrane. Once the embryos settle, immediately begin to rehydrate the embryos through a methanol/H_2O series (9:1, 7:3, 5:5, 3:7, 1:9, 5Ð10 minutes each) followed by 100% buffer. (Embryos left for an extended time in methanol become overextracted and float instead of sinking during the hybridization procedure.)

There are at least two reasons that other embryo fixation procedures might be used, however: (1) Cytoskeletal elements, particularly microtubules, are not preserved well by the method above and (2) mutations affecting the chemistry of the vitelline membrane may preclude successful devitellinization. In either case, a one-step fixation/devitellinization in methanol/EGTA solution may be acceptable, although the nuclear morphology is not as well stabilized by this procedure.

Egg Chambers

Drosophila egg chambers are more challenging to fix than embryos. They are easily overfixed such that probes will not penetrate well into the oocyte nucleus, even if some hybridization is detected in the follicle and nurse cells closer to the surface. If late-stage oocytes are to be examined, the tissue must also be protected against osmotic shock before and during fixation, because this will activate metaphase-arrested nuclei to undergo the metaphase-anaphase transition. The large-scale preparatory method described by Matthies et al. (this volume) for immunostaining may be used, but reduce the formaldehyde solution to 3.7% for younger egg chambers and 5Ð8% to optimize hybridization to older (stage 10 and above) chambers. Warming the buffered formaldehyde to 30¼C immediately prior to fixation tends to enhance probe penetration. In contrast to reported results with immunostaining, FISH is equally successful whether fixation is performed with freshly prepared formaldehyde or commercial formaldehyde solutions.

Manual dissection of fixed ovarioles gives a much higher yield per adult than the blender technique (facilitating analysis of mutants), and younger egg chambers in particular will be enriched, although the procedure itself is more laborious. A procedure for manual dissection and fixation of egg chambers is given in Protocol 2.6.

Other Tissues

Protocol 2.7 should serve as a starting point for other tissues and is a general approach to fixing tissue or cells onto microscope slides in a manner compatible with whole-mount FISH analysis. The procedure includes a simple formaldehyde fixation in buffer, followed by postfixation in cold ethanol. Postfixation of the tissue in methanol or ethanol can markedly improve permeability. In some instances, it may be preferable to fix tissue onto coverslips, because microscope optics are usually designed to optimize imaging immediately adjacent to the coverslip. On the other hand, coverslips are much more fragile and harder to manipulate.

To evaluate the success of a particular fixation procedure, the hybridized sample should be compared to identically fixed tissue that has not undergone FISH. In particular, the appearance of condensed mitotic or meiotic chromosomes with and without hybridization can provide a useful criterion for deciding whether preservation is adequate (Figure 2.3). Underfixation will result in deterioration of morphology; overfixation will

lead to weak or undetectable FISH signals and high fluorescent background. With some tissues, the middle ground may be a challenge to find. The ultimate goal is to maintain the organization found in the living state. However, this requirement can be relaxed somewhat if the question being asked does not address chromosome organization, e.g., if only the presence or absence of particular sequences is being assayed. As with any cytologi procedure, consideration should be given to ensuring that results do not represent fixation artifacts. When different fixation procedures converge to yield the same answer, confidence in the experimental results is enhanced.

PROTOCOL 2.6

Manual Dissection and Fixation of Egg Chambers

Materials

Supplies and Equipment

Dissecting tools

- dissecting surface; e.g., a silicone rubber dissecting pad made with Sylgard silicone elastome (Dow Corning) cured in a large petri plate, or a siliconized multiwell slide (see below)
- fine dissecting forceps, such as DuMont et Fils #5 (Roboz)
- stereo dissecting microscope

Pasteur pipette

Polypropylene tube(s) (0.5 ml), such as those used for PCR

Preparation of Siliconized Glass Slides and Coverslips

Immerse slides or coverslips briefly in a 1–2% solution of Surfasil (Pierce) in chloroform. Rinse once with chloroform and allow to air dry. Store at room temperature.

Solutions and Reagents

Ether or CO_2

1x Modified Robb's saline (MRS) (see Matthies et al. [Protocol 4.1])

Cacodylate Fixative Buffer (see Matthies et al. [Protocol 4.1])

2x SSCT

0.3 M NaCl
0.03 M sodium citrate
0.1% Tween-20

Preparation of 37% Formaldehyde

To a 100-ml Pyrex screw-cap tube, add 1.85 g of EM grade paraformaldehyde (Polysciences) and 3.5 ml of H_2O. Cap loosely and place in a boiling water bath.

Add 90 l of 1 N NaOH and agitate for approximately 1 minute. The solution should become nearly clear. Cool by running the bottom of the tube under a stream of tap water. Pass the solution through a nonsterile 0.22-m syringe filter into a clean screw-cap airtight vial. Total volume is ~5 ml. Use the same day.

CAUTION: cacodylate, chloroform, CO_2, ethanol, ether, NaOH, paraformaldehyde (see Appendix 4)

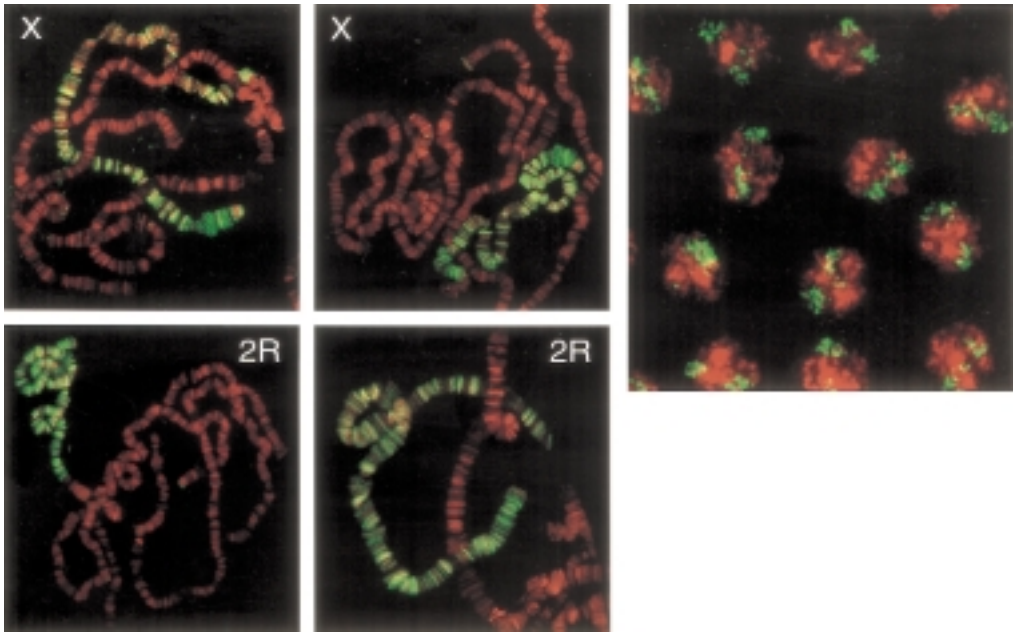

Figure 2.3. Chromosome painting probes on polytene and embryonic chromosomes. These images show different regional probes generated by microdissection and DOP-PCR (panels at left). Two different probes, one to the X chromosome and one 2R, are hybridized to polytene chromosome squashes to assess their coverage and specificity. (Green) Hybridization signal; (red) DNA counterstain (DAPI). These successful probes give very good coverage of complete or nearly complete euchromatic arms. Very limited cross-hybridization is detected on other chromosome arms, mostly in telomeric regions. (Right) Whole-arm probe (here 3L) hybridized in situ to a whole-mount blastoderm embryo. In most of the nuclei, two separate large signals are detected, representing the separate domains occupied by the two homologous arms. In one nucleus, the homologous signals are either immediately adjacent to each other or interspersed with each other. At later nuclear cycles, homologous pairing is much more extensive (Hiraoka et al. 1993), and the two homologous signals are rarely separate.

Method

1. Anesthetize four to eight females with carbon dioxide or ether. Transfer to a 100-drop of MRS on a silicone rubber dissecting plate or siliconized slide.
2. Rupture the abdomens with forceps, and transfer the intact ovaries to a fresh 100-drop of MRS.
3. When all the ovaries have been dissected, transfer them to a 100-drop of fixative (5% formaldehyde made by diluting freshly prepared 37% solution in cacodylate buffer, prewarmed to 37¼C) on the dissecting surface. Start a timer set for 4 minutes.
4. During the fixation (4 minutes), begin to separate individual ovarioles by teasing them apart with forceps.
5. At the end of the fixation period, transfer the ovaries individually to a large drop of 2x SSCT. Continue separating the ovaries into individual ovarioles. This process will take several minutes.
6. Use a pasteur pipette or forceps to transfer the ovarioles into a 0.5-ml tube containing 2x SSCT. Wash with two changes of 2x SSCT.

7. Repeat steps 1Ð6 until sufficient material for hybridization has been obtained. Proceed with hybridization as described in Protocol 2.8.

PROTOCOL 2.7

Formaldehyde Fixation onto Slides for Whole-mount FISH

Materials

Supplies and Equipment

Dissecting tools

dissecting surface; e.g., a surface made with Sylgard silicone elastomer (Dow Corning), a siliconized multiwell slide, or siliconized coverslips (see p. 43)
fine dissecting forceps, such as DuMont et Fils #5 (Roboz)
scalpel or razor blade
stereo dissecting microscope

Siliconized coverslips (18×18 mm; see p. 43)

Glass slides. For some tissues, it is helpful to first treat slides with agents that improve adhesion, including polylysine solutions, aminoalkylsilane, and gelatin "subbing" solutions. Commercially available treated slides such as Superfrost Plus (Fisher) may also be useful.

Whatman #3 filter paper

Hemostats, the tips covered with short pieces of silicone rubber tubing, for handling slides

Solutions and Reagents

Dissecting buffer (typically a buffered saline solution such as RingerÕs or phosphate-buffered saline [PBS]; see Appendix 3)

Formaldehyde (37%) prepared fresh from paraformaldehyde

Liquid nitrogen in a Dewar flask, or an aluminum block dry ice, to freeze slides

Ethanol (95%), chilled to Ð20¼C in a Coplin jar or other slide-staining container

2x SSCT (see p. 43)

CAUTION: aminoalkylsilane, dry ice, ethanol, formaldehyde, liquid nitrogen, silane (see Appendix 4)

Method

1. On a dissecting surface or siliconized glass coverslip, dissect or otherwise isolate th desired tissue in a suitable buffer, e.g., RingerÕs solution or PBS. Transfer the tissue a 20Ð30-l drop of buffer on a siliconized coverslip and remove large contaminants with forceps.
2. Add an equal volume of x2 formaldehyde in dissecting buffer (typically 3Ð8% formaldehyde made by diluting freshly prepared 37% solution into buffer).
3. Remove all but 10Ð20 of the fixative by careful pipetting.
4. Touch the center of a glass slide to the drop of fixative solution and invert carefully to sandwich the tissue between the slide and coverslip.

5. Wick away excess buffer with pieces of absorbent paper, e.g., Whatman #3 filter paper.

   Note: The aim of this step is to press the tissue gently against the slide, but not to flatten it.

6. Fix for a total period of 5Ð10 minutes.

7. Immerse the slide and coverslip into liquid nitrogen, or place on a flat aluminium block on dry ice, to freeze the sample. Once frozen, crack the coverslip using a new single-edged razor blade, and quickly transfer the slide to a staining jar containing 95% ethanol at Ð20¼C. Store in ethanol at Ð20¼C if hybridization is not performed immediately.

8. Repeat steps 1Ð7 until sufficient material has been obtained. When sufficient samples have been prepared, allow the ethanol to rise to above 0¼C (i.e., until frost on outside of container melts). Transfer samples to a container filled with 0.5 xSSCT. Proceed with hybridization as described in Protocol 2.9.

HYBRIDIZATION METHODS

The general strategy for FISH is to equilibrate the tissue in buffered formamide, to add the probe(s), and to denature both the chromosomal DNA and probe together by heat treatment. The probe is then allowed to anneal for several hours at an appropriate temperature, unbound probe is washed away, and secondary detection (if required) is performed. The nuclear DNA is then usually counterstained, and the sample is mounted for microscopy. For experiments in which immunolocalization of other cellular components is desired, these staining steps can conveniently be performed after hybridization.

The key strategic decision that must be made is whether the tissue can be handled in suspension or whether it would be better fixed to microscope slides. Procedures for hybridizing tissues in suspension and on microscope slides are given.

Hybridization to Tissues in Suspension

Samples such as *Drosophila* embryos or egg chambers are easily manipulated in suspension in 0.5-ml PCR tubes (Protocol 2.8), in which solutions are changed by allowing the tissue to settle to the bottom of the tube, aspirating the liquid carefully, and replacing with 400Ð500 l of the new solution. This is by far the most convenient way to perform the hybridization and requires a minimum volume for all washing steps. The denaturation step is also easy to perform consistently, because the sample can be heated and annea in a thermal cycler or conventional water bath.

Hybridization to Tissues on Microscope Slides

Other tissues may be too large, delicate, transparent, buoyant, or have other physical properties that make fixation on slides a more suitable approach. If the tissue is not amenable to handling in suspension, it can be fixed directly onto a microscope slide or coverslip, which is then carried through the procedure in Coplin jars, larger staining jars, or coverslip-staining jars (Thomas Scientific). This procedure has been useful for spermatocytes, spermatids, and imaginal disks (see Protocol 2.9). Coplin jars require 50Ð60 ml of solution to cover the sample, larger staining jars require 200 ml, and coverslip-staining jars hold about 7Ð10 ml. Following hybridization, staining with antibodies or other detection

reagents can be performed in a humid chamber. Pipette 25–50 staining solution directly on to the tissue and cover with a cut piece of Parafilm. Wash in Coplin or staining jars, allowing the Parafilm to float away from the glass.

PROTOCOL 2.8

Hybridization to Tissue in Liquid Suspension

Materials

Supplies and Equipment

Thermal cycler or other means of heating tubes to 91¼C and incubating at 37¼C
Aspirator with fine tip and liquid trap
Glass slides
Coverslips (22 x 22 mm)

Solutions and Reagents

Fixed material in standard or thin-walled 0.5-ml polypropylene tubes (Protocol 2.6)
2x SSCT (see p. 43)
20x SSC
Tween-20 (a 10% v/v stock)
2x SSCT with increasing formamide concentrations is made from 20x SSC, 10% Tween-20, and formamide in H_2O
(Optional) RNase A (10 mg/ml stock in H_2O; used at a final concentration of 10–100 g/ml)
Formamide (Fluka 47670)
Labeled probe(s)
Reagents for immunostaining and secondary detection
- primary antibody
- 0.5% protein blocking solution
- fluorescent detection reagents, e.g., fluorescently labeled streptavidin, antibiotin, or antidigoxigenin antibodies (see Table 2.1)

Mounting medium. Mounting solutions for fluorescence microscopy are typically buffered 90% glycerol solutions containing an antifading agent such as DABCO (1,4-diazabicyclo-[2,2,2]-octane), PPD (p-phenylenediamine), or NPG (n-propyl gallate). A commercially available mounting medium such as VectaShield (Vector) could be used. For preparation of glycerol/NPG, see below.
Nail polish

Preparation of 1.1x Hybridization Solution

Add the following components to a 15-ml conical plastic tube with volume markings:

dextran sulfate	1 g
20x SSC (see Appendix 3)	1.5 ml
formamide	5 ml

Make up the final volume to 9.0 ml with H_2O. Dextran sulfate will take several hours to dissolve at room temperature; the hybridization solution may be stored for months at 4¼C.

Preparation of Glycerol/NPG

To make an inexpensive all-purpose mounting medium, dissolve 4% NPG (w/v) in high-quality glycerol. Agitate for several hours. The solution may be stored at room temperature indefinitely.

Adjust the pH by adding 70 l of 2 M Tris base (no HCl) and 30 l of H_2O to 900 l of glycerol/NPG. Mix thoroughly. This pH-adjusted mountant should be used within a day or two.

CAUTION: DABCO, DAPI, DNA counterstains (e.g., DAPI, propidium iodide), formamide, phenylenediamine, propidium iodide, n-propylgallate (see Appendix 4)

Method

1. Wash the tissue three times with 2x SSCT. Incubate for at least 10 minutes per wash.
2. (Optional) Treat the sample with 10 g/ml boiled RNase A in 2x SSCT for 30 minutes.

   Note: This step is usually dispensable, but probes to highly transcribed sequences, e.g., rDNA, may also bind to RNA. Therefore, this step may serve as a useful control. This treatment is also useful if the nuclear DNA will be counterstained with propidium iodide or another dye that binds to RNA (see step 13, below).

3. Step the tissue gradually into 2x SSCT containing 50% formamide, by adding sequentially and incubating for 10 minutes in each of the following solutions:

   2x SSCT containing 20% formamide
   2x SSCT containing 40% formamide
   2x SSCT containing 50% formamide

4. Add fresh 2x SSCT/50% formamide, place samples at 37¼C, and incubate for at least 30 minutes.

   Note: This prehybridization incubation can have a marked impact on the permeability of some tissues and should be increased to as long as several hours if inconsistent probe penetration or high background is observed.

5. Aspirate as much of the prehybridization solution as possible. Take care not to aspirate the tissue. Add the Probe Solution. Mix gently by flicking the tube.

Probe Solution

Add 25Ð500 ng of each probe (depending on complexity) to 36 1.1x hybridization buffer. Adjust the final volume of the probe mixture to 40 with H_2O and mix thoroughly by repeated pipetting. The final buffer composition should be 1.6x SSC, 50% formamide, 10% dextran sulfate.

Note: The probe concentration must be optimized empirically, but it is usually 0.5Ð10 ng/ Beyond an optimal level, increasing the concentration of double-stranded probe molecules may preferentially enhance their ability to self-anneal. This is because the kinetics for self-annealing follow the square of probe concentration, whereas hybridization to the chromosomes should increase only linearly with probe concentration.

6. Denature the probe and chromosomal DNA by heating the sample to 91¼C for 2 minutes

   Note: The denaturation temperature has been optimized by examining signal/noise and morphological preservation in formaldehyde-fixed samples (see Figure 2.4). With fixatives other than formaldehyde, denaturation at 70Ð80¼C may be sufficient.

7. Reduce the sample temperature to 37¼C and allow the probe to anneal for several hours (typically overnight).

   Note: This stringency will work for most probes, but for very AT-rich sequences, if hybridization fails, annealing temperatures of 30Ð37¼C should be tested.

8. Add 500 l of 2x SSCT/50% formamide to the sample, mix, and allow to settle.

9. Wash the sample with three changes of 2x SSCT/50% formamide at the annealing temperature (see step 7 and Note, above) over a total period of at least 1 hour.

10. Step the sample back out of formamide by washing for 10 minutes each with:

    2x SSCT/40% formamide
    2x SSCT/20% formamide

11. Wash with three changes of 2x SSCT (without formamide).

12. (For secondary detection or immunostaining.) For avidin and many antibodies (including anti-digoxigenin F[ab] fragments from Boehringer Mannheim), perform staining in 2x SSCT, but if another buffer (e.g., PBS, TBS [Tris-buffered saline]) is preferred, it is simple to switch at this point. Perform the following steps:

    a. Block the sample with a 0.5% (w/v) protein solution (e.g., BSA or normal serum proteins). Incubate the sample in blocking solution for 30 minutes.
    b. Add diluted antibody (typical dilutions, 1:200Ð1:500) and/or avidin (1:2000 dilution). Incubate the sample with antibody/avidin solution for 2 hours, protected from light, with agitation on a nutator or similar device.
    c. Wash tissue with three changes of buffer for at least 10 minutes each wash to remove unbound detection reagents.

13. Counterstain DNA with DAPI or another fluorescent counterstain in the second wash of step 12c.

    Note: For nucleic acid dyes that also bind to RNA (e.g., propidium iodide), an RNase step before counterstaining is crucial (see step 2, above).

14. Mount the samples as follows:

    a. Exchange the tissue into mounting medium and then transfer to a slide or coverslip. Alternatively, pipette the tissue in buffer onto a slide or coverslip (poly-lysine-treated glass may facilitate this), gently remove most of the buffer, and overlay with mounting medium.
    b. If the sample is on a coverslip, hold a glass slide horizontally over the drop of mounting medium and then touch the center of the slide to the drop. If the sample is on a slide, invert the slide and touch the drop to a coverslip lying flat on a benchtop.
    c. Allow the liquid to spread out between the two layers of glass, then invert carefully. If necessary, remove excess mounting medium from the edge of the coverslip by blotting or gentle aspiration. Use clear nail polish to seal the edges of the coverslip to the slide.

       Notes: A minimal volume of mounting medium should be used, so that the samples are as close to the coverslips as possible and are immobilized by sandwiching between the two layers of glass.

       Samples tend to deteriorate over time in glycerol and should therefore be stored in buffer that has been used to perform the staining and washing steps (i.e., PBS, TBS, etc., made to 1 mM EDTA) at 4¼C until shortly before imaging.

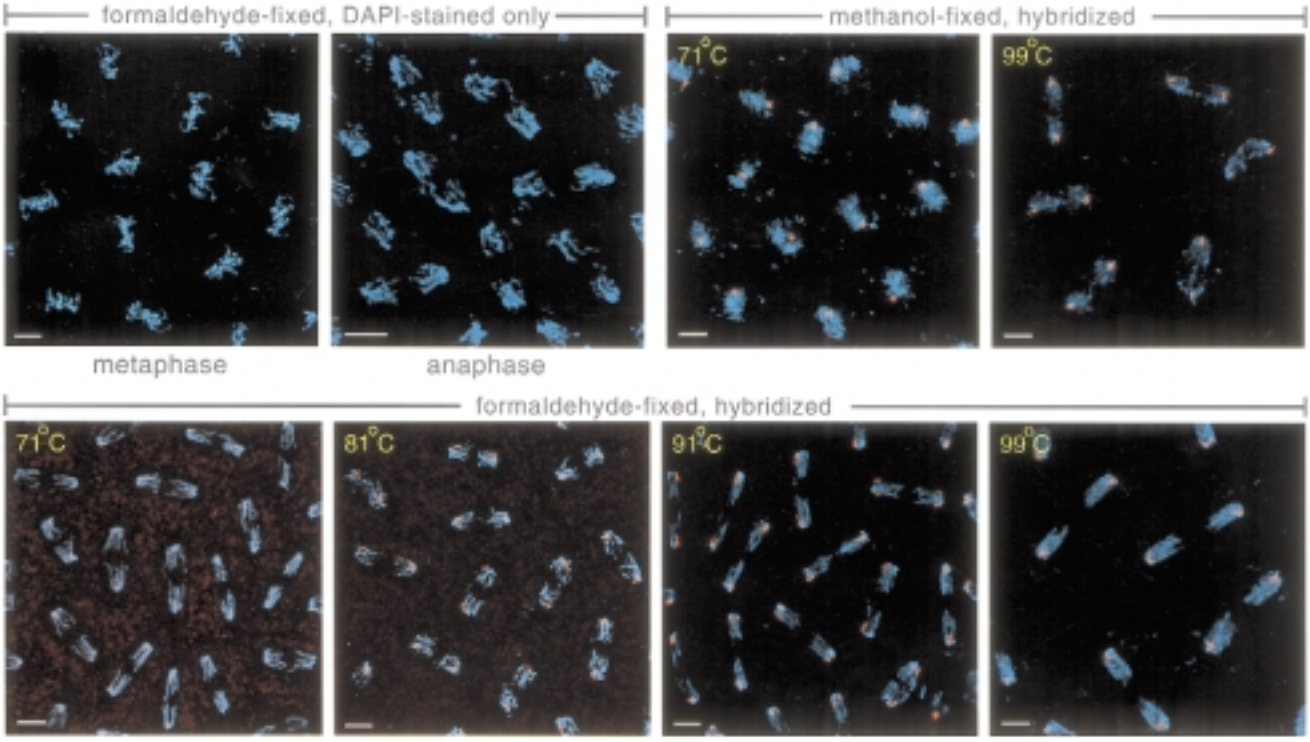

Figure 2.4. Comparison of the effects of different fixation methods and hybridization temperatures on chromosome morphology following FISH. Two panels (top left) show mitotic chromosomes that have been fixed with formaldehyde according to standard procedures, and not subjected to hybridization. Their appearance defines the standard to which chromosome morphology shou be compared following hybridization. It is easier to compare mitotic chromosomes to interphase nuclei, which have diffuse DNA staining even under optimal conditions. The remaining six panels show chromosomes hybridized with a probe to the 359-b repeat on the X chromosome. This is a very abundant sequence, and the probe can easily be detected even under suboptimal co ditions. When embryos are fixed with methanol alone (two panels, top right), hybridization is successful and signal/noise is very good, even with relatively low denaturation temperatures. However, chromosome morphology is better preserved by formaldehyd fixation (four panels, bottom). Following fixation with 3.7% formaldehyde, the denaturation step is crucial for achieving good signal/background. In 50% formamide, as the denaturation temperature is increased from about 70¼C to 91¼C, the signal gradu improves, and the cytoplasmic background fluorescence is reduced, to an optimal point at about 91¼C. Beyond this, higher den uration temperatures do not improve the signal/noise and will begin to degrade the chromosome morphology perceptibly. (Reprinted, with permission, from Spector et al. 1998.)

PROTOCOL 2.9

Hybridization to Tissue on Slides or Coverslips

Materials

Supplies and Equipment

Coplin or slide-staining jars (VWR or other supplier) or coverslip-staining jars (Thomas Scientific)

Aspirator with fine tip and liquid trap

Coverslips (22 x 22 mm)

Kimwipes

Humidified denaturation chamber. A simple homemade version may be constructed by setting an aluminium heat block to 94¼C and covering the block with a plastic lid containing a layer of Kimwipes moistened with H_2O around the inner rim (Figure 2.5).

Humidified incubation chamber, consisting of a watertight plastic container (e.g., Tupperware) containing a few layers of paper towels moistened with 2x SSC, overlaid with a sheet of Parafilm

Glass slides

Hemostats, the tips covered with short pieces of silicone rubber tubing, for handling slides

Solutions and Reagents

Fixed tissue on slides or coverslips (from Protocol 2.7)

2x SSCT (see p. 43)

(Optional) RNase A (10 mg/ml in H_2O)

Labeled probe(s)

1.1x Hybridization Solution (see p. 47)

Formamide (Fluka 47670)

Reagents for immunostaining and secondary detection

- primary antibody
- 0.5% protein blocking solution
- fluorescent detection reagents, e.g., fluorescently labeled streptavidin, antibiotin, or antidigoxigenin antibodies (see Table 2.1)

Mounting medium (see p. 48)

DNA counterstains (e.g., DAPI, propidium iodide; see Appendix 3)

CAUTION: DAPI, formamide, propidium iodide (see Appendix 4)

Method

1. Place the slide(s) in a Coplin jar containing 2x SSCT. Incubate for at least 10 minutes. Repeat wash with two changes of 2x SSCT.

2. (Optional) Treat the sample with RNase A (final concentration 10–100 μg/ml).

   Note: RNase may be added to the buffer in the Coplin jar; or smaller volumes can be used by transferring the slide(s) to a humid chamber, pipetting the RNase solution directly onto the sample, and covering with a piece of Parafilm or a coverslip.

3. Step the tissue gradually into 2x SSCT containing 50% formamide, by transferring sequentially and incubating in each of the following solutions for 10 minutes:

   2x SSCT containing 25% formamide
   2x SSCT containing 50% formamide

4. Transfer samples to fresh 2x SSCT/50% formamide, place the staining jar in a 37¼C water bath, allow the temperature to equilibrate, and incubate for at least 30 minutes before addition of the probe.

   Note: This prehybridization incubation can have a marked impact on the permeability of some tissues and should be increased to as long as several hours if inconsistent probe penetration or high background is observed.

5. Carefully remove the slides/coverslips from the prehybridization solution and drain on paper towels. (Save the prehybridization solution for the first posthybridization wash.) Wearing gloves, use the tip of an aspirator and/or Kimwipes to remove as much of the prehybridization solution as possible without damaging the tissue. Wipe the back of the glass dry with Kimwipes.

6. Pipette the Probe Solution onto a clean 22 x 22-mm coverslip or, if the sample is on a coverslip, pipette it onto a slide. Touch the drop of solution to the sample and invert the slide.

Probe Solution

Add 100–200 ng of each probe (depending on complexity) to 1 ml Hybridization Solution and adjust volume to 15 ml with H_2O. The final buffer composition should be 2 x SSC, 50% formamide, 10% dextran sulfate.

Note: The probe concentration must be optimized empirically, but it is usually 0.5–10 ng/ml. Beyond an optimal level, increasing the concentration of double-stranded probe molecules may preferentially enhance their ability to self-anneal. This is because the kinetics for self-annealing follow the square of probe concentration, whereas hybridization to the chromosomes should increase only linearly with probe concentration.

7. Denature probe and chromosomal DNA by placing the slide onto a hot block pre-equilibrated to 94¼C. Cover with a humidifying lid (Figure 2.5) and incubate for 2 minutes.

Note: As an alternative to constructing a humidified denaturation chamber, seal the coverslip to the slide with a gasket of rubber cement, most easily applied using a syringe with a wide-bore needle. Allow the rubber cement to dry, and then denature the slide directly on a heat block at 94¼C.

8. Transfer the slides to a humidified chamber at 37¼C and incubate for several hours to overnight.

9. Transfer slides to a Coplin or staining jar containing 2 x SSCT/50% formamide at the annealing temperature.

Note: If the samples are on slides, allow the coverslips to fall off and discard them. If rubber cement was used, this should be carefully peeled off using forceps while the slide is immersed in washing solution. If the samples are on coverslips, retrieve them carefully using flat-tipped forceps (Millipore) and transfer to coverslip-staining jars containing 2 x SSCT/50% formamide, at the annealing temperature.

10. Wash the sample with one further change of 2 x SSCT/50% formamide at the annealing temperature over a total period of at least 1 hour.

11. Transfer the sample into 2 x SSCT/25% formamide at room temperature and incubate for at least 10 minutes.

12. Wash with three changes of 2 x SSCT (without formamide).

13. (For secondary detection or immunostaining.) For avidin and many antibodies (including antidigoxigenin and antidigoxin antibodies), perform staining in 2 x SSCT, but if another buffer (e.g., PBS and TBS) is preferred, it is simple to switch at this point. For staining steps (below), transfer the slides into a humid chamber, pipette the blocking or staining solution onto the sample, and overlay with a piece of Parafilm cut to approximately 25 x 25 mm (or as needed to cover the specimen).

    a. Block the sample with a 0.5% (w/v) protein solution (e.g., BSA and normal serum proteins). Incubate the sample in blocking solution for 30 minutes.

    b. Add diluted antibody (typical dilutions, 1:200 to 1:500) and/or avidin (1:2000 dilution). Incubate the sample with antibody/avidin solution for 2 hours, protected from light.

    c. Wash tissue with three changes of 2 x SSCT for at least 10 minutes each wash to remove unbound detection reagents.

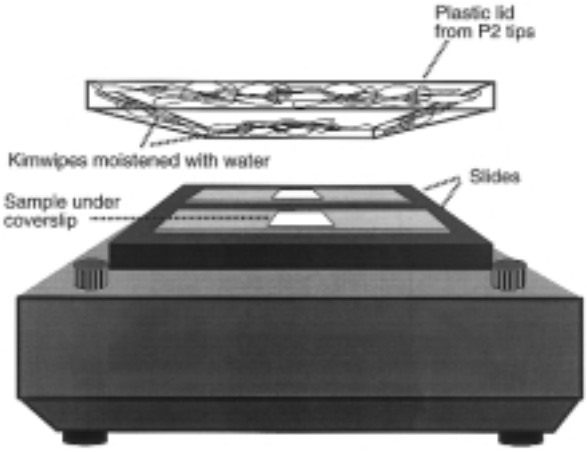

Figure 2.5. Denaturation of FISH samples on slides using a humidified heat block. This diagram illustrates a simple way to heat-denature samples on glass slides using a conventional heating device (VW or other supplier) with a removable aluminum block. The heat block is first equilibrated at 94¼C using a thermometer placed in one of the wells, after which, the aluminum block is then inverted so that its flat side faces up. A humidifying chamber can then be assembled using a shallow plastic box; the lids from racks of P2 Pipetman tips are perfectly sized for this. Kimwipes are placed around the interior rim of the lid and moistened with water (salt would damage the anodized aluminum surface). The lid is placed over the slides during the 2-minute denaturation period. This type of block accommodates two slides at a time. (Reprinted, with permission, from Dernburg 1999 [Oxford University Press].)

