

# Manipulating the Mouse Embryo

A LABORATORY MANUAL

FOURTH EDITION



## ALSO FROM COLD SPRING HARBOR LABORATORY PRESS

*Antibodies: A Laboratory Manual*, Second Edition

*Gastrulation: From Cells to Embryo*

*Molecular Cloning: A Laboratory Manual*, Fourth Edition

*Mouse Hematology: A Laboratory Manual*

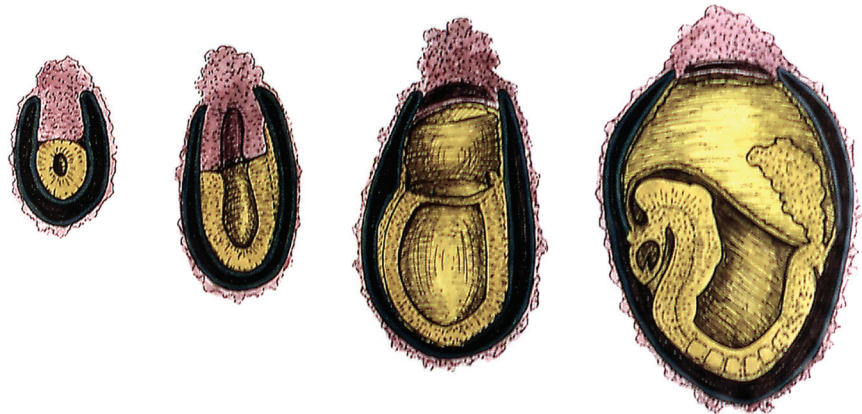
*Mouse Models of Cancer: A Laboratory Manual*

*Mouse Phenotypes: A Handbook of Mutation Analysis*

# Manipulating the Mouse Embryo

A LABORATORY MANUAL

FOURTH EDITION



Richard Behringer

*University of Texas  
M.D. Anderson Cancer Center*

Marina Gertsenstein

*Toronto Centre for Phenogenomics  
Transgenic Core*

Kristina Vintersten Nagy

*Samuel Lunenfeld Research Institute  
Mount Sinai Hospital, Toronto*

Andras Nagy

*Samuel Lunenfeld Research Institute  
Mount Sinai Hospital, Toronto*



COLD SPRING HARBOR LABORATORY PRESS  
Cold Spring Harbor, New York • [www.cshlpress.org](http://www.cshlpress.org)

# MANIPULATING THE MOUSE EMBRYO

A LABORATORY MANUAL  
FOURTH EDITION

All rights reserved

© 2014 by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York  
Printed in the United States of America

<b>Publisher</b>	John Inglis
<b>Acquisition and Managing Editor</b>	Judy Cuddihy
<b>Director of Editorial Development</b>	Jan Argentine
<b>Developmental Editors</b>	Kaaren Janssen, Andrea R. Russo, and Michael Zierler
<b>Project Managers</b>	Mary Cozza and Inez Sialiano
<b>Permissions Coordinator</b>	Carol Brown
<b>Production Editor</b>	Kathleen Bubbeo
<b>Production Manager</b>	Denise Weiss
<b>Cover Designer</b>	Ed Atkeson

*Front cover artwork:* Mouse embryos at the blastocyst stage, just before implantation, with three distinct cell types: trophoctoderm cells, which will form the placenta, in light blue; pluripotent epiblast cells, which will give rise to all cells of the animal body, in navy; and primitive endoderm cells, which will differentiate into the yolk sac, in pink. (Courtesy of Mubeen Goolam and Magdalena Zernicka-Goetz, University of Cambridge, United Kingdom.)

*Title page illustration:* Early postimplantation stages of mouse embryogenesis. Illustration by Rosa Beddington. (Printed, with permission, from John Skehel, Medical Research Council.)

Library of Congress Cataloging-in-Publication Data  
Behringer, Richard.

Manipulating the mouse embryo : a laboratory manual / Richard Behringer, University of Texas, M.D. Anderson Cancer Center [and three others]. -- Fourth edition.

pages cm

Includes bibliographical references and index.

ISBN 978-1-936113-00-2 (hardback) -- ISBN 978-1-936113-01-9 (paper)

1. Mice--Embryos--Laboratory manuals. 2. Mice--Genetic engineering--Laboratory manuals. 3. Transgenic mice--Laboratory manuals. 4. Mice as laboratory animals. I. Title.

QL737.R6M2468 2013

616.027333--dc23

2013023915

10 9 8 7 6 5 4 3 2 1

Students and researchers using the procedures in this manual do so at their own risk. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in this manual and has no liability in connection with the use of these materials. All registered trademarks, trade names, and brand names mentioned in this book are the property of the respective owners. Readers should please consult individual manufacturers and other resources for current and specific product information.

With the exception of those suppliers listed in the text with their addresses, all suppliers mentioned in this manual can be found on the BioSupplyNet website at [www.biosupplynet.com](http://www.biosupplynet.com).

All World Wide Web addresses are accurate to the best of our knowledge at the time of printing.

Procedures for the humane treatment of animals must be observed at all times. Check with the local animal facility for guidelines.

Certain experimental procedures in this manual may be the subject of national or local legislation or agency restrictions. Users of this manual are responsible for obtaining the relevant permissions, certificates, or licenses in these cases. Neither the authors of this manual nor Cold Spring Harbor Laboratory assume any responsibility for failure of a user to do so.

