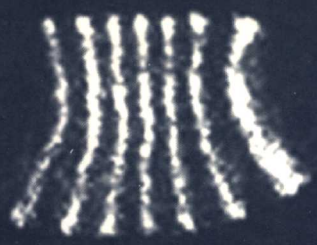

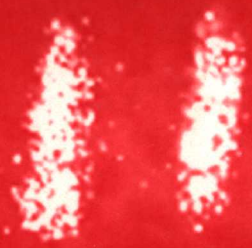

TISSUE IN SITU HYBRIDIZATION

Methods in Animal Development

Trevor Jowett

An EMBO Practical Course

Trevor Jowett

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Methods in Animal Development

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List of Protocols

Foreword

The European Molecular Biology Organization (EMBO) practical courses play an important role in the training of scientists in diverse techniques. The reality is that very many cannot participate in these courses because of practical limitations on the number that can be correctly trained in a laboratory. This series of EMBO practical books is designed to extend the number of those who can benefit from the work which goes into preparing an EMBO practical course to a much wider audience.

The first book in this series focussed on DNA sequencing. This current book on in situ hybridization covers an equally important and fast growing area of activity. As more genes with potentially interesting functions are identified, it becomes very important to see exactly which tissue expresses the gene. For larger tissues this can be performed in a straightforward manner using Northern blots. However, for many tissues and different cell types in situ hybridization is the only method which can give information on gene transcription at the cellular level. The methods associated with in situ hybridization have been growing in popularity over the last decade and now have reached the stage where very many laboratories, both large and small, need to be able to carry out experiments of this type.

This book, which has been compiled by Trevor Jowett and his colleagues, contains a number of important assets which go beyond a simple combination of useful recipes. As is indicated in this book, different methodological approaches have been developed in various laboratories. The practical course brought together people from different groups who pooled their knowledge and experience, thereby allowing comparisons of different methods to be made. It is a characteristic of EMBO practical courses that experts come from different laboratories, and one of the fruits of this is clearly to be found in this book. The other aspect which is particularly notable in this book is the fact that the methods are described for a variety of different species. It is therefore possible to get very practical guidance from this book on how to adapt some of the generally used methods to the organism of your choice.

I believe that this book is a very appropriate addition to the series on EMBO practical courses and will be of benefit to many.

Professor Frank Gannon
Executive Secretary of EMBO
Heidelberg, July 1996

Preface

This book is based on an EMBO Practical Course for those wishing to apply the method of in situ hybridization to their research in developmental biology. The course was the last of three such courses organized by T. Jowett and held in the Medical School at the University of Newcastle upon Tyne since 1991. Over the past decade the technique of in situ hybridization has been an essential tool for the developmental biologist. During this time the technique has significantly changed. Originally it was only performed on tissue sections with isotopically labeled probes, and signals were visualized by autoradiography. The most popular approach now is to use non-isotopically labeled probes which allows in situ hybridization to whole-mount embryos as well as tissue sections. The hybridized probes are visualized using antibodies. Introduction of immunocytochemical techniques has led to the development of double labeling to allow the localization of multiple transcripts. This book describes the latest techniques for performing two-color in situ hybridization with different chromogenic enzyme substrates and with different fluorochromes.

The EMBO Practical Courses have allowed a direct comparison of different in situ protocols developed independently by different researchers. This has led, in some cases, to improvement in the methods and to the identification of critical parameters in the procedure. This book therefore represents the combined experience of several research laboratories all actively using the technique.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
AP	alkaline phosphatase
ATP	adenosine 5′-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIO	biotin
BSA	bovine serum albumin
CHAPS	3-(3-cholamidopropyl)-dimethylammonio-1-propane sulphonate
CTP	cytidine 5′-triphosphate
DAB	diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DIG-11-UTP	digoxigenin-11-uridine 5′-triphosphate
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) also N,N,N′,N′-tetraacetic acid
ELF™	Enzyme Labeled Fluorescence
Fab fragment	fragment of immunoglobulin prepared by papain treatment
FITC	fluorescein isothiocyanate
FLU	fluorescein
FITC-12-UTP	fluorescein-12-uridine 5′-triphosphate
GTP	guanosine 5′-triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]
HRP	horseradish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
INT	2-(4-iodophenyl)-5-(4-nitrophenyl)-3-phenyltetrazolium chloride
MABT	0.1 M maleic acid, 150 mM NaCl, 0.1% Tween-20
MABTB	MABT +2% Blocking powder
MEMFA	0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde
MEMPFA	0.1 M MOPS, pH 7.4, 2 M EGTA, 1 mM $MgSO_4$ and 3.7% paraformaldehyde
MOPS	3-[N-morpholino]propanesulphonic acid