16. Counterstain with DAPI or another fluorescent dye in the second wash.
17. Mount samples for microscopy as follows:
    a. Pipette 12Ð15 l of mounting medium onto a clean 22 x 22-mm coverslip.
    b. Transfer the slide briefly to a staining jar containing 50 mM Tris-Cl (pH 7.5) to remove most of the salt.
    c. Remove slide and drain briefly on paper towels. Use Kimwipes and/or an aspirator to remove as much buffer as possible from the slide without damaging the sample or allowing it to dry out.
    d. Touch the sample to the drop of mounting medium, invert the slide, and seal with clear nail polish.

TROUBLESHOOTING

The most common problem in hybridization experiments is the absence of a detectable signal. This is also the most difficult situation upon which to improve, because the source of the problem is hard to diagnose and may lie with the fixation method, probe synthesis, or other experimental factors. It is extremely helpful to embark on any attempt at hybridization using the type of probe most likely to work: an oligonucleotide probe to a simple, abundant, repetitive sequence. The *Drosophila* genome contains a wide choice of such sequences, including some that are restricted to specific chromosomal loci (Table 2.2). After labeling, test the probe on an acid-fixed mitotic chromosome preparation using methods such as those described by Pimpinelli et al. (this volume). Chances are high that such a probe, if labeled successfully, will produce at least a weak signal in embryos or other whole-mount tissues, allowing the experimental conditions to be refined and optimized.

Single-copy probes can also be tested on squash preparations of polytene chromosome (see Pardue, this volume).

More often than not, disappointing or inconsistent results are due to over- or under-fixation of the tissue. Potential problems attributable to fixation include failure to detect the probe, nonspecific binding of probe or detection reagents, and unacceptable morphological preservation. If the probe can be readily detected on flattened or acid-fixed preparations, then effort should be focused on improving the whole-mount fixation.

MICROSCOPY AND IMAGE ANALYSIS

FISH experiments frequently require the detection of faint signals at several different wavelengths within thick biological samples. High-quality oil immersion lenses, well-designed fluorescence filters, and sensitive detection devices such as cooled CCD camer all help to extend the sensitivity and versatility of the technique.

Diploid Drosophila nuclei are typically about 3–5 m in diameter, only one order of magnitude greater than the resolution limit of traditional optical microscopes. Questions regarding the subnuclear distribution of specific sequences are thus close to the edge o what can be accomplished with fluorescent detection. To achieve high resolution, it is valuable to apply techniques that remove out-of-focus information from fluorescence images. Two widely used methods are confocal laser-scanning microscopy (CLSM) and wide-field deconvolution microscopy, in which the out-of-focus light is collected but later restored to its proper position within the object using computational algorithms.

ACKNOWLEDGMENT

This work was supported in part by a postdoctoral fellowship (DRG-1392) from the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation.

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3

BrdU Labeling of Chromosomes

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THE RAPID ADVANCES IN UNDERSTANDING the regulation of the cell cycle in organisms from yeast to humans have included the need to determine accurately what part of the cell cycle is being experimentally regulated. Presented in this chapter is an easy method fo marking and detecting the S phase in *Drosophila* embryos. This technique involves the incorporation of the thymidine analog, BrdU (5-bromo-2«-deoxyuridine), into DNA and its detection by immunofluorescence or histochemistry (see Figure 3.1) (Edgar and OÕFarrell 1990; Duronio and OÕFarrell 1994). Also presented is a description of labelin larval, pupal, and adult tissues (Truman and Bate 1988; Awad and Truman 1997; Britton and Edgar 1998). These methods can be expanded with a double label of different parts of S phase using a combination of BrdU and a second analog, IdU (5-iodo-2«-deoxyuridine) (Aten et al. 1994). In addition, combining BrdU labeling with in situ hybridization enables determination of what sequences are being replicated (Calvi et al. 1998; A.W Shermoen and P.H. OÕFarrell, in prep.).

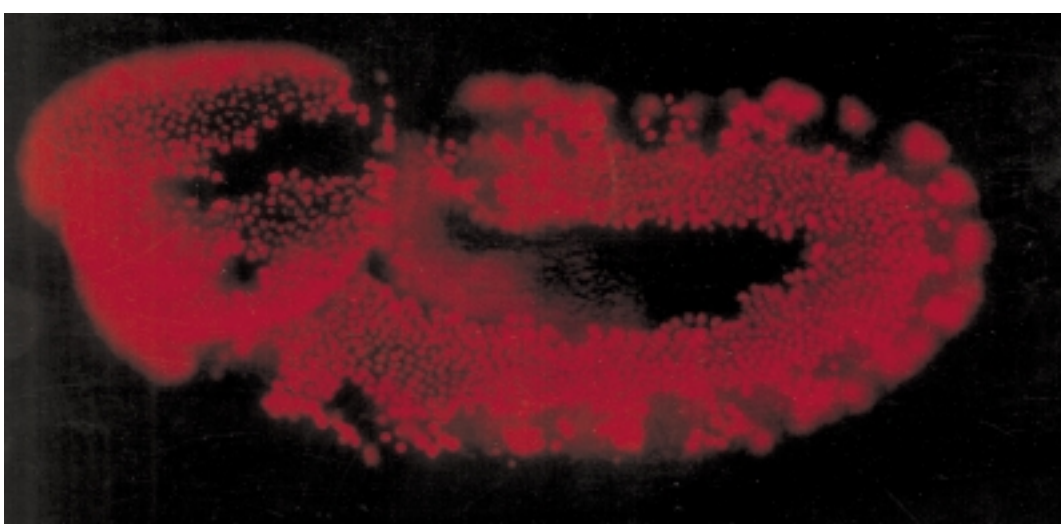

Figure 3.1. Stage-11 embryo labeled for 16 minutes with BrdU and stained with monoclonal antibody anti-BrdU and a goat anti-mouse-Rho secondary antibody. The image was taken using a 20× objective on a Leica DMRD microscope.

FLOW CHART 1

BrdU LABELING

Embryos

Collect embryos → 50% bleach x 2 minutes → Rinse with H_2O x ~1 minute → Blot x 15–30 seconds → Octanex 4 minutes → Blot x 30–45 seconds → BrdU labeling medium x 5 minutes → Fix in 5% formaldehyde/1xPBS x 20 minutes → methanol x 20 seconds → Store at –20°C in methanol.

Tissues (Imaginal Discs, Salivary Glands, CNS)

Clean larvae with H_2O → Dissect tissue in Schneider's medium (or PBS) → Transfer the tissue to a basket with fine-mesh Nitex (not more than 45-hole; it is useful to leave discs attached to other tissue if using a larger-mesh Nitex) in Schneider's medium → BrdU labeling medium x 10 minutes → 5% formaldehyde x 30 minutes → PTX (3x 10 minutes) → May be stored in PTX at 4°C overnight.

PROTOCOL 3.1*

BrdU Labeling

A detailed procedure for BrdU labeling of embryos is given below. Flowchart 1 presents a summary of this procedure for embryos and other tissues.

Materials

Media

Grape agar plates (for preparation, see Appendix 3)
Schneider's medium (GIBCO BRL 11720-034)

Supplies and Equipment

Culture dishes (60 x 15 mm; Fisher 8-757-13A)
A black surface under the embryos to allow easier visibility
Squirt bottle containing H_2O
Paint brush (#2)
Kimwipes
Pipetman (1 ml)
Watch glass (2) (Fisher 15-355; these beveled-edge watch glasses are deeper)
Dissecting microscope
Glass scintillation vials (20 ml) or Eppendorf tubes for fixation and devitellinization
Pasteur pipette
Dissecting tools for BrdU labeling of larvae (see Flowchart 1)

- Dumont tweezers (#5; Ted Pella 5622)
- micro slides (75 x 50 mm, Corning 2947-7550); Sigmacote (Sigma SL-2) or RAIN-X (UNELKO Corp.; commonly sold in auto supply stores) is used to coat the slide to ensure that the dissecting medium beads into drops and traps the larvae to be dissected

*For all protocols, H_2O indicates glass distilled and deionized.

Preparation of Baskets for Egg Collections, Treatments, and Incubations

The key consideration of what size basket to use is that the embryos must be approximately one monolayer or more. Described below are two baskets used by the author for large numbers of embryos. For descriptions of other baskets, see Rothwell and Sullivan (this volume).

The first basket is made from a 50-ml plastic screw-cap centrifuge tube, 2.5 cm in height. Cut a hole in the cap as large as possible, but still maintaining enough of the structure to be able to screw the cap on firmly. Cut an 8-cm square piece of Nitex (~70-mesh, Sefar America, Inc. [4221 NE 34th St., Kansas City, Missouri 64117 [Phone: 800-283-8182]). Screw the top on with the mesh between the top and body as taut and flat as possible. Use somewhat greater volumes than used for a commercial basket. For preparation of baskets for larval tissues, use fine-mesh Nitex (not more than 45-hole).

Commercial embryo baskets may be obtained from Labworks, Inc. (721 Bolero Ct., Novato, California 94945 [Phone: 707-484-7998; Web site: www.labworksinc.com], LW-401; customer must specify size). Dimensions: 3.2 cm (inner diameter), 3.8 cm (outer diameter), 2 cm (height).

Solutions and Reagents

Bleach (50%)
Octane (Sigma O 2001)
1x Phosphate-buffered saline (PBS) (pH 7.2; for preparation, see Appendix 3)
Formaldehyde (37%; Sigma F 1635) or dilute to 5% formaldehyde in 1x PBS
Heptane
Methanol

BrdU-labeling Medium

Prepare 1 mg/ml (BrdU; Sigma B 5002) in SchneiderÕs medium. Alternatively, use 1x PBS, which is just as effective.

CAUTION: bleach, formaldehyde, heptane, methanol, octane (see Appendix 4)

Method

1. (Embryo collection) Collect the embryos as follows:
   a. Squeeze H_2O from a squirt bottle onto the grape agar plate containing eggs. Use a paintbrush to loosen the embryos from the surface of the agar.
   b. Pour the H_2O containing the embryos into a basket. Rinse the grape agar plate with more H_2O. Transfer the rinse to the basket.
   c. Use Kimwipes to blot the embryos dry from the bottom of the basket.

   Note: A cage of approximately 5 g of adult flies will produce approximately 40Ð60 embryos/minute (or more than double that amount obtained on the first day with fresh flies, properly fed).
2. (Dechorionation) Immerse the embryos in 50% bleach and agitate occasionally for 2 minutes. Wash these embryos in running H_2O (deionized or distilled) until there is no longer any bleach odor (~1 minute).
3. Use Kimwipes to blot the H_2O from the inner and outer sides. Then blot the underside of the basket to remove H_2O from around the embryos.

Note: The time for blotting depends on the number of embryos obtained—a monolayer should be blotted for approximately 15 seconds, whereas more than a monolayer of embryos must be blotted for approximately 30 seconds. This is a critical step because too much blotting will collapse the vitelline membrane against the embryo. These desiccated embryos do not devitellinize efficiently. In addition, too little blotting slows down the uptake of BrdU and makes the start of labeling more heterogeneous among the embryos.

4. (Permeabilization) Immerse the embryos in octane (in a watch glass) to permeabilize them. Use a 1-ml Pipetman to spurt 1 ml of octane on the embryos to agitate them. Continue this agitation occasionally for the duration of this step (4 minutes). During this period, add approximately 2 ml of the BrdU-labeling Medium to a 60×15-mm culture dish (see step 6, Notes, below).

   Note: The state of the embryos may be monitored under a dissecting microscope during the agitation. Ensure that there is no sheen around the embryos. The presence of a sheen would be indicative of an H_2O envelope preventing the octane from permeabilizing the embryo.

5. After 4 minutes, lift the basket from the octane, blot the underside with Kimwipes, and blow gently on the embryos until there is no octane odor (~30–45 seconds).

6. (BrdU labeling) Perform BrdU labeling as follows:

   a. Place the basket into the 60×15-mm culture dish containing BrdU-labeling Medium (see step 4, above).

   b. Pour about 6–8 ml of the BrdU-labeling Medium over the embryos (the embryos should be ~1–4 mm below the surface of the liquid). Again monitor the embryos with a dissecting microscope while spritzing the labeling medium onto the embryos with a 1-ml Pipetman. Take up the medium from the culture dish outside of the basket and use a strong spurt to dislodge the embryos from the bottom of the basket (the embryos should at least cycle through a monolayer condition at the surface).

      Notes: Ensure that there is no sheen over the embryos, which would be indicative of a coating of octane that would act as a barrier to the entry of BrdU. During the labeling period, occasionally spurt medium over the embryos to agitate them and keep them off the sides, and rotating in the middle of the basket. For optimal labeling times, see Troubleshooting below.

      If precise timing of labeling is important, start step 6a with SchneiderÕs medium alone or 1x PBS. After the embryos are on the surface and there is no octane ÒsheenÓ around them, transfer the basket to BrdU-labeling Medium.

      If different periods of labeling from the same sample are desired, collect these samples by using a small square of Nitex to scoop up the quantity of embryos needed and transfer these to a vessel containing heptane and formaldehyde (proceed to step 7, below).

7. Fix and devitellinize the embryos as follows:

   a. Transfer the embryos to a container containing equal volumes of formaldehyde and heptane. The container should be sufficiently large so that the embryos are at the interface when rocking. Fix the embryos while rocking (3–4 minutes for 37% formaldehyde or 20 minutes for 5% formaldehyde in 1x PBS). At the end of this period, use a pasteur pipette or Pipetman to withdraw the formaldehyde from below the heptane layer (the embryos will form a layer at the interface between the two solutions).

   b. Now add about 2–2.5 volumes of methanol and shake vigorously for 20 seconds. The devitellinized embryos will fall to the bottom of the container.

   c. Aspirate the embryos at the interface and the upper layer of heptane, and then add as much of the methanol as possible without disturbing the embryos. Transfer the embryos to a new Eppendorf tube and rinse twice with fresh methanol.

      Note: These embryos may be stored indefinitely at −20°C in methanol.

PROTOCOL 3.2

BrdU Detection

Presented here is an in-depth description of the BrdU detection procedure for embryos. Flowchart 2 provides a summary of this procedure for embryos as well as for larval, pupal, and adult tissues with the use of fluor-conjugated secondary antibody.

The choice of secondary antibody is between a fluorescently labeled antibody or an antibody that leaves a histochemical deposition for the detection of the BrdU, such as alkaline phosphatase (AP) deposition of nitroblue tetrazolium (NBT) or horseradish peroxidase (HRP) deposition of diaminobenzidine (DAB). These histochemical detection methods are about ten times more sensitive than that of normal fluorescently labeled antibodies. However, one consequence of the chemical deposit is to quench any costaining with a fluorescently labeled antibody in the region of BrdU label. Both the AP reaction, which leaves a blue precipitate, and the HRP reaction, which leaves a brown precipitate for a double-label could be used. One shortcoming of this approach is the difficulty in detecting subcellular localization, or the colocalization of two targets. Both of these methods are outlined in this protocol.

Materials

Supplies and Equipment

Eppendorf tubes (0.5 ml and 1 ml)
Sequencing tips
Aluminum foil
Multiwell culture dish (24-well; Fisher 08-772-4G)
Nutator
P-200 Pipetman
Coverslips (22 x 22 mm)
Micro slides (25 x 75 mm, Fisher 12-518-100B) for mounting embryos
Nail polish

Solutions and Reagents

Sodium tetraborate (100 mM)
Normal goat serum (NGS; Jackson ImmunoResearch Laboratories 005-000-121)
Hoechst 33258 (Molecular Probes H-1398)
Mountant (e.g., Fluoromount-G, Southern Biotechnology Associates 100-01)
Primary antibody. Monoclonal antibody (Mab) anti-BrdU (Becton Dickinson 347580); for a table of primary antibodies, see Dolbeare and Shelden (1994)

FLOW CHART 2

BrdU DETECTION

Embryos and Tissues (Imaginal discs, Salivary Glands, CNS)

HCl treat x 30 minutes Borax (2 x 2 minutes) PTX (2 x 3 minutes) 10% NGS/PTX x 45 minutes Mab anti-BrdU x 1.5 hours PTX x 2 minutes 10% NGS/PTX (3 x 20 minutes) Goat anti-mouse Rho x 1 hour PTX (1 x 2 minutes, 2 x 20 minutes) Hoechst x 2 minutes PTX (2 x 5 minutes) Mount.

Secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.); use either:
- Anti-mouse ¥ Fluor, Goat anti-mouse ¥ Rho (115-026-072)
- Anti-mouse secondary antibodies for histochemical detection
  - (1) anti-mouse ¥ AP (115-056-072) or
  - (2) anti-mouse ¥ HRP (115-036-006)

Sodium azide (5%)

HCl/Triton X-100 Solution

2.2 N HCl
0.1% Triton X-100
Use freshly prepared solution.

PTX Solution

1x PBS
0.1% Triton X-100

10% NGS/PTX

Prepare a solution of 10% NGS in PTX. Alternatively, use 5% milk (dried) in PTX instead of 10% NGS/PTX. These solutions are used for blocking and for dilution of antibodies.

Preadsorbed Antibodies

Dilute the commercial concentration of the secondary antibody 30–50 times with 10% NGS/PTX (to make it 1x concentration). Mix the antibody solution with an equal volume of embryos at room temperature for 1–2 hours.

The embryos are usually from an overnight collection, which is dechorionated, fixed, devitellinized, and stored at –20¼C in methanol for such a purpose. The embryos are equilibrated with PTX before use. These stock antibodies can be used for months if stored with the embryos at 4¼C.

AP Detection Solution 1

100 mM NaCl
50 mM $MgCl_2$
100 mM Tris-Cl (pH 9.5)
1 mM Levamisole
0.1% Triton X-100

AP Detection Solution 2

This solution comprises AP Detection Solution 1 (above) containing 4.5 ml NBT (Boehringer Mannheim 1 383 213) and 3.5 ml BCIP (5-bromo-4-chloro-3-indolyl phosphate; Boehringer Mannheim 1 383 221).

Staining Solution for HRP Detection

1x PBS
0.5 mg/ml DAB
0.003% H_2O_2
0.05% $NiCl_2$
Use freshly prepared staining solution.

CAUTION: BCIP, BrdU, DAB, Fluoromount-G, HCl, H_2O_2, methanol, $MgCl_2$, NBT, $NiCl_2$, sodium azide, sodium tetraborate (see Appendix 4)

Method

1. Aspirate the methanol from the embryos (use a Pipetman with a sequencing tip to withdraw the last of the methanol). Start with approximately 25–50 l of labeled embryos (200–8000 embryos).
2. Denature the DNA as follows:
   a. Add at least 10 volumes of freshly prepared HCl/Triton X-100 Solution to the embryos.
   b. Rock on a Nutator for 15 minutes. Allow the embryos to settle to the bottom of the Eppendorf tube.
   c. Withdraw most of the HCl/Triton X-100 Solution. Repeat steps 2a and 2b (above). Withdraw all of the HCl/Triton X-100 Solution.
3. Neutralize the embryos as follows:
   a. Add approximately 10 volumes of 100 mM sodium tetraborate (borax) to the embryos.
   b. Rock the embryos in the solution for approximately 2 minutes. Allow the embryos to settle to the bottom of the Eppendorf tube.
   c. Aspirate most of the borax solution. Repeat steps 3a and 3b (above). Withdraw all of the borax solution.
4. Rinse the embryos twice with PTX for 3 minutes. In preparation for antibody detection of BrdU, incubate the embryos in 10% NGS/PTX or 5% milk/PTX for 45 minutes.
5. Dilute the stock monoclonal antibody anti-BrdU 1/20 with 10% NGS/PTX. Add 100 l of antibody for 20–40 l of embryos (the minimum volume of antibody should be 100 l). Rock the embryos on a Nutator for 1.5 hours. Withdraw the monoclonal antibody anti-BrdU, which may be saved at 4¼C and used a second time.
6. Rinse the embryos with PTX briefly (<2 minutes). Then rinse again with 10% NGS/PTX or 5% milk/PTX (x3 20 minutes). Aspirate off the blocking solution. Withdraw the final volume of solution using a P-200 and a sequencing tip, to avoid losing embryos.
7. Add 100–200 l of the preadsorbed secondary antibody at a final dilution of approximately 1/300.
8. There is a choice of secondary antibody detection using either of the following methods (for a discussion of these methods, see above):
   a. Fluorescent detection using Goat anti-mouse ¥ fluor (Rho, FITC, Cy5; Jackson ImmunoResearch Laboratories). These are usually used at about 1/300–1/500 in 10%NGS/PTX or 5% milk/PTX at a final volume of 100–200
      i. Wrap the tubes containing embryos and fluorescent antibodies in aluminum foil to prevent any bleaching of signal and rock at room temperature for 1 hour. Allow the embryos to settle and withdraw the secondary antibody. (This secondary antibody may also be stored at 4¼C and used again.)
      ii. Fill the Eppendorf tube with PTX and rock the embryos several times and allow them settle. Aspirate off most of the PTX using a sequencing pipette tip over the tip of a pasteur tip for greater control.

iii. Refill the tube with PTX and rock on the Nutator for 20 minutes. Repeat this wash a second time.

iv. Stain these embryos with 1 g/ml Hoechst 33258 for 2 minutes. Aspirate the Hoechst solution and rinse with PTX twice for 5 minutes each.

Notes: The Hoechst stain is for quality control of the DNA denaturation step. The euchromatin of interphase chromosomes produces a fuzzy Hoechst stain if properly denatured. Heterochromatin and mitotic chromosomes stain fairly well, despite the denaturation.

Additional stains that may be used are antibodies to the nuclear pore such as wheat germ agglutinin (WGA) conjugated with a fluor (WGA-fluorescein; Molecular Probes W-834); antibodies to chromosomes in mitosis from prophase to telophase (rabbit anti-phosphohistone 3; Upstate Biotechnology 06-570; this antibody is used 1/1000 and is preadsorbed and leaves no background); or antibodies to in situ hybridization probes (see Dernberg, this volume; Calvi et al. 1998; A.W. Shermoen and P.H. OÕFarrell, in prep.). Another option for a fluorescent signal is to use the tyramide signal amplification system (TSA) developed by New England Nuclear. This method uses HRP activity to deposit a tyramide-fluor at and in the immediate region of the target antibody with easily a fivefold or greater amplification (Wilkie and Davis 1998; Speel et al. 1999).

b. Histochemical detection of BrdU using either goat anti-mouse ¥ AP or goat anti-mouse ¥ HRP:

Goat anti-mouse ¥ AP

i. Add 100–200 l (final diluted volume) of preadsorbed antibody diluted 1x to 1 with 10% NGS/PTX. Incubate on a Nutator for 1 hour and then withdraw the secondary antibody.

ii. Rinse the embryos briefly with PTX (several rocks in the hand). Aspirate off most of the PTX and wash with three changes, 20 minutes each, of PTX.

iii. Rinse the embryos twice for 5 minutes each with AP Detection Solution 1.

iv. Stain with AP Detection Solution 2.

Note: Staining is usually performed in a multiwell culture dish covered with aluminum foil; check the staining reaction periodically (after 10 minutes initially) under a dissecting microscope. There is less background if the staining is done wrapped in aluminum foil.

Goat anti-mouse ¥ HRP

i. Perform steps i and ii, above, as for goat anti-mouse ¥ AP.

ii. Withdraw the PTX from the embryos. Add 300 to 1 ml of the Staining Solution for HRP detection. Check the staining every few minutes. When the stain has developed sufficiently, stop the reaction by adding approximately 25 l of 5% sodium azide.

9. Mount embryos as follows:

a. Withdraw the embryos using a Pipetman (P-200) with a cut-off pipette tip and deposit them on a coverslip (22 22 mm). Withdraw the PTX using a sequencing tip on a P-200.

b. Cover the embryos with 35–40 of mountant (the volume will vary with the number of embryos). Spread the embryos with a sequencing tip. Gently touch the middle of a slide to the mountant/embryos. Let the mountant spread to the sides of the coverslip (if it does not spread, add a bit more mountant at the side of the coverslip).

c. Seal the edges of the coverslip with nail polish (this will prevent mountant from mixing with the oil used with oil-immersion lens, an optically fatal condition).

TROUBLESHOOTING

Poor yield at the devitellinization step (step, step 7 in Protocol 3.1). Experience has shown that this is invariably due to too much drying at the step between the dechorionation and the octane permeabilization (i.e., step 3 in Protocol 3.1). The second most common problem is too much drying at the step between octane permeabilization and the immersion into BrdU labeling medium (i.e., step 5 in Protocol 3.1).

Gradient of BrdU labeling. This problem usually occurs from inadequate clearing of octane from part of the surface of the embryo (see step 4 in Protocol 3.1). A gradient of labeling may also be seen if the vitelline membrane is only partially removed. The vitelline membrane, which is left, will adhere closely to the embryo and may be seen as a diffraction pattern around the embryo using differential interference contrast (DIC) optics.

Differences in labeling efficiency between embryos of different ages. Older embryos (nuclear cycles 15 and 16; stages 8–11) label much more efficiently (5 minutes), whereas the younger embryos take longer to label (8 minutes).

ACKNOWLEDGMENTS

I thank Justine Melo, Smruti Vidwans, and Tin Tin Su for comments on this procedure description. The work during which the previously published methods were modified was supported by National Institutes of Health grant RO1-GM-37193 to Patrick H. O'Farrell.

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4

Analysis of Meiosis in Fixed and Live Oocytes by Light Microscopy

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DROSOPHILA FEMALE MEIOSIS HAS SERVED NOT ONLY AS THE CLASSIC SYSTEM for understanding the fundamental basis of the inheritance of genetic material (Bridges 1916), but also as an excellent model system for studying the mechanics of female meiosis (for reviews, see Hawley et al. 1993; Orr-Weaver 1995). The success of the meiotic analysis of Drosophila females reflects both the wealth of existing meiotic mutants (Hawley 1993) and the fact that chromosome behavior can easily be monitored using marked chromosomes. Characteristics of many of these mutants have been analyzed by molecular (Zhang et al. 1990; Kerrebrock et al. 1992; Rasooly et al. 1994; Hari et al. 1995; Sekelsky et al. 1995; McKim et al. 1996; Bickel et al. 1997; McKim et al. 1998) and biochemical (McDonald et al. 1990; Walker et al. 1990; Afshar et al. 1995) studies, leading to a mechanistic understanding of certain aspects of meiosis. To directly visualize the effects of these mutations on the meiotic machinery, procedures have been developed that allow study of Drosophila female meiosis by classical immunocytochemical (Theurkauf and Hawley 1992), FISH (fluorescent in situ hybridization; Dernburg et al. 1996; see Dernburg, this volume), and GFP (green fluorescent protein; Wang and Hazelrigg 1994; Endow and Komma 1996, 1997; Moore et al. 1998; also see Hazelrigg, this volume) methods.

Much has been learned about Drosophila meiosis by immunocytochemical studies of whole-mount oocytes lacking functional meiotic proteins and by localization of some of the relevant proteins (McKim et al. 1993; White-Cooper et al. 1993; Afshar et al. 1995; Endow and Komma 1996; Matthies et al. 1996; Moore et al. 1998). However, none of these classic methods easily point out kinetic problems, nor are the earliest points of deviation always readily obvious by static methods. For these reasons, and because the behavior of the highly dynamic components of the meiotic spindle may be perturbed in meiotic mutants, methods have been developed to directly observe the cytoskeleton and chromosomes in living egg chambers (Theurkauf 1994a; Endow and Komma 1996; Matthies et al. 1996).

These cytochemical and live imaging methods are discussed in this chapter. For additional protocols on immunocytochemical analysis of oogenesis, see Theurkauf (1994b)

and Verheyen and Cooley (1994), and for a protocol on in vitro activation of oocytes, see Page and Orr-Weaver (1997). Finally, oogenesis has been thoroughly studied by electron microscopy (for reviews, see Mahowald and Kambysellis 1980; Spradling 1994).

ANATOMY AND CELL BIOLOGY OF *DROSOPHILA* OOGENESIS: A BRIEF SUMMARY

Several reviews written about oogenesis should be referred to for more details and references (see, e.g., Mahowald and Kambysellis 1980; Spradling 1994). The purpose of this cursory summary is to aid in early attempts at applying the methods described below and identifying the morphological features of the egg chamber stages. These stages are arbitrary, but they are useful for discussions and evaluations of oogenesis.

Figure 4.1 presents a schematic representation of the female reproductive system. Egg chambers develop in a pair of ovaries (Ov) (consisting of parallel ovarioles (Ovl) in anterior (germarium, Grm) and posterior (vitellarium, Vtl) compartments. Egg chamber development begins with the division of the stem cells in the germarium, and all of the subsequent stages are, in a more or less linear and chronological array, in the vitellarium with the latest stage closest to the lateral oviduct (Ovd). The first asymmetric stem-cell division leads to a new stem cell and a developing cystoblast. The cystoblast goes through four mitotic divisions with incomplete cytokinesis, generating a 16-cell cyst in which the cells are connected by cytoplasmic bridges called ring canals. These ring canals can be nicely visualized by the actin stain, phalloidin.

The 16-cell cyst is surrounded by somatically derived epithelial cells (the follicle cells), which eventually divide mitotically four to five times. In a single view through the mid-section of an egg chamber, the follicle cells stained with DAPI (4«,6-diamidino-2-phenylindole) are seen as brightly staining dots at the perimeter (Figure 4.2).

The entire cyst moves into and eventually down the vitellarium. Two of the cells in the cyst begin to differentiate into oocytes, and the synaptonemal complex begins to be laid down in either of these stage-2 cells (Carpenter 1975). Eventually, 1 cell becomes an oocyte, with the remaining 15 cells becoming metabolically active polyploid nurse cells.

Division of the follicle cells ceases in stage-6 egg chambers, and they become polyploid during the next stage. Since both the nurse and follicle cells become increasingly polyploid during various stages, whereas the oocyte nucleus remains diploid, it is easy to understand why the oocyte nucleus, in comparison, becomes relatively weakly marked by DNA indicators (dyes or GFP-histone).