The materials and methods in this manual may infringe the patent and proprietary rights of other individuals, companies or organizations. Users of this manual are responsible for obtaining any licenses necessary to use such materials and to practice such methods. COLD SPRING HARBOR LABORATORY MAKES NO WARRANTY OR REPRESENTATION THAT USE OF THE INFORMATION IN THIS MANUAL WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHT.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Cold Spring Harbor Laboratory Press, provided that the appropriate fee is paid directly to the Copyright Clearance Center (CCC). Write or call CCC at 222 Rosewood Drive, Danvers, MA 01923 (978-750-8400) for information about fees and regulations. Prior to photocopying items for educational classroom use, contact CCC at the above address. Additional information on CCC can be obtained at CCC Online at [www.copyright.com](http://www.copyright.com).

For a complete catalog of all Cold Spring Harbor Laboratory Press publications, visit our website at [www.cshlpress.org](http://www.cshlpress.org).

*This edition is dedicated to Anne McLaren (1927–2007) who was a pioneer in mouse genetics and embryology. Anne took particular interest in talking with young scientists about their research and providing them with valuable feedback and encouragement. She also said, “always keep in mind that the embryos are so precious. Never waste a single one, there is always another unanswered question it can solve.” Years ago we were among those fortunate young scientists who were inspired by Anne.*

RICHARD BEHRINGER  
MARINA GERTSENSTEIN  
KRISTINA VINTERSTEN NAGY  
ANDRAS NAGY

### **General Safety and Hazardous Material Information**

This manual should be used by laboratory personnel with experience in laboratory and chemical safety or students under the supervision of such trained personnel. The procedures, chemicals, and equipment referenced in this manual are hazardous and can cause serious injury unless performed, handled, and used with care and in a manner consistent with safe laboratory practices. Students and researchers using the procedures in this manual do so at their own risk. It is essential for your safety that you consult the appropriate Material Safety Data Sheets, the manufacturers' manuals accompanying equipment, and your institution's Environmental Health and Safety Office, as well as the General Safety and Disposal Information in Appendix 3 for proper handling of hazardous materials described in this manual. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in this manual and has no liability in connection with the use of these materials.

All registered trademarks, trade names, and brand names mentioned in this book are the property of the respective owners. Readers should consult individual manufacturers and other resources for current and specific product information. Appropriate sources for obtaining safety information and general guidelines for laboratory safety are provided in the General Safety and Hazardous Material Information Appendix of this manual.

### **Experimental Animals**

Procedures for humane treatment of animals must be observed at all times. Consult your local institutional guidelines.

---

# Contents

Preface	xxi
<b>CHAPTER 1</b>	
<b>Genetics and Embryology of the Mouse: Past, Present, and Future</b>	<b>1</b>
Introduction	2
Mendelian Inheritance and Linkage: The Beginnings of Mouse Genetics	3
Origins of the Laboratory Mouse	4
Creation of Inbred Strains and Other Resources of Mouse Genetics	4
Origins of Developmental Genetics of the Mouse	9
A Heritage of Experimental Mouse Embryology	11
Manipulating the Mouse Genome	14
Systematic Mutagenesis and Phenotyping of the Mouse	15
<b>CHAPTER 2</b>	
<b>Summary of Mouse Development</b>	<b>23</b>
INTRODUCTION	24
Gestation and Embryo Staging	24
Oocyte Maturation and Ovulation	27
Spermatogenesis	32
Fertilization	34
Cleavage: Zygote to Eight-Cell Uncompacted Morula	35
Morula Compaction and Blastocyst Formation: The First Differentiation Events	35
Implantation in the Uterus	37
Trophectoderm and Its Derivatives	39
Formation of the Primitive Endoderm and Epiblast: The Second Round of Differentiation Events	41
The Epiblast Lineage	41
<i>Epiblast Cells Divide Rapidly</i>	42
<i>The Epiblast Is a Pluripotent Tissue</i>	44
The Pregastrula Embryo	45
Gastrulation: Formation of Mesoderm and Definitive Endoderm	45

Fate Maps of Early Postimplantation Embryos	49
Origin of the Germline	49
Generation of Regional Diversity in the Mesoderm	51
The Anterior Visceral Endoderm	52
The Node	52
The Tail Bud	54
Embryonic Turning	54
Somites and Their Derivatives	55
Lateral Plate and Intermediate Mesoderm and Their Derivatives	58
Sex Determination and Differentiation	60
Limb Formation	61
Neurulation: Formation of the Nervous System	61
Regional Diversification in the Neural Tube	62
Neural Crest	65
Formation of the Branchial Arches and the Pharyngeal Region	66
Gut Development	67
Extra-Embryonic Tissues	68
Visceral Endoderm	70
Parietal Endoderm	71
Differentiation of the Extra-Embryonic Mesoderm	72
The Structure and Function of the Chorioallantoic Placenta	72
The Adult Mouse	74
<i>Mouse Coat Color and Its Genetics</i>	74
<i>Morphological and Behavioral Mutants</i>	78

### CHAPTER 3

## A Mouse Colony for the Production of Transgenic and Chimeric Animals 85

---

INTRODUCTION	85
Pathogen Control in Experimental Mouse Colonies	86
Location of the Embryo Manipulation Laboratory	87
Establishing a Breeding Colony to Supply Embryo Donor and Recipient Mice	88
Embryo Donors	89
<i>Female Embryo Donor Mice for Pronuclear Microinjection</i>	89
<i>Female Embryo Donor Mice for Generation of ES Cell Chimeras</i>	90
<i>Fertile Stud Male Mice</i>	91
<i>Production of Embryo Donors</i>	91
Embryo Recipients	92
<i>Female Mice to Serve as Pseudopregnant Recipients</i>	92
<i>Sterile Male Mice to Induce Pseudopregnancy in Females</i>	93
<i>Production of Embryo Recipients</i>	93
Pregnancy with Manipulated Embryos	94
Setting up Natural Matings	94