NBT	nitroblue tetrazolium
NTMT	0.1 M NaCl, 0.1 M Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20
PAP complex	peroxidase antiperoxidase complex
PBS	phosphate buffered saline
PBT	PBS + 0.1% Tween-20
PEM	0.1 M Pipes pH 7.0, 2 mM EGTA pH 8.0, 1 mM $MgSO_4$, adjusted to pH 6.95
PIPES	piperazine-N,N′-bis[2-ethanesulphonic acid] also 1,4-piperazinediethanesulphonic acid
POD	horseradish peroxidase
PVA	polyvinyl alcohol
20× SSC	3 M NaCl, 0.3 M sodium citrate
TBST	140 mM NaCl, 27 mM KCl, 250 mM Tris-HCl pH 7.5, 0.1% Tween-20
TESPA	3-aminopropyltriethoxysilane
TKM	0.1 M Tris-HCl pH 8.2, 150 mM KCl, 10 mM $MgCl_2$
TMB	3,3′,5,5′-tetramethylbenzidine
TN	0.1 M Tris-HCl pH 7.4, 150 mM NaCl
TNBT	tetranitroblue tetrazolium
TNBTr	TN buffer + 2 mg/ml BSA, 0.1% Triton X-100
TNT	0.1 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.1% Tween-20
TNTr	50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.18 Triton X-100
TRITC	tetramethylrhodamine isothiocyanate
UTP	uridine 5′-triphosphate

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Chapter 1
Principle of the Technique

1.1 Generalized Procedure for Nonisotopic In situ Hybridization

Techniques for the localization of mRNAs in situ were first developed in the late 1970s and are now widely used in many areas of biological research. They provide an extremely powerful tool for analyzing patterns of gene expression, both temporally and spatially. Although other very sensitive methods are available for assaying transcript levels in different tissues and at different developmental stages or under different physiological conditions, in situ hybridization is unique in its ability to reveal patterns of gene expression with cellular resolution.

The principles of the technique are very straightforward and over recent years protocols have evolved that make its practice equally simple. The objective is to fix the tissue so that all the mRNA being transcribed at the time is retained within the cells. The mRNA transcribed by a specific gene is then detected in situ by hybridization with a probe labeled so that it can be detected either by autoradiography, chromogenic stains, or fluorescence. All that is required is a supply of the appropriate stages of the organism in which the gene of interest is expressed and a cloned fragment of this gene.

The procedure of in situ hybridization can be divided into the following component steps.

1. Fixation of the tissue and storage of tissue.
2. Synthesis of labeled DNA or antisense RNA probe(s).
3. Pretreatment to permeabilize the tissue and block nonspecific binding of probe.
4. Hybridization of the probe.
5. Washing to remove unbound probe.
6. Incubation in blocking solution to prevent nonspecific binding of the antibodies.
7. Incubation in antibody against the probe-hapten.
8. Washing to remove unbound antibody.
9. Visualization of the bound antibody either with chromogenic stain or by epifluorescent microscopy.

1.1.1 FIXATION

Tissue samples are fixed using a cross-linking fixative, the most commonly used being paraformaldehyde, formaldehyde, and glutaraldehyde. After fixation the tissues may be dehydrated and stored at −20°C for prolonged periods before in situ hybridization.

Depending upon its size, tissue may be hybridized either intact or after sectioning; the former has the advantage of simplicity and provides a good overall impression of the pattern of gene expression for instance in an intact embryo. However, it is often desirable to prepare sections of the material, especially for the analysis of particularly large specimens, where probe penetration may become a problem, or for the analysis of internal organs and structures with complex morphology. Although tissues can be frozen and sectioned using a cryostat, it is preferable to use paraffin wax as an embedding medium. Paraffin-embedded material is easier to handle and retains superior morphology in comparison with frozen sections.

1.1.2 PROBES

Transcripts are detected using either DNA or RNA probes. DNA probes can be prepared from cloned DNA in a number of ways, such as nick translation, random priming, or polymerase chain reaction (PCR) amplification. RNA probes (riboprobes) may be prepared by in vitro transcription of the cloned DNA using the bacteriophage SP6, T3 or T7 polymerases; promoters for these polymerases are incorporated into a number of commonly used and highly versatile cloning vectors such as Bluescript™ (Stratagene). RNA probes prove to be the most sensitive and reliable and for this reason these are the only ones used in the methods described in this book.

In the earliest protocols (Akam 1983; Hafen *et al.* 1983; Ingham *et al.* 1985), DNA or RNA probes were labeled isotopically, usually employing ^{3}H- or ^{35}S-labeled nucleotides, and detected following hybridization by autoradiography. Apart from the safety considerations always associated with radioactive materials, the autoradiographic method of signal detection presents a number of other drawbacks. Preparation of materials for autoradiography is an expensive and time-consuming procedure; moreover, depending upon the abundance of the transcript to be detected, exposure times can be relatively long and must be determined empirically. The higher energy emissions of ^{35}S compared to ^{3}H significantly reduces exposure times but at the same time the longer path length decreases resolution; even with ^{3}H, the signal only approaches single cell resolution. The use of radioactive probes required that the hybridizations were performed on tissue sections rather than whole-mounts and also only allowed probing for single transcripts on individual sections. Studying the expression pattern of two transcripts required the separate probing of adjacent sections. Despite these limitations, radioactive in situ hybridization was an essential tool in determining the genetic hierarchy of the genes controlling development of

the *Drosophila* embryo (Ingham 1988) and in early studies of gene expression in mammalian development.