At stage 8, the oocyte is readily distinguished as it begins the endocytic uptake of the yolk (stage 8, Figure 4.2, top). Stage-9 egg chambers can be recognized by the position of the oocyte nucleus, which at this time is adjacent to the follicle cell/nurse cell interface (see weakly stained dot in DAPI image of stage 9, Figure 4.2, bottom). During stage 11, the nurse cells dump their contents into the oocyte. The volume of the oocyte dramatically increases at the expense of the nurse cellÕs cytoplasm (see stage 11, Figure 4.2, bottom). stage 12, the nurse cells begin to degenerate and at the beginning of stage 13, only 15Ð nurse cell nuclei are remaining. The oocyte chromatin of stage-13 oocytes is much less intensely stained by DNA indicators than the nurse cells. To clearly denote the position of the karyosome in stage-13 oocytes, the image intensities of the karyosome in the example of this stage were exaggerated (Figure 4.2, bottom, stage 13). The karyosome is readily

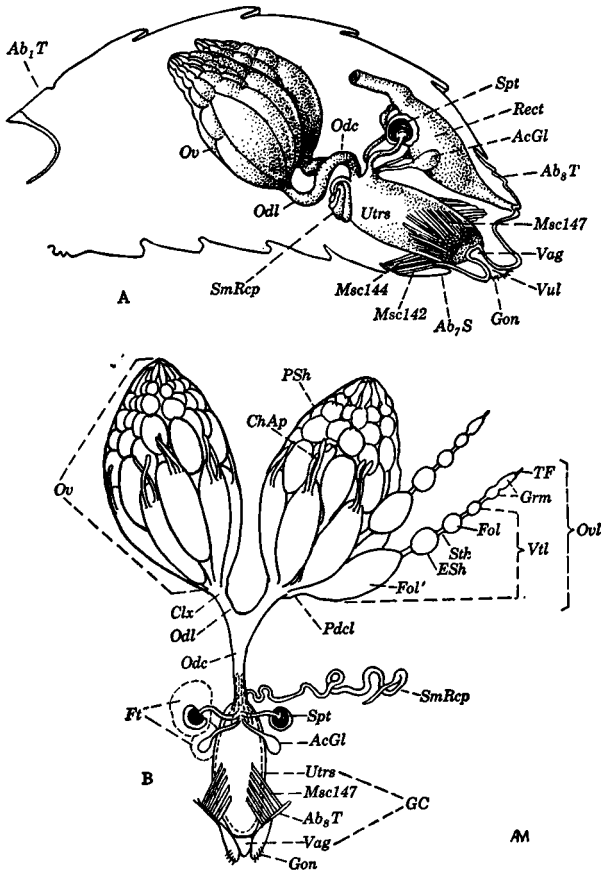

Figure 4.1. Female reproductive system. (Top) Side view of the abdomen with the left wall removed. (Ov) Ovary; (Odl) lateral oviduct; (Odc) common oviduct; (Utrs) uterus; (Vag) vagina; (Vul) vulva; (Gon) gonopod; (Rect) rectum; (Ab_1T and Ab_8T) first and eighth abdominal tergites. (Bottom) Dorsal view of the reproductive organs in isolation. The abbreviations for the bottom image are the same with the addition of the following: (PSh) Peritoneal sheath; (ChAp) chorionic appendage or dorsal appendage; (Ov) ovariole; (TF) terminal filament; (Grm) germarium; (Vtl) vitellarium; (Fol) follicle; (Stk) interfollicular stalk; (ESh) epithelial sheath; (Fok) basal follicle; (Pdc) pedicle; (SmRcp) seminal receptacle; (Spt) spermatheca; (AcG) accessory gland. (Reprinted from Miller 1950.)

obvious at the dorsal-anterior part of the oocyte, adjacent to one of the dorsal appendages or filaments (compare Nomarski and DAPI images).

Around stage 13, the oocyte nuclear envelope breaks down and the meiotic spindle assembles around the chromatin. The meiotic spindle arrests at metaphase I until fertilization. The metaphase I spindle is highly tapered and lacks astral microtubules. The chromatin is stretched on the spindle but is held together by chiasmata. The always nonexchange fourth chromosomes are found adjacent to the main mass that contains the exchange chromosomes.

In fixed cells, the chorion and follicle cells are removed, so the investigator must be able to recognize the shape of the stage-14 oocyte. In intact oocytes, the dorsal appendag are generated by a particular class of follicle cells during stage 13 and are completed alor with the chorion during stage 14. Study of these appendages will allow the investigator to recognize the overall morphology of the oocyte (~500 μm in length), because this is the region where the metaphase I spindle (12–15 μm in length) is located.

Stage	Length	Identification	Nomarski	DAPI
2	8 hr	average size: 25 × 25 ; karyosome has not yet formed (arrow); oocyte and nurse cell nuclei similar in size		
3	8 hr	average size: 35 × 35 ; karyosome (k) and endobody are visible in germinal vesicle		
4	6 hr	average size: 40 × 50 ; nurse cell nuclei contain similar amounts of DNA and appear polytene (arrow)		
5	5 hr	average size: 55 × 75 ; nurse cell nuclei no longer polytene (arrow); posterior nurse cells have more DNA than anterior ones		
6	3 hr	average size: 60 × 85 ; follicle cell mitoses cease; nurse cell ploidy equal		
7	6 hr	average size: 70 × 115 ; more elongated in shape than previous oval-shaped stages; nurse cells have higher ploidy at posterior; no yolk visible in oocyte		
8	6 hr	yolk visible in oocyte (arrow); follicle cell layer still uniform		

Stage	Length	Identification	Nomarski	DAPI
8	6 hr	yolk visible in oocyte; follicle cell layer uniform		
9	6 hr	follicle cells in process of migration over oocyte, resulting in anterior-posterior gradient of cell thickness (arrows); oocyte is ~1/3 of egg chamber		
10A	6 hr	follicle cells form columner epithelium over oocyte; centripetal migration not yet visible; oocyte is 1/2 of egg chamber		
10B	4 hr	centripetal migration in progress (arrows); dorsal follicle cells thicker than ventral (triangle); vitelline membrane extends into opercular region		
11	0.5 hr	oocyte larger than nurse chamber due to onset of nurse cell dumping, but dumping still incomplete		
12	2 hr	dumping complete; 15 nurse cell nuclei remain at anterior; dorsal filaments not visible in nurse chamber		
13	1 hr	some nurse cell nuclei still remain; dorsal filaments visible at anterior end		
14	>2 hr	no nurse cell nuclei remain; dorsal filaments complete their elongation		

Figure 4.2. Stages of oogenesis. (Top) Stage 2 to stage 8. The various stages are shown viewed by Nomarski and fluorescence (DAPI) optics. Stage durations are from transplantation studies into host females. (Bottom) Stage 8 to stage 14. (Reprinted, with permission, from Spradling 1994.)

METHODS TO VISUALIZE OOGENESIS AND FEMALE MEIOSIS

Described in this chapter are protocols and reagents used for the analysis of fixed or live egg chambers using light microscopy. Methods for fixing egg chambers are the same as standard fixation methods, but with additional steps to remove vitelline and chorion layers. Depending on the ease of obtaining a reasonable number of mothers, egg chambers can be isolated either by a mass method (see Protocol 4.1) or, if necessary, by hand dissection (see Protocol 4.2). Protocol 4.3 describes procedures for removal of the chorion and vitelline membranes from late-stage egg chambers and permeabilization of the vitelline membrane of early-stage egg chambers. The protocols for fixation can also be used for enhancer-trap experiments utilizing the *lacZ* gene, which is part of the P-*lac* W element used in many mutant screens. This allows the identification of the expression pattern of a P-element-disrupted gene required for oogenesis by a standard in situ β-galactosidase assay (see Protocol 4.5).

Large numbers of mutants can be screened by using dissected, live egg chambers observed by differential interference optics (DIC) (Figure 4.2) (see Verheyen and Cooley 1994), fluorescence microscopy, or time-lapse laser confocal microscopy. The overall structure and development of the egg chambers can be quickly monitored in unfixed egg chambers, by taking advantage of the autofluorescence of chorion and by the use of DNA-binding dyes. These dyes can penetrate live, early-stage egg chambers. The overall state chromatin, tubulin, and actin can be rapidly determined using flies expressing GFP-histone2AvD, GFP-ncd (Endow and Komma 1997), or GFP-moesin (Edwards et al. 1997). However, the GFP-labeled moesin and ncd do not label all structures equally, so labeled actin, tubulin, and/or DNA-binding dyes can be injected into living egg chambers to directly observe dynamic events. Protocol 4.4 describes procedures for labeling egg chambers with antibodies or DNA-binding dyes.

IMAGING WHOLE-MOUNT FIXED EGG CHAMBERS

The methods described in this section were developed by Bill Theurkauf in Bruce Alberts' laboratory (Theurkauf and Hawley 1992), and further refined in the Hawley (McKim et al. 1993) and Goldstein laboratories (Matthies et al. 1996).

PROTOCOL 4.1*

Mass Isolation and Fixation of Egg Chambers

Egg chambers are obtained essentially according to the procedure of Theurkauf and Hawley (1992). Two alternative methods for mass isolation can be used and are described below. These protocols were developed to allow antibodies to penetrate the oocyte and to avoid premature activation of the oocyte, as hypotonic buffers lead to activation (Mahowald et al. 1983). Method 1 describes the isolation and subsequent fixation of egg chambers. Method 2 is essentially similar to Method 1, but involves isolating egg chambers in the presence of fixative. When oocytes are isolated in fixative, there is not much difference in the overall morphology except that the fourth chromosomes are more consistently found attached to the chromosomal mass. An early indicator of activation is fourth chromosome movement to the poles. In both cases, but especially for the second method, speed is the most critical component.

*For all protocols, H_2O indicates glass distilled and deionized.

Materials

Media

Bottles Containing Yeast Paste

Prepare standard corn molasses fly food (for preparation, see Appendix 3) in half-pint jars. Combine 2.7 g of yeast in 6 ml of H_2O Mix to form a paste. Apply generously to one side of the bottle. This amount of yeast should be sufficient for approximately 10 bottles.

Supplies and Equipment

Modified containers; use either:
- 50-ml Falcon tubes, each with the bottom removed and a hole cut into the cap
- 100-ml plastic beakers with the bottoms removed

Nitex screen (350m, 250 m, 125 m, 86 m, and 64 m, each 2 sq. inches)
(Optional) Rubber bands
Ring stands (2)
Funnels (2)
Beakers (2) (500 ml)
CO_2 station
Blender. Use either an 8-ounce or a 5-cup container. An 8-ounce blender container minimizes the surface area, but the standard, 5-cup blender containers are satisfactory.
Dissecting microscope
Falcon tubes (15 ml)

Solutions and Reagents

(Optional) Ether
1x PBS (see Appendix 3)
Formaldehyde (16%, EM grade, methanol-free; Ted Pella)

5x Modified RobbÕs Medium

275 mM sodium acetate
40 mM potassium acetate
100 mM sucrose
10 mM glucose
2.2 mM $MgCl_2$
0.05 mM $CaCl_2$
100 mM HEPES (pH 7.4)

1x RobbÕs Medium Containing 1% Bovine Serum Albumin (BSA)

Prepare 400 ml of 1x RobbÕs Medium from the 5x stock containing 1% BSA. Use as a freshly prepared solution.

This solution is required for performing Method 1.

4% Paraformaldehyde in 1x RobbÕs Medium

Prepare 400 ml of 1x RobbÕs Medium from the 5x stock containing 4% paraformaldehyde. Warm to 30¼C. Use as a freshly prepared solution.

This solution is required for performing Method 2.

EBR (1% BSA)

130 mM NaCl
4.7 mM KCl
2.2 mM $MgCl_2$
1.9 mM $CaCl_2$
10 mM HEPES (pH 7.1)

2x Fix Buffer (pH 7.2)

200 mM potassium cacodylate
200 mM sucrose
80 mM sodium acetate
20 mM EGTA

1x Fix Buffer

Combine 1 part of 2x Fix Buffer with 1 part of 16% formaldehyde (i.e., 1:1 ratio). The final concentration of formaldehyde is 8%.

PBST

1x PBS
0.1% Triton X-100

CAUTION: $CaCl_2$, CO_2, ether, formaldehyde, KCl, $MgCl_2$, paraformaldehyde, potassium cacodylate (see Appendix 4)

Method 1

Isolation and Subsequent Fixation of Egg Chambers

1. Hold several hundred 2Ð3-day-old female flies with males for 2Ð4 days in well-yeasted bottles.

   Note: This procedure causes the flies to maximize egg production and laying. When fewer flies are kept in the bottle, all stages of oogenesis are found in proportion to the duration of each stage (see Figure 4.2). In more crowded bottles, a much higher percentage of stage-14 oocytes is observed.

2. Set up filters to separate ground flies from egg chambers and to collect purified egg chambers at the desired stage. Attach the Nitex filters of the appropriate mesh size to the top of a modified 50-ml Falcon tube by screwing on the cap or by using a rubber band at the bottom of a modified 100-ml beaker.

   a. To separate ground flies from egg chambers, attach a 350-μm filter to either a modified Falcon tube or a beaker on a ring stand. Set up a funnel above this unit and place a 500-ml beaker below to collect the filtrate.

   b. To collect purified egg chambers at the desired stage, set up another funnel above two more filters in series (a 250-μm filter followed by a 125-μm filter) on a ring stand and place a 500-ml beaker below.

      Notes: This assembly allows the investigator to quickly pour the ground flies onto the filters and then regrind the flies retained on the 350-μm filter at least once.

      The 125-μm filter retains mostly stage-11Ð14 egg chambers. If earlier stages are desired, an 86-μm filter will trap stages 9Ð14, allowing stages 1Ð8 to flow through; these can be trapped by a 64-μm net.

3. Pour 150 ml of freshly prepared 1x RobbÕs Medium containing 1% BSA into the blender.

   Notes: The BSA helps minimize egg chamber losses and helps in the purification step, as many fly parts float in this buffer, whereas egg chambers sink.

   If the main focus of the study is not meiosis, it may be better to use EBR (containing 1% BSA), which is iso-osmotic, rather than RobbÕs Medium, which is hypertonic.

4. Anesthetize the flies with either CO_2 or ether. Transfer flies to the blender.
5. Pulse the flies three to five times at the lowest setting (2 seconds each time).
6. Pour all of the mix from the blender onto the 350-m filter. The egg chambers flow through and are collected in the beaker.
7. Remove the 350-m Nitex filter and rinse the flies into the blender container with another 150 ml of 1x RobbÕs Medium containing 1% BSA. Blend again as in step 5.

   Note: It is tempting to blend for too long, but the result is that more legs are isolated and the eyes can become disrupted. Verify the efficiency of releasing the egg chambers and optimize the blending by looking at the filtrate using a dissecting microscope. Use the least blending possible to get a reasonable quantity of egg chambers. Blending too vigorously produces more legs with their sticky bristles, making egg chamber rolling difficult (see Protocol 4.3).

8. Pour the filtrate through the 350-m filter. Collect the filtrate in the beaker containing the filtrate from step 6.
9. Quickly transfer the filtrate through the funnel above the 250- and 125-m filters. Rinse the eggs on the filters with another 50 ml of 1x RobbÕs Medium containing BSA. Then rinse the contents from the 125-m filter into a 15-ml tube with a minimum of PBS. Allow the egg chambers to settle for approximately 5 minutes (there is no need to wait longer because the remnants that settle slower are not egg chambers).
10. Aspirate the buffer and add 1x Fix Buffer (warmed to 25¼C). Fix with gentle rocking for no more than 4Ð5 minutes.
11. Allow the eggs to settle for 1 minute and then quickly remove the fix and add 10 ml of PBST. Allow the eggs to settle and repeat this three times.

    Notes: If the aim is to study germaria through stage-10b egg chambers, skip the next step (i.e., Protocol 4.3 [Removal of the Chorion and Vitelline Membranes]). Proceed to washing egg chambers (Protocol 4.3 [Washing and Permeabilizing]).

    A good approach to monitor fixation is by looking at the extensive microtubules found in well-fixed follicle cells. In overfixed egg chambers, histone antibodies do not label nurse cell nuclei very well.

Method 2

Simultaneous Isolation and Fixation of Egg Chambers

1. Follow steps 1Ð6 and step 9 as described above in Method 1 using 4% paraformaldehyde in 1x Robbs Medium.

   Note: In Method 2, flies are blended only once, poured through the 350-m filter, and subsequently over the 250-m and 125-m filters (see step 2, below). This should take less than 1 minute.

2. Perform steps 10Ð11 as described in Method 1.

PROTOCOL 4.2

Hand Dissection of Egg Chambers

When it is difficult to generate large numbers of females, hand dissection is an option. Egg chambers from 16 flies are dissected in Modified RobbÕs Medium. If the fly is healthy, the ovaries will take up a substantial portion of the abdomen.

Materials

Media

Bottles containing yeast paste (see p. 72)

Supplies and Equipment

Dissecting surface, either slides or spot plates (Pyrex)
Black surface for placing slides or spot plates
Fiber-optic light source
Dissecting tools
- dissecting microscope
- Dumont #5 tweezers (2 pairs)

Kimwipes

Solutions and Reagents

1x RobbÕs Medium containing 1% BSA (see p. 72)

Fixative
- 1x Fix Buffer (from 2x stock, see p. 73)
- 4% fresh paraformaldehyde

CAUTION: paraformaldehyde (see Appendix 4)

Method

1. Raise flies as described in Protocol 4.1, step 1.
2. Add a drop of Modified RobbÕs Medium either onto a slide or onto spot plates (Pyrex), placed on a black surface and illuminated from the two sides with a fiber-optic light source.
3. Anesthetize two flies at a time and collect the ovaries from each fly as follows:
   a. Use a pair of Dumont #5 tweezers to grab the constriction at the junction of the thorax and abdomen (see Figure 4.1, around p. 1). Use another pair of tweezers to pull at the tip of the posterior abdomen (region that appears as a lip around the vulva in Figure 4.1) while the fly is on its dorsal side. The uterus and any oocyte about to emerge will be attached to a piece of the abdomen and some internal organs will also come along.
   b. Pull until the organs rupture, wipe the tweezers on a Kimwipe, and remove the remainder of any exposed organs.

c. While still maintaining the grip on the most proximal abdomen, T1 (A1 region), remove a portion of the cuticle. At this point, the paired ovaries will come out of the fly, but sometimes a little pressure behind the ovaries will facilitate this. Dissect the other fly.

4. Put both pairs of ovaries in a well with Fixative for 4 minutes. During this time, remove the muscle-containing sheath surrounding the ovarioles and tease them apart into separate egg chambers.

5. Proceed with the next appropriate step (see Protocol 4.3) depending on the stage of interest, i.e., removing the outer layers (rolling to remove both chorion and vitelline membranes for late-stage egg chambers) or washing and permeabilizing the egg chambers (detergent extraction for stages where the vitelline membrane is still extractable up to stage 13).

PROTOCOL 4.3

Outer Membrane Removal of Egg Chambers

The follicle cells, chorion, and vitelline layers are removed from late-stage egg chambers by a process called Òrolling.Ó This allows antibodies to penetrate the eggs. For early-stage egg chambers, the vitelline membrane can be permeabilized with a nonionic detergent.

Materials

Supplies and Equipment

Slides, end-frosted
Dissecting microscope

Solutions and Reagents

PBST (see p. 73)

Method

Removal of the Chorion and Vitelline Membranes

1. Transfer the fixed egg chambers to the frosted portion of a slide. Remove most of the buffer.

2. Place the frosted portion of another slide on top and roll the egg chambers between the slides. Alternatively, place a 22-mm coverslip on top of the embryos and roll the egg chambers between the coverslip and slide.

Notes: Roll the eggs hard enough to remove both the chorion and the vitelline layers, to allow antibodies to penetrate. The eggs are fairly tough, but the trick is to have just the right amount of eggs and to remove most of the buffer. Too few eggs tend to get damaged; on the other hand, if there are too many eggs, it is difficult to remove the vitelline layer. At the point when the egg chambers have been rolled enough, some will appear to be breaking open.

In the beginning, learn to recognize the vitelline membrane in a dissecting microscope. If necessary, look at the eggs at 488 nm under a fluorescent microscope; under 488-nm excitation, the vitelline membrane is obvious and appears as an autofluorescent green sheet. Vitelline membrane that tends to come off in small pieces indicates that the formaldehyde is getting old.

Washing and Permeabilization

1. Rinse the egg chambers into a 15-ml Falcon tube with PBST and allow them to settle by gravity.
2. Remove the PBST. Add 5 ml of PBST and gently rock the egg chambers for 30 minutes.
3. Allow the egg chambers to settle. Remove PBST and rinse three times with 10 ml of PBST.

PROTOCOL 4.4

Labeling the Egg Chambers

Materials

Supplies and Equipment

Microcentrifuge tubes (0.5 ml; Eppendorf)
Coverslips (#1.5)
Disposable transfer pipettes
Nail polish

Solutions and Reagents

Primary antibodies
- For labeling of - and -tubulin:
  - DM1A, anti- -tubulin, mouse monoclonal (Sigma T 9026)
  - DM1B, anti- -tubulin, mouse monoclonal (Amersham N357)
  - YL1/2, anti- -tubulin, rat monoclonal (Accurate)
  - YOL1/34, anti- -tubulin, rat monoclonal (Accurate)
- For direct labeling of - and -tubulin:
  - Cy3-labeled TUB2.1, anti-tubulin, mouse monoclonal (Sigma)
  - FITC-labeled DM1A, anti--tubulin, mouse monoclonal (Sigma)
- For histone labeling:
  - anti-core histone, monoclonal IgG2 (Chemicon MAB052; monoclonal antibody to histone H1 + core proteins)

Secondary antibodies
1x PBS (see Appendix 3)
(Optional) DNA-binding dyes (e.g., OliGreen, SYBR Green I, or PicoGreen; Molecular Probes)
(Optional) Rhodamine-phalloidin (Molecular Probes R-415), 10 aliquot at Ð20¼C

Blocking Solution
- PBST (see p. 73)
- Blocking reagent
- Some examples of blocking reagents include BSA (0.1Ð5%); normal serum from the same host species as that in which the secondary antibodies were raised (serum from various species is available from Jackson ImmunoResearch Laboratories); Blotto (prepared by dissolving powdered milk [0.1Ð5%] in PBS and briefly centrifuged).

Mounting Medium
1x PBS
90% glycerol
1 mg/ml p-phenylenediamine

CAUTION: p-phenylenediamine (see Appendix 4)

Method

1. Transfer the egg chambers to a 0.5-ml microcentrifuge tube. Incubate the egg chambers in Blocking Solution for 1 hour. Then rinse the egg chambers five times with Blocking Solution.

   Note: If only microtubules, chromosomes, or actin are being labeled, this blocking step can be skipped with little degradation of the signal. However, for other antibodies, the minimum amount and best blocking reagent must be determined empirically. Too much blocking can sometimes obscure the signal, but careful examination is necessary to determine background versus genuine labeling. Preimmune sera for affinity-purified antibodies, preadsorbing antibody with antigen, and secondary antibody alone are good controls, but can be misleading. The best control and method is to use a null mutant; it is simple to determine the best blocking conditions and antibody concentration using null mutants.

2. Add the appropriate primary antibodies at empirically determined concentrations (usually 0.1Ð10g/ml) in 0.2Ð0.5 ml of Blocking Solution. Incubate either at room temperature for 4 hours or at 4¼C overnight.

   Notes: When performing double-labeling of microtubule-associated proteins and tubulin, it is sometimes better to add the nontubulin antibody first, incubate, and then add the tubulin antibody; however, adding both simultaneously also works.

   Chromosomes can be visualized using DNA-binding dyes or histone antibodies. For most purposes, dyes are sufficient and the relevant methods are discussed below (see Protocol 4.4 Notes), but antibody labeling gives a crisper outline of the edges of the chromatin. A mouse monoclonal antibody against core histones sold by Chemicon yields excellent results. To label microtubules, several monoclonal antitubulin (or -subunit) antibodies have been used, including mouse monoclonal antibodies DM1A and DM1B and rat monoclonal antibodies YL1/2 and YOL1/34. The rat monoclonal antibodies label meiosis I spindles slightly better, but Sigma sells an FITC-conjugated anti- -tubulin antibody that works very well and eliminates the need for secondary antibodies. Alternatively, directly label both the core histone antibody and one of the tubulin antibodies by standard methods (Harlow and Lane 1988) or buy a kit sold by several companies (e.g., Sigma and Amersham). The secondary antibodies are thus avoided, which makes the procedure faster and gives less background. Generally, direct immunofluorescence is successful for antigens that are abundant or highly concentrated at one location.

3. Wash the egg chambers six times with Blocking Solution for 15 minutes (each wash).

4. Add the appropriate secondary antibodies in Blocking Solution. Incubate at room temperature for 4 hours. Wash egg chambers as described in step 3 (above).

5. Rinse three times with 500 of PBS.

6. (Optional) To label actin, incubate with fluorescently conjugated phalloidin as follows:

   a. Place a 10l previously frozen aliquot of rhodamine-phalloidin in a SpeedVac and dry for approximately 5 minutes. Resuspend in 100 PBS (final concentration 1 g/ml).

b. Incubate at room temperature for 20 minutes.

c. Rinse three times with 500 of PBS.

7. After the final wash, allow egg chambers to settle and then remove as much as possible of the PBS. Add 22–40 of Mounting Medium. Make sure that the Mounting Medium and whatever PBS is left are mixed by gently stirring with the tip of a disposable transfer pipette, and then transfer to the surface of a clean #1.5 coverslip.

Notes: It is important to remove as much of the PBS as possible because the refractive index of the mounting media (1.515) is designed to match the refractive index of glass (PBS has a much lower refractive index).

Most lenses are designed to image through 170 m coverslips; therefore, the common practice of using #1 coverslips (the thickness of #1.5 coverslips is ~150 m) can be considered to be major tradeoff for the increase in working distance compared to the substantial degradation of the thick egg chamber image. Put a few pieces of broken coverslip into the mounting media on the coverslip to avoid compressing the egg chambers. This also makes it easy to mount the coverslip and slide in a parallel manner. Put the slide over the coverslip and seal with nail polish.

PROTOCOL 4.4 NOTES

If histone antibodies are not used, DNA can be detected by staining with DAPI (0.1 g/ml) or with some of the DNA-binding dyes sold by Molecular Probes. DAPI is reliable and resistant to bleaching, but it requires detection with UV, and the oocyte (but not the earlier egg chambers) is autofluorescent in this range. Molecular Probes sells many DNA-binding dyes that work well. Several dyes including TOTO-3 have been used as recommended by Molecular Probes, but SYBR Green or PicoGreen are much less expensive and are very bright even when used at a dilution of 1:10,000. Many *Drosophila* laboratories use OliGreen at 1:5000 to 1:10,000 dilution to label egg chambers treated with 20 g/ml of RNase A for 30 minutes. Label with DNA-binding dyes after the next to final wash.

PROTOCOL 4.5

-Galactosidase Activity in *Drosophila* Ovaries

Materials

Supplies and Equipment

Water bath at 37¼C
Depression slide
Dissecting microscope
Slides
Coverslips (#1.5)

Solutions and Reagents

1x PBS (see Appendix 3)
X-gal (8%) in DMSO, freshly prepared
Mounting Medium without *p*-phenylenediamine (see p. 78)

-Galactosidase Staining Solution

10 mM sodium phosphate
150 mM NaCl
1 mM $MgCl_2$
3.3 mM $K_4[Fe^{II}(CN)_6]$
3.3 mM $K_3[Fe^{III}(CN)_6]$
0.3% Triton X-100

Immediately before use, incubate the staining solution and 8% X-gal solution at 37¼C. To 1 ml of warm staining solution, add 25 of warm X-gal solution. This is done to increase the solubility of X-gal.

CAUTION: DMSO, $K_3[Fe^{III}(CN)_6]$, $K_4[Fe^{II}(CN)_6]$, $MgCl_2$, p-phenylenediamine, sodium phosphate, X-gal (see Appendix 4)

Method

1. Obtain egg chambers by hand dissection and fix as described in Protocol 4.2. Permeabilize the egg chambers as described in Protocol 4.3 (Washing and Permeabilization).
2. Incubate in -galactosidase Staining Solution at 37¼C. Observe the egg chambers in a depression slide with a dissecting microscope. After egg chambers have been stained sufficiently, rinse with PBS.

   Note: The time for staining should be determined empirically and will range from 5 minutes to overnight. Some lines that express high levels of activity may need to be rinsed with PBS after several minutes. Others take much longer.
3. Mount the egg chambers in Mounting Solution without the p-phenylenediamine.
4. Photograph with a conventional light microscope.

DATA COLLECTION

The best images are obtained when a confocal microscope is used to avoid out-of-focus light, and images should be obtained by whatever averaging methods are available (at least 9 averages). Images with the least amount of aberrations can be obtained by looking at structures that are close to the surface of the egg chamber on the side next to the coverslip. Lenses should be used with the highest available numerical aperture (NA). To observe structures deep in the egg chambers, use lenses that have a coverslip correction collar. Careful examination of deep structures and a reiterative adjustment of this collar will improve the quality of these images. Data collection should be optimized by taking advantage of the full dynamic range of the data-collection equipment, i.e., set the black level just below the lowest signal and adjust the gain to avoid saturation of the part of the image that will be recorded. Most digital image-collection devices have look-up tables that use displays in pseudo-coloring, making this part of data collection trivial. The same effect can

be achieved by postcollection data-processing methods; however, the data lose resolution because fewer data points exist between each intensity value.

LIVE ANALYSIS OF MEIOSIS

The tradition of observing living *Drosophila* cells injected with fluorescent cellular proteins was initiated in the Alberts laboratory at the University of California, San Francisco. Doug Kellogg injected fluorescent cytoskeletal elements into early embryos (Kellogg et al. 1988), Jon Minden injected labeled histones (Minden et al. 1989), and Bill Theurkauf began injecting egg chambers with labeled tubulin (Theurkauf et al. 1994a). Later, in collaboration with the Theurkauf and Goldstein laboratory, these methods were used to study meiosis in wild-type and *mei-41*-null mutants (Matthies et al. 1996).

Tubulin injection and subsequent imaging of live, early egg chambers have been reported by Theurkauf (1994a), but will not be discussed here. No one has reported injecting actin into egg chambers, but this has been done in early embryos, and the tubulin method discussed below should work for fluorochrome-conjugated actin. The overall gross morphology of live egg chambers dissected as described below can be observed with any microscope equipped with DIC optics. OliGreen works well for observing the chromatin by fluorescence. The early stages quickly pick up the dye and label the DNA; however, later stages need to be injected. OliGreen can be diluted 1:100, and a small aliquot can be mixed in the endogenous fluid around the dissected egg chambers on the coverslip. Rhodamine-conjugated tubulin labeled as described by Kellogg et al. (1988) or commercially available conjugated tubulin from either Molecular Probes or Cytoskeleton has been used. All work well, and purchasing the labeled tubulin saves a lot of time. The tubulin from Molecular Probes is brighter, but the tubulin from Cytoskeleton is less expensive.

Chromatin can be imaged by injecting various DNA-binding dyes, but the effect of the dyes on chromatin in vivo is not always easy to determine. Therefore, to facilitate the study of chromosome behavior, we modified a 4.0-kb *Drosophila* His2AvD genomic fragment to encode a fusion protein containing nearly all of His2AvD (an H2AZ class variant histone) fused to the GFP of the jellyfish *Aequorea victoria* (Clarkson and Saint 1999). Since this construct is under the control of endogenous His2AvD regulatory sequences within the genomic fragment, it is expressed in a pattern indistinguishable from that of His2AvD and complements an His2AvD null allele. Preliminary studies with flies bearing this GFP-histone fusion in the presence of wild-type levels of His2AvD show some slight female meiotic chromosome misbehavior.

PROTOCOL 4.6

Analysis of Meiosis in Live Egg Chambers

Materials

Supplies and Equipment

Injection apparatus
Syringe (10 or 20 ml)
Plastic tubing to connect micropipette holder to syringe

Glass needles (filament: 4 inches long, 1.0 mm diameter)
Coverslips (#1.5; 50 x 22 mm)
Inverted microscope linked to a confocal
CO_2 station
Dissecting tools
    Dumont #5 forceps
    dissecting microscope
Microcentrifuge tubes
Microcentrifuge or Beckman T100 tabletop ultracentrifuge

Solutions and Reagents

Halocarbon oil
Rhodamine tubulin (10-l aliquot at Ð20¼C; Molecular Probes or Cytoskeleton)
(Optional) Fluorescein or rhodamine actin (Cytoskeleton)
OliGreen (10-l aliquot at Ð20¼C; Molecular Probes)

CAUTION: CO_2 (see Appendix 4)

Method

Pipette Pulling and Setting Up the Injection Apparatus

1. Set up the injection apparatus on the microscope.

    Notes: Any standard, simple injection apparatus will suffice; use plastic tubing between the needle holder and a simple 10- or 20-ml syringe.

    Injection needles are pulled similar to the needles pulled for P-element transformation, but the tips are stubbier.

2. Break open the tip of a sealed, pulled needle as follows:

    a. Place a coverslip on the stage of the inverted microscope linked to the confocal. Focus on the middle of the edge of a coverslip.

    b. Move the stage with the mounted coverslip to the side, and bring the needle into focus (the injection holder approaches the stage at a fairly shallow angle). Gently tap the needle against the side of the coverslip and visually verify that the needle tip breaks. Lift the needle holder straight up; this allows the needle to come back into focus quickly.