Inducing Superovulation	95
<i>Strain Background</i>	96
<i>Age and Weight</i>	96
<i>Dose of Gonadotropins</i>	97
<i>Time of Administration of the Gonadotropins</i>	97
<i>Reproductive Performance of Stud Males</i>	97
Animal Identification and Tissue Biopsies	97
<i>Ear Punching or Notching</i>	98
<i>Toe Clipping</i>	98
<i>Tattooing</i>	98
<i>Ear Tags</i>	99
<i>Microchip Implants</i>	100
<i>Tail-Tip Excision</i>	100
<b>PROTOCOLS</b>	
1 Selecting Females in Estrus and Checking Plugs	103
2 Preparation of Gonadotropins and Superovulation	105
3 Intraperitoneal (i.p.) Injection	107

## CHAPTER 4

### Recovery and In Vitro Culture of Preimplantation Embryos 109

---

<b>INTRODUCTION</b>	109
Culture Media for Preimplantation Embryos	109
<i>History</i>	109
<i>Two-Cell Block</i>	110
<i>Media for the Development from Zygotes to Blastocysts</i>	112
Critical Components of Preimplantation Embryo Culture	113
<i>Glucose</i>	113
<i>Amino Acids</i>	114
<i>Osmolarity</i>	115
<i>Oxygen Tension, CO<sub>2</sub>, pH, Light, and Temperature</i>	115
<i>Embryo Incubation Volume and the Role of Growth Factors</i>	117
Effect of Embryo Culture on Gene Expression	117
Important Considerations for Successful In Vitro Embryo Culture	118
<i>Medium Choice and Experimental Outcome</i>	118
<i>Reagents</i>	118
<i>Embryo Collection and Culture</i>	119
<i>Quality Control</i>	119
Preparation of Embryo Culture Media	120
Pipettes for Embryo Handling	121
Collection of Preimplantation Embryos	123
<b>PROTOCOLS</b>	
1 Preparation of Media for Embryo Handling and Culture	126

2	Setting Up Microdrops Culture	132
3	Making Embryo-Handling Pipettes from Hard Glass Capillaries	134
4	Preparation of Siliconized Pipettes	136
5	Opening the Abdominal Cavity and Locating Female Reproductive Organs	137
6	Collection of Zygotes and Removal of Cumulus Cells with Hyaluronidase	139
7	Collection of Two-Cell- to Morula-Stage Embryos	143
8	Collection of Blastocysts	146

**CHAPTER 5**

**Isolation, Culture, and Manipulation of Postimplantation Embryos 149**

---

	<b>INTRODUCTION</b>	149
	Isolating Postimplantation Embryos	150
	<i>Visualizing Early Embryo Implantation Sites</i>	150
	<i>Isolating Postimplantation Embryos</i>	150
	<i>Isolating Extra-Embryonic Membranes</i>	150
	<i>Separating Postimplantation Germ Layers</i>	151
	<i>Germ-Layer Explant Recombination Culture</i>	151
	<i>Isolating Germ Cells from the Genital Ridge</i>	151
	Culturing Postimplantation Embryos	152
	<i>Preparing Embryos</i>	152
	<i>Roller Culture of Postimplantation Embryos</i>	153
	<i>Static Culture of Postimplantation Embryos</i>	153
	<i>Static Culture of Postimplantation Embryos for Imaging</i>	154
	Introducing Nucleic Acids into Postimplantation Embryos by Electroporation	154
	<b>PROTOCOLS</b>	
1	Visualizing Early Embryo Implantation Sites by Dye Injection	157
2	Isolating Postimplantation Embryos	159
3	Isolating Extra-Embryonic Membranes	167
4	Separating Postimplantation Germ Layers	170
5	Germ-Layer Explant Recombination Culture	173
6	Isolating Germ Cells from the Genital Ridge	175
7	Roller Culture of Postimplantation Embryos	178
8	Static Culture of Postimplantation Embryos	182
9	Static Culture of Postimplantation Embryos for Imaging	184
10	Electroporation of Postimplantation Embryos In Vitro	187
11	Electroporation of Postimplantation Embryos In Utero	190

**CHAPTER 6**

**Surgical Procedures 195**

---

	<b>INTRODUCTION</b>	195
	General Guidelines for Mouse Surgeries	196
	Anesthesia and Analgesia	197
	<i>Anesthetics</i>	197

<i>Analgesics</i>	198
Vasectomy	199
Embryo Transfer	199
<i>Background</i>	199
<i>Role of Zona Pellucida in Embryo Transfer</i>	200
<i>Number of Embryos to Transfer</i>	200
<i>Recipient Females</i>	201
<i>Technical Aspects</i>	202
Caesarean Section and Fostering	203
Tissue Transplantation	203
Tissue Biopsy	203
Ovariectomy and Castration	203
<b>PROTOCOLS</b>	
1 Preparation of Tribromoethanol (Avertin)	206
2 Vasectomy of Mice	208
3 Oviduct Transfer of Mouse Embryos	211
4 Uterine Transfer of Mouse Embryos	216
5 Caesarean Section and Fostering of Mice	220
6 Transplantation of Adult or Embryonic Tissues under the Kidney Capsule of Mice	222
7 Subcutaneous Injection of Pluripotent Stem Cells in Mice	225
8 Tissue Biopsies in Mice	227
9 Blood Collection by Tail Bleeding in Mice	230
10 Ovariectomy of Mice	232
11 Castration of Mice	234

