Electron microscopy in molecular biology

a practical approach

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Preface

The integration of microscopic and chemical analyses has provided the basis of our present understanding of the biology of cells. For the molecular biologist, the visual dimension is provided generally by high resolution electron microscopy. The electron microscope as a tool for analysing molecular structure, interactions and processes is one that can be used increasingly with confidence, particularly in conjunction with biochemical and biophysical studies. Detailed, yet simply-applied, methods devised and elaborated upon by many specialists over the past 25 years have made ultrastructural analysis accessible to a widening group of investigators, to the extent that visual representation is considered now to be an essential component in virtually all areas of study.

In this book we have attempted to gather together those methods which we consider to be most generally applicable to current studies in molecular biology. Each chapter contains a set of related practical protocols provided by experts with first-hand knowledge of the techniques that they describe. The limited size of the book has forced us to be selective of topics and to make substantial revision of some chapters to avoid undue repetition. We acknowledge the understanding of the authors in our dissection of their original manuscripts and we hope that the resulting cross-references between chapters prove to be effective for the reader.

The individual chapters are grouped according to similarities in the specimen material and methodology described. A progression can be followed from the preparation and analysis of relatively well-defined structures such as nucleic acids (Chapters 1 and 2) and proteins (Chapters 6 and 7), to simple complexes formed between them (Chapter 3), to, at a higher level of complexity, chromatin (Chapter 4) and its forms active in transcription (Chapter 5). Finally techniques are described which make use of probes designed to identify individual molecules in complex situations. Specifically, these are: the production and application of immunoreagents tagged with electron-dense markers (Chapter 8); the detection of radiolabelled components by high resolution autoradiography (Chapter 9); and the localization of specific nucleic acid sequences within chromosomes and chromatin by *in situ* hybridization (Chapter 10). While many of the basic methods in specimen and grid preparation are described in the earlier chapters, particularly in Chapter 1, this book is not a manual on the use of the electron microscope. (The reader is referred to one of the many existing texts for this instruction.) Rather this book is intended as an introduction to special techniques for the preparation of biological macromolecules for ultrastructural analysis.

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Abbreviations

AAF	2-acetylaminofluorenyl
Anthrabis	(1,4-bis[3-(benzyldimethylamino)propylamine]9,10-anthraquinone dichloride
ARG	autoradiography
BAC	benzyldimethylalkylammonium chloride
bio-NTP	biotinylated nucleoside triphosphate
BPV	bovine papillomavirus
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DAB	3,3′-diaminobenzidine
DBP	DNA-binding proteins
DHSS	dinitrophenol–hapten sandwich staining
DMSO	dimethyl sulphoxide
DNFB	dinitrofluorobenzene
DNP	dinitrophenol
DNP-N-IE	3-[2,4-dinitrophenylamino]proprionimidate HCl
DRB	5,6-dichloro-1-β-ribofuranosyl benzimidazole
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
Eg	geometric error
EM	electron microscopy
Ep	photographic error
F	actin filaments
GEA	gold latensification - Elon-ascorbic acid
GLAD	gold-labelled antigen detection
HB	hybridization buffer
HD	half distance
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
hnRNA	heterogeneous nuclear RNA
HSV	herpes simplex virus
IgG	immunoglobulin G
IgM	immunoglobulin M
IF	intermediate-sized filaments
IR	inverted repeat
MAP	microtubule-associated protein
MBS	modified Barth's saline
NP-40	Nonidet P-40
NTS	non-transcribed spacer
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase–anti-peroxidase
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PEI	polyethylene imine
Pipes	piperazine-N-N′-bis(2-ethanesulphonic acid)
PLL	poly-L-lysine
PMSF	phenylmethylsulphonyl fluoride

PTA	phosphotungstic acid
PVS	polyvinyl sulphate
rDNA	DNA containing ribosomal RNA genes
RNP	ribonucleoprotein (complex of RNA and protein)
γ-s-ATP	γ-thioadenosine triphosphate
SDS	sodium dodecyl sulphate
ss	single-stranded
SSB	single-strand binding
SSC	standard sodium citrate buffer
SV40	simian virus 40
T_m	melting temperature
T_{max}	temperature for maximum rate
T_{ss}	strand separation temperature
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TE	0.01 M Na_2 EDTA, 0.1 M Tris-HCl pH 8.5
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethyl glycine
TU	transcription unit
UF	urea−formamide

Contents

CHAPTER 1

Preparation of nucleic acids for electron microscopy

LESLEY W.COGGINS

1. INTRODUCTION

Electron microscopy has proved to be an increasingly powerful tool in the study of nucleic acids. It provides qualitative and quantitative data on the size and structure of native and experimentally manipulated DNA and RNA molecules. Such molecules in solution are three-dimensional flexible random coils. To be displayed for visualization in the electron microscope (EM) they must first be converted to a two-dimensional array of unaggregated molecules. The most successful method for achieving this was introduced by Kleinschmidt and Zahn (1) about 25 years ago. A solution of nucleic acid and a basic protein is used to form a monolayer at a water–air interface. The resulting complex is adsorbed to a supporting film mounted on an EM grid. A disadvantage of this method is that any proteins naturally associated with the nucleic acid are obscured by the added protein. Several methods of overcoming this problem have been developed for investigating nucleic acid–protein interactions. The Kleinschmidt–Zahn method together with protein-free techniques are described in this chapter (see also Chapter 3 for applications of non-protein methods), whilst experimental manipulations which provide further information about nucleic acid molecules are discussed in Chapter 2.

2. PROTEIN MONOLAYER METHODS

2.1 **Principles**

The Kleinschmidt–Zahn technique (1,2) is a simple, rapid and reliable method