Hand Dissection of Egg Chambers

1. Maintain well-fed, 2Ð3-day-old flies as described for the fixed-oocyte preparations (see step 1 in Protocol 4.1).

2. Anesthetize several females with CO_2 and immerse in halocarbon oil.

    Note: The halocarbon oil allows for high oxygen levels and helps keep the oocytes bathed in their native extracellular fluid.

3. Remove ovaries as described in step 3 of Protocol 4.2 and tease apart with two forceps on the surface of a clean #1.5 coverslip (50 x 22 mm).

4. Remove whatever loose particles are visible and rotate the oocytes in the appropriate position in a line of 5Ð10 oocytes.

Note: In the next step, the oocytes will be observed through an inverted microscope linked to a confocal; thus, the goal is to orient the late stage-12 to early stage-13 oocyte in such a way as to position the intact nucleus as close as possible to the coverslip. At this stage, the nurse cells have begun to degenerate, but 15Ð13 nurse cell nuclei can usually still be seen. The nurse cells can be seen as spheres at the most anterior portion of the oocyte. The dorsal appendages are usually not visible, but will extend during the experiment.

5. Observe the oocytes on the inverted microscope linked to the confocal using either DIC or phase-contrast optics.

Note: If using GFP-histone-expressing flies, the oocyte karyosome in the nucleus is seen as a dim green spot relative to intensely bright, nurse cell nuclei or even the follicle cells. With these optics, the nucleus (~35 m) is seen as a clearing of the oocyte cytoplasm. It lies on the posterior and most anterior side of the oocyte. If the oocyte is not positioned right, the nucleus is not as clearly visible because it is not as close as possible to the coverslip. Go back to the dissecting microscope and reorient the oocyte.

Injection

1. To prepare the dye mixture for injection, quickly thaw out 10 aliquots of rhodamine-labeled tubulin and OliGreen. Dilute the OliGreen 1:10 in H_2O. Add 1 l of 1:10 dilution of OliGreen to 1 l of rhodamine-tubulin. Centrifuge in a microcentrifuge at 12,000 g at 4¼C for 10 minutes or in a Beckman T100 tabletop ultracentrifuge at 20,000 g.
2. Mount the coverslip on the stage, and pipette 1 l of the rhodamine-labeled tubulin onto the oil. It is important to perform these steps quickly to avoid tubulin polymerization in the needle. Use a 10x or 20x lens to lower the needle into the tubulin and draw some into the needle.
3. Lift up the needle and bring the first oocyte of the column into focus. Lower the needle and push it into the oocyte until the tip of the needle lies just next to the nucleus and push on the syringe. A small clearing will be seen in the cytoplasm. Pull the needle out and inject the remaining oocytes.

DATA COLLECTION

Set the confocal to collect every 30 seconds, and choose an oocyte that looks the best usi either a 40x or 60x lens. Look for a nice clear circular nuclear outline and the best orientation gauged by the sharpness of the best focus. If the oocyte is lying on the dorsal side par allel to the coverslip, the nucleus will appear perfectly round. If the oocyte is on its side with the nucleus closest to the coverslip, the nucleus can be seen to lie next to the membrane ar will appear as a half-circle. The second orientation is more difficult to image, but different aspects can be detected. When nuclear envelope breakdown approaches, the nuclear env lope begins to pucker. Quickly begin collecting data every 4Ð10 seconds. Take either a sing scan or two scans and average. Averaged scans look crisper, but time-resolution is obvious ly lost. Most confocals are capable of doing time-lapse Z-series, and this makes it easier t follow the karyosome. Soon after the nuclear envelope breaks down, the karyosome move toward the middle of the nucleoplasm, and thus it is necessary to refocus every 10 second until the position of the karyosome has stabilized. If able to observe in four dimensions as many confocals can, this is less of a problem. Eventually, the tubulin signal can be seen around the chromatin. Data are best collected on the hard drive, and data presentation of single images can be carried out using Adobe Photoshop. Videos from the digital images ca also be generated with commercially available software packages sold by Adobe Systems.

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5

Cytological Analysis of Spermatocyte Growth and Male Meiosis in *Drosophila melanogaster*

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Drosophila male meiosis is a highly suitable system for the genetic and molecular dissection of the meiotic process. In the past few years, many genes involved in the regulation and execution of male meiosis have been identified and characterized (for review, see Maines and Wasserman 1998). Due to their large size, meiotic cells of *Drosophila* males have proved to be excellent for the cytological examination of mutant phenotypes and immunolocalization of meiotic gene products (Gunsalus et al. 1995; Williams et al. 1995, 1996; Hime et al. 1996; Molina et al. 1997; Basu et al. 1998; Bonaccorsi et al. 1998; Carmena et al. 1998; Giansanti et al. 1998). It is also of interest that meiotic division can be viewed in testes of third-instar larvae. This allows examination of meiosis in animals homozygous for mitotic mutations that die at the larval/pupal boundary (Ripoll et al. 1985; Gonzales et al. 1988; Sunkel and Glover 1988; Casal et al. 1990; Gunsalus et al. 19 Molina et al. 1997). In this chapter, we describe the main procedures for the cytological analysis of premeiotic and meiotic stages of *Drosophila* males. To facilitate the understanding of these procedures, an overview of *Drosophila* male meiosis is given below.

OVERVIEW

Male meiosis occurs in the context of a complex developmental process, called spermatogenesis, that leads to the formation of 64 spermatozoa from each primary gonial cell (for reviews, see Lindsley and Tokuyasu 1980; Fuller 1993; Maines and Wasserman 1998). *Drosophila melanogaster* spermatogenesis begins in the germinal proliferation center, an area located at the apex of the testis which comprises 5Ð9 germ-line stem cells and 9Ð somatically derived, cyst progenitor cells (Hardy et al. 1979; for review, see Lindsley and Tokuyasu 1980). The asymmetric division of a stem cell generates a primary spermatogonium and another stem cell, which remains anchored at the testis apex. Concomitantly with the formation of this primary gonial cell, the asymmetric division of two neighboring cyst progenitor cells generates two cyst cells, which encyst the primary spermatogonium and its progeny until completion of spermatogenesis.

The primary gonial cell undergoes four rounds of mitotic divisions, giving rise to 16 primary spermatocytes. These cells, after the premeiotic DNA synthesis, enter a growth

phase that lasts approximately 90 hours and results in a 25-fold increase in nuclear volume (for review, see Lindsley and Tokuyasu 1980; Fuller 1993; Cenci et al. 1994). Nuclear growth is accompanied by the formation of the Y chromosome lampbrush-like loops generated by the kl-5, kl-3, and ks-1 fertility factors. At the end of the growth period, the Y loops degenerate, and primary spermatocytes divide meiotically producing 64 spermatids (Bonaccorsi et al. 1988; Cenci et al. 1994).

In both gonial and meiotic divisions, the execution of cytokinesis does not lead to complete separation of the daughter cells, which remain connected by cytoplasmic bridges called the ring canals (Lindsley and Tokuyasu 1980; Hime et al. 1996). Moreover, in cysts containing spermatogonia or immature primary spermatocytes, all of the germ-line cells are linked together by the fusome, a prominent, branched cytoplasmic structure enriched in both F actin and -spectrin, which passes through the ring canals. The fusome drastically regresses in mature spermatocytes and remains almost absent during meiotic divisions but reforms after meiosis in spermatid cysts (Gunsalus et al. 1995; Hime et al. 1996; Giansanti et al. 1999). A fusome has also been observed in *Drosophila* females, and several genes have been identified that specify fusome components (Robinson and Cooley 1996). Yet, the precise function of this structure is still poorly defined.

Degeneration of the Y-chromosome loops marks the beginning of meiosis and is concomitant with the formation of a very prominent spindle, particularly amenable to cytological analysis (see, e.g., Cenci et al. 1994; Gunsalus et al. 1995; Bonaccorsi et al. 199; Carmena et al. 1998; Giansanti et al. 1998). Both meiotic divisions occur within a double nuclear membrane that is invaginated and possibly fenestrated at positions underlying the asters but does not otherwise appear to disassemble (Tates 1971; Church and Lin 1982). In addition, during most phases of meioses I and II, the nuclei are surrounded by two additional systems of membranes. Three to five layers of so-called parafusorial membranes lie parallel to the spindle axis, whereas several layers of umbrella-shaped astral membranes cup the nuclear poles associated with the asters (Tates 1971). The actual biological role of these two membrane systems is not currently understood.

During meioses I and II, mitochondria line up along the parafusorial membranes and are equally partitioned between the two daughter cells at each division. If no meiotic errors occur, each spermatid receives the same haploid chromosome complement and the same amount of mitochondria (Tates 1971; Fuller 1993). After completion of the second meiotic division, the mitochondria inherited by each cell fuse to form a complex structure, called the Nebenkern, containing multiple layers of mitochondrial membranes. Thus, immediately following meiosis, at the so-called onion stage, a cyst is composed of 64 spermatids, each containing a single nucleus associated with a single Nebenkern. Onion-stage spermatids can be easily analyzed in living preparations viewed by phase-contrast optics where nuclei and Nebenkern are phase-light and phase-dark, respectively. In wild type, onion-stage nuclei and Nebenkern have a highly regular appearance and consistently exhibit similar sizes. Deviations from this regularity are diagnostic of failures in the fidelity of meiotic divisions. For example, because the volume of an onion-stage nucleus is proportional to its chromatin content (Gonzalez et al. 1989), the presence of nuclei of different dimensions is indicative of errors in chromosome segregation. On the other hand, spermatids composed of an abnormally large Nebenkern associated with two or four regular-sized nuclei are diagnostic of failures in meiotic cytokinesis (Fuller 1993; Castrillon and Wasserman 1994; Gunsalus et al. 1995; Williams et al. 1995; Basu et al. 1998; Carmena et al. 1998; Giansanti et al. 1998).

In our description of the procedures for cytological analysis of meiosis, we first delineate the methods for preparation of living testes. We then provide a detailed account of the

fixation procedures for indirect immunofluorescence with various types of antibodies. Next, we outline the squashing and staining techniques for meiotic chromosome preparations. Finally, we present brief descriptions of both Y-loop development and male meiosis, providing a photographic documentation of these processes. We believe that these pictures facilitate recognition of the various premeiotic and meiotic stages and help in defining departures from normality observed in meiotic mutants.

PREPARATION OF LIVING TESTES

The easiest and fastest method for the analysis of spermatocyte growth and male meiosis is the examination of living testis squashes in aqueous solutions. These preparations allow unambiguous recognition of most cell types of Drosophila spermatogenesis, as well as most phases of male meiosis (see, e.g., Fuller 1993; Cenci et al. 1994; Maines and Wasserman 1998). Living testes squashes can be successfully carried out using a variety of aqueous solutions: Testis Buffer (see below; Hennig 1967; Pisano et al. 1993; Cenci et al. 1994); insect RingerÕs (Lifschytz and Hareven 1977; Kemphues et al. 1980; Castrillon et al. 1993); TB1 (7 mM K_2HPO_4, 80 mM KCl, 16 mM NaCl, 5 mM $MgCl_2$, 1% PEG 6000; Regan and Fuller 1990); PBS (Castrillon et al. 1993); and 0.7% NaCl (Sunkel and Glover 1988; Gonzalez and Glover 1993). Although these solutions give very similar results, in our laboratory, we routinely use testis buffer (TB). Protocol 5.1 is a standard procedure for preparation of living testes squashes.

PROTOCOL 5.1*

Preparation of Live Testis Squashes

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needles
- dissecting microscope

Nonsiliconized coverslips (20 × 20 mm)
Slides
Blotting paper

Solutions and Reagents

Testis Buffer (TB)

Component and final concentration	Amount to add to make 100 ml
183 mM KCl	1.364 g
47 mM NaCl	0.274 g
10 mM Tris-HCl	0.121 g
1 mM EDTA	0.037 g
1 mM PMSF	0.017 g

Dissolve in approximately 80 ml of H_2O. Adjust to pH 6.8 and bring to final volume of 100 ml with H_2O. Sterilize by filtration.

CAUTION: KCl, PMSF (see Appendix 4)

*For all protocols, H_2O indicates glass distilled and deionized.

Method

1. Dissect testes in TB from either third-instar larvae, pupae, or young adults.
2. Transfer testes into a 1-drop of TB on a clean, nonsiliconized 22×22-mm coverslip. Tear open the adult testes using either very thin forceps or tungsten needles.
3. Place a clean slide over the coverslip without pressing and invert the sandwich. If desired, a very mild squashing can be obtained by removing excessive buffer from the edges of the coverslip with a piece of blotting paper.

   Note: Steps 1–3 are usually performed at room temperature.
4. Analyze preparations with a ×40 objective, using phase-contrast optics.

PREPARATION AND STAINING OF FIXED TESTES

Fixation Procedures

Several fixation procedures have been developed to make testis preparations for the purpose of immunostaining. These protocols and their relative advantages and disadvantages are described below.

Protocol 5.2 describes a methanol-acetone fixation technique (Pisano et al. 1993; Cenci et al. 1994) which results in very good preservation of cell morphology. Fixed cells viewed by phase-contrast optics exhibit most of the structural details that can be seen in live material. This allows analysis of unstained fixed preparations and selection of the most suitable ones for immunostaining. Remarkably, the Y loops, which are usually faint and labile in living preparations, become clearly apparent after this type of fixation. Moreover, this fixation protocol results in excellent microtubule preservation for immunostaining with antitubulin antibodies. The main disadvantage of this technique is a poor preservation of chromosome structure. In most instances, the chromosomes do not exhibit a distinct morphology and tend to coalesce into one or more masses of chromatin.

Protocol 5.3 has proved to be particularly suitable for tubulin and centrosomin immunostaining (Bonaccorsi et al. 1998). It results in preparations having the same characteristics as those obtained by using Protocol 5.2.

Protocol 5.4 describes a fixation procedure (Gunsalus et al. 1995; Hime et al. 1996; Giansanti et al. 1998) that preserves F-actin-containing structures because it does not involve the use of methanol. Thus, it is particularly suitable for contractile ring and fusome visualization. However, it does not preserve cell morphology and microtubules as efficiently as Protocol 5.2 and does not permit a clear detection of the Y loops.

Protocol 5.5 (Williams 1993; Giansanti et al. 1999) was originally developed by Cayetano Gonzalez (EMBL, Heidelberg). It preserves chromosome morphology very well, allowing a clear visualization of the chromosome condensation-decondensation cycle. It also results in excellent preservation of microtubules for immunostaining. The main disadvantage of this fixation technique is its poor preservation of cell morphology, as viewed by phase-contrast optics. In addition, because it involves a rather hard squashing of formaldehyde-fixed testes, meiotic figures result flatter and larger than those obtained using the above fixation protocols.

Staining Procedures

Immunostaining with a Single Antibody

Testis preparations fixed according to Protocols 5.2 through 5.5 can be immunostained with a variety of antibodies. The concentration of the primary antibody will vary with both the type of antibody and the type of incubation and should be determined empirically each time. Protocol 5.6 describes a general procedure for immunostaining.

Double Immunostaining

Double immunostaining can be carried out using two different primary antibodies raised in different species, such as mouse or rabbit. These primary antibodies can be detected separately using species-specific secondary antibodies conjugated with different fluorochromes. One of these secondary antibodies should be conjugated with dyes such as rhodamine or Texas Red, which emit a red fluorescence, and the other with fluorescein, which produces a green fluorescence. In our laboratory, double immunostaining is carried out by repeating twice the steps described in Protocol 5.6 (see Notes).

Detection of double immunofluorescence requires the absence of Òbleed throughÓ through the filters used for red and green fluorescence. In addition, double immunofluorescence is greatly facilitated by the use of a cooled charged-coupled device (CCD) that permits separate collection of the fluorescein and rhodamine signals as gray-scale digital images. These images can be then converted into Photoshop format, pseudocolored, and merged (see, e.g., Gunsalus et al. 1995; Bonaccorsi et al. 1998; Giansanti et al. 1998).

F-actin Staining

Preparations fixed according to Protocol 5.4 can be stained for F actin according to Protocol 5.7. This staining procedure proved to be highly suitable for staining the male fusome and the cytokinetic contractile ring (Gunsalus et al. 1995; Hime et al. 1996; Giansanti et al. 1998).

Chromatin Staining

To visualize DNA, preparations obtained using Protocols 5.2 through 5.5 can be stained with several DNA-binding dyes (see Protocol 5.8). If the slides are to be examined by a fluorescence microscope equipped with filters that permit UV (ultraviolet) excitation, the most suitable dyes for DNA staining are Hoechst 33258 or DAPI (see, e.g., Bonaccorsi et al. 1988; Cenci et al. 1994; Hime et al. 1996; Carmena et al. 1998). If the slides are to be analyzed with a confocal microscope not equipped with a UV laser, DNA can be stained with either propidium iodide or TOTO-3 iodide (Gonzalez and Glover 1993; Carmena et al. 1998).

Multiple Stainings

Testis squashes can be stained with antibodies directed to specific proteins, with rhodamine-phalloidin to visualize F actin, and with DNA-binding dyes. Best results are obtained when these stainings are carried out in the following order: immunostaining, F-actin staining, and DNA staining. This implies that multiply stained preparations are all mounted in the media described for DNA staining (see Protocol 5.8, step 2).

PROTOCOL 5.2

Methanol-Acetone Fixation Technique I

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needles
- dissecting microscope

Nonsiliconized coverslips (20 × 20 mm)
Slides
Blotting paper
Razor blade

Solutions and Reagents

TB (for preparation, see p. 89)
Liquid nitrogen
Methanol, at Ð20¼C
Acetone, at Ð20¼C

1x PBS

Component and final concentration	Amount to add to make 1 liter
8.0 mM Na_2HPO_4	1.14 g
1.5 mM KH_2PO_4	0.20 g
137.0 mM NaCl	8.00 g
2.7 mM KCl	0.20 g

Combine all components in approximately 800 ml of H_2O and stir to dissolve; adjust pH to 7.4; bring to final volume of 1 liter; sterilize by autoclaving. We usually prepare a 10x stock solution of PBS and dilute to 1x.

PBS/Acetic Acid/Triton X-100 Solution

1x PBS
0.5% acetic acid
1% Triton X-100

CAUTION: acetic acid, acetone, KCl, KH_2PO_4, liquid nitrogen, methanol, Na_2HPO_4 (see Appendix 4)

Method

1. Perform steps 1Ð3 of Protocol 5.1 at room temperature.
2. Freeze the slides in liquid nitrogen.
3. Remove the coverslips with a razor blade, immediately immerse the slides into methanol at Ð20¼C, and leave for 5 minutes.
4. Transfer the slides into acetone at Ð20¼C and leave for 1Ð2 minutes.

5. Transfer the slides into PBS/Acetic Acid/Triton X-100 Solution and leave at room temperature for 10 minutes.
6. Wash the slides twice in PBS (5 minutes each wash).

PROTOCOL 5.3

Methanol-Acetone Fixation Technique II

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needles
- dissecting microscope

Nonsiliconized coverslips (20 x 20 mm)
Slides
Blotting paper
Razor blade

Solutions and Reagents

TB (for preparation, see p. 89)
Liquid nitrogen
Methanol, at Ð20¼C
Acetone, at Ð20¼C
1x PBS (see p. 92)

PBS/Acetic Acid/Tween-20 Solution
- 1x PBS
- 0.1% acetic acid
- 0.1% Tween-20

PBS/Tween-20 Solution
- 1x PBS
- 0.1% Tween-20

CAUTION: acetic acid, acetone, liquid nitrogen, methanol (see Appendix 4)

Method

1. Perform steps 1Ð3 of Protocol 5.1 at room temperature.
2. Freeze the slides in liquid nitrogen.
3. Remove the coverslips with a razor blade, immediately immerse the slides into methanol at Ð20¼C, and leave for 15 minutes.
4. Transfer the slides into acetone at Ð20¼C and leave for 30 seconds.

5. Transfer the slides into PBS/Acetic Acid/Tween-20 Solution and leave for 10 minutes.
6. Wash the slides three times in PBS/Tween-20 Solution (10 minutes each wash).
7. Wash in PBS for 5 minutes.

PROTOCOL 5.4

Fixation with Paraformaldehyde

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needles
- dissecting microscope

Nonsiliconized coverslips (20×20 mm)
Slides
Blotting paper
Razor blade

Solutions and Reagents

TB (for preparation, see p. 89)
Liquid nitrogen
Ethanol, at Ð20¼C
1x PBS (see p. 92)
Paraformaldehyde (4%) dissolved in 1x PBS; use as a freshly prepared solution
PBT (1x PBS containing 0.1% Triton X-100)

CAUTION: ethanol, liquid nitrogen, paraformaldehyde (see Appendix 4)

Method

1. Perform steps 1Ð3 of Protocol 5.1 at room temperature.
2. Freeze the slides in liquid nitrogen.
3. Remove the coverslips with a razor blade, immediately immerse the slides into ethanol at Ð20¼C, and leave for 10 minutes.
4. Fix slides in 4% paraformaldehyde in PBS at room temperature for 7 minutes.
5. Wash the slides twice in PBS at room temperature (5 minutes each wash).
6. Immerse the slides for 30 minutes in PBT (PBS containing 0.1% Triton-X) at room temperature.
7. Wash the slides twice in PBS (5 minutes each wash).

PROTOCOL 5.5

Formaldehyde Fixation

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needles
- dissecting microscope

Nonsiliconized coverslips (20 × 20 mm)
Slides
Blotting paper
Razor blade

Solutions and Reagents

Saline (0.7% NaCl in H_2O)
Acetic acid (45% and 60%)
Liquid nitrogen
Ethanol, at Ð20¼C
PBT (1x PBS containing 0.1% Triton X-100)
1x PBS (see p. 92)
Formaldehyde (3.7%) dissolved in 1x PBS

CAUTION: acetic acid, ethanol, formaldehyde, liquid nitrogen (see Appendix 4)

Method

1. Dissect larval or adult testes in saline.
2. Fix testes in 3.7% formaldehyde in PBS at room temperature for 30 minutes.
3. Transfer testes into 45% acetic acid and leave for 30 seconds.
4. Transfer testes into a drop (4 l) of 60% acetic acid placed on a 22 × 22-mm coverslip and keep them at room temperature for 2Ð3 minutes.
5. Invert the sandwich and squash testes applying a moderate pressure.
6. Freeze the slides in liquid nitrogen.
7. Remove the coverslips with a razor blade, immediately immerse the slides into ethanol at Ð20¼C, and leave for 15 minutes.
8. Transfer the slides into PBT and leave at room temperature for 10 minutes.
9. Wash the slides twice in PBS at room temperature (5 minutes each wash).

PROTOCOL 5.6

Immunostaining of Fixed Testes Preparations

Materials

Supplies and Equipment

Slides, prepared as described in Protocols 5.2Ð5.5
Humid chamber (e.g., a slide box containing two layers of blotting paper soaked with H_2O)
Rubber cement

Solutions and Reagents

1x PBS (see p. 92)
PBT (1x PBS containing 0.1% Triton X-100)
Vectashield mounting medium H-1000 (Vector Laboratories)

PBS/BSA
- 1x PBS
- 1% BSA

PBT/BSA
- 1x PBS
- 0.1% Triton X-100
- 1% BSA

Antibodies

Primary antibodies, diluted in PBS/BSA or PBT/BSA
Secondary antibodies, diluted in PBS

Method

1. After the final PBS washes described in Protocols 5.2 through 5.5, immerse the slides in PBS/BSA and leave at room temperature for 45 minutes.
2. Remove excess of PBS from around the specimen. (The squashed testes appear as opaque area on the slide.) Cover with a 20-l drop of primary antibody diluted in PBS/BSA. Alternatively, dilute the primary antibody in PBT/BSA.
3. Incubate the slides in a humid chamber for the desired time (e.g., either at room temperature for 2 hours or at 4¼C overnight).
4. Wash the slides twice in PBS (5 minutes each wash). If the primary antibody was dissolved in PBT/BSA, perform two washes in PBT (5 minutes each) and one wash in PBS for 5 minutes.
5. Remove excess of PBS from around the specimen (see step 2) and cover the preparation with 20 l of the secondary antibody diluted in PBS.

Note: We routinely use the following secondary antibodies: FLUOS-conjugated anti-mouse sheep IgG F(ab«)2 fragment (FLUOS: 5[6]-carboxyfluorescein N-hydroxysuccinimide ester), and rhodamine-conjugated anti-mouse sheep IgG, F(ab«)2 fragment, both from Boehringer Mannheim,

and both diluted 1:10; FITC-conjugated anti-rabbit goat IgG and TRITC-conjugated anti-rabbit goat IgG, both from Cappel (Durham), and both diluted 1:50.

6. Incubate the slides in the dark in a humid chamber at room temperature for 1 hour.
7. Wash the slides twice in PBS (5 minutes each wash).
8. If preparations are not to be stained with other reagents, mount either in PBS or in Vectashield. However, mounting in Vectashield results in poor cell morphology when slides are examined by phase-contrast optics.
9. Seal with rubber cement. Omit this step if slides are mounted in Vectashield.

PROTOCOL 5.6 NOTES

In our laboratory, double immunostaining is carried out as follows: Immunostain the slides with one of the primary antibodies and detect it with either a fluorescein- or a rhodamine-conjugated secondary antibody, following steps 1Ð7 (above). Then immunostain the slides with the second primary antibody and detect it with the appropriate secondary antibody (steps 2Ð8, above).

PROTOCOL 5.7

F-Actin Staining

Materials

Supplies and Equipment

Slides, prepared as described in Protocol 5.4
Humid chamber (e.g., a slide box containing two layers of blotting paper soaked with H_2O)
Rubber cement

Solutions and Reagents

1x PBS (see p. 92)
Vectashield H-1000 mounting medium (Vector Laboratories)

Preparation of Rhodamine-labeled Phalloidin

Dissolve 300 units of rhodamine-labeled phalloidin (Molecular Probes) in 1.5 ml of methanol. This stock solution can be stored at Ð20¼C.
Dry 100 l of this stock solution under vacuum and resuspend in 200 l of PBS.

CAUTION: methanol (see Appendix 4)

Method

1. Remove the excess of PBS from around the specimen and cover it with 10 l of Rhodamine-labeled Phalloidin.
2. Incubate the slides in the dark in a humid chamber at 37¼C for 1.5 hours.
3. Rinse the slides in PBS for 1Ð2 minutes.
4. Mount the slides either in PBS or in Vectashield H-1000 medium.
5. Seal with rubber cement. Omit this step if slides are mounted in Vectashield.

PROTOCOL 5.8

Chromatin Staining

Materials

Supplies and Equipment

Slides, prepared as described in Protocols 5.2–5.5
Rubber cement

Solutions and Reagents

1x PBS
Hoechst 33258 (0.5 g/ml), dissolved in Hoechst Buffer
Hoechst 33258 (5 g/ml), dissolved in Hoechst Buffer
DAPI (0.2 g/ml), dissolved in 1x PBS

Hoechst Buffer

Component and final concentration	Amount to add to make 1 liter
10 mM Na_2HPO_4	1.42 g
150 mM NaCl	8.76 g
30 mM KCl	2.23 g

Dissolve in approximately 800 ml of H_2O. Adjust to pH 7.0 and bring to final volume of 1 liter with H_2O. Sterilize by autoclaving.

CAUTION: DAPI, Hoechst 33258, KCl, Na_2HPO_4 (see Appendix 4)

Method

Hoechst or DAPI Staining

1. After final PBS washes, air-dry slides.
2. Stain with Hoechst or DAPI as follows:
   a. For Hoechst staining: Stain with 0.5 g/ml Hoechst 33258 dissolved in Hoechst Buffer for 10 minutes. Rinse for 1 minute and mount in Hoechst Buffer. Alternatively, directly mount slides in 5 g/ml of Hoechst 33258 dissolved in Hoechst Buffer.
   b. For DAPI staining: Stain with 0.2 g/ml DAPI dissolved in PBS for 10 minutes. Rinse for 1 minute and mount in PBS. Alternatively, directly mount slides in 0.2 g/ml of DAPI dissolved in PBS.

      Note: Hoechst 33258- and DAPI-stained slides can also be mounted in Vectashield H-1000. However, as mentioned earlier, Vectashield affects the appearance of cells viewed by phase-contrast optics.
3. Seal with rubber cement.

Staining with Propidium Iodide or TOTO-iodide

To stain preparations with propidium iodide or TOTO-iodide, add these dyes to 90% glycerol, 2.5% n-propyl gallate mounting medium, to a final concentration of 1 g/ml and 1/200, respectively (Gonzalez and Glover 1993; Carmena et al. 1998).

PREPARATION OF MEIOTIC CHROMOSOMES

Several techniques have been developed for preparation of male meiotic chromosomes (see, e.g., Cooper 1965; Ashburner 1989; Miyazaki and Orr-Weaver 1992). All of these techniques allow a clear visualization of chromosomes, but not of other cellular structures such as the cytoskeleton and the spindle. We describe here three of these techniques that in our hands give good reproducible results. The first technique, developed by Lifschytz and Hareven (1977) and described below in Protocol 5.9, is particularly useful for the analysis of adult testes. The other two techniques (Ripoll et al. 1985; Cenci et al. 1997) are routinely used in our laboratory for larval and pupal testis preparations. The technique described in Protocol 5.10 is a very simple method for preparing aceto-orcein-stained chromosomes. The other technique outlined in Protocol 5.11 allows preparation of unstained chromosomes that can be subsequently stained with various dyes such as Giemsa, Hoechst 33258, and DAPI, or processed for in situ hybridization.

PROTOCOL 5.9

Preparation of Meiotic Chromosomes from Adult Testes

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needle
- dissecting microscope

Slides
Coverslips (20 × 20 mm)
Blotting paper
Nail polish

Solutions and Reagents

Acetic acid (45% and 60%)
Orcein (3%), dissolved in 60% acetic acid (for preparation of 2% aceto-orcein, see p. 100)

Ringer's Solution

Component and final concentration	Amount to add to make 1 liter
182 mM KCl	13.6 g
46 mM NaCl	2.7 g
3 mM $CaCl_2$	0.33 g $CaCl_2 \cdot 2H_2O$
10 mM Tris-HCl	1.21 g

Combine all components in approximately 800 ml of H_2O and stir to dissolve. Adjust to pH 7.2 and bring to a final volume of 1 liter with H_2O. Sterilize by autoclaving.

Lacto-Aceto-orcein

Lactic acid: 3% orcein in 60% acetic acid (1:1 ratio)

CAUTION: acetic acid, $CaCl_2$, KCl, lactic acid (see Appendix 4)

Method

1. Dissect testes in RingerÕs Solution.
2. Fix testes in 45% acetic acid for 1 minute.
3. Stain in 3% orcein dissolved in 60% acetic acid for 5 minutes.
4. Quickly rinse the testes in 60% acetic acid and transfer them into a small drop of 60% acetic acid placed on a slide.
5. Cut testes under dissecting scope to release testis content. Add a drop of LactoÐAceto Orcein (1:1 lactic acid to 3% orcein in 60% acetic acid).
6. Gently lower a coverslip over the specimen; remove excess liquid from the edges of the coverslip with a piece of blotting paper.
7. Seal with nail polish.

PROTOCOL 5.10

Aceto-Orcein Preparation of Meiotic Chromosomes from Larvae or Pupae

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needle
- dissecting microscope

Coverslips (20 x 20 mm)
Slides
Blotting paper
Nail polish

Solutions and Reagents

Saline (0.7% NaCl in H_2O)

2% Aceto-Orcein

Boil orcein powder (Gurr, BDH Laboratory Supplies, Poole, Dorset BH15 1TD England; Phone: +44 1202 660444; Fax: +44 1202 666856; Web site: http://www.bdh.com) in 45% acetic acid for 30Ð45 minutes in a reflux condenser. We usually make 5% orcein and subsequently dilute it to 2% with 45% acetic acid. Before use, remove particulate matter by either filtration or centrifugation in a microcentrifuge.