## CHAPTER 7

### Production of Transgenic Mice by Pronuclear Microinjection 237

---

<b>INTRODUCTION</b>	238
Applications of Transgenic Mouse Technology	238
Gene Transfer into the Mouse Genome by Pronuclear Microinjection	240
Designing Transgenes	242
<i>Effects of Prokaryotic Vector Sequences</i>	242
<i>Length of DNA Construct</i>	242
<i>Coinjection of Two (or More) Transgenes</i>	243
<i>Distinguishing Expression of Transgenes and Endogenous Genes</i>	243
<i>Reporter Genes</i>	244
<i>Using Previously Identified Tissue-Specific Regulatory Sequences in Transgene Constructs</i>	244
<i>Expression of cDNAs and the Role of Introns in Transgene Expression</i>	245
<i>Using “Housekeeping Gene” Promoters to Direct Ubiquitous Expression</i>	245
<i>Factors Affecting the Efficiency of Gene Transfer</i>	245
<i>General Considerations about Large DNA Constructs</i>	247
Isolation and Purification of DNA	247
<i>General Considerations</i>	247

Contaminants and Methods for Their Removal	247
<i>Commercially Available Kits for Plasmid Preparation and DNA Purification</i>	249
<i>Preparation of Standard-Sized Plasmid DNA from Bacterial Cultures</i>	249
<i>Digestion of Plasmid DNA for Release of Genetic Construct Fragment</i>	251
<i>Gel Separation</i>	251
<i>Recovery of DNA Fragments from Agarose Gels</i>	251
<i>Fragment Purification</i>	251
Preparing DNA for Microinjection	252
<i>Determining and Maintaining High DNA Quality</i>	252
<i>Determining Concentration of DNA for Injection</i>	253
<i>Filtering the DNA Solution</i>	254
<i>Storage of Prepared DNA</i>	254
Preparation of Technical Equipment and Zygotes	254
<i>Choice of Mouse Strain</i>	254
<i>Preparation of Zygotes</i>	255
<i>Making Holding Pipettes</i>	256
<i>Making Injection Pipettes</i>	257
<i>Microinjection Setup</i>	258
Microinjection	262
<i>Timing of Pronuclear Injection</i>	262
<i>Microinjection of Mouse Zygotes</i>	262
<i>After Microinjection</i>	263
Establishment and Maintenance of Transgenic Mouse Lines	263
<b>PROTOCOLS</b>	
1 Simple, Reliable Steps for DNA Fragment Isolation and Purification	269
2 Isolating BAC DNA from Bacterial Cultures Using the NucleoBond System	271
3 Purification of BAC DNA Prepared with the NucleoBond System	273
4 Large-Scale Preparation of Yeast Agarose Plugs to Isolate YAC DNA	274
5 Purification of YAC DNA Using a Two-Gel Electrophoresis Procedure	279
6 Purification of YAC DNA Using PFGE and Ultrafiltration	286
7 Preparing Injection Buffer for Standard-Sized DNA	289
8 Preparing Injection Buffer for BAC/YAC DNA	290
9 Preparation of In Vitro–Translated RNA for Microinjection	292
10 Making Holding Pipettes	294
11 Making Injection Pipettes	296
12 Microinjection Setup	298
13 Microinjection of Mouse Zygotes	301
14 Microinjection of RNA into Mouse Zygotes	306
15 Microinjecting Lentivirus into Mouse Embryos	308
16 Infecting Mouse Embryos by Coculturing with Lentivirus	312
<b>DISCUSSION</b>	
Troubleshooting Guide for Microinjection of Mouse Zygotes and Production of Transgenic Mice	315

<b>CHAPTER 8</b>	
<b>Embryo-Derived Stem Cell Lines</b>	<b>321</b>
<hr/>	
INTRODUCTION	321
Derivation and Culture of Mouse Embryonic Stem Cells	322
<i>Historic Background and New Developments</i>	322
<i>General Considerations for Optimal ES Cell Culture</i>	325
<i>Media and Reagents</i>	327
<i>Serum and Serum Replacement</i>	328
<i>Leukemia Inhibitory Factor (LIF)</i>	329
<i>Trypsin/EDTA</i>	330
Feeder Cells	330
<i>Primary Mouse Embryonic Fibroblasts</i>	330
<i>STO Fibroblasts</i>	330
<i>Mycoplasma</i> and Pathogen Testing	331
Karyotype	332
In Vitro Differentiation of ES Cells	333
Trophoblast and Extra-Embryonic Endoderm Stem Cells	333
Epiblast Stem Cells	334
Progenitor Cells from the Central Nervous System	334
 PROTOCOLS	
1 Preparation of Primary Mouse Embryonic Fibroblasts (MEFs)	338
2 Preparation of Feeder Cell Layers from STO or MEF Cells	341
3 Passage of ES Cells	343
4 Cryopreservation of ES Cells	345
5 Testing Serum Batches	348
6 Derivation of ES Cell Lines Using Serum-Containing Culture Conditions	350
7 Derivation of ES Cell Lines Using Small-Molecule Inhibitors of Erk and Gsk3 Signaling (2i)	355
8 Chromosome Counting	363
9 Differentiating ES Cells into Embryoid Bodies by Suspension Culture	366
10 Differentiating ES Cells into Embryoid Bodies by Hanging-Drop Cultures	368
11 Differentiating ES Cells into Embryoid Bodies in AggreWell Plates	370
12 Derivation and Culture of TS Cell Lines	373
13 Derivation and Culture of XEN Cell Lines	379
14 Derivation and Culture of EpiSC Lines	382
15 Isolation, Culture, and Differentiation of Progenitor Cells from the Central Nervous System	389
16 Isolation, Propagation, and Differentiation of Radial Glia-Like Neural Progenitor Cells in Adherent Cultures	395
 CHAPTER 9	
<b>Germline-Competent Stem Cells Derived from Adult Mice</b>	<b>403</b>
<hr/>	
INTRODUCTION	403
Spermatogonial Stem Cells	404
<i>Culture of Spermatogonial Stem Cells</i>	404

