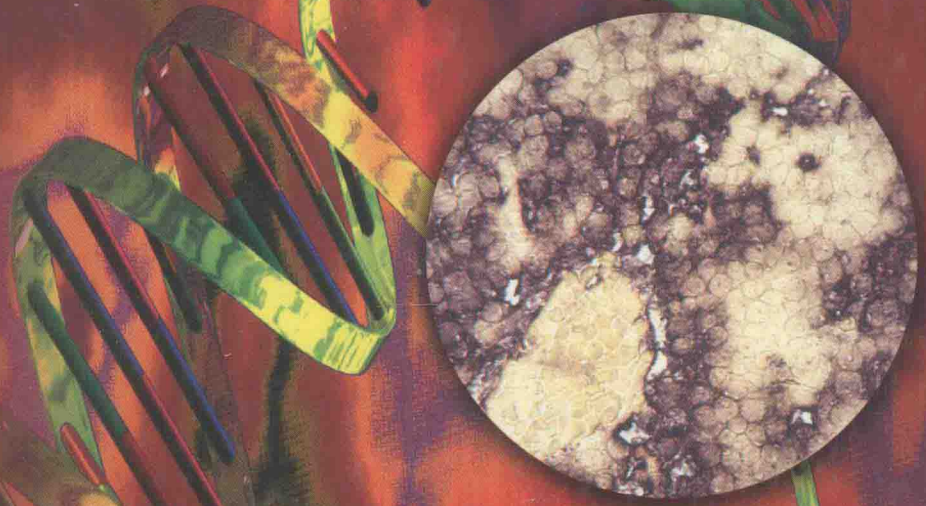
METHODS IN VISUALIZATION SERIES

Gérard Morel
Mireille Raccurt



PCR/RT-PCR *in situ* LIGHT and ELECTRON MICROSCOPY

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Methods in Visualization

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PCR/RT - PCR In Situ Light and Electron Microscopy
Gérard Morel and Mireille Raccurt

SERIES PREFACE

Visualizing molecules inside organisms, tissues, or cells continues to be an exciting challenge for cell biologists. With new discoveries in physics, chemistry, immunology, pharmacology, molecular biology, analytical methods, etc., limits and possibilities are expanded, not only for older visualizing methods (photonic and electronic microscopy), but also for more recent methods (confocal and scanning tunneling microscopy). These visualization techniques have gained so much in specificity and sensitivity that many researchers are considering expansion from in-tube to *in situ* experiments. The application potentials are expanding not only in pathology applications but also in more restricted applications such as tri-dimensional structural analysis or functional genomics.

This series addresses the need for information in this field by presenting theoretical and technical information on a wide variety of related subjects: *in situ* techniques, visualization of structures, localization and interaction of molecules, and functional dynamism *in vitro* or *in vivo*.

The tasks involved in developing these methods often deter researchers and students from using them. To overcome this, the techniques are presented with supporting materials such as governing principles, sample preparation, data analysis, and carefully selected protocols. Additionally, at every step we insert guidelines, comments, and pointers on ways to increase sensitivity and specificity, as well as to reduce background noise. Consistent throughout this series is an original two-column presentation with conceptual schematics, synthesizing tables, and useful comments that help the user to quickly locate protocols and identify limits of specific protocols within the parameter being investigated.

The titles in this series are written by experts who provide to both newcomers and seasoned researchers a theoretical and practical approach to cellular biology and empower them with tools to develop or optimize protocols and to visualize their results. The series is useful to the experienced histologist as well as to the student confronting identification or analytical expression problems. It provides technical clues that could only be available through long-time research experience.

Gérard Morel, Ph.D.
Series Editor

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She has worked in a number of different laboratories, forming the basis for her knowledge and expertise in the fields of cellular and molecular biology. She has published more than 20 papers in the field of protein and nucleic acid detection from normal and pathological tissues. She has taught histology at the Lyon School of Medicine from 1976 to 1989. Moreover, she has regularly organized and taught the theory and practical courses of immunocytology, *in situ* hybridization, and PCR in training courses at light and electron microscopic levels in different European countries. She has organized six meetings or workshops.

Her expertise and current research interests include the localization and regulation of hormone and receptor gene expressions, correlated with signaling molecules in normal and tumoral states. Most recently, she has become interested in extrapituitary expression of growth hormone in fetal and adult tissues and its regulation in mammary gland.

GENERAL INTRODUCTION

Over the past decade, no new procedure in molecular biology has achieved such an exceptional degree of biotechnical acceptance as the polymerase chain reaction (PCR). This *in vitro* enzymatic amplification of particular genetic sequences is now a basic tool in experimental work, and has become a highly standardized process. More recently, improved enzymes, automated routines, and the possibility of carrying out the reaction on DNA chips have opened entirely new applications for the method. In addition to its impact in laboratory research and medical diagnostics, PCR holds promise for significant advances in quality control for agricultural products, and has become the most powerful weapon in the arsenal of forensic science.

Powerful as PCR may be, however, it still involves the destruction of cells and tissue, and morphologists whose interests are associated with intact structures have remained unsatisfied. What they needed was a way to adapt PCR methods to undamaged cells or tissue sections to detect small numbers of copies of DNA or RNA *in situ*, while still preserving morphology. And in 1990, Haase et al. actually succeeded in amplifying lentiviral DNA in infected cells and detecting the amplification product by *in situ* hybridization. And so it was that *in situ* PCR came into being. This demonstrates that a "mere" technical innovation is sometimes all that is needed to give a fresh impetus to research.