CAUTION: acetic acid (see Appendix 4)

Method

1. Dissect larval or pupal testes in saline.
2. Transfer testes into a drop of 2% Aceto-Orcein placed on a coverslip and leave for 2 minutes.

3. Lower a slide over the coverslip and invert the sandwich; squash gently between two sheets of blotting paper.
4. Seal with nail polish.

PROTOCOL 5.11

Preparation of Unstained Meiotic Chromosomes from Larvae or Pupae

Materials

Supplies and Equipment

Dissecting tools
- fine forceps or tungsten needle
- dissecting microscope

Siliconized coverslips (20×20 mm)
Slides
Blotting paper
Razor blade

Solutions and Reagents

Acetic acid (45%)
Dry ice or liquid nitrogen

CAUTION: acetic acid, dry ice, liquid nitrogen (see Appendix 4)

Method

1. Perform steps 1–3 of Protocol 5.10 but use 45% acetic acid instead of 2% aceto-orcein, and use siliconized coverslips.
2. Freeze the slides either on dry ice or in liquid nitrogen.
3. Remove the coverslip with a razor blade and let the slides air-dry.

   Note: Air-dried slides can be stained with a variety of dyes, processed for chromosome banding, or used for in situ hybridization. For a detailed account of these techniques, see Pimpinelli et al. (this volume).

SPERMATOCYTE GROWTH AND Y-LOOP DEVELOPMENT

Spermatocyte growth and Y-loop unfolding are illustrated in Figure 5.1, which shows cells fixed by the methanol/acetone procedure described in Protocol 5.2 and sequentially stained for tubulin and chromatin. Stage subdivision has been proposed by Cenci et al. (1994) and is based only on morphological criteria. Each stage corresponds to a phase of spermatocyte development that is unambiguously distinguishable from both the preceding and the following one.

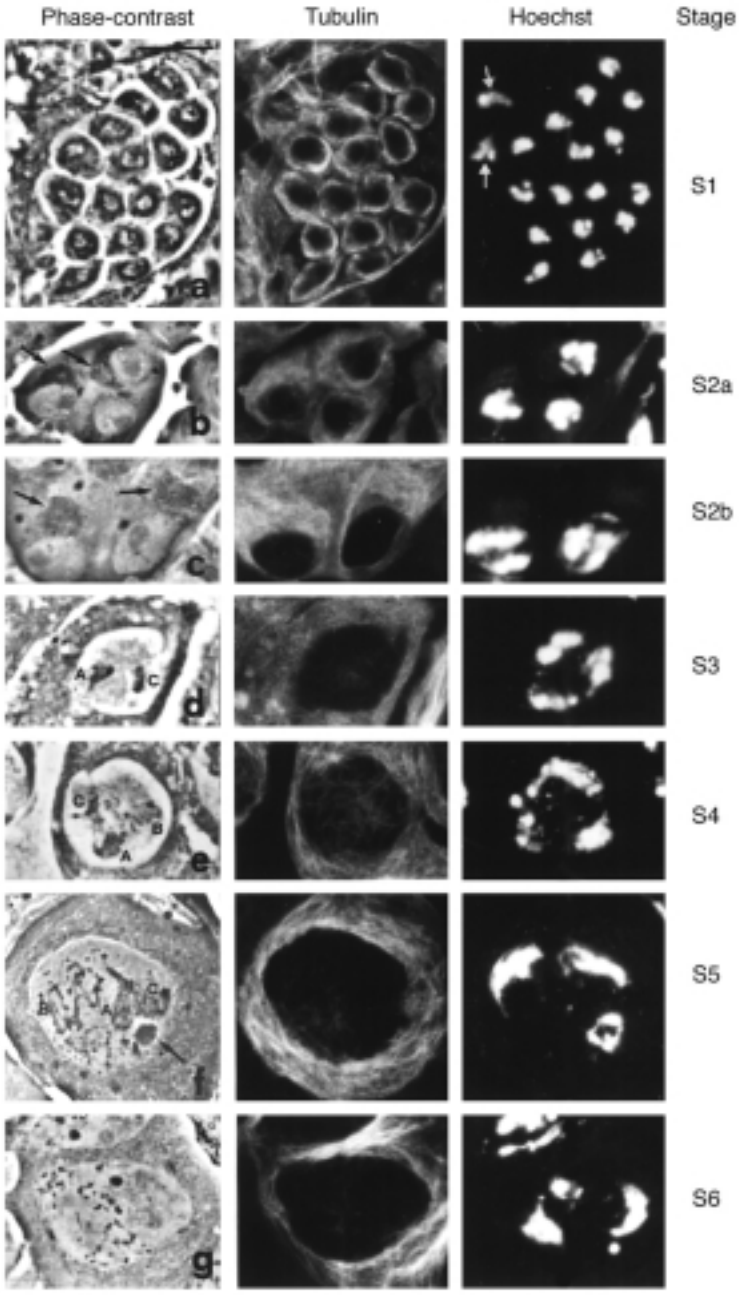

Figure 5.1. Primary spermatocyte growth and Y-loop development. (a) A complete cyst of 16 young primary spermatocytes; the arrows point to the cyst cells. (b, c) Polar spermatocytes; the arrows point to the clusters of mitochondria. (d) Young apolar spermatocyte showing the primordia of the Y-loop (A) and the 1 (C) Y-chromosome loops. (e) Primary spermatocyte in the S4 stage in which some filaments of the 3 loop (B) have become apparent. (f) Mature primary spermatocyte in the S5 stage showing fully developed Y-chromosome loops. (g) Primary spermatocyte in the S6 stage with disintegrating Y loops. Bar, 10 m. (Reprinted, with permission, from Cenci et al. 1994.)

Primary spermatocytes that have just completed premeiotic DNA synthesis (stage S1) are similar in size and morphology to spermatogonia. As the S1 spermatocyte enters the growth period, its nucleus assumes an eccentric position within the cytoplasm, and the mitochondria cluster at the cell pole opposite to the nucleus (stages S2a and S2b). During

the S2 stage (also called the polar spermatocyte stage; Tates 1971; Fuller 1993), the nucleus enlarges, and the chromatin that initially occupies the entire nuclear space begins to subdivide into three masses or clumps (stage S2). Two of these masses correspond to the paired large metacentric autosomes and the other corresponds to the sex chromosomes. The paired fourth chromosomes cannot be readily distinguished and are usually associated with the sex chromosome clump (Cooper 1965; Cenci et al. 1994).

As spermatocyte growth continues, the nucleus resumes a central position within the cell and the mitochondria disperse; the chromatin clumps expand moderately, while remaining apposed to the inner nuclear envelope, so that the nuclear space not occupied by the chromatin progressively increases. This space becomes filled by the Y chromosome loops elaborated by the *kl-5* (loop A), *kl-3* (loop B), and *ks-1* (loop C) fertility factors. These structures develop asynchronously with a characteristic sequence, providing useful markers for staging spermatocyte growth (Bonaccorsi et al. 1988; Cenci et al. 1994). The first appearance of the Y loops marks the S3 spermatocytes, which exhibit two phase-dark masses that correspond to the primordia of the *kl-5* and *ks-1* loops. The *kl-3* loop begins to unfold later during the S4 stage. Then, the three loops enlarge steadily and reach their maximum size in mature spermatocytes (stage S5), where they occupy most of the nuclear space. Although the *kl-5* and *ks-1* loops are composed of a thick thread, and the *kl-3* loop of a thinner and less folded filament, in many cases, the Y chromosome loops cannot be distinguished from each other. However, the Y loops can be identified by both cytological and genetic methods.

Cytologically, the Y loops can be differentiated by either Giemsa staining or immunostaining with loop-specific antibodies. Preparations obtained following Protocol 5.2 can be stained with 2% Giemsa (Merck) dissolved in borate buffer (Merck), rinsed in tap water, air-dried, and then mounted in Euparal. In these Giemsa-stained preparations, the *kl-5* and *ks-1* loops are selectively stained, whereas the *kl-3* loop and the chromatin clumps remain unstained (Bonaccorsi et al. 1988). Alternatively, the Y loops can be differentiated by immunostaining with (1) the S5 monoclonal antibody (Saumweber et al. 1980), which reacts strongly with the *kl-5* loop and weakly with the *ks-1* loop, but fails to decorate the *kl-3* loop (Melzer and Glatzer 1985; Bonaccorsi et al. 1988); (2) the T53-1 antibody, which specifically recognizes the *kl-3* loop (Pisano et al. 1993); and (3) either the anti-RB97 antibody (Heatwole and Haynes 1996) or the anti-Boule antibody (Eberhart et al. 1996; Cheng et al. 1998), which specifically bind the *ks-1* loop.

The Y loops can also be distinguished by genetic methods. For example, the use of fertile X-Y translocations with one breakpoint within the X heterochromatin and the other between two adjacent Y-chromosome fertility factors (Kennison 1981) permits construction of males carrying complementary sets of Y loops (Hardy et al. 1981; Bonaccorsi et al. 1988). Because the two elements of the T(X;Y) segregate regularly, X/Y^DX^P and $X^DY^P/0$ males can be obtained by crossing T(X;Y) males to regular and to attached-X/0 females, respectively. Thus, if the T(X;Y) used is broken between *kl-5* and *kl-3*, the spermatocytes of X/Y^DX^P males will only exhibit the *kl-5* loop, whereas those of $X^DY^P/0$ males will carry both the *kl-3* and *ks-1* loops. Males missing one or two specific Y loops can also be constructed using Y chromosomes that are deleted for the corresponding loop-forming fertility factors. These Y deficiencies are usually kept in combination with XáYL or XáYS. Thus, if XáYL/Df(YL)* or XáYS(Df(YS)* males [Df(YL)* and Df(YS)* denote genetically and cytologically defined Y deficiencies] are crossed to regular females, the male progeny will only exhibit the Y loops carried by Df(YL)* and Df(YS)*, respectively.

The disintegration of the Y loops marks the end of the growth phase of primary spermatocytes and the beginning of meiosis. During stage S6, the Y loops fall apart into pieces

the nucleolus disappears, and the chromatin clumps begin to condense while remaining apposed to the nuclear envelope.

THE MEIOTIC DIVISION

The meiotic division, as well as its subdivision into stages (Cenci et al. 1994), is illustrated in Figures 5.2 and 5.3, which show meiotic cells fixed by methanol/acetone (Protocol 5.2), immunostained for tubulin, and then stained with Hoechst 33258. We believe that these figures can provide a useful guide for recognition of meiotic stages and definition of the departures from normality observed in meiotic mutants. The sequence of meiotic phases presented in Figures 5.2 and 5.3 is, in most instances, self-explanatory. We thus focus only on some aspects of the meiotic process that are not immediately obvious from these figures. For a detailed description of the events illustrated in Figures 5.2 and 5.3, see Cenci et al. (1994).

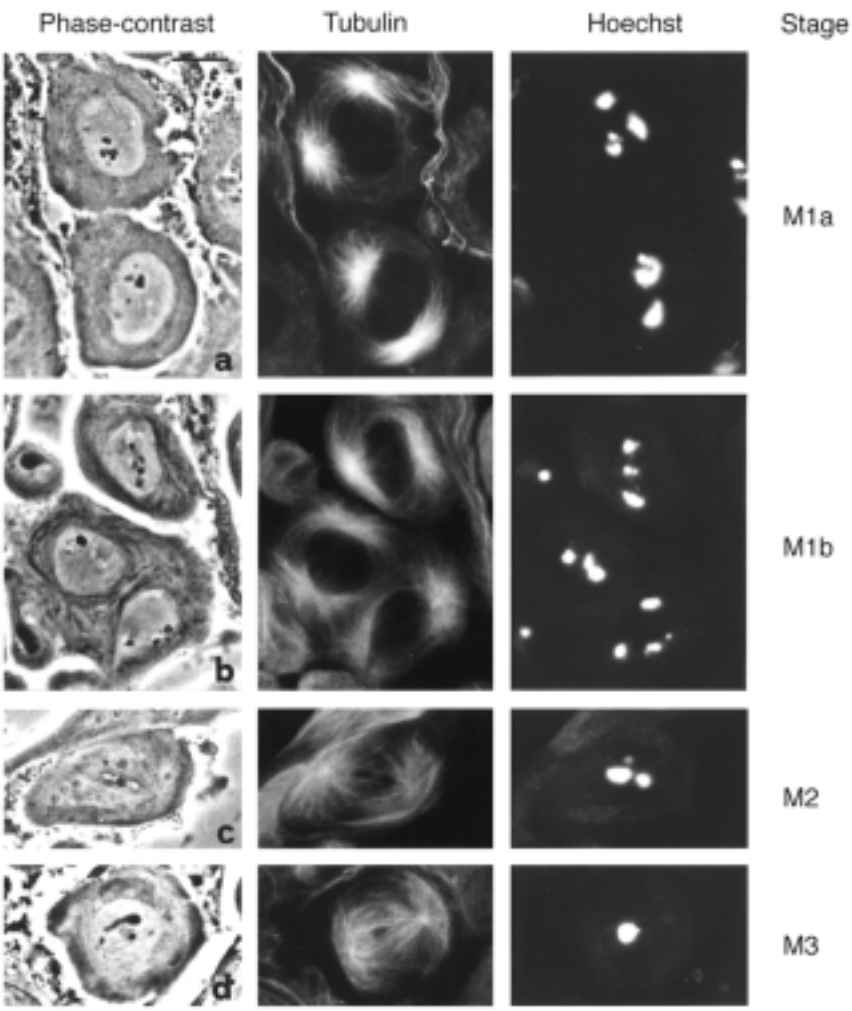

Figure 5.2. First meiotic division in D. melanogaster males. (a) Late prophase nuclei in the M1a stage showing phase-dense granules resulting from disintegration of the Y loops; (b) prometaphase nuclei; (c) late prometaphase nucleus in which the nuclear-cytoplasmic demarcation is no longer visible; (d) metaphase I with congregated bivalents. (Continued on facing page.)

The disintegration of the Y loops and the onset of the meiotic division are accompanied by a substantial shrinking of the spermatocyte nucleus. Late prophase cells in the M1 stage have a nuclear diameter 20% shorter than that of mature spermatocytes in the S5 stage. The M1 stage is also characterized by a high degree of chromatin condensation and by the presence of two prominent asters. Interestingly, the process of chromatin condensation, which occurs between the S6 and M1 stages, is rather rapid and no leptotene-pachytene stages can be seen in *Drosophila* primary spermatocytes (Cooper 1965; Cenci et al. 1994). Although the M1a spermatocytes are in late prophase, the M1b cells are probably in early prometaphase; in M1b cells, a few spindle fibers irradiating from the asters appear to reach the bivalents that occupy variable positions within the nucleoplasm. During the transition between early (M1b stage) and late (M2 stage) prometaphase, the nuclear-cytoplasmic demarcation evident in the M1 stages progressively disappears. This phenomenon is not related to the breakdown of the nuclear membrane, but rather to the dispersion of the nuclear lamins (White-Cooper et al. 1993; Eberhart and Wasserman 1995).

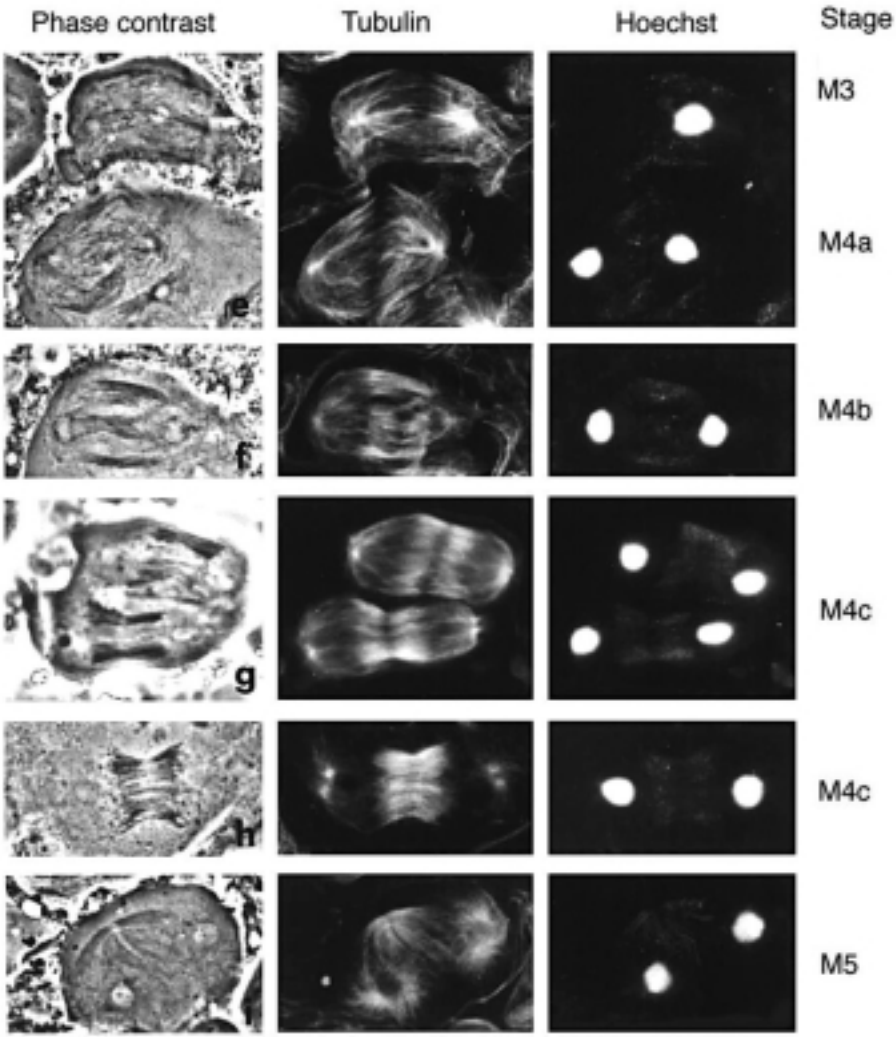

Figure 5.2. (Continued). (e) Metaphase II (top) plus early anaphase (bottom); (f) mid-anaphase; (g, h) late anaphases showing prominent central spindle; (i) telophase. Bar, 10 μm. (Reprinted, with permission, from Cenci et al. 1994.)

From late prometaphase I (stage M2) to telophase I (stage M5), the spindle undergoes dramatic transformations. In prometaphase (stage M2), metaphase (stage M3), and anaphase A (stage M4a), the spindle consists of two closely apposed, umbrella-shaped half-spindles. During anaphase B (stages M4b and M4c), the spindle poles move apart and spindle microtubules reorganize: The astral microtubules shorten and the density of

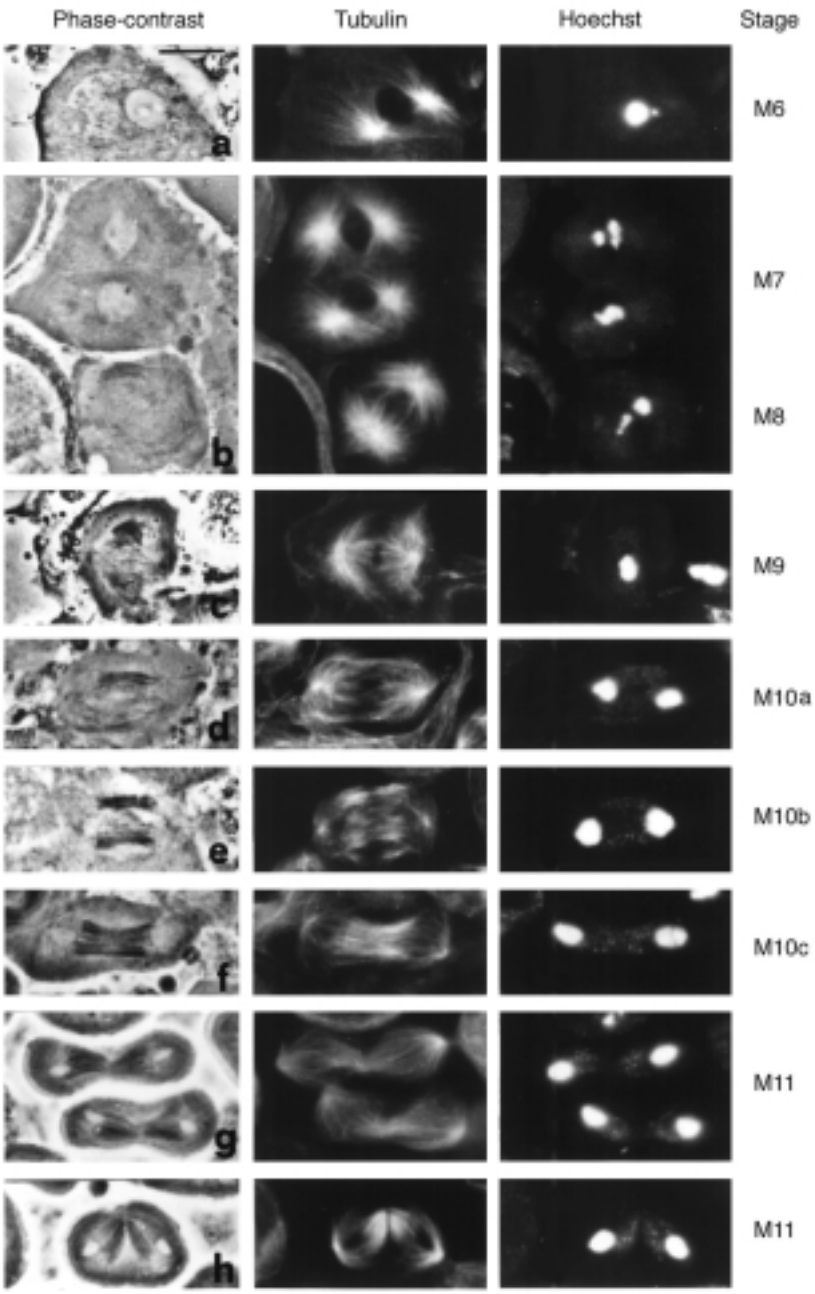

Figure 5.3. Second meiotic division in D. melanogaster males. (a) Prophase secondary spermatocyte nucleus. (b) Two prometaphase secondary spermatocyte nuclei (top) plus a prometaphase cell (bottom) without nuclear-cytoplasmic demarcation. (c) Metaphase; (d) early anaphase; (e) mid-anaphase; (f) late anaphase; (g, h) telophases. Bar, 10 μm. (Reprinted, with permission, from Cenci et al. 1994.)

microtubules between the segregating sets of chromosomes progressively increases. Th latter microtubules form a dense bundle, called the central spindle, which is pinched in the middle during telophase when cytokinesis occurs (stage M5).

After completion of the first meiotic division and a very short prophase (stage M6), secondary spermatocytes enter prometaphase (stages M7 and M8) and then metaphas (stage M9). Here again, nuclear-cytoplasmic demarcation disappears between the M7 and M8 stages, due to the dispersion of nuclear lamins. Subsequent spindle transformations are very similar to those that occur during the first meiotic division. After anaphase A (stage M10a), aster microtubules shorten and a prominent central spindle assembles (stages M10b and M10c), which is pinched in the middle during telophase II (stage M11).

During ana-telophase I and II, the mitochondria appear to line up along the central spindle. The occurrence of cytokinesis divides these organelles into two equal groups a each meiotic division. As a consequence, all of the spermatids receive the same amount o mitochondria and develop Nebenkern of identical sizes.

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6

Preparation and Analysis of Polytene Chromosomes

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ALTHOUGH THE LARGE POLYTENE CHROMOSOMES of diptera were originally described in the late 1880s, it was not until the early 1930s that their significance to the study of the genome of *Drosophila* was realized. Theophilus Painter (1933, 1934) first characterized the polytene chromosomes of *Drosophila* and emphasized their importance in studying the structure of chromosomes and the localization of genes. Calvin Bridges (1935, 1937) quickly took up the task of making extremely detailed maps of *Drosophila* salivary gland polytene chromosomes and correlating these maps with the known genetic maps. Bridges (1938) began revising the polytene chromosome maps to include more detail, but died after completing only the *X* chromosome. The remaining revised maps were finished by his son, Philip (Bridges and Bridges 1939; Bridges 1941a,b, 1942). Both the original and revised maps have been reprinted (Lindsley and Grell 1968; Lindsley and Zimm 1992) and continue to be the standards for the field.

Polytene chromosomes are found in several larval and adult tissues, but preparations are usually made of the chromosomes in the larval salivary glands, because the glands are easily dissected and the polytene chromosomes are large.

PROTOCOL 6.1*

Dissection of Larval Salivary Glands and Polytene Chromosome Preparation

Materials

Supplies and Equipment

Deep depression slide or shallow dish for dissection. Hanging drop slides (Fisher) or depression culture microscope slides (VWR) work well. Alternatively, use a siliconized microscope slide (the siliconized surface keeps the liquid from spreading too much; for preparation of siliconized slides, see below).

*For all protocols, H_2O indicates glass distilled and deionized.

Very sharp forceps (2 pairs). Forceps with bent or broken tips make the dissections much more difficult. Fine-point forceps can be obtained from VWR (25607-856) or from Roboz (Web site: www.roboz.com).

Dissecting microscope

Glass coverslips (22 × 22 mm; also called cover glasses) siliconized if the coverslip is to be removed for a permanent preparation or if the chromosomes are to be used for another procedure, such as in situ hybridization (for preparation of siliconized coverslips, see below).

Glass microscope slides need not be treated, but must be brushed clear of any lint or dust just before use. Both microscope slides and coverslips can be obtained from almost any scientific supply company, such as Fisher, Thomas Scientific, or VWR.

Dissecting needle (VWR 25778-000) or other pointed object for tapping on the preparation; the object should not be too sharp or it will break the coverslip when tapped.

Bibulous paper (VWR 28511-007) for blotting out the excess stain

(Optional) Razor blade, if the coverslip is to be removed.

Preparation of Siliconized Slides and Coverslips

Any siliconizing solution, such as Sigmacote (Sigma SL-2), will work. Dip the slides or coverslips in the siliconizing solution in a chemical fume hood, allow to air-dry, and clean by rubbing with a soft cloth or tissue.

Siliconized slides can be stored indefinitely. Make sure to remove all dust and lint just before use. Use a soft paintbrush to remove lint while blowing across the coverslip.

Solutions and Reagents

Saline (0.7% NaCl)

Acetic acid (45%)

(Optional) Liquid nitrogen, a block of dry ice, or an ethanol/dry-ice bath, if the coverslip is to be removed

(Optional) Ethanol, 95% and absolute, if the slides are to be made permanent

(Optional) Mounting medium, e.g., Permount (Fisher), if the slides are to be made permanent

Staining Solution of Lactic Acid, Acetic Acid, and Orcein

3 parts glacial acetic acid (Sigma A 6283)

2 parts lactic acid (Sigma L 1250)

1 part H_2O

2% powdered orcein (1 g per 50 ml of solution; Sigma O 7380)

The final concentrations of acetic acid and orcein are 50% and 2%, respectively. Although differing concentrations of lactic acid (between 16% and 30%) are used, the lactic acid concentration suggested above by Lefevre (1976) is preferred.

Heat the staining solution (but do not boil) and filter. If stored tightly capped, it will last for many years. The same solution, but without the orcein stain, can be used to prepare polytene chromosomes for in situ hybridization.

CAUTION: acetic acid, dry ice, ethanol, lactic acid, liquid nitrogen (see Appendix 4)

Method

Dissecting Larval Salivary Glands

Salivary glands are dissected from wandering third-instar larvae. The cultures should be well fed (adding live yeast paste every couple of days helps) and not very crowded. Although larvae raised at 18¼C are preferred, larvae raised at higher temperatures are fine if large and healthy. Select large larvae that have crawled out of the medium, but have not yet stopped and everted their spiracles.

1. Place the larvae in saline in a deep depression slide or small dish. At this stage, determine the sex of the larvae if chromosomes from a particular sex are desired. Alternatively, select larvae of the desired genotype.

   Notes: The testes develop faster than the ovaries and are visible in living larvae as two clear spheres on either side of the body cavity, approximately two thirds of the way from the anterior to posterior. If the gonads are not clearly visible, the larva is a female.

   Marker mutations can also be used to pick larvae of particular genotypes. For example, *Tubby* is a dominant mutation that makes the larvae short and fat. It is carried by some balancer chromosomes. The homozygous, nonbalancer-carrying larvae can be selected as *Tubby* mutants. Loss-of-function mutations in *yellow* cause the mouth hooks to be pigmented brown instead of black. Homozygous *red* mutants have dark red granules deposited in the Malpighian tubules (wild-type Malpighian tubules are yellow in color). There are also balancer chromosomes available from the Bloomington *Drosophila* stock center (http://flystocks.bio.indiana.edu/) with transposons carrying GFP (green fluorescent protein) (Brand 1995) that can be used to select larvae of desired genotypes.

2. Perform the dissection in either 45% acetic acid or a saline solution.

   Note: Salivary glands can be dissected directly in 45% acetic acid if done quickly (this solution is used for fixing the chromosomes; see step 1). It is usually easier to dissect the salivary glands in a saline solution first, so that there is less need to rush. A 0.7% saline solution is good for dissection, because it is hypotonic and causes the cells to swell.

3. Grasp the mouth hooks firmly with one pair of forceps. Then grasp the body of the larva approximately midway down with the other pair of forceps. Figure 6.1A shows the positions of the forceps. Slowly pull the two pairs of forceps apart. The mouth hooks should separate from the body, with the salivary glands and little else attached (Figure 6.1B). The paired salivary glands are large, clear sausage-shaped cylinders connected at one end by a common duct. A pair of dissected salivary glands is shown in Figure 6.1C.

4. Try to keep the glands attached to each other by the duct, to make it easier to transfer them together. Remove the mouth hooks (they prevent the glands from flattening completely) and any other unwanted tissue by gently teasing with the forceps.

Fixation and Staining

1. Transfer the dissected salivary glands to a drop of 45% acetic acid for approximately 30 seconds.

   Note: If the glands are left too long in the acetic acid solution, they fall apart before transfer (step 2, below). If they are not left long enough, the nuclei will not break open and the chromosomes will not spread well. The exact timing of fixation should be determined by trial and error.

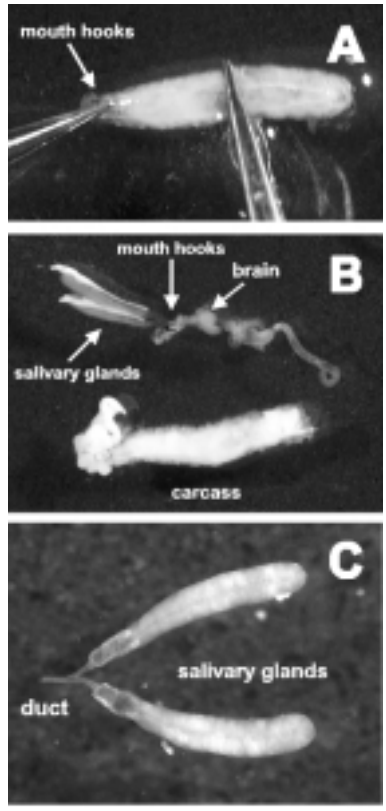

Figure 6.1. Dissection of larval salivary glands. (A) Position of the forceps for removing mouth hooks and salivary glands from larva. Anterior end of larva with mouth hooks is to the left. (B) Dissected mouth hooks with associated tissues, including salivary glands, and the remaining carcass of the larva (below). (C) Completely dissected salivary glands still joined at the common duct.

2. Place a drop (~25 l) of staining solution containing lactic acid, acetic acid, and orcein on a siliconized coverslip. After the glands are fixed sufficiently, transfer them to the drop of staining solution. Allow the glands to sit in the staining solution for several minutes.

   Note: I allow the salivary glands to sit in staining solution for only approximately 2 minutes; however, the time should be determined by trial and error. If not stained sufficiently, the chromosomes appear very light. If stained for too long, the polytene bands are be very dark and refractory. Understained chromosomes are usually easier to analyze than overstained chromosomes.