<i>Transplantation of Spermatogonial Stem Cells</i>	405
Induced Pluripotent Stem Cells	406
<i>Reprogramming Vectors</i>	407
<i>Testing Pluripotency and Germline Competence of Established iPS Cell Lines</i>	408

#### PROTOCOLS

1 Isolation of the Spermatogonial Stem Cell–Containing Fraction from Testes	410
2 Culture and Expansion of Primary Undifferentiated Spermatogonial Stem Cells	413
3 Cryopreserving and Thawing Spermatogonial Stem Cells	416
4 Spermatogonial Stem Cell Transplantation to the Testis	418
5 Integrating <i>piggyBac</i> Transposon Transgenes into Mouse Fibroblasts by Electroporation	424
6 Integrating <i>piggyBac</i> Transposon Transgenes into Mouse Fibroblasts Using Chemical Methods	426
7 Reprogramming Mouse Fibroblasts with <i>piggyBac</i> Transposons	428

### CHAPTER 10

<b>Vector Designs for Pluripotent Stem Cell–Based Transgenesis and Genome Alterations</b>	<b>431</b>
---	------------

---

INTRODUCTION	431
Glossary of Elements	432
<i>Genomic and Gene Elements</i>	432
<i>Selectable Markers</i>	434
<i>Reporters</i>	436
<i>Site-Specific Recombinases</i>	437
<i>Inducible Systems</i>	439
<i>Reporters to Detect Recombinase Action</i>	440
ES Cell–Mediated Transgenesis	440
Gene Targeting	442
Gene and Promoter Trap	445
Homologous Recombination–Replaceables	446
Site-Specific Recombination-Mediated Insertions	448
Induced Site-Specific Chromosomal Aberrations	449
Isolating ES Cells Homozygous for Specific Mutations	451
PROTOCOLS	
1 In Vitro Screen for Widespread Transgene Expression	455
2 In Vitro Screen to Identify Silent but Activatable (S/A) Integration Sites for a Tet-Inducible Transgene	458

### CHAPTER 11

<b>Introduction of Foreign DNA into Embryonic Stem Cells</b>	<b>461</b>
--	------------

---

INTRODUCTION	461
General Considerations	462
<i>Culture Conditions</i>	462

<i>Genotyping Assays</i>	462
<i>Transfection Methods</i>	462
<i>Targeting Approaches</i>	463
Purifying DNA for Introduction into the ES Cell Genome	464
Electroporating DNA into ES Cells and Selection Methods	464
Isolating Individual ES Cell Colonies by Picking, Replica-Plating, and Freezing ES Cell Lines	465
<b>PROTOCOLS</b>	
1 Electroporating DNA into ES Cells	468
2 Isolating Individual ES Cell Colonies by Picking	472
3 Passaging and Freezing ES Cells in 96-Well Plates	475
4 Thawing ES Cells from a 96-Well Plate	478
5 Rapid Preparation of DNA from Cells in 96-Well Tissue Culture Plates	480
6 Preparation of DNA from Cells in 24-Well Tissue Culture Plates	483
7 Genotyping ES Cell Colonies before Picking	485

**CHAPTER 12****Production of Chimeras 489**


---

<b>INTRODUCTION</b>	489
General Considerations	490
Types of Chimeras	500
<i>Diploid Embryo ↔ Embryo Aggregation Chimeras</i>	500
<i>ES Cells ↔ Diploid Embryo Aggregation and Injection Chimeras</i>	501
<i>Diploid ↔ Tetraploid Embryo Aggregation Chimeras</i>	502
<i>ES Cells ↔ Tetraploid Embryo Aggregation and Injection Chimeras</i>	502
Preparation of Embryos for Injection or Aggregation	503
ES Cell Injection Chimeras	505
<i>The Microinjection Setup</i>	505
<i>Preparation of the Injection and Holding Pipettes</i>	506
Transfer of Chimeric Blastocysts	506
Breeding Chimeric Mice	507
General Troubleshooting for Chimera Production and Breeding for Germline Transmission	509
<b>PROTOCOLS</b>	
1 Making ES Cell Injection Needles	514
2 Assembling the Microinjection Setup	518
3 Preparation of ES Cells for Injection	520
4 Injecting Blastocysts	522
5 Injecting Eight-Cell-Stage Embryos	526
6 Preparation of ES Cells for Aggregation	531
7 Preparation of the Aggregation Plate	534
8 Removal of the Zona Pellucida	536
9 Production of Tetraploid Embryos	539
10 Assembly of the Aggregates	542