The limitations of radioactive in situ hybridization are obviated by using non-isotopically labeled probes such as digoxigenin or fluorescein, and since the publication in 1989 of a reliable method for hybridizing whole *Drosophila* embryos using digoxigenin-labeled probes (Tautz and Pfeifle 1989), such labels have largely replaced their radioactive counterparts. The protocols described in the following chapters have been developed specifically for the use of probes labeled with either digoxigenin or fluorescein.

The development of non-isotopic methods has allowed significant advances to be made in the technique of in situ hybridization. It is now possible to perform hybridizations on whole tissues or organisms and detect transcripts present at lower copy numbers. The improvements have mainly been achieved through advances in probe design and synthesis. Antisense RNA probes are more sensitive than single-stranded DNA probes because they form double-stranded RNA with the target transcripts which are considerably more stable than DNA/RNA hybrids. This has the advantage that more stringent conditions can be used for the hybridization which reduces problems with nonspecific background signals. The antisense RNA probes contain non-isotopically labeled ribonucleotides and are more stable and cheaper to produce. The signals are subsequently detected with immunological reagents which recognize the haptens linked to the ribonucleotides incorporated into the probes. The antibodies used for detection are conjugated either with enzymes such as alkaline phosphatase and horseradish peroxidase or to fluorochromes. Antibodies conjugated with enzymes are detected by chromogenic or fluorescent substrates.

1.1.3 PRETREATMENTS AND HYBRIDIZATION

Prior to hybridization, the tissue must be rehydrated and permeabilized to allow easy penetration of the probe. This is usually performed by incubation with proteinase K which punctures the cell membranes. In small, delicate specimens such as whole mounts of young zebrafish embryos this may not be necessary and indeed may be detrimental to the morphology. It is essential, however, for larger vertebrate embryos such as mouse, chick and *Xenopus*. It is also important for paraffin-embedded sections as the wax appears to mask the transcripts reducing the efficiency of hybridization. Treatment with proteinase K is followed by a second fixation step to stabilize the material and prevent overdigestion by the protease.

The tissue is then prehybridized in the hybridization solution using the same conditions as for hybridization but without the probe. This step serves to block nonspecific binding sites for the probe. It may be found that in some cases this prehybridization may be omitted but it is always advisable to include it in initial experiments. After prehybridization the tissue is incubated in hybridization solution containing probe overnight.

After hybridization the unbound probe is removed by several washes in a series of low salt concentration solutions. This strips off probe which is weakly bound to partially complementary transcripts.

1.1.4 IMMUNOLOCALIZATION OF TRANSCRIPTS

The bound probes are recognized immunologically with antibody raised against the hapten used to label the probe. In the same way as nonspecific binding sites must be blocked prior to hybridization, the tissue must be preincubated in a blocking solution before it is challenged with the antibody. In addition it may be desirable to preabsorb the antibody prior to its use. The material for preabsorption may be an acetone powder made from the same species as is being hybridized. Incubation with the antibody may be relatively short (1–2 h) or overnight. Following incubation the material must be thoroughly washed to remove unbound antibody.

1.1.5 VISUALIZATION OF THE SIGNAL

The hybridization signal may be visualized with chromogenic agents or by fluorochromes. For the former, the antibody used is conjugated with either alkaline phosphatase or horseradish peroxidase and the material is incubated in a solution of an enzyme substrate which is converted from a colorless, water-soluble compound to a colored precipitate. Several different enzyme substrates are available which are of varying sensitivity and produce differently colored precipitates.

If fluorescent signals are required, the antibody is incubated with an enzyme substrate which is converted to a fluorescent precipitate or the antibody used is conjugated with a fluorochrome instead of an enzyme. The latter method is less sensitive because there is no enzyme-mediated amplification of the signal. Alternatively, a degree of amplification can be achieved by using a secondary fluorochrome-conjugated antibody.

1.2 Detection of Multiple Transcripts

Once the expression pattern of a gene is established, it is often necessary to relate it to that of other genes expressed at the same period of development. For example, transcripts from different genes may be expressed in complementary or overlapping domains. Relating the temporal and spatial patterns of expression of different genes is essential if one is studying genes involved in the same developmental pathway or genes which are involved in intercellular signaling. Also, understanding how particular mutations upset normal development may require studying the effect of the mutation on the expression of other genes. Thus, it is desirable to perform in situ hybridization with multiple probes on the same tissue.