3. Hold a clean microscope slide horizontally above the coverslip and slowly lower it until the slide touches the surface of the drop of stain. Capillary action should lift the coverslip up to the slide. Quickly flip the slide and coverslip over, so that the coverslip is now on top.

Spreading the Chromosomes

1. Use a pointed object, such as a dissecting needle, to tap on the middle of the coverslip, being very careful not to move the coverslip with respect to the slide. Begin tap-

ping in the middle of the coverslip and slowly work out from the center in a spiral. The preparation should be tapped sufficiently to disperse the cells and spread the chromosomes.

Note: It is important that the dissecting needle be held vertical while tapping. When the dissecting needle hits the coverslip, the tap compresses the stain and forces it away from the center of the preparation. As the dissecting needle moves away, the coverslip moves back up to its original position. The bouncing of the coverslip on the slide caused by the tapping sets up waves of stain that spread the chromosome arms away from each other.

2. Place the slide between two layers of bibulous paper and press firmly on the coverslip to remove the excess stain. Be careful not to move the coverslip across the microscope slide at this stage. The preparation should be squashed as flat as possible. If enough force cannot be applied with the thumb to completely flatten the preparation, place it overnight at 4¼C. The stain will evaporate at the edges and flatten the preparation. Do not let it evaporate too long, or the preparation will begin to dry out and air will appear under the edges of the coverslip.

Making the Preparation Permanent

1. To freeze the preparation, place the slide either in liquid nitrogen, in an ethanol/dry ice bath, or on top of a flat block of dry ice.
2. After several minutes, when the preparation is completely frozen, insert the edge of a razor blade between the edge of the coverslip and the slide, and flip the coverslip off quickly.
3. Dehydrate in 95% ethanol for 10 minutes and then in absolute ethanol for 10 minutes. Air-dry.
4. Place a drop of mounting medium on the slide and add a coverslip. Place a weight on top to flatten the coverslip and allow to dry before examination.

PROTOCOL 6.1 NOTES

If large dry areas appear between the coverslip and slide around the edges of the preparation immediately after squashing (i.e., following step 2 in Spreading the chromosomes above), there is probably lint (or debris such as mouth hooks) in the preparation. Such preparations cannot be flattened sufficiently and should be discarded. Make sure that all dust and lint are removed from the microscope slides and coverslips before use. Also make sure that the mouth hooks and any other chitinous larval debris have been completely removed from the dissected salivary glands (see step 4 in Dissecting Larval Salivary Glands above).

If the polytene chromosomes are not distinct, i.e., they are puffy with indistinct bands, the glands have not been fixed sufficiently before staining. In addition to the poor morphology, underfixed salivary gland nuclei will also not break open very well and the chromosomes will appear enclosed in small spheres. Add 5 to 10 seconds to the fixation time in the 45% acetic acid before staining (see step 1 in Fixation and Staining above). If the chromosomes are brittle and break into a large number of small frag-

ments upon squashing, the glands have been fixed for too long. This is seen less often as the glands tend to fall apart and cannot be transferred to the staining solution if left too long in the 45% acetic acid.

EXAMINATION AND ANALYSIS OF POLYTENE CHROMOSOMES

Preparations are best examined on a compound microscope with phase-contrast optics. I prefer to start at a lower magnification (400× final magnification), but switch to a higher magnification (630× or 1000× final magnification) for detailed analyses. Bridges (1935) divided the euchromatic portions of the genome into 102 numbered divisions. Divisions 1 through 20 are on the left arm of the X chromosome. Divisions 21 through 60 and 61 through 100 comprise the second and third chromosomes, respectively, with the centromeres between divisions 40 and 41 for the second chromosome and between divisions 80 and 81 for the third chromosome. The small fourth chromosome includes divisions 101 and 102. Each division is subdivided into six lettered subdivisions (letters A through F). In the revised maps, each polytene chromosome band is numbered. Thus, polytene chromosome band 21C2 refers to the second band in the third subdivision of the first numbered division of the left arm of the second chromosome. Lefevre (1976) published photographic interpretations of Bridges maps that are extremely useful. These photographs have been reprinted in Lindsley and Zimm (1992) and are currently available together with copies of Bridges original polytene chromosome maps from Academic Press (ISBN: 0-12-450991-6). Photographs of portions of the polytene chromosomes can also be found on the images location of FlyBase at: http://flybase.bio.indiana.edu:82/images/.

It is difficult at first to recognize the chromosome arms, but with practice, it becomes much easier. One good method for learning to recognize the various chromosome arms without instruction is to obtain stocks with simple, described chromosome rearrangements. These serve as good landmarks and allow the identification of those regions with certainty.

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7

In Situ Hybridization to Polytene Chromosomes

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IN SITU HYBRIDIZATION TO POLYTENE CHROMOSOMES can be used to answer several kinds of questions. For instance, the technique is frequently used to find the chromosomal site of a cloned DNA sequence, as well as to investigate the distribution of families of repeated sequences in the chromosome set and to measure the amount of a repeated sequence different sites. These different experimental goals are best accomplished by variations o the technique.

Simple mapping of genes and even gene fragments of a few hundred base pairs is ea ily done using nonradioactive probes and by omitting several steps in slide preparation (these steps are marked ÒoptionalÓ in the protocol). On the other hand, quantitation of sequences at different chromosomal sites is best done using radioactive probes where th signal is measured directly from the bound probe, rather than after the one or two subsequent steps (antibody or streptavidin binding, enzymatic color production) that are required to detect nonradioactive probes. In addition, tritium probes can reliably detect sequences significantly shorter than those detected by nonradioactive probes on polyten chromosomes. For quantitative studies, results are improved by following the complete protocol for slide preparation.

PREPARATION OF LABELED NUCLEIC ACID PROBES

The simplest and most successful probes are made by random primer labeling (Feinberg and Vogelstein 1983, 1984) of gel-purified fragments of cloned DNA. The DNA can be labeled while still in the gel slice or it can be purified with an Elutip-d minicolumn (Schleicher and Schuell), using the manufacturerÕs directions.

Nonradioactive Probes We prefer digoxigenin for nonradioactive labeling. However, biotin-labeled probes have been used by a number of laboratories. Biotin probes

can be used in double-label experiments with digoxigenin (DIG) (Hartmann and Jackle 1995). A procedure for labeling gel-purified DNA fragments with digoxigenin is given below (see Protocol 7.1).

Radioactive Probes. Tritium (^3H) is by far the best radioisotope for in situ hybridization because of the very low energy of the beta particle that is emitted. These beta particles travel less than 1 μm through autoradiographic emulsion, giving a precise localization for the probe. A procedure for ^3H labeling of DNA in agarose is given in Protocol 7.2.

PROTOCOL 7.1*

Random Primer Labeling of Gel-purified DNA Fragments with Digoxigenin

Materials

Supplies and Equipment

Elutip-d minicolumn (Schleicher and Schuell)
DIG DNA-labeling Kit (Boehringer Mannheim 1175 033)

Solutions and Reagents

Hybridization buffer (see step 5, Protocol 7.4)

Method

1. Isolate the DNA fragment of interest from the low-melt agarose gel using an Elutip-d minicolumn.
2. Denature 0.5 μg of the DNA by boiling for 2 minutes. Chill on ice.
3. Label the DNA with the DIG DNA-labeling Kit (Boehringer Mannheim) as directed by the manufacturer. Allow the labeling reaction to proceed at room temperature overnight.
4. Stop the reaction by boiling for 10 minutes. There is no need to remove unincorporated nucleotides. Adjust the volume of solution to that desired for hybridization by adding H_2O and salts (and formamide, if desired) (see step 5, Protocol 7.4).

   Note: The reaction will yield enough probe for at least 20 preparations.
5. Immediately before applying the hybridization mix to the slides, boil the mixture for 3 minutes and quickly chill.
6. The mix can be stored at Ð20¼C, but should be reboiled each time prior to use.

*For all protocols, H_2O indicates glass distilled and deionized.

PROTOCOL 7.2

Random Primer Labeling of DNA in Agarose with Tritium

Materials

Supplies and Equipment

Elutip-d minicolumns (Schleicher & Schuell)

Solutions and Reagents

Unlabeled dNTP stocks (dATP, dCTP, and dGTP [each 100 μM in Buffer 1, see below]; alternatively, use 100 μM dNTP stocks from Boehringer Mannheim)
^{3}H-labeled TTP
Bovine serum albumin (BSA; 10 mg/ml)
DNA polymerase I, large (Klenow) fragment
Salmon sperm DNA
Absolute ethanol
Hybridization buffer (see step 5, Protocol 7.4)

Buffer 1

200 μM Tris-Cl (pH 8.0)
25 μM $MgCl_2$
50 μM β-mercaptoethanol
If commercially prepared 100 μM dNTP stocks are used, this buffer will not be required.

Buffer 2

1 mM Tris-Cl (pH 7.5)
1 mM EDTA

Solution X

1.25 M Tris-Cl (pH 8.0)
0.125 M $MgCl_2$

Solution A

1 ml of Solution X
18 μl of β-mercaptoethanol
5 μl each dATP, dCTP, dGTP (100 mM stocks, see above)

Solution B

2 M HEPES (pH 6.6)
Adjust pH with 4 M NaOH.

Solution C

Hexadeoxyribonucleotides (90 OD units/ml in Buffer 2)

OLB

Mixture of Solutions A:B:C (ratio 100:250:150)

CAUTION: β-mercaptoethanol, ethanol, NaOH, radioactive substances (see Appendix 4)

Method

1. Excise DNA from the gel in as narrow a band as possible (~100Ð500 ng of DNA per gel slice). Transfer to a preweighed Eppendorf microcentrifuge tube. Determine weight of the gel slice and add 3 ml of CH_2 per gram of gel to the tube.
2. Boil for 7 minutes. Then incubate at 37¼C for 10 minutes.

   Note: The fragment can be stored at Ð20¼C after boiling, but it should be reboiled for 3 minutes prior to use.
3. Add 5 Ci of ^{3}H-labeled TTP to an Eppendorf microcentrifuge tube. Allow to dry.
4. Add the following (in the order shown) to the Eppendorf tube (step 3, above):

H_2O	to give a final volume of 50 l
OLB	10 l
10 mg/ml BSA	2 l
DNA in agarose	up to 32.5 l (25Ð100 ng)
DNA polymerase I, large (Klenow) fragment	5 units

   Note: For random primer labeling of gel-purified DNA, see Protocol 7.2 Notes, below.
5. Incubate at room temperature from 3 hours to overnight.

   Note: Overnight labeling is preferred because it gives a higher yield of labeled product to unlabeled template.
6. Purify the product with an Elutip-d minicolumn, as directed by the manufacturer. Count 2 l of the eluate in a scintillation counter to determine yield.
7. Add salmon sperm DNA to 50 g/ml and precipitate with 2.5 volumes of ethanol. Centrifuge in a microcentrifuge to pellet the DNA. Dry the pellet. Dissolve in hybridization buffer to give about 100,000 cpm/15 l of mix (this is the amount applied to each slide). Immediately before use, boil for 3 minutes and quickly chill. This probe can be stored at Ð20¼C, but it does not have a long storage life.

PROTOCOL 7.2 NOTES

For purified DNA, the standard random primer reaction involves the following steps:

Heat DNA to 100¼C for 2 minutes and quick cool.
Follow Protocol 7.2 for gel slices, but add 50Ð100 ng of denatured DNA in step 4.

POLYTENE CHROMOSOME PREPARATIONS

The most important step for in situ hybridization is making the salivary gland squashes. The quality of the chromosome preparations determines both the ease of identifying the chromosome landmarks and the efficiency of binding the probe. Unfortunately, criteria that promote good cytology are generally opposed to those that promote high levels of hybridization, so the most useful chromosomes are a compromise. Protocol 7.3 describes the preparation of polytene chromosome squashes from salivary glands. Details of this technique can also be found in Kennison (this volume).

PROTOCOL 7.3

Preparation of Polytene Chromosome Squashes

Materials

Supplies and Equipment

Dissecting tools
Plastic test tube tops
Coverslips (18 mm^2, siliconized)
Razor blade
Sturdy dissecting needle (VWR 25 778-000)
Phase-contrast microscope
Dry, dust-free box for holding slides

Preparation of Subbed Slides

Wash slides, dip in subbing solution, and allow to dry in a dust-free place. These slides may be stored indefinitely in a dry, dust-free container. Subbed slides give a better retention of cytological preparations.

Siliconized Coverslips

Dip coverslips (18 mm^2) in Sigmacote (Sigma SL-2), rinse well, and dry. Siliconization prevents adherence of cytological material to coverslips.

Solutions and Reagents

Acetic acid (45%)
Liquid nitrogen
Ethanol (95%)

Subbing Solution

This is an aqueous solution of 0.1% gelatin and 0.01% chrome alum (chromium potassium sulfate: $CrK[SO_4]_2 \cdot 12H_2O$]. Dissolve the gelatin in hot water. Allow the solution to cool, and then add chrome alum. Subbing solution can be stored at 4¼C for long periods.

DPBS

130 mM NaCl
7 mM Na_2HPO_4
3 mM KH_2PO_4

CAUTION: acetic acid, ethanol, KH_2PO_4, liquid nitrogen, Na_2HPO_4 (see Appendix 4)

Method

1. Grow larvae in moderately crowed vials with added yeast. Choose larvae that are climbing up the sides of the tube.

   Note: Female larvae have better polytene chromosomes than males and also have twice the amount of X chromosome DNA.

2. Dissect the larvae in DPBS. Remove the salivary glands and gently clean off as muc fat body as possible.

   Note: Take care to avoid injuring the salivary glands. It is better to leave fat body than to injure the glands, because chromosomes from injured glands spread poorly.

3. Place a small drop (~15 μl) of 45% acetic acid on a dust-free siliconized 18-mm² coverslip. Transfer the glands to this drop, being careful not to carry any DPBS to dilute the acid. Cover the drop with a plastic test tube top to prevent evaporation and let sit for 2Ð5 minutes. (The optimal time may vary from stock to stock.)

4. Invert a subbed slide over the coverslip and press it down lightly.

   Note: Place the glands at approximately the same position on each slide, so that they can be found easily as they are carried through the procedure.

5. Turn the slide over so that the coverslip is on top and tap lightly over the glands with a sturdy dissecting needle. The aim is to break the nuclei and to spread the chromosomes. Check spreading with a phase-contrast microscope.

   Notes: The coverslip can move around slightly during this step. The amount of liquid should permit gentle movement without shearing chromosomes.

   Nuclei from overlapping chromosomes will not have good morphology after the hybridization.

6. When chromosomes are well spread, place the slide on a paper towel with the coverslip side down and press firmly to flatten the chromosomes. Do not move the coverslip sideways at this point or chromosomes will be stretched.

7. Submerge the preparation in liquid nitrogen at least till bubbling ceases. Remove, flip coverslip off with a razor blade, and immediately plunge slide in 95% ethanol. Leave for 10 minutes. Repeat washes in 95% ethanol twice (10 minutes each) and air-dry the slides.

8. If planning to use ^{3}H-labeled probe, make several (5Ð10) slides and develop after different autoradiographic exposures to obtain optimal labeling of the preparation. This is especially important if the aim is to quantify label over different sites comparatively. Exposures that give good grain counts at different sites can be chosen and ther counts corrected to the same exposure time.

PROTOCOL 7.3 NOTES

Preparations can be checked dry with a phase microscope. The best chromosomes are fl and gray with no refractivity. Chromosomes with a clearly recognizable banding pattern at this stage will have good morphology at the end of the experiment. Slides may be kep for weeks in a dry, dust-free box.

PROTOCOL 7.4

Pretreatment and Hybridization

Materials

Supplies and Equipment

Moist chamber. During several steps, preparations are incubated with small amounts of liquid under coverslips. To avoid having to seal the coverslips, place the slides in a moist chamber. A plastic sandwich box makes a good chamber. Place paper towels on the bottom, soaked with a solution. The solution in the chamber should have the same salt concentration as the solution under the coverslip, to prevent distillation and a change in concentration of hybridization buffer. (If the hybridization buffer contains formamide, use 20x SSC in the chambers. It is less expansive and balances the hybridization buffer well.) Slides are supported above the moist paper towels on two parallel glass rods.

Dish to hold slides

Magnetic stirrer

Magnetic stirring bar

Solutions and Reagents

20x SSC (see Appendix 3)

2x SSC

Triethanolamine (0.1 M, pH 8.0)

Acetic anhydride

RNase A (100 g/ml in 2x SSC)

Ethanol (70% and 95%)

NaOH (0.07 N; prepared fresh immediately prior to use)

Hybridization buffer (usually 2x or 4x TNS, see below)

1x TNS
- 0.15 M NaCl
- 10 mM Tris-Cl (pH 6.8)

CAUTION: acetic anhydride, ethanol, NaOH, triethanolamine (see Appendix 4)

Method

1. Treat the slides as follows:
   a. Heat the preparations in 2x SSC at 70¼C for 30 minutes.
   b. Rinse in 2x SSC at room temperature.
   c. Dehydrate in 70% ethanol for 10 minutes (twice) and then in 95% ethanol for 5 minutes. Air-dry.

      Note: This step helps chromosomes adhere to the slide.
2. (Optional) Remove endogenous RNA as follows:
   a. Insert slides in a moist chamber and place approximately 50 µl of RNase A (100 µg/ml in 2x SSC) on top of the squash. Cover preparation with a coverslip and incubate at room temperature for 2 hours.

b. Remove the coverslips by dipping in a beaker of 6x SSC. Perform three washes in 2x SSC (5 minutes each wash).

c. Dehydrate through ethanol as in step 1c (above).

Note: This step improves hybridization if there are significant endogenous transcripts in the vicinity of the target sequence (a situation that holds for the ribosomal genes and many puffed regions). Endogenous transcripts are seldom troublesome in simple mapping experiments, but their removal may be important for rigorous quantitative analysis of sequences.

3. (Optional, to reduce background from radioactive probes) Acetylate the preparation with acetic anhydride (5 ml/liter in 0.1 M triethanolamine [pH 8.0]). Place the slides in a rack and suspend in a dish over a magnetic stirring bar. Cover the slides with triethanolmine and then add acetic anhydride while stirring. When acetic anhydride is completely dissolved, turn off the stirrer and let sit for 10 minutes. Dehydrate through ethanol as in step 1c.

4. Denature DNA of preparation by placing slides in freshly made 0.07 M NaOH for 3 minutes. Wash through three changes of 70% ethanol (10 minutes each) and then two changes of 95% ethanol (5 minutes each). Air-dry.

5. Set up the hybridization reaction. Place 10 µl of probe directly over each preparation and cover with an 18-mm^2 coverslip. Place in a moist chamber made with the hybridization buffer (or 2x SSC, if hybridization buffer contains formamide).

Note: Choose the same hybridization buffers and temperatures as those for filter hybridization. We usually use 2x TNS and hybridize overnight at 68¼C. For cross-species hybridization, we increase the salt concentration to 4x or 6x. For the high probe concentrations that we use for nonradioactive experiments, an incubation for 4Ð6 hours is all that is needed. Much lower probe concentrations are used for radioactive probes and hybridizations are incubated for 12Ð16 hours.

6. Remove the coverslips by dipping into 2x SSC. Perform 3 washes (10 minutes each) to remove unhybridized probe.

Note: For radioactive probes, these washes are best done at 60¼C; but for nonradioactive probes, room temperature washes seem to work.

7. Slides with nonradioactive probes are now ready for hybrid detection (see Protocol 7.5). Slides with radioactive probes should be dehydrated through ethanol, as in step 1c, and air-dried for autoradiography (see Protocol 7.6).

PROTOCOL 7.5

Detection of Digoxigenin Probes

Digoxigenin probes are detected with an antibody, which is coupled to an enzyme or to a fluorescent dye. This protocol describes use of the antibody coupled to alkaline phosphatase.

Materials

Supplies and Equipment

Moist chamber (see p. 125)
Phase-contrast microscope

Solutions and Reagents

NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) stock solution (Boehringer Mannheim 1681451)
Anti-DIG–alkaline phosphatase conjugate (Boehringer Mannheim 1093274)
Giemsa stain
Phosphate buffer (0.01 M, pH 6.8)

PBT
- DPBS
- 0.1% Tween-20

pH 9 Buffer
- 100 mM NaCl
- 50 mM $MgCl_2$
- 100 mM Tris-Cl (pH 9.0)
- 0.1% Tween-20

CAUTION: $MgCl_2$, NBT/BCIP (see Appendix 4)

Method

1. After washing off nonhybridized probe (see step 6 in Protocol 7.4), wash slides three times in PBT (10 minutes each).
2. Drain each slide and wipe around the chromosomes, but do not allow them to dry. Apply 20 l of antibody solution (1:200 dilution in PBT) over the squash, cover with a coverslip, and place in moist chamber. Incubate at room temperature for 1 hour.
3. Remove coverslips by dipping in PBT. Wash slides as follows:
   a. Wash three times in PBT (3 minutes each).
   b. Wash three times in pH 9 buffer (3 minutes each).
4. Make alkaline phosphatase reaction solution by adding 20 l stock NBT/BCIP solution to 1 ml of pH 9 Buffer. Put 50 l over the preparation and cover with a large coverslip. Allow color to develop at room temperature. Check color development under the microscope. When signal is adequate, stop the reaction by washing in H_2O.

   Note: The time necessary is usually 15 minutes to 2 hours.
5. Stain the chromosomes with Giemsa stain diluted 1:20 with 0.01 M phosphate buffer (pH 6.8) or in H_2O. Stain for 2–3 minutes, rinse in H_2O, and check color under the microscope. If desired, return to staining solution to increase color.

   Note: Alternatively, slides can be viewed unstained with phase. Place a drop of H_2O over the chromosomes and add a coverslip to view. Remove coverslip and store slides dry.

PROTOCOL 7.6

Autoradiography for Detection of Radioactive Probes

Materials

Supplies and Equipment

Dark room, completely light-tight with a double door to allow exit and entry without exposing slides that are drying

Safelight (Wratten series II; Kodak)

Plastic or glass dipping chamber, to fit a microscope slide with minimal waste of emulsion

Rack for drying slides

Water bath at 45¼C

Light-tight black microscope slide boxes to hold exposing slides

Black electrical tape

Silica gel

Solutions and Reagents

Developer (Kodak D-19)

Fixer (Kodak Rapid Fix)

Kodak NTB-2 autoradiographic emulsion diluted 1:1 with H_2O and stored in 10-ml aliquots in a light-tight box. When diluting emulsion, mix gently to avoid making bubbles.

Giemsa stain

Phosphate buffer (0.01 M, pH 6.8)

Method

1. In the dark, with one safelight, melt an aliquot of the emulsion for 15 minutes in the 45¼C water bath. Pour into a dipping chamber, prewarmed at 45¼C, taking care to avoid forming bubbles.
2. Dip each slide slowly into the emulsion, drain, and place on the rack to dry for approximately 2 hours.
3. Place the dried slides in the light-tight boxes containing silica gel. Wrap with black electrical tape and store in a refrigerator. (The refrigerator should not contain either radioactive materials or chemicals that might affect the emulsion.)

   Note: Time of exposure depends on the probe and the target. For single-copy genes on polytene chromosomes, a 24-hour exposure is usually enough. Prepare several slides so that different exposures can be checked.

4. Develop slides in Kodak D-19 for 2 minutes, rocking gently for 6-second periods interspersed by 6-second rests. Stop the reaction by immersing slides in H_2O for 30 seconds and then fix in Rapid Fix for 5 minutes. The room lights may be turned on after slides have been fixed.
5. Wash five times in H_2O (5 minutes each).
6. Stain slides with Giemsa diluted 1:20 with phosphate buffer for 2–3 minutes. Wash with H_2O and air-dry.

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8

Mapping Protein Distributions on Polytene Chromosomes by Immunostaining

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THE FORMIDABLE SIZE AND STRUCTURE OF POLYTENE CHROMOSOMES allow mapping of chromosomal protein distributions at very high resolution. Immunological methods were already used in the early 1970s to identify the chromosomal association of abundant nuclear proteins, such as histones or RNA polymerase II. However, an absolute requirement for a successful and refined mapping of protein distributions is the reproducibility of the polytene-banding patterns throughout the procedure. An important improvement in this respect was the introduction of the modifications described by Silver and Elgin (1976), which resulted in highly reproducible banding patterns comparable to acid-treated chromosome preparations. A further technical advance was the introduction of enzyme-coupled secondary antibodies. Unlike the fading fluorescent signals, this alternative means of detection yielded stable preparations that allowed an extensive and thus more precise cytological mapping of the binding sites of many different regulatory proteins (Zink and Paro 1989). Currently, immunostaining polytene chromosomes is most often used for achieving the following goals:

- Mapping distributions of chromosome-associated proteins. For map positions of some regulatory factors, see FlyBase at: http://flybase.bio.indiana.edu:82/allied-data/lk/cytofeatures.
- Identifying and characterizing cis-regulatory DNA elements bound by particular proteins. Transgene constructs containing the element will create a new protein-binding site at the integration site (Saumweber et al. 1990; Zink et al. 1991).
- Mapping functional protein domains necessary for chromosomal binding or other activities, such as the interaction with other partner proteins (Kuroda et al. 1991; Messmer et al. 1992; Rastelli et al. 1993; Platero et al. 1995).

PROTOCOL 8.1*

Preparation of Chromosome Squashes from Third-instar Larvae

Materials

Media

Nutrient-rich Fly Medium

agar	8 g
dried yeast	18 g
soybean meal	10 g
molasses	7 g
malt extract	80 g
cornmeal	80 g

Adjust volume to 1 liter with H_2O and boil extensively on a hot plate until all ingredients are well dissolved. Add 6.3 ml of propionic acid as a mold inhibitor. Pour into 175-ml bottles and allow to solidify. To each bottle, add a large drop of live bakerÕs yeast on the surface of the medium.

Supplies and Equipment

Dissecting tools
- tweezers
- dissecting microscope

Siliconized coverslips (Corning or equivalent quality, 22 x 22 mm)

Pencil with eraser end

Filter paper (Whatman 3MM)

(Optional) Squashing apparatus; for extended chromosome-spreading sessions, we use the custom-made squashing apparatus illustrated in Figure 8.1.

Microscope equipped for phase contrast

Diamond pen

Razor blade

Preparation of Poly-L-lysine-coated Slides

Place 100Ð200 slides in racks.

In general, high-quality slides do not require any pretreatment with detergent. However, in some cases, pretreatment with a strong detergent may be necessary, to ensure a homogeneous wetting of the surface by the poly-L-lysine solution. Wash slides for 2 hours in a strong detergent, and then wash for 2 hours in running tap water and rinse twice in H_2O. Dip slides twice in 95% ethanol and air-dry.

Dip slides into a poly-L-lysine solution (slide adhesive solution, 0.1% w/v in H_2O; Sigma P 8920). After withdrawal of the rack, the solution should wet the glass surface uniformly and stay on slides. Air-dry slides and store at 4¼C.

Solutions and Reagents

Liquid nitrogen

(Optional) Methanol

(Optional) Ammonium sulfate (50% w/v)

*For all protocols, H_2O indicates glass distilled and deionized.

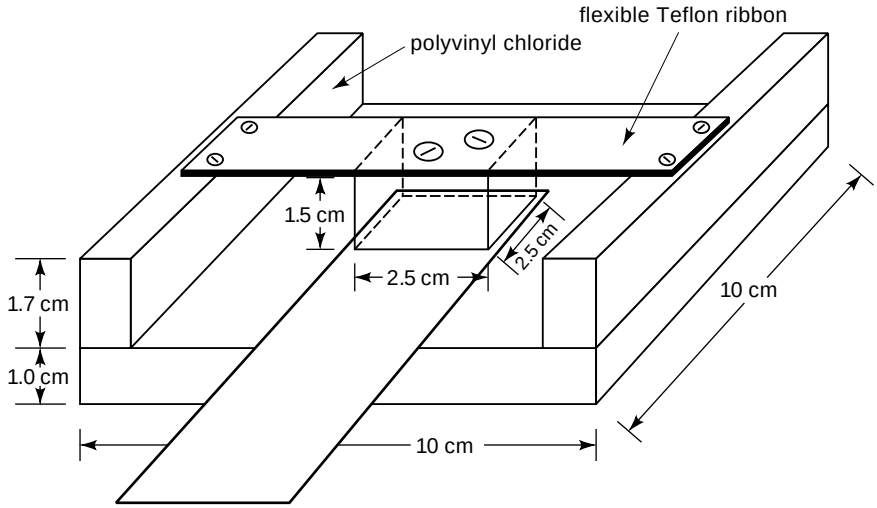

Figure 8.1. Schematic diagram of the squashing apparatus. We use this apparatus to ease the effort whe squashing chromosomes over an extended period, and to produce more homogeneous pressure over th entire coverslip. A small block made of polyvinyl chloride (2.5 x 1.5 cm) is attached to a flexible Teflon ribbon as shown. The block should hang approximately 2Ð3 mm above the plane of the holding block (also made of polyvinyl chloride). A filter paper (i.e., Whatmann 3MM) and the slide with the coverslip are then positioned on the holding block and held in place by the suspended block.

1x Phosphate-buffered Saline (PBS; pH 7.5)

137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4 \cdot 7H_2O$
1.4 mM KH_2PO_4

Fixing Solution

glacial acetic acid	2.25 ml
37% formaldehyde stock solution	0.25 ml
H_2O	2.50 ml

Important: Prepare Fixing Solution fresh every 2Ð3 hours!

37% Formaldehyde Stock Solution

To 1.85 g of paraformaldehyde, add H_2O to a final volume of 5 ml. Add 70 l of 1 N KOH. Boil to dissolve the paraformaldehyde, filter, and store in aliquots at Ð20¼C.

CAUTION: acetic acid, ammonium sulfate, formaldehyde, KCl, KH_2PO_4, KOH, liquid nitrogen, methanol, Na_2HPO_4, paraformaldehyde (see Appendix 4)

Method

Raising Third-instar Larvae

1. Raise flies in 175-ml bottles containing Nutrient-rich Fly Medium.

   Note: The quality of the chromosomes depends critically on the state of nutrition and well-being of the larvae. This is particularly important to consider in cases where the genetic background (e.g., unhealthy mutants or certain transgenic lines) places a heavy toll on the development of the larvae.

2. Allow the flies to lay eggs just to the point where larvae will hatch under uncrowded conditions (i.e., <100 larvae in a 175-ml bottle). Let the larvae develop at 18¼C.
3. For salivary gland preparations, use third-instar larvae that are still crawling and have not yet started to pupate.

Dissecting Larval Salivary Glands and Fixing Chromosomes

1. To prepare one slide, remove two larvae from the bottle and wash in PBS. With tweezers, dissect out the two pairs of salivary glands in PBS (for details on dissection of larval salivary glands, see Kennison, this volume). Carefully try to get rid of most of the attached white fat-body cells, without damaging or separating the two glands.
2. Hold the salivary gland pairs at the common duct with tweezers and transfer the glands to a drop of fixing solution on a siliconized coverslip. Incubate the glands for 10Ð20 minutes, occasionally stirring with the tip of the tweezers to ensure homogeneous fixation.

   Note: The fixation time is an important parameter and needs to be adjusted for every antigen tested. Aim for the shortest time necessary to visualize the signal, as extended fixation times will result in difficulties later in spreading the chromosomes appropriately.

Preparing Chromosome Squashes

1. Take up the coverslip with a poly-L-lysineÐtreated slide.

   Note: At this point, the spreading of the chromosomes begins. Unlike the sole treatment with acids (see Kennison, this volume), the inclusion of formaldehyde keeps the chromosome sticky, making it more difficult to obtain preparations with well-spread chromosome arms.
2. Tap the coverslip with the eraser end of a pencil until the cells are broken up.

   Note: This can be monitored best when done against a black background.
3. Move the eraser end of the pencil over the coverslip to spread the chromosomes. To avoid extensive movement of the coverslip, hold down the coverslip with the tip of a finger (use latex gloves to prevent acid burns). Do not continue to spread the specimen if the coverslip sticks to the slide, as this will result in shearing of the chromosomes.