11	Disaggregating Cleavage-Stage Embryos and the Inner Cell Mass of Blastocysts into Individual Cells	547
12	Immunosurgery: Isolating the Inner Cell Mass of Blastocysts	549
<b>CHAPTER 13</b>		
<b>Genotyping</b>		<b>551</b>
<hr/>		
	<b>INTRODUCTION</b>	<b>551</b>
	Tissue Samples and DNA Preparation for Genotyping or Characterization of Transgenes	551
	Detection and Analysis of Transgene Produced by Pronuclear Injection	552
	Detection and Analysis of ES Cell–Mediated Gene/Genome Alterations	554
	Cloning Transgene/Host DNA Junctions	555
	Identifying Homozygous Transgenic Mice or Embryos	555
	<i>In Situ Hybridization to Interphase Nuclei</i>	556
	<i>Test Breeding</i>	556
	<i>Southern Blot Analysis Using a Flanking Probe</i>	556
	<i>PCR Analysis Using a Flanking Primer</i>	557
	<b>PROTOCOLS</b>	
1	Isolation of High-Molecular-Weight DNA from Mouse Tail Tips	558
2	Isolation of High-Molecular-Weight DNA from Embryonic Tissues, Yolk Sac, Umbilical Cord, and the Like	560
3	Preparation of Tissue Lysates for PCR Template	562
	• Alternative Protocol: Preparation of Template from Embryonic and Adult Tissue Samples	564
	• Alternative Protocol: Preparation of Template from Mouse Tail Tissue	566
4	Polymerase Chain Reaction	567
5	Preparation of DNA for Southern Blot Analysis or PCR from ES or Other Cultured Cells	569
<b>CHAPTER 14</b>		
<b>Parthenogenesis, Pronuclear Transfer, and Mouse Cloning</b>		<b>571</b>
<hr/>		
	<b>INTRODUCTION</b>	<b>571</b>
	Generating Parthenogenotes	571
	Performing Pronuclear Transfer	572
	Cloning Mice	572
	<b>PROTOCOLS</b>	
1	Parthenogenetic Activation of Oocytes	574
2	Pronuclear Transplantation in the Mouse Embryo	577
3	Cloning Mice	583
<b>CHAPTER 15</b>		
<b>Assisted Reproduction: Ovary Transplantation, In Vitro Fertilization, Artificial Insemination, and Intracytoplasmic Sperm Injection</b>		<b>593</b>
<hr/>		
	<b>INTRODUCTION</b>	<b>593</b>
	Approaches to Bypass Mouse Infertility	593



<i>Female Infertility</i>	594
<i>Male Infertility</i>	595
Strategies to Rescue the Germline of Mice	596
<i>Ovary Transplantation</i>	596
<i>In Vitro Fertilization</i>	596
<i>Artificial Insemination</i>	597
<i>Intracytoplasmic Sperm Injection</i>	598

#### PROTOCOLS

1 Ovary Transplantation	601
2 In Vitro Fertilization	604
3 Nonsurgical Artificial Insemination	615
4 Determining the Stage of the Estrous Cycle by Vaginal Smear	617
5 Surgical Artificial Insemination	619
6 Intracytoplasmic Sperm Injection	623

### CHAPTER 16

<b>Cryopreservation, Rederivation, and Transport of Mouse Strains</b>	<b>631</b>
---	------------

---

<b>INTRODUCTION</b>	<b>631</b>
Embryo Cryopreservation	632
<i>Factors to Consider in Embryo Cryopreservation</i>	632
<i>Equilibrium Methods of Cryopreservation</i>	633
<i>Nonequilibrium Methods of Cryopreservation</i>	634
Sperm Cryopreservation	635
Oocyte and Ovary Cryopreservation	636
Storage and Records	637
Rederivation of Mouse Strains	637
Transport of Mouse Embryos and Germ Plasm	640

#### PROTOCOLS

1 Embryo Cryopreservation by Slow Freezing	646
2 Embryo Cryopreservation by Rapid Cooling	652
3 Embryo Cryopreservation by High-Osmolality Vitrification	658
• Alternative Protocol: High-Osmolality Vitrification of Embryos in Insemination Straws	662
4 Sperm Cryopreservation Using Cryoprotectant Containing Monothioglycerol (MTG)	665
5 Sperm Cryopreservation Using Cryoprotectant Containing L-Glutamine	671
6 Ovary Cryopreservation	676
7 Shipment of Live Preimplantation-Stage Embryos	679

### CHAPTER 17

<b>Techniques for Visualizing Gene Products, Cells, Tissues, and Organ Systems</b>	<b>683</b>
--	------------

---

<b>INTRODUCTION</b>	<b>684</b>
Visualizing Gene Products	684
<i>Isolating Total RNA from Mouse Embryos or Fetal Tissues</i>	684