One of the first successes achieved by the new technique was to confirm the relationship between HIV and AIDS. Initially, it had been found that only 1% of CD4 cells in the blood of asymptomatic seropositive subjects was infected by HIV, and it was difficult to see how such a small number of infectious particles could be responsible for such a serious condition. In 1992, however, several teams, including those of G. Nuovo and O. Bagasra, then that of Kominoth, began developing *in situ* PCR, which combined the amplifying power of the PCR with the ability of *in situ* hybridization to localize target sequences. Using this technique, it was demonstrated that in reality 30 to 40% of circulating CD4 cells were infected by HIV, thus making it clear that AIDS was essentially a viral disease, and underscoring the need for direct therapy against viral replication. *In situ* PCR and RT-PCR provided the first means of detecting minute quantities of DNA or RNA in nondisrupted cells and tissue, with the possibility of subsequently detecting the amplified product at the site of origin. But if *in situ* PCR is to be successful, it requires considerable optimization—not just of the PCR cycling but also of the fixation step and tissue processing, the PCR tools and reagents used for the amplification, and the detection procedure. The objective of this book is to give scientists (morphologists, pathologists, or molecular biologists) the information they need about the basic approach to histological analysis, biochemistry of PCR, and to provide molecular biologists with a practical approach to histological analysis.

Chapter 1 is a general presentation of *in situ* PCR/RT-PCR and the variants. The parameters for fixation, tissue processing, and enzyme digestion are set out in Chapters 2 and 3. Reverse transcription (RT) and amplification techniques are described, with theoretical considerations and practical step-by-step guidance, in Chapters 4 and 5. Detection procedures are discussed in Chapters 6 and 7. And, given that *in situ* PCR can be combined with electron microscopy, the basic principles of these methods are presented in detail in Chapter 8. Numerous controls are, of course, needed to check for diffusion and potential causes of background, negative, or nonspecific signals. Finally, the causes of the false positives and false negatives associated with the different techniques are outlined in Chapter 9, with recommendations on how to avoid them. Chapter 10 provides guidelines that should help experimenters work out their own

in situ PCR/RT-PCR protocol, although in the last analysis it is the empirical conditions themselves that dictate the precise details of any given protocol. Some examples of results are illustrated in Chapter 11. Methods for preparing the different reagents are given in the Appendices.

Finally, it is the hope of the authors, who are themselves actively involved in the development of *in situ* PCR/RT-PCR, that the present work will provide practical solutions to some of the problems encountered by experimenters in the implementation of these complex, still-evolving techniques.

ABBREVIATIONS

AEC	⇨ 3-amino-9-ethylcarbazole
ATP	⇨ adenosine triphosphate
BCIP	⇨ 5-bromo-4-chloro-3-indolyl phosphate
bp	⇨ base pair
cDNA	⇨ complementary deoxyribonucleic acid
CTP	⇨ cytosine triphosphate
DNA	⇨ deoxyribonucleic acid
DAB	⇨ 3′-diaminobenzidine tetrahydrochloride
dATP	⇨ deoxy-adenosine triphosphate
dCTP	⇨ deoxy-cytosine triphosphate
DEPC	⇨ diethyl-pyrocarbonate
dGDP	⇨ deoxyguanosine-5′-diphosphate
dGMP	⇨ deoxyguanosine-5′-monophosphate
dGTP	⇨ deoxyguanosine 5′-triphosphate
DNase	⇨ deoxyribonuclease
dNTP	⇨ deoxynucleoside triphosphate
DTT	⇨ dithiotreitol
dUTP	⇨ deoxyuridine-5′-triphosphate
EDTA	⇨ ethylene diamine tetraacetic acid
Fab	⇨ immunoglobulin fragment obtained by proteolysis (papaine)
$(Fab')_2$	⇨ immunoglobulin fragment obtained by proteolysis (pepsine)
Fc	⇨ immunoglobulin fragment obtained by proteolysis (papaine)
FITC	⇨ fluorescein isothiocyanate
GTP	⇨ guanosine triphosphate
Ig	⇨ immunoglobulin
IgG	⇨ immunoglobulin G
kb	⇨ kilobase
kDa	⇨ kilodalton
MM	⇨ molecular mass
mRNA	⇨ messenger ribonucleic acid
Mw	⇨ molecular weight
NBT	⇨ nitroblue tetrazolium
NTP	⇨ nucleoside triphosphate
Oligo (dT)	⇨ oligo-deoxythymidine
PBS	⇨ phosphate buffer saline
PCR	⇨ polymerase chain reaction
PF	⇨ paraformaldehyde
RNA	⇨ ribonucleic acid
RNase	⇨ ribonuclease
rRNA	⇨ ribosomic ribonucleic acid

rt	⇨ room temperature
RT	⇨ reverse transcription
RT-PCR	⇨ reverse transcription–polymerase chain reaction
SSC	⇨ standard saline citrate
T_H	⇨ hybridization temperature
Tm	⇨ melting temperature
tRNA	⇨ transfer ribonucleic acid
Tw	⇨ washing temperature
U	⇨ unit (enzymatic activity)
UDP	⇨ uridine-5′-diphosphate
UTP	⇨ uridine-5′-triphosphate
v/v	⇨ volume/volume
w/v	⇨ weight/volume

CONTENTS

Chapter 1

General Principles

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