   Note: The chromosomes are very brittle at this point of the process. The extent of spreading depends on the constitution and size of the chromosomes, as well as on the time of fixation.
4. Invert the slides and place over one layer of blotting paper. Apply firm pressure with the thumb; the chromosomes become flattened and pressed to the slide.

   Note: The squashing apparatus (see Figure 8.1) can substantially ease this step, because it allows a more firm and homogeneous pressure on the chromosomes without the risk of moving the coverslip.
5. Examine the preparation under phase contrast. Use only preparations with well-spread chromosome arms showing high-contrast banding patterns. Mark the position of the coverslip with a diamond pen.
6. Freeze slides in liquid nitrogen and remove coverslip with a razor blade (wear appropriate eye protection).
7. Dip slides immediately in PBS and wash for 15 minutes, slowly shaking the rack. Repeat wash in PBS.
8. Proceed with the immunostaining as described in Protocol 8.2. Alternatively, keep the slides (up to 1 week) in 100% methanol or in 50% (w/v) ammonium sulfate at 4¼C. Be aware, however, that certain antigens might be affected by exposure to methanol.

PROTOCOL 8.2

Immunostaining

Immunostaining can be performed by either of the following three methods. Method 1 describes a procedure for immunostaining using enzyme-coupled secondary antibodies. Where only weak signals are observed with Method 1, amplification with the biotin-avidin system might be helpful (Method 2). Secondary antibodies tagged with a fluorochrome can be used to speed up the detection procedure, as well as for double-labeling experiments (Method 3).

Materials

Supplies and Equipment

Rack for holding slides
Slide jars
Aluminium foil

Solutions and Reagents

1x PBS (see p. 133)

Primary antibodies. Affinity-purified primary antibodies are diluted in PBS/BSA (bovine serum albumin). The dilutions must be adjusted for each individual primary antibody. Typical dilutions for rabbit polyclonal antibodies range between 1:50 and 1:500. For double-labeling experiments, use primary antibodies raised in two different species.

Secondary antibodies. Secondary antibodies are diluted in PBS containing 2% normal serum, which is obtained from the same species as the secondary antibody. Depending on the immunostaining method used, the following antibodies are used:

- Method 1: Anti-rabbit IgG (Fc) horseradish peroxidase (HRP) conjugate; 1:100 dilution.
- Method 2: Biotin-conjugated secondary antibody and biotin-avidin amplification system (Vectastain Elite ABC Kit; Vector Laboratories PK 6100).
- Method 3: Fluorescence-conjugated secondary antibody; for double-labeling experiments, use fluorescence-conjugated secondary antibodies raised to the corresponding type of primary antibody; the dilution of each antibody needs to be determined individually.

Solution containing 0.5 mg/ml DAB (3,3«-diaminobenzidine tetrahydrochloride; Sigma D 5637) and 0.01% H_2O_2 (Merck 107210)

(Optional) DAB enhancement kit (metal amplification system for peroxidases; Sigma FASTDAB with metal enhancer D 0426)

Blocking solution

Add a small spoon of nonfat dry milk to 40 ml of PBS.

Wash Solution 1

1x PBS
300 mM NaCl
0.2% NP-40 (Nonidet P-40)
0.2% Tween-20Ð80

Wash Solution 2
- 1x PBS
- 400 mM NaCl
- 0.2% NP-40
- 0.2% Tween-20Ð80

PBS/Triton X-100
- 1x PBS
- 1% Triton X-100

PBS/BSA
- 1x PBS
- 1% BSA

PBT
- 1x PBS
- 0.1 % BSA

CAUTION: DAB, H_2O_2 (see Appendix 4)

Method

Method 1: Use of Enzyme-coupled Secondary Antibodies

1. Wash stored slides twice in PBS (10 minutes each wash) and once in PBS/Triton X-100 for 10 minutes. Incubate in Blocking Solution at room temperature for 1 hour or at 4¼C overnight, slowly shaking the jar containing the slides. Drain off excess solution.
2. Add 40 l of diluted affinity-purified primary antibody to each slide. Cover with a coverslip and incubate at room temperature for 1 hour and also at 4¼C overnight in a humid chamber.
3. Rinse slides in PBS to remove the coverslips and place in rack. Wash three times in Blocking Solution (5 minutes each wash). Shake rack with slides thoroughly during the washing procedure.
4. Rinse slides in PBS and remove excess solution. Add 40 l of secondary antibody (i.e., anti-rabbit IgG [Fc] HRP conjugate, 1:100 dilution) to each slide. Cover with a coverslip and incubate at room temperature for 1 hour in a humid chamber.

   Note: Often, commercially available secondary antibody preparations show a high affinity for *Drosophila* nuclear proteins, resulting in some excellent signals on the chromosomes. As such, a corresponding control experiment should always be included before a new batch is utilized. Cross-reactivity of secondary antibody preparations can often be substantially reduced by preincubating a lower dilution of the serum overnight with formaldehyde-fixed embryos (for preparation of preadsorbed antibodies, see Rothwell and Sullivan [this volume], Protocol 9.3).
5. Rinse slides in PBS and place in rack. Wash 15 minutes in Wash Solution 1 and 15 minutes in Wash Solution 2. Shake rack thoroughly during the washing procedure.

   Note: If background problems persist, the NaCl concentration in Wash Solution 2 can be raised to 500 mM.
6. Rinse slides in PBS. Add 100 l of a solution containing 0.5 mg/ml DAB and 0.01% H_2O_2. Follow the appearance of the signals under the microscope using bright-field

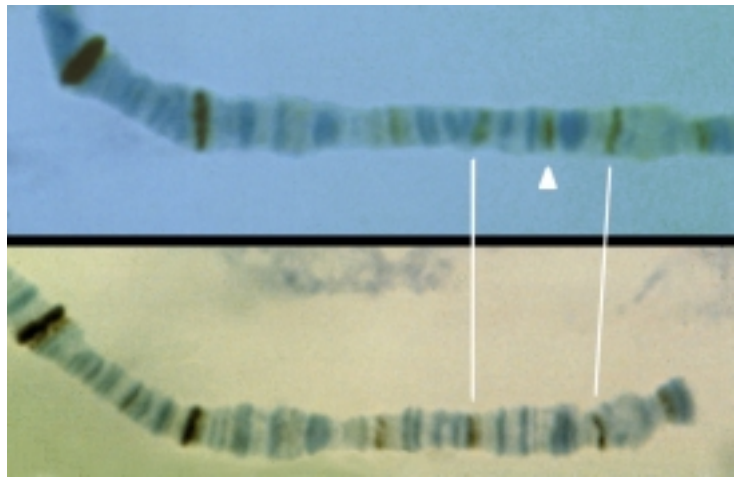

Figure 8.2. Distribution of the Polycomb protein at section 43Ð49 of the second chromosome visualized with enzyme-conjugated (HRP) secondary antibodies (Top) Chromosome with a transgene construct containing a Polycomb-binding site (PRE) at 44E (arrowhead) (bottom) wild-type chromosome.

optics to prevent overstaining. Stop the reaction by dipping slides in PBS. Wash in PBS for 10 minutes.

Note: Silver treatment of the preparation turns the brown color of a DAB signal to black, thus substantially improving the contrast, which is very convenient when black and white photography is used for documentation. We use the DAB enhancement kit from Sigma and follow the instructions given by the supplier, except that the metal amplification step is shortened to approximately 1 minute.

7. Counterstain with Giemsa as described in Protocol 8.3.

Method 2: Use of Biotin-conjugated Secondary Antibodies and Enzymatic Detection with the Avidin/Biotin System

1. Perform steps 1Ð3 as described in Method 1 (above).
2. Perform step 4 of Method 1 using a secondary antibody conjugated to biotin.
3. During secondary antibody incubation, mix the biotin solution and the avidin-HRP solution to allow the formation of complexes. We use reagents from the Vectastain Elite ABC kit. Mix 40 l of solution A and 40 l of solution B in 1 ml of PBT at room temperature for 10 minutes.
4. After incubation with secondary antibodies, rinse slides twice in PBT (10 minutes each). Remove excess liquid and place 150 l of the biotin-avidin-HRP mix on the preparation and cover with a coverslip. Incubate the slides at room temperature for 40 minutes in a humid chamber.
5. Wash slides in Wash Solution 1 for 15 minutes and then in Wash Solution 2 for 15 minutes.

   Note: If background problems persist, the NaCl concentration in Wash Solution 2 can be raised to 500 mM.

6. Perform the color reaction as described in Method 1, step 6.

Method 3: Use of Fluorescence-coupled Secondary Antibodies

1. Perform steps 1–3 described in Method 1, above.

   Note: For double-labeling experiments, both primary antibodies (raised in two different species) can be applied simultaneously at the appropriate concentrations.

2. Rinse slides in PBS and remove excess solution. At this point, perform all steps under dimmed-light conditions. During incubations, wrap the slide jars in aluminum foil to minimize bleaching of fluorochromes. Add 40 of diluted fluorescence-conjugated secondary antibody solution. Cover with a coverslip and incubate at room temperature for 1 hour in humid chamber.

   Note: Also during this step, both secondary antibodies of the appropriate type can be applied simultaneously for double-labeling experiments.

3. Rinse slides in PBS and place in a rack. Wash slides in Wash Solution 1 for 15 minutes and then in Wash Solution 2 for 15 minutes.

   Note: If background problems persist, the NaCl concentration in Wash Solution 2 can be raised to 500 mM.

4. Store slides in PBS at 4¼C in the dark.

5. Counterstain with Hoechst 33258 as described in Protocol 8.4.

PROTOCOL 8.3

Giemsa Staining

Chromosomes with HRP signals should be counterstained with Giemsa as described below.

Materials

Supplies and Equipment

Slide jars

Solutions and Reagents

Giemsa (Merck 109204)
Sodium phosphate buffer (10 mM, pH 6.8)
Glycerol (99.5%)
(Optional) Permanent mounting solutions, e.g., Entelan (EM Science)

CAUTION: Giemsa, sodium phosphate (see Appendix 4)

Method

1. Prepare a 1:130 dilution of Giemsa in 10 mM sodium phosphate buffer (pH 6.8). Stain slides for 30 seconds to 1 minute. Rinse slides by dipping several times into H_2O.

   Note: If chromosome bands appear too weak in a microscope with bright-field optics, repeat staining.

2. Mount in 99.5% glycerol with a coverslip and immediately examine the slides under the microscope.

   Note: Giemsa stain will fade within a few hours. However, chromosomes can be washed in PBS and restained. For storage, slides can be frozen at –20¼C. Entelan (EM Science) can be used as a permanent mounting solution.

PROTOCOL 8.4

Staining with Hoechst 33258

Chromosomes with fluorescent signals can be counterstained with Hoechst 33258 as described below. However, banding patterns produced by the fluorescent DNA stains are often difficult to compare with the published polytene chromosome-banding patterns. We find it more convenient to simply use phase contrast to correlate the fluorescent signal with the corresponding chromosome bands.

Materials

Solutions and Reagents

Hoechst Staining Solution

10 g/ml Hoechst 33258
1x PBS

Preparation of the Stock Solution of MowiolÐDABCO 2.5%

Combine 2.4 g of Mowiol (Hoechst), 6 g of glycerol, and 6 ml of H_2O and mix for 3 hours. Add 12 ml of 0.2 M Tris-Cl (pH 8.5) and incubate with mixing at 50¼C for 10 minutes. Centrifuge at 500 g for 15 minutes to pellet insoluble material. Add DABCO (1,4-diazabicyclo-[2,2,2]-octane; Merck 803456) to final 2.5% to the solution as antibleaching agent. Store in 500- l aliquots at Ð20¼C.

CAUTION: DABCO (see Appendix 4)

Method

1. Stain slides in Hoechst staining solution for 5 minutes.
2. Mount preparations with fluorescent signals in MowiolÐDABCO 2.5%.

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Cell Biology

9

Fluorescent Analysis of Drosophila Embryos

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CELLULAR ANALYSIS IN *DROSOPHILA* is frequently being applied to problems that were once addressed only through genetic, molecular, and biochemical approaches. A numb of factors have contributed to this renaissance in *Drosophila* cell biology: Many of the procedures for cellular analysis are efficient, inexpensive, and relatively easy to perform; t generation of highly specific antibodies for immunofluorescent analysis is now routine (see Rebay and Fehon, this volume); most *Drosophila* researchers have ready access to conventional, confocal, or deconvolution fluorescent microscope systems; and finally, a var ety of high-quality fluorescent probes are now commercially available (Table 9.1). Mor recently, the availability of green fluorescent protein (GFP)-tagged proteins has made li fluorescent analysis possible (see Hazelrigg, this volume).

The early *Drosophila* embryo is particularly amenable to fluorescent analysis. Large numbers of specifically staged embryos are easily collected from normal and muta stocks. There is a wealth of molecular and genetic reagents that make the cellular anal particularly powerful. The morphological and cellular events of embryogenesis have bee extensively characterized (Foe and Alberts 1983; Foe et al. 1993). For example, dire after fertilization, the embryo proceeds through a series of rapid nuclear divisions tha rely on the highly coordinated dynamics of the microtubules, microfilaments, and other cytoskeletal components (Warn et al. 1984; Karr and Alberts 1986; Kellogg et al. 198 During this time, critical events that establish the axis and patterning in the embryo ar occurring (for review, see St Johnston and NŸsslein-Volhard 1992). These events ha been thoroughly described through fluorescent analysis and provide an excellent resou in which to analyze the primary cellular defect in newly isolated mutations.

As many of these early events are under maternal control, much of this analysis h been performed with maternal-effect mutations (Sullivan et al. 1993). With the develop ment of the FLP-FRT (site-specific FLP recombinase–FLP recombination target) syste it is now possible to efficiently generate germ-line clones of zygotic lethal mutations ar analyze the maternal effect of these mutations (Golic 1991; Dang and Perrimon 1992 Approximately 70–80% of the genes that mutate to zygotic lethality are also required the early embryo. Consequently, this provides a general alternative approach towa examining the cellular basis of some of the more intractable zygotic lethal mutation

Table 9.1. Commercially Available Reagents

Structure	Probe [a]	Vendor/Part no. [b]
Nucleus/DNA	DAPI	Sigma/D 9542
	PI	Sigma/P 4170
	anti-histone	Chemicon/MAB052
	Hoechst 33258	Molecular Probes
	OliGreen[a]	Molecular Probes
Cytoskeleton		
F-actin	FITC-phalloidin	Sigma/P 5282
	TRITC-phalloidin	Sigma/P 1951
actin	anti-actin	ICN Biomedicals/clone C4
tubulin	-tubulin	Chemicon/MAB380
	-tubulin	Sigma/T 9026
Membrane	FITC-ConA	Molecular Probes
	anti-py99	Santa Cruz Biotechnology
	-spectrin	Sigma/S 1390
Motors	dynein	Chemicon/MAB1618
	kinesin	DSHB/SUK4,5
Other	adducin-related protein	DSHB/1B1
	even-skipped	DSHB/2B8
	Wingless	SHB/4D4
	engrailed	DSHB/4D9
	syntaxin	DSHB/8C3
	cyclin B	DSHB/F2F4
Secondaries	FITC goat anti-mouse	Chemicon/AP124F
	FITC goat anti-rabbit	Chemicon/AP132F
	TRITC goat anti-mouse	Chemicon/AP124R
	TRITC goat anti-rabbit	Chemicon/AP132R
	Cy5 goat anti-mouse	Chemicon/AP124S
	Cy5 goat anti-rabbit	Chemicon/AP132S

[a]Abbreviations: (ConA) concanavalin A; (DAPI) 4,6-diamidino-2-phenylindole; (FITC) fluorescein isothiocyanate; (PI) propidium iodide; (py99) phosphotyrosine; (TRITC) tetramethylrhodamine isothiocyanate.
[b]DSHB is the Developmental Studies Hybridoma Bank (University of Iowa).

(Garcia-Bellido and Robbins 1983; Perrimon et al. 1984, 1989; Perrimon and Mahowald 1986). Transformants bearing GFP-tagged proteins are now frequently used for live cellular analysis (see Hazelrigg, this volume). Fluorescent analysis of fixed samples complements this approach. Fixed analysis allows many more embryos to be examined in a single session on the microscope, and the fixed preparations are stable for long periods. Antiquenching reagents in the mounting media allow extensive documentation of the samples without deterioration of the fluorescent signal (see p. 156). Double- and triple-labeling for colocalization studies are easily performed (see Figure 9.4B,C). Fixed samples also enable one to record the images over multiple planes for three-dimensional reconstructions.

This chapter describes the most common and generally applicable procedures for fixed fluorescent analysis of the *Drosophila* embryo. Pioneers in this field such as Rabinowitz (1941), Zalokar and Erk (1977), Foe and Alberts (1983), and Mitchison and Sedat (1983) laid the groundwork for many of the techniques described here. *Drosophila* embryos are protected by an outer chorion layer as well as an impermeable and opaque vitelline membrane (Figure 9.1). Consequently, preparation of *Drosophila* embryos proceeds as follows: chorion removal, vitelline membrane permeabilization, fixation, and finally, vitelline membrane removal. These procedures as well as procedures for collecting, staining, and mounting the embryos are described below.

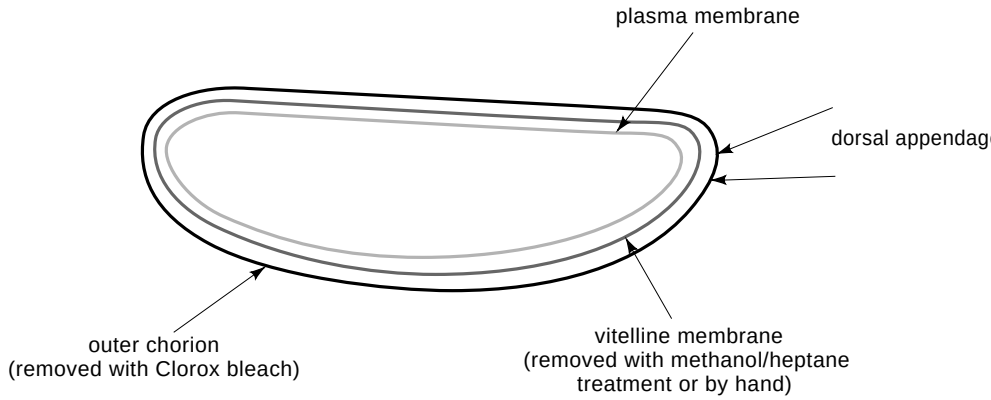

Figure 9.1. Membranes of the *Drosophila* embryo. Each embryo contains a plasma membrane, vitelline membrane, and outer chorion.

PROTOCOL 9.1*

Embryo Collection

Materials

Supplies and Equipment

Plastic fly ÒcollectionÓ bottle (6 ounce; Applied Scientific AS-355N)
Lids from 35 x 10-mm disposable plastic tissue-culture dishes
Screw-cap microcentrifuge tubes (1.5 ml)
Razor blades
Synthetic mesh (120 m, 206 821 7345; Research Nets Inc., 14207 100th Ave Ne, Bothell, Washington 98011-5126. Telephone: 425-821-7345)
Small fine-haired paint brush
Squirt bottle containing H_2O

Preparation of Grape or Apple Juice Egg Plates

Plastic-ware and ingredients:

- 10 x 35-mm plastic petri dish lids
- 700 ml of H_2O (deionized)
- 300 ml of juice concentrate (grape or apple)
- 0.5 g of methylparaben dissolved in 10 ml of ethanol
- 30 g of agar (American Bioorganics 00-58010)

Add agar to the H_2O and autoclave for 40 minutes. While autoclaving, spread the tops of the petri dishes face up on a series of trays. Add methylparaben solution to juice concentrate Quickly and thoroughly mix the concentrate into the autoclaved solution. Immediately begin dispensing media into dishes. Once the juice plates have cooled and hardened, store at 4½ This procedure makes 1 liter of solution and yields approximately 170 juice plates.

Yeast Paste

Mix 1 g of dry yeast and 1.3 ml of H_2O to yield a paste.

CAUTION: ethanol (see Appendix 4)

*For all protocols, H_2O indicates glass distilled and deionized.

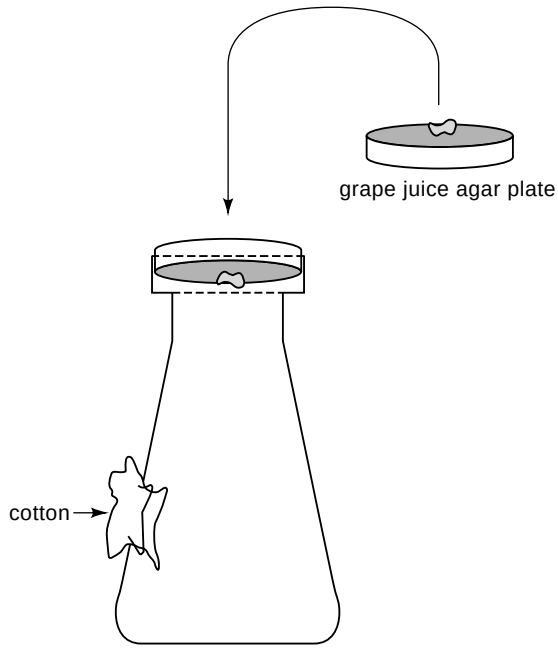

Figure 9.2. Collecting bottle and egg plate (see text for details).

Method

1. Place 200Ð400 flies in a 6-ounce plastic collection bottle fitted with a small cotton-filled breathing hole. Cover the bottle with a 35×10-mm disposable, plastic petri dish lid containing grape juice agar and a small dollop of thick yeast paste (see Figure 9.2). The eggs are deposited on the grape juice agar. Avoid opening the incubator during the egg-laying period, as the flies prefer quiet.

   Note: Typically, a single bottle is set up for each line being examined. However, if embryos of short time points (<1 hour) are being collected and/or the line does not lay many embryos, several bottles can be used.

2. While the flies are laying, prepare egg baskets as follows:

   a. Cut 1.5-ml screw-cap microcentrifuge tubes in half (see Figure 9.3). Also cut off the top of each screw cap, so that it forms an open ring that can be screwed back onto the microcentrifuge tube.

   b. Place a piece of 120-m synthetic mesh around the threaded end of each microcentrifuge tube.

   c. Use the ring made from the screw cap to hold the mesh in place, thus creating a basket.

   Notes: If larger quantities of eggs are being fixed, larger egg baskets must be constructed. A good rule of thumb is that enough surface area should be provided so that the embryos can lie in a monolayer on the mesh.

   In general, a single basket is used for each timed embryo collection. Between collections, the egg basket is thoroughly washed with H_2O, and the mesh is replaced.

3. Obtain embryo collections at appropriate time points. Transfer the embryos from the agar plates into the egg basket using H_2O and a small paintbrush. If there are relatively few embryos, rapidly pick the embryos from the plate with forceps and transfer them in clusters to the egg basket. In either case, wash all the embryos into the mesh at the

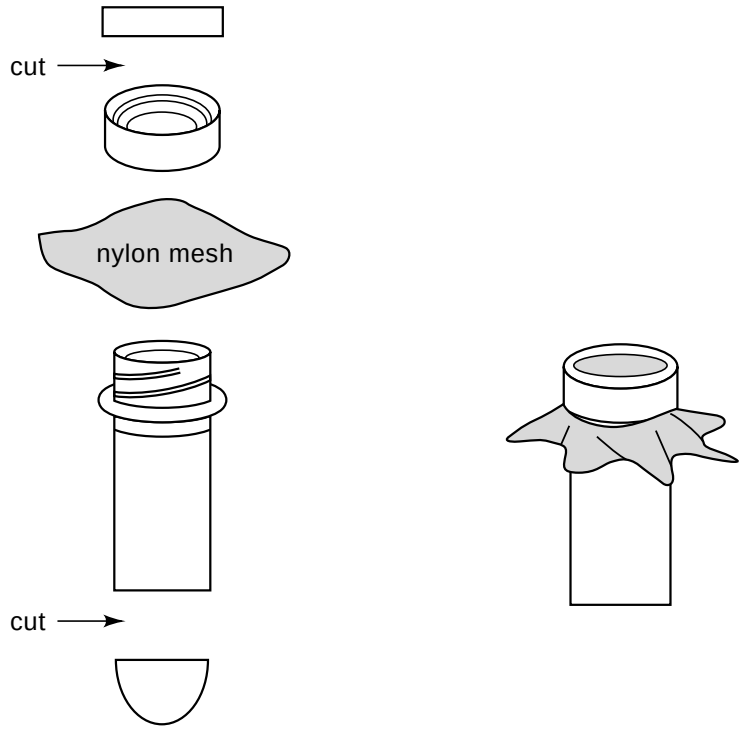

Figure 9.3. Constructing an egg basket (see text for details).

bottom of the basket using copious amounts of H_2O. Proceed rapidly with the washing to avoid anoxia.

Note: For embryo collections from various time points, dechorionation should follow immediately for each collection (see Protocol 9.2).

PROTOCOL 9.2

Embryo Dechorionation

Materials

Supplies and Equipment

Dissecting microscope
Glass petri dish
Pasteur pipette

Solutions and Reagents

Squirt bottle containing 1x Embryo Wash Solution
Bleach (Clorox)

10x Embryo Wash Solution

7% NaCl
0.5% Triton X-100
Dissolve 70 g of NaCl and 5 ml of Triton X-100 in sufficient H_2O. Adjust to 1 liter with H_2O.

CAUTION: bleach (see Appendix 4)

Method

1. Place the egg baskets in a glass petri dish partially filled with a 50% bleach (Clorox) solution (the level should be just below the rim of the basket). Use a pasteur pipette to continually rinse the embryos with the bleach solution.

   Note: Because the potency of the bleach varies, monitor the dechorionation process by observing the embryos under a dissecting microscope. When the dorsal appendages have dissolved in 80% of the embryos (~1Ð3 minutes), immediately proceed to step 2, below (extensive washing). Monitoring prevents damaging the embryos through overexposure to bleach.

2. Immediately wash the basket of embryos using a squirt bottle containing Embryo Wash Solution. Wash the inner edges of the basket, so that all of the embryos lie on the mesh.

EMBRYO FIXATION

Protocol 9.3 presents the following fixation procedures:

Formaldehyde-based Fixation Techniques. The slow formaldehyde fix method (see Protocol 9.3, Method 1) is a modification of the Mitchison and Sedat protocol (1983) and is excellent for preserving many structures deep within the embryo because it allows time for the fix to permeate the embryo. In general, use reagent-grade formaldehyde. However, preservation of some cellular components may require higher-quality (EM-grade) formaldehyde.

The fast formaldehyde fix method (see Protocol 9.3, Method 2) designed by Theurkauf (1992) is a modification of the slow formaldehyde fixation procedure. It preserves cortical structures and is excellent for fixing dynamic cytoskeletal structures such as microtubules.

Fixation with Methanol. This is a relatively harsh procedure that destroys membranes (see Protocol 9.3, Method 3). In addition, in early embryos, the pole cells are often lost. However, it adequately preserves many structures, such as microtubules. The methanol fix has the advantage that virtually all of the embryos are devitellinized. This is important when analyzing embryos that are limited in quantity.

Fixation by Boiling. The boiling fix method (see Protocol 9.3, Method 4) is often tried when more standard fixations fail. For example, immunofluorescent analysis of a number of centrosome proteins requires this fixation method. This procedure also results in devitellinization of virtually all of the embryos.

Fixation without Methanol (Hand Devitellinization of Embryos). Often, the highest quality preparations are obtained by not exposing the embryos to methanol, which destroys membrane and other structures. In addition, some reagents such as fluorescently labeled phalloidin will not work if the embryos have been exposed to methanol. Foregoing the methanol, however, necessitates removing the vitelline membrane by hand. We find this procedure to be most satisfactory (see Protocol 9.3, Method 5). For examples of embryos prepared by this method, see Figure 9.4B,C.

The appropriate technique depends on a number of variables, including the preservation qualities of the cellular components being examined, the position of the components within the embryos, and the fluorescent probe used. Some general guidelines are presented, but for a new antibody or reagent, it may be necessary to try all of these techniques to determine which one is the most appropriate.

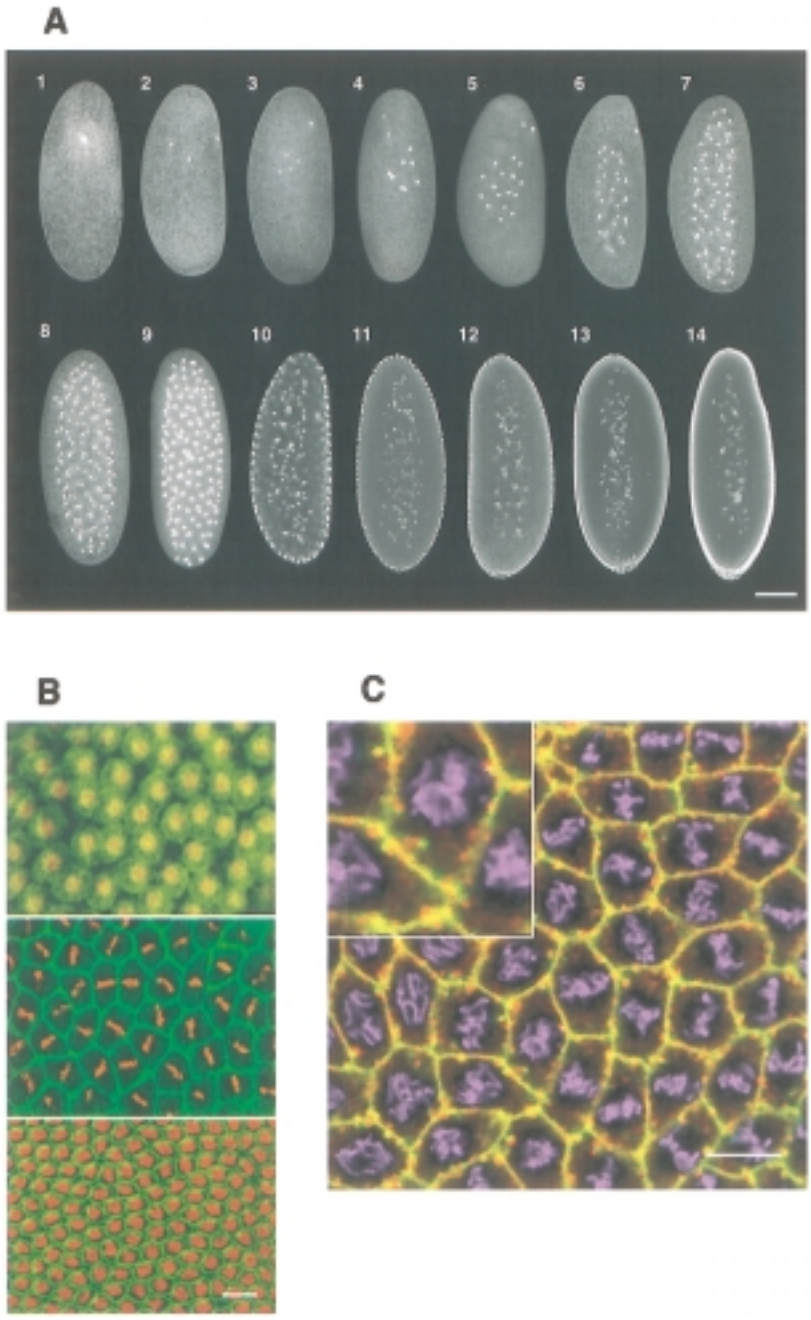

Figure 9.4. Confocal micrographs of wild-type Drosophila embryos. (A) Whole-mount Drosophila embryos stained with propidium iodide to visualize the nuclear division cycle. Shown are syncytial embryos in nuclear division cycles 1–13 and interphase of nuclear cycle 14 (cellularization). Bar, 1 m. (B) Surface views of Drosophila embryos prepared by hand devitellinization and double-stained for actin (fluorescein phalloidin, green) and DNA (propidium iodide, red). Interphase actin caps (top panel) and actin-based metaphase furrows (middle panel) are shown for embryos in nuclear division cycle 13. (Bottom panel) Embryo at cellularization. Actin-based cellularization furrows surround each of the nuclei. Bar, 10 m. (C) Surface view of a Drosophila embryo in late metaphase/early anaphase of nuclear division cycle 13. The embryo was prepared by hand devitellinization and triple-stained for actin (fluorescein phalloidin, green), DNA (propidium iodide, purple), and the furrow component, Dah (anti-Dah, Cy5-labeled secondary, red). Both actin and Dah localize to the furrows and appear to colocalize in some regions (yellow staining, inset). Bar, 10 m. (Inset) 2x magnification.