<i>General Techniques for Immunohistochemistry and In Situ Hybridization of Mouse Embryo Sections</i>	684
<i>Immunohistochemistry of Embryo Sections</i>	686
<i>Immunohistochemistry of Whole-Mount Embryos</i>	686
<i>Fluorescent Analysis of Whole-Mount Embryos and Fetal Organs</i>	686
<i>In Situ Hybridization of Embryo and Tissue Sections with RNA Probes</i>	686
<i>In Situ Hybridization of Whole-Mount Embryos with RNA Probes</i>	687
<i>Staining for <math>\beta</math>-Galactosidase Activity</i>	691
<i>Staining for Alkaline Phosphatase Activity</i>	691
Visualizing Cells	692
<i>Visualizing Fluorescent Proteins</i>	692
Visualizing Tissues and Organ Systems	693
<i>Visualizing the Mouse Skeleton Using Histochemical Stains</i>	693
<i>Visualizing the Fetal Vasculature by India Ink Injection</i>	694
<i>Visualizing the Fetal Great Blood Vessels by Plastic Casting</i>	694
<b>PROTOCOLS</b>	
1 Isolating Total RNA from Mouse Embryos or Fetal Tissues	695
2 Preparing Tissue Fixation Solutions	698
• Alternative Protocol: Bouin's Fixative	699
3 Handling Blastocysts for Fixation	700
4 Embedding Samples in Wax	701
• Additional Protocol: Embedding Small Embryos	703
5 Preparing Glass Slides and Coverslips for In Situ Hybridization	704
6 Cutting Wax Sections Using a Microtome	706
• Alternative Protocol: Transferring Sections onto Water Droplets	708
7 Cutting Thick Sections Using a Vibratome	709
• Alternative Protocol: Modifications for Live Specimens	712
8 Dewaxing and Rehydrating Sections before In Situ Hybridization or Staining	714
9 Immunohistochemistry of Embryo Sections	715
10 Immunohistochemistry of Whole-Mount Embryos	719
11 DAPI Staining of Whole-Mount Embryos or Fetal Organs	722
12 Immunofluorescent Staining of Whole-Mount Embryos	724
13 Mounting Embryos for Microscopic Observation and Imaging	726
14 In Situ Hybridization of Embryo and Tissue Sections with Radiolabeled RNA Probes	728
• Additional Protocol: Autoradiography	733
15 In Situ Hybridization of Whole-Mount Embryos with Nonradiolabeled RNA Probes	735
16 Imaging Embryos after Whole-Mount In Situ Hybridization	741
17 Sectioning Embryos after Whole-Mount In Situ Hybridization	742
• Alternative Protocol: Wax Sections	744
18 Staining Whole Embryos and Sections for $\beta$ -Galactosidase (LacZ) Activity	745
• Alternative Protocol: Staining Frozen Sections	748
19 Staining for Alkaline Phosphatase Activity	751
20 Visualization of Fluorescent Protein Expression in Whole-Mount Postimplantation-Stage Embryos	753

21	Observation of Fluorescent Proteins in Living Cells	755
22	Intracellular Observation of Fluorescent Proteins in Fixed Cells	756
23	Fixation and Paraffin Embedding for GFP Visualization	757
24	Alcian Blue Staining of the Fetal Cartilaginous Skeleton	759
25	Alcian Blue/Alizarin Red Staining of Cartilage and Bone	760
26	Alizarin Red Staining of Postnatal Bone	762
27	Visualizing the Fetal Vasculature by India Ink Injection	763
28	Visualizing the Fetal Great Blood Vessels by Plastic Casting	765

## CHAPTER 18

### Setting Up a Micromanipulation Laboratory 767

---

INTRODUCTION	767
The Micromanipulation Laboratory and Its Environment	767
<i>General Considerations</i>	767
<i>Inbred, Outbred, Hybrid, and Genetically Modified Mice</i>	768
General Equipment	769
<i>Refrigerators and Freezers</i>	769
<i>Incubators</i>	769
<i>Laminar Flow Hoods and Biological Safety Cabinets</i>	770
Microscopes	771
<i>Stereomicroscopes</i>	771
<i>Inverted Microscopes</i>	772
<i>Temperature Controllers for Heating and Cooling</i>	
<i>Microscope Stages</i>	772
<i>Vibration-Free Tables</i>	773
Micromanipulation	773
<i>Micromanipulators</i>	773
<i>Microinjection Systems</i>	773
<i>Piezo Impact and Laser Systems</i>	774
Production of Microtools from Glass Capillaries	774
<i>Pullers</i>	775
<i>Microforges, Bevelers, and Grinders</i>	775
Electrofusion	775
Surgical Instruments	776
Cryopreservation	777

## APPENDIX 1

### Buffers and Solutions 779

---

Acidic Tyrode's Solution for Removing Zona Pellucidae from Preimplantation Embryos	780
Alkaline Phosphatase Buffers (NTMT)	780
Alkaline Phosphatase Staining Solution	780
Chicago Sky Blue 6B, Also Called Pontamine Sky Blue	781
Hyaluronidase	781

Mannitol (0.3 M)	781
Methylene Blue Solution	781
Pancreatin/Trypsin Solution for Separating Germ and Tissue Layers	781
PBSMT for Immunohistochemistry of Whole-Mount Embryos	782
PBT	782
Phenol:Chloroform Solution for Isolating Total RNA from Mouse Embryos or Fetal Tissues	782
Phosphate-Buffered Saline (PBS)	782
Pronase Solution	783
Saline/EDTA Buffer plus Glucose for Isolation of Germ Cells and Tissue Culture	783
SSC (20×)	783
SSPE (20×)	783
TAE (Tris-Acetate/EDTA) Buffer (1× Solution)	783
TE (Tris-EDTA) Buffer	783
Trypsin (0.25%) in Tris-Saline for Tissue Culture	784
Trypsin-EDTA Solution	784
Tyrode Ringer's Saline (pH 7.6–7.7), Ca <sup>2+</sup> /Mg <sup>2+</sup> -Free	784
<b>APPENDIX 2</b>	
<b>WWW Resources</b>	<b>785</b>
<hr/>	
<b>APPENDIX 3</b>	
<b>General Safety and Hazardous Material Information</b>	<b>789</b>
<hr/>	
<b>Index</b>	<b>795</b>