PROTOCOL 9.3

Embryo Fixation

Materials

Supplies and Equipment

Scissors
Glass vials (5 ml)
Pasteur pipette
Conical tube (50 ml)
3MM Whatman paper
Double-stick tape
Needle (23 gauge)
Syringe (3 ml)
Petri dish lid (35 x 10 mm)

Solutions and Reagents

Heptane
Formaldehyde (reagent and EM grade)
Methanol
Embryo Wash Solution (for preparation, see p. 145)

PEM Buffer (Karr and Alberts 1986)

0.1 M PIPES
1 mM $MgCl_2$
1 mM EGTA
Adjust pH to 6.9 with KOH.

10x PBS Solution (Sambrook et al. 1989)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Dissolve all components in 800 ml of H_2O. Adjust the pH to 7.4 with HCl. Store at room temperature. It is not necessary to sterilize this solution for working with *Drosophila* embryos.

PBTA Solution (1x PBS, 1% BSA, 0.05% Triton X-100, 0.02% Sodium Azide)

Mix the following components:

10x PBS	50 ml
BSA	5 g
Triton X-100	250 l
Sodium azide	0.1 g

Adjust volume to 500 ml with H_2O.

Preparation of Heptane Saturated with 37% Formaldehyde

1. Combine equal volumes of heptane and 37% formaldehyde in a scintillation vial (for small volumes of 2Ð10 ml total) or in a 100-ml glass bottle with plastic screw-top (for larger volumes of 25Ð80 ml total).
2. Secure the lid and shake the mixture vigorously for 15 seconds. Let the solution settle into the 2 phases.

3. Repeat this mixing procedure several (at least three) times before using the solution t ensure that the heptane becomes saturated with the formaldehyde. The saturated heptan is the upper phase in the 1:1 heptane:formaldehyde stock.

It is best to prepare the solution the day before it is to be used, shaking the vial or bottle peri odically throughout the day. Because formaldehyde is sensitive to light, wrap the bottle in aluminum foil. The mixture is stored at room temperature and remains active for several months. If crystals form in the mixture upon addition of the formaldehyde, the solution will not work properly and should be discarded. Use formaldehyde from another source to prepare fresh solution.

CAUTION: formaldehyde, HCl, heptane, KCl, KH_2PO_4, KOH, methanol, $MgCl_2$, Na_2HPO_4, sodium azide (see Appendix 4)

Method 1

Slow Formaldehyde Fix

1. Remove the mesh containing the dechorionated embryos from the egg basket. T embryos should be in the center of the mesh. Cut away the excess mesh and extr excess liquid by gently blotting with a paper towel.
2. Place the mesh (embryos facing out) on the inner edge of a 5-ml glass vial. Use a p teur pipette filled with approximately 1 ml of heptane to wash the embryos off the mesh into the vial (contaminating bits of agar will preferentially stick to the mesh).
3. Remove the mesh, and immediately add an equal volume of 3.7% formaldehyde PEM buffer. Screw the lid on tightly and shake vigorously for 15 seconds.
4. Let the vial stand at room temperature for 20 minutes.

   Note: At the end of this period, the embryos will lie at the interface between the lower formaldehyde and the upper heptane layer. Be aware of the time, as longer fixation times result in cross-linking of the plasma membrane and the vitelline membrane and thus significantly decrease the efficiency of devitellinization.
5. Carefully remove the bottom formaldehyde phase of fluid. Use a yellow pipette tip and angle the vial to remove as much formaldehyde as possible without removing t embryos.
6. Add 1.0 ml of methanol. Cap the vial, shake vigorously for 15 seconds, and let star for 1 minute after shaking.

   Note: Usually about half of the embryos are devitellinized and these sink to the bottom of the vial.
7. Remove the upper heptane layer, along with the embryos that did not sink. Add methanol to the embryos remaining until the vial is approximately two-thirds full. Store these embryos in methanol at 4¼C.

   Note: Storing the embryos overnight at 4¼C before staining allows time for clearing and often results in higher-quality images.

Method 2

Fast Formaldehyde Fix

Perform steps 1Ð7 as described for the Slow Formaldehyde Fix (Method 1, abov However, at step 3, add 37% formaldehyde and fix for 5 minutes at room temperatur

(step 4). As with the Slow Formaldehyde Fix procedure, longer fix times decrease the yield because of poor devitellinization.

Method 3

Methanol Fix

1. Perform steps 1 and 2 of the Slow Formaldehyde Fix (Method 1, above).
2. Remove the mesh and add 1.0 ml of methanol. Cap the vial, shake vigorously for 15 seconds, and let stand for 1 minute after shaking.

   Note: Almost all of the embryos are devitellinized and sink to the bottom of the vial.
3. Remove the upper heptane layer and most of the methanol, leaving the embryos at the bottom of the vial. Add fresh methanol until the vial is approximately two-thirds full. Store these embryos in methanol at 4¼C.

Method 4

Boiling Fix

1. Heat 5 ml of Embryo Wash Solution to 90Ð100¼C in a 50-ml conical tube. Add dechorionated embryos to the heated Embryo Wash Solution.

   Note: This step causes the rapid fixation of the embryos.
2. Immediately add 40 ml of ice-cold Embryo Wash Solution and place on ice. Allow the embryos to sink to the bottom of the tube (~1 minute).
3. Use a pasteur pipette to transfer the fixed embryos in approximately 1 ml of Embryo Wash Solution to a 5-ml glass vial. Allow the embryos to sink to the bottom of the vial (~30 seconds).
4. Remove as much of the Embryo Wash Solution as possible, leaving the embryos in the bottom of the vial. Proceed with the devitellinization by adding 1 ml of heptane followed by 1 ml of methanol to the embryos. Cap the tube, shake it vigorously for 15 seconds, and let stand for 1 minute after shaking.

   Note: Almost all of the embryos are devitellinized and sink to the bottom of the vial.
5. Remove the upper heptane layer and most of the methanol, leaving the embryos at the bottom of the vial. Add fresh methanol until the vial is approximately two-thirds full. Store these embyros in methanol at 4¼C.

Method 5

Embryo Fixation without Methanol (Hand Devitellinization of Embryos)

1. Wash dechorionated embryos into a 5-ml glass vial with approximately 1 ml of heptane saturated with 37% formaldehyde. Incubate the embryos in this solution at room temperature for 40 minutes.
2. Use a pasteur pipette to transfer the embryos to a small piece of 3MM Whatman paper and allow the heptane to evaporate (~30 seconds).

   Note: Ensure that the embryos stay in the neck of the pipette when transferring them. Embryos entering the body of the pipette tend to stick to the sides and are lost. Also, the embryos tend to sink quickly toward the tip of the pipette. It is best to allow them to sink and to transfer them in 1Ð2 drops onto the Whatman paper.

3. Transfer the embryos from the Whatman paper to double-stick tape positioned on the bottom of the lid of a small petri dish (35 x 10 mm) by gently placing the Whatman paper embryo-side down onto the tape and very gently tapping the paper until the embryos stick to the tape. Remove the Whatman paper and immediately cover the embryos with PBTA Solution.
4. Remove the vitelline membrane by hand under a dissecting scope using a 23-gauge needle mounted on a 3-ml syringe. This is best achieved by poking a small hole in the vitelline membrane at one end of the embryo and gently ÒpushingÓ the embryo out through the hole by applying pressure from the opposite end. The membrane will remain stuck to the tape and the devitellinized embryo will float up into the PBTA Solution.
5. Transfer the devitellinized embryos in PBTA Solution to a 5-ml glass vial. Store embryos in PBTA at 4¼C or proceed immediately with staining of embryos (see pp. 153Ð156).

STORAGE OF FIXED EMBRYOS

Hand-devitellinized embryos are stored in PBTA Solution (see p. 148) at 4¼C. For all other fixes, store the embryos in methanol at 4¼C. Overnight storage in methanol clears the embryo and often improves image quality. However, loss of image quality does occur if the embryos are stored in methanol for extended periods of time (weeks). The extent of the deterioration depends on the probe and the antigen being examined.

REHYDRATION OF EMBRYOS STORED IN METHANOL

Embryos stored in methanol must be rehydrated before staining. Rehydration methods differ slightly for embryos fixed in methanol and embryos fixed in formaldehyde (Methods 1 and 2, below). If a new probe or antibody is being used, a gentle rehydration method is recommended (Method 3, below).

PROTOCOL 9.4

Rehydration of Embryos

Materials

Supplies and Equipment

Microcentrifuge tube (1.5 ml)
Pasteur pipette

Solutions and Reagents

Methanol
PBTA Solution (for preparation, see p. 148)
1x PBS (for preparation of 1x PBS, see p. 148)

CAUTION: methanol (see Appendix 4)

Method 1

Rehydration of Embryos Fixed in Methanol

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Add 250 l of methanol to the embryos. Gently add 250 l of PBTA Solution, taking care not to shake the tube.

   Note: Do not shake the PBTA:methanol mixture, because bubbles that form interfere with the ability of the embryos to sink to the bottom of the tube.
3. Add PBTA Solution until the tube is two-thirds full. Invert the tube gently two to three times and let the embryos sink to the bottom of the tube.
4. Remove the solution, leaving the embryos in the bottom of the tube, and add 500 l of PBTA Solution.
5. Allow the embryos to rehydrate in the PBTA Solution at room temperature for 15 minutes on a rotator.

Method 2

Rehydration of Embryos Fixed in Formaldehyde

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Add 500 l of PBTA Solution. Allow embryos to rehydrate in this solution at room temperature for 15 minutes on a rotator.

Method 3

Alternative Rehydration Procedure

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Perform 5-minute washes in the following series of methanol:PBS solutions: 80%:20%, 60%:40%, and 20%:80%.
3. Immerse the embryos in 100% PBS, and then 100% PBTA.
4. Incubate the embryos in PBTA at room temperature for 30 minutes on a rotator.

EMBRYO STAINING

Embryos can be stained with specific fluorescent probes or antibodies through either direct or indirect immunofluorescence. Extensive washing with frequent solution changes reduces background and is key to high-quality preparations. Consequently, any extended break in the preparation (such as leaving overnight) should be made during a washing step. Washes more than 2 hours should be performed at 4¼C.

Nuclear Stains

Several effective probes exist for visualizing DNA (see Table 9.1). Embryo staining procedures are described below for the following:

Staining with propidium iodide (see Protocol 9.5)
Staining with 4,6-diamidino-2-phenylindole (DAPI; see Protocol 9.6)

Other fluorescent DNA probes are processed as described for DAPI. Table 9.1 provide list of commonly used probes and compatible fixations.

Antibody Staining

Unlabeled primary antibodies require the use of a labeled secondary antibody. As me tioned above, extensive rinsing and washing is the key to good preparations. A procedu for staining using primary unlabeled antibodies is given in Protocol 9.7. Variations of this procedure are discussed in Protocol 9.7 Notes.

PROTOCOL 9.5

Propidium Iodide Staining

Propidium iodide is a nucleic acid stain that is added to the mounting medium (for preparation, see p. 156; see also Figure 9.4A–C). Before staining with propidium iodid the embryos must be treated with RNase to remove the RNA.

Materials

Solutions and Reagents

RNase (10 mg/ml)
PBTA Solution (for preparation, see p. 148)

PBS-Azide
1x PBS (for preparation of 1x PBS, see p. 148)
0.02% sodium azide

CAUTION: sodium azide (see Appendix 4)

Method

1. Allow the rehydrated embryos to settle to the bottom of the tube and remove as muc of the PBTA Solution as possible.
2. Add enough 10 mg/ml RNase to cover the embryos and incubate at 37¼C for 2 hou
3. Remove the RNase (store this RNase at 4¼C; it can be reused many times). Wash embryos several times in PBTA.
4. Wash embryos with PBS-Azide and mount as described in Protocol 9.8. Alternativel treat embryos with another probe or antibody.

PROTOCOL 9.6

DAPI Staining

DAPI is a commonly used DNA-binding dye. Because it is specific for double-strande DNA, no prior RNase treatment is required. When working with DAPI, wear gloves because it is a potential mutagen.

Materials

Solutions and Reagents

PBTA Solution (for preparation, see p. 148)

100x DAPI

Dissolve 10 mg of DAPI in 100 ml of methanol. Store in the dark at Ð20¼C.

CAUTION: DAPI, methanol (see Appendix 4)

Method

1. Allow the rehydrated embryos to settle to the bottom of the tube and remove as much of the PBTA Solution as possible.
2. Add 495 l of PBTA and 5 l of 100x DAPI to the embryos. Incubate on a rotator for 5 minutes. To avoid quenching, the tube must be protected from light during and after this staining step.
3. Remove the DAPI solution and discard it as hazardous waste.
4. DAPI-stained embryos require extensive rinsing. Quickly rinse the embryos three times in PBTA Solution, allowing the embryos to settle between rinses. Wash the embryos in PBTA for 1 hour. Repeat this rinse/wash cycle at least once. It is beneficial to include an overnight wash at 4¼C.

PROTOCOL 9.7

Unlabeled Primary Antibody staining

Materials

Supplies and Equipment

Pasteur pipette
Microcentrifuge tubes (0.5 ml; Eppendorf)

Solutions and Reagents

PBS-Azide (see p. 153)
PBTA Solution (for preparation, see p. 148)
Primary and secondary antibodies

Method

1. Use a pasteur pipette to transfer the embryos (in PBTA) to a 0.5-ml microcentrifuge tube (Eppendorf). Allow embryos to settle to the bottom of the tube. Remove the PBTA Solution and add primary antibody (diluted in PBTA) to the embryos. (If using a new antibody, try concentrations of 0.1 g/ml, 1 g/ml, and 10 g/ml.) Incubate the embryos in primary antibody at room temperature for 1 hour on a rotator (for best results, incubate the embryos at 4¼C overnight).

2. Remove the primary antibody and rinse the embryos three times with PBTA, allow ing the embryos to settle between rinses. Wash the embryos for at least 1 hour at roo temperature. Longer washes and more rinses usually produce cleaner image Overnight washes should be performed at 4¼C.

   Note: The primary antibody usually can be reused several times and should be stored at 4¼C.

3. Add fluorescently labeled secondary antibody (usually at 1 g/ml) and incubate at room temperature for 1 hour. Make sure it is directed against the same species which the primary antibody was generated.

   Note: Once the fluorescent secondary antibody is added, the embryos should be kept out of the light as much as possible.

4. Remove the secondary antibody. Proceed through three rinse/wash cycles described in step 2, above.

5. At this point, either prepare the embryos for mounting (steps 6Ð7) or counterstain the embryos for another probe (see Protocol 9.7 Notes, Double Labeling).

6. Rinse the embryos four times in PBS-Azide to remove the detergent (Triton X-100) Proceed to Protocol 9.8.

PROTOCOL 9.7 NOTES

Double Labeling. When performing a double-immunofluorescent analysis, it is possible to save time by mixing the primary antibodies together in step 1 and mixing the secondary antibodies in step 3 of the unlabeled primary antibody staining procedure (above). However, by performing the staining in sequence, the primary antibodies are not mixed and can be reused many times. To perform the staining in sequence, simply repeat the primary and secondary staining (step 1Ð4). Make sure to wash the embryos thoroughly between stains to avoid contamination of the primary and secondary antibodies. The following rules apply for double labeling:

1. When examining whether two structures colocalize, the clearest results are obtained when the separate components are stained with fluorescein and Cy (Figure 9.4C). Bleed-through between these channels is avoided because the emission maxima lie far apart (519 nm and 670 nm, respectively).
2. When double staining with two primary antibodies of the same species, one of the antibodies must be directly labeled (see below).

Directly LabeledÐAntibody Staining. Coupling a fluorophore directly to a primary antibody is a simple procedure (Francis-Lang et al. 1999) and produces excellent images. Directly labeled antibodies produced in this way are often used when staining embryos with two antibodies generated in the same species. When counter staining with an unlabeled primary antibody, take care to stain with the directly labeled antibody last. This ensures that the secondary used to visualize the unlabeled primary does not bind to the directly labeled antibody. To stain embryos with a directly labeled antibody, perform the following steps:

1. Use a pasteur pipette to transfer the embryos (in PBTA) to a 0.5-ml microcentrifuge tube (Eppendorf) and let the embryos settle at the bottom of the tube.

2. Remove as much PBTA as possible and add 300–500 ml of the labeled antibody (diluted in PBTA) to the embryos.
3. Incubate the embryos in the labeled antibody at room temperature for 1 hour on a rotator in the dark.

Using Dyes and Fluorescently Labeled Molecules for Multichannel Labeling. Dyes, fluorescently labeled molecules, and antibodies can be used together to generate effective double- and triple-labeled images (Figure 9.4B,C). When performing a triple-label stain, take care to remove as much bleed-through from neighboring channels as possible. If two components under investigation are closely positioned and need to be resolved, be sure to stain them with fluorophores with very different emission maxima (e.g., fluorescein and Cy5, see Figure 9.4C).

PROTOCOL 9.8

Mounting and Storage of Embryos

Materials

Supplies and Equipment

Coverslips (22 x 22 mm or 22 x 30 mm)
Glass slides
Nail polish

Solutions and Reagents

Glycerol-based Mounting Medium

For the stock, prepare 10 mg/ml 1,4-phenylenediamine (Aldrich P2,396-2) in 1 x PBS (see p. 148). Combine 10 ml of the stock with 90 ml of glycerol. Store in 1-ml aliquots at –20¼C.

Mounting Medium with Propidium Iodide

Prepare mounting medium as described above. Add propidium iodide to a final concentration of 1 mg/ml. Store in the dark at –20¼C.

CAUTION: phenylenediamine, propidium iodide (see Appendix 4)

Method

Before proceeding with mounting the embryos, be sure that steps 6 and 7 of the procedure for unlabeled primary antibodies (see Protocol 9.7) have been completed.

1. Remove as much of the PBS-Azide solution as possible and add 10–40 ml of glycerol-based mounting medium to the embryos.

   Note: The mounting medium should be kept on ice, and the Mounting Medium with Propidium Iodide should be kept in the dark. Mounting medium with or without propidium iodide can be used depending on the choice of stain.

2. Transfer the embryos (in mounting medium) onto a glass microscope slide using a P-200 Pipetman with the yellow tip cut at an angle to allow pipetting of the viscous solution. Place a coverslip over the embryos and seal with nail polish.

Note: It is important that the correct amount of mounting solution (containing embryos) is placed on the slide. For example, a 22 × 22-mm coverslip requires 40 μl of mounting media. Less than this volume results in bubbles and more than this volume results in a floating coverslip that cannot be sealed with nail polish.

3. Store the slides flat at Ð20¼C in the dark.

Note: When stored at Ð20¼C, the embryos are stable in this medium for many days with little loss of image quality.

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10

Imaginal Discs

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THE IMAGINAL DISCS ARE SET ASIDE DURING EMBRYONIC DEVELOPMENT and do not partici-
pate appreciably in larval life. During metamorphosis, however, the larval epidermis i
converted into the pupal case, and the imaginal discs (along with the histoblast nest
form the outer covering of the developing adult. Many different approaches have bee
used to analyze the roles of specific gene products in the patterning of imaginal tissu
This chapter describes methods for generating genetic mosaics, by which genetic char
can be limited to small groups of imaginal cells, and presents immunohistological tech
niques for monitoring gene expression.

Imaginal disc primordia appear in embryos as clusters of 20Ð40 cells, first identifiab
by the expression of various marker genes, that invaginate from the embryonic epithel
um. Each disc is a single-layered epithelial sheet or sac that stays connected to the em
onic and larval epithelium by a thin stalk. Eventually, each sac flattens and the two sid
of the sac take on different characteristics, forming on one side the thicker, highly folc
ed disc epithelium and on the other side, the thinner, unfolded peripodial membrane
Most of the ectodermal adult structures are derived from the disc epithelium. The disc
undergo extensive proliferation during the three larval instars, and by late in the thirc
instar, just before metamorphosis, each disc contains tens of thousands of cells. Figu
10.1 schematically shows the locations and shapes of the imaginal discs in the third ins
larva.

Just prior to metamorphosis, at approximately 108 hours after egg laying (AEL) a
25¼C, the third-instar larva ÒwandersÓ out of the food and up the side of the vial or b
tle; the length of this stage is approximately 12 hours, but varies considerably. Toward t
end of this stage, the anterior spiracles evert and, at approximately 120 hours AEL, t
larva pupariates: The larva stops wandering and the larval epithelium forms the barre
shaped body wall of the puparium (the white prepupal stage, WPP). After an hour or so
the body wall tans to a dark brown color. For the first 5Ð6 hours after pupariation (AP)
the fly is still a prepupa, and the imaginal cells have yet to secrete the pupal cuticle. Duri
the prepupal stages, the imaginal discs of the appendages (leg, wing, haltere, etc.)
through the dramatic morphological changes of eversion. For instance, the leg disc
unfold and lengthen into a tube, and the wing discs lengthen and flatten to form the win

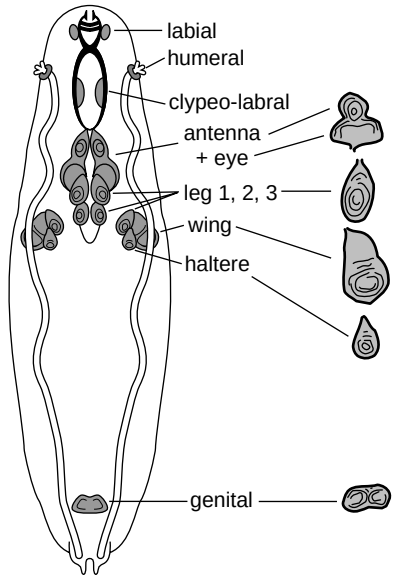

Figure 10.1. Schematic representation of late third-instar larvae, showing the approximate locations and shapes of the imaginal discs. (Reprinted, with permission, from Bodenstein 1950.)

blade. During eversion, the disc epithelia break through the peripodial membrane. At approximately 5Ð6 hours AP, the disc epithelia begin to secrete the pupal cuticle. Later, at about 16 hours AP, the pupal cuticle lifts off the surface of the disc epithelium (apolysis) in preparation for secretion of the adult cuticle, which begins at approximately 40 hours AP. Cell division, epithelial patterning, and morphological changes occur at all stages up to the time of secretion of the adult cuticle and differ from disc to disc. Space limits prevent a detailed description of those changes here, but for several useful reviews, see Bodenstein (1950), Cohen (1993), Fristrom and Fristrom (1993), and Blair (1995, 1999).

Each stage described above presents different advantages and disadvantages for observations. A great deal of patterning takes place during the early stages of disc development, from embryogenesis through mid-third instar. However, at early stages, the imaginal discs are small and difficult to identify. At wandering third instar, discs are easy to isolate. However, the extensive folding of the disc epithelia at wandering third instar obscures some events, and several patterning processes are not yet complete. At prepupal and pupal stages, eversion unfolds some structures, and by 24Ð40 hours AP, many (although not all) developmental processes are complete. However, once secreted, the pupal and adult cuticle can block the penetration of most probes (although X-gal staining for galactosidase activity can penetrate cuticle). Therefore, this chapter concentrates on three easily isolated stages, the wandering third-instar disc, prepupal stage (0Ð6 hours AP), and pupal stage from 24Ð40 hours AP, when the pupal cuticle can be removed.

The techniques described below are our standard laboratory protocols for generating mosaic clones and visualizing those clones in wandering larval stage imaginal discs and pupal tissues. The immunohistological techniques are also generally applicable to the description of protein expression. To visualize mitotic recombinant clones in adults, see Lawrence et al. (1986) for appropriate genetic markers.

GENETIC MOSAICS

Techniques for Generating Clones

Means exist for both removing and adding back gene funtion to imaginal cells (for review see Blair 1995, 1999). Particularly useful has been the generation of genetic mosaics, u either mitotic recombination or the FLPout technique (Figure 10.2). In mitotic recombination, homozygotic cells are generated in heterozygotic flies by inducing recombinatio between homologous chromosomes. This is done either by irradiating heterozygotic la vae with X-rays or-rays or by using heat-shock-induced expression of FLPase to induc recombination between FLPase recombination targets (FRTs) that were inserted in

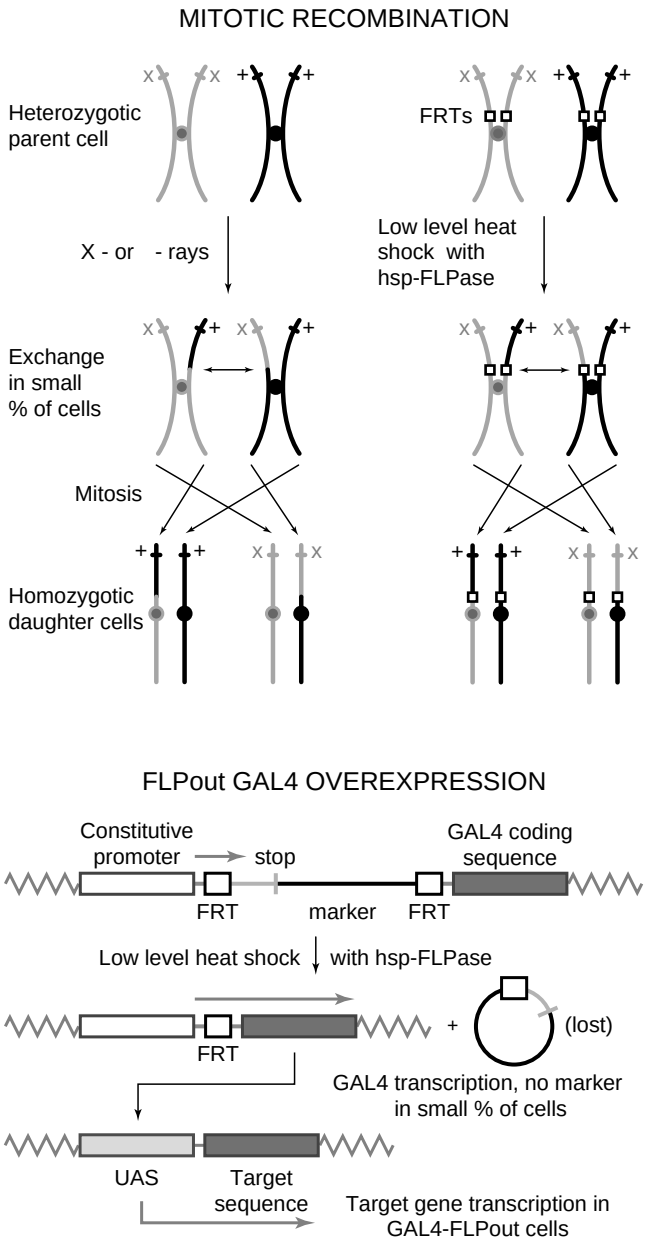

Figure 10.2. Schematic representation of mitotic recombination and GAL4 FLPouts. See text for details (Reprinted, with permission, from Blair 1995.)

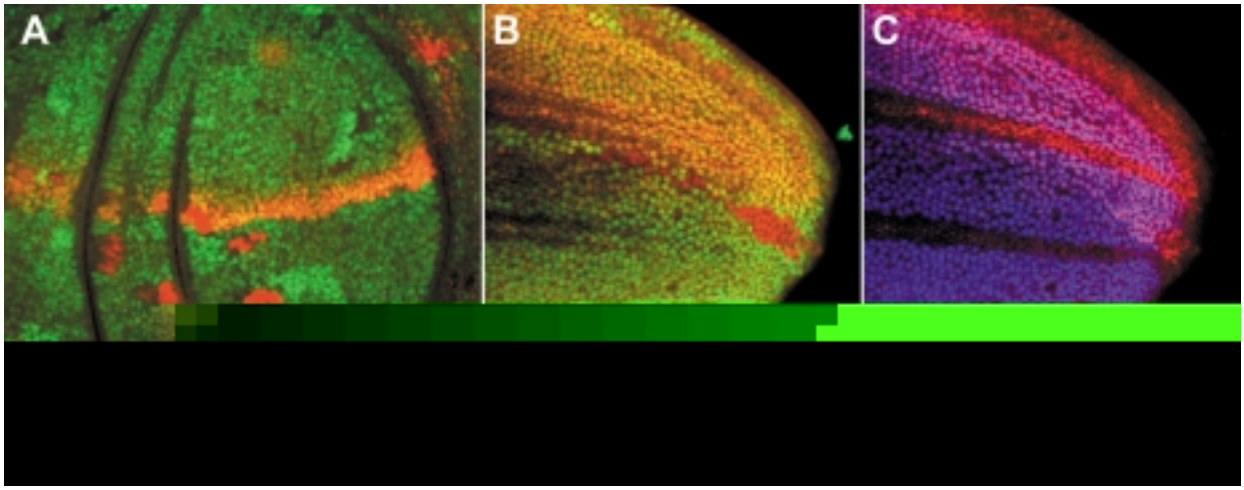

Figure 10.3. Mitotic recombinant clones lacking the posterior Òselector Ó transcription factor gene *ci* and invected (*en-inv*^{P}) in late third-instar wing discs (*A*) or approximately 30-hr AP pupal wings (*B*, *C*). Clones are identified by the absence of the Myc epitope marker construct, as followed using anti-Myc (*green*). The disc in *A* also contains the *patched-xho* LacZ (*ptc-xho*) promoter construct, whose expression was followed using anti-galactosidase (*red*). The *ptc-xho* construct is normally expressed in a stripe just anterior to the anterior-posterior compartment boundary, but is ectopically expressed in most posterior *en-inv*^{P} clones. The pupal wing in *B* and *C* contains the *cubitus interruptus*-LacZ enhancer trap (*ci-LacZ*, *red*) that is normally repressed in the posterior, but is ectopically expressed in a posterior *en-inv*^{P} clone. The wing has also been stained with anti-DSRF (*blue*), which is not expressed in vein nuclei (*C*). Note the ectopic unstained region surrounding the *en-inv*^{P} clone, indicating the induction of ectopic vein-like development in neighboring wild-type cells.

selected chromosome arms (Xu and Rubin 1993). In the FLPout technique, heat-shock-induced expression of FLPase joins a ubiquitous promoter to a selected coding sequence by removing blocking DNA flanked by FRTs (Struhl and Basler 1993). A useful variant on the FLPout technique couples it with the upstream activation sequence (UAS)-GAL4 technique of Brand and Perrimon (1993). A GAL4 FLPout clone expresses GAL4, which in turn drives the expression of any gene coupled to the UAS promoter (de Celis and Bray 1997; Pignoni and Zipursky 1997).

With appropriate levels of irradiation or heat shock, mitotic recombination or FLPouts can be generated in a small percentage of disc cells at any stage; each cell then divides and normally forms a coherent patch or ÒcloneÓ of genetically altered tissue (see Protocol 10.1). However, the location of such clones is random; thus, clones must be identified using genetic or histological markers (see Figure 10.3)

Identifying Sexes and Genotypes

As most crosses for generating mosaic clones generate several different genotypes, it is often more efficient to screen out the inappropriate larvae before dissection and staining.

Sexing Larvae. From mid-third instar to WPP stages, the genital discs are visible through the body wall as clear spaces on each side of the abdomen, approximately one quarter of the way from the posterior end. Place larvae in CO_2 in a dish on a dark surface, illuminate with light from the side, and examine under a dissecting scope. The male genital discs are large and obvious, but the female genital discs are small and often difficult to see (see Figure 10.4).

Balancers. Some balancer chromosomes contain useful larval markers. The dominant mutation Tubby (Tb) is present on some versions of the third-chromosome balancers TM6 and TM6B and on the second-third Ò double balancer SM5a-TM6B