## Preface

IT HAS BEEN MORE THAN 10 YEARS SINCE THE PUBLICATION of the third edition of *Manipulating the Mouse Embryo*. Ten years ago, many of the basic methods of mouse embryology and genetic manipulation were routine, including zygote injection, embryonic stem (ES) cell culture, homologous recombination, and blastocyst injection to generate chimeras. Indeed many of these methodologies had been relegated to institutional core facilities. Many of the “important” genes had been knocked out and their phenotypes characterized. The mouse genome (C57BL/6 J) had been sequenced, assembled, and annotated, and scientists could order cDNA and genomic clones without having to screen libraries. Fluorescent proteins of many different colors were shining in the cells of transgenic mouse embryos and tissues and dancing in time-lapse movies. Much was known about the molecular embryology of the mouse. At the time, it seemed there would only be small incremental advances in new embryological and genetic methods to manipulate the mouse. Since then, new stem cell lines were derived, including epiblast stem and XEN cells, creating new cellular resources to understand pluripotency and differentiation. Large-scale ethylnitrosourea (ENU) mutagenesis programs created many new alleles in the mouse identified by phenotype. This was succeeded by the International Knockout Mouse Consortium (IKMC), which aims to mutate all protein-coding genes in the mouse genome by gene trapping or gene targeting. Scientists can now order ES cells with mutations for their favorite gene and have their core facilities generate mutant mice. Many of these mutant ES cell lines are now being turned into mice for standardized phenotyping by the International Mouse Phenotyping Consortium (IMPC).

Certainly the advances in DNA sequencing technologies have facilitated many experiments and opened up new opportunities for genome analysis. However, the biggest breakthrough during the previous decade was the derivation of induced pluripotent stem (iPS) cells by the expression of a discrete set of transcription factors in somatic cells. Here again, the mouse led the way because of the foundation of knowledge of ES cells—their culture, genetic manipulation, and assays of pluripotency. The ability to reprogram somatic cells to a state of pluripotency has made a tremendous impact on our concepts of stem cells and differentiation, including their relevance to cellular therapies for a variety of human diseases. As we complete this fourth edition, new targeted gene manipulation technologies have been reported that may supplant ES cells as vehicles to generate mutant mice because RNA/DNA constructs can be injected into zygotes to achieve gene targeting, bypassing the need to generate chimeras. In 1989, Brinster et al. reported achieving homologous recombination by zygote injection of a gene-targeting construct; however, the efficiency was prohibitively low. The new TALEN and CRISPR/Cas methods exploit the basic biology of plants and bacteria for very efficient gene targeting in mice by expression in zygotes achieved by microinjection. Once again, there seem to be no limits for manipulating the mouse embryo to address fundamental biological questions and provide novel biomedical insights for human biology and disease.

As the field of mouse developmental genetics progresses, the “Mouse Manual” continues to evolve. The fourth edition is built upon the foundation of the previous editions—notably the efforts of the original editors, Brigid Hogan, Frank Costantini, Elizabeth Lacy, and subsequently Rosa Beddington. This new edition has been updated, incorporating many new methods since the publication of the third edition in 2003. New chapters and protocols have been added, including the most up-to-date assisted reproduction techniques for sperm and embryo cryopreservation; generation of induced pluripotent stem cells; isolation, generation, and transplantation of spermatogonial

stem cell lines; in utero electroporation of gene constructs into postimplantation embryos; vibratome sectioning of live and fixed tissues for imaging thick tissue sections; and whole-mount fluorescent staining methods for three-dimensional visualization.

We are very grateful to the many people who generously helped us to produce the present edition. They provided new and updated protocols, figures, and images and served as an incredibly helpful source of expert information. We thank (in alphabetical order): Vernadeth Alarcon, Wojtek Auerbach, Ralph Brinster, Gabrielle Brons, Jorge Cabezas, Abel Carcagno, Tracy Carroll, Andrew Corso, Thomas DeChiara, Michael Dewey, Amanda Duselis, Gabriel Gonzalez, Shaun Goodyear, Mubeen Goolam, Anna-Katerina Hadjantonakis, Cheng-Chiu Huang, Scott Hutton, Kimberly Inman, Angelo Iulianella, Kenneth Jones, Min Kang, Yusuke Marikawa, Maria Mileikovskaia, Keiji Mochida, Claudio Monetti, Lluís Montoliu, Naomi Nakagata, Jennifer Nichols, Mark Nolte, Jon Oatley, Jan Parker-Thornburg, Shirley Pease, Jaime Rivera-Pérez, Larysa Pevny, Peter Rugg-Gunn, Nestor Saiz, Lisa Sandell, Thomas Saunders, Jillian Shaw, Allison Stewart, Robert Taft, Patrick Tam, Paul Tesar, Peter Tonge, Paul Trainor, Balázs Varga, Monkia Veres, Paul Vrana, Knut Woltjen, Yojiro Yamanaka, and Magda Zernicka-Goetz.

We also thank the many individuals at Cold Spring Harbor Laboratory Press who have helped to make this new edition a reality, including John Inglis, Jan Argentine, Denise Weiss, Kaaren Janssen, Michael Zierler, Andrea R. Russo, Inez Sialiano, and Kathleen Bubbeo. We especially thank our editor Judy Cuddihy for her enthusiasm, endless patience, friendly encouragement, and creative insights. Finally, as with the third edition, it is our hope that the newest edition of this manual will help train the future leaders and innovators of the mouse developmental genetics and molecular embryology fields.

RICHARD BEHRINGER  
MARINA GERTSENSTEIN  
KRISTINA VINTERSTEN NAGY  
ANDRAS NAGY

## REFERENCE

Brinster RL, Braun RE, Lo D, Avarbock MR, Oram F, Palmiter RD. 1989. Targeted correction of a major histocompatibility class II E  $\alpha$  gene by DNA microinjected into mouse eggs. *Proc Natl Acad Sci* 86: 7087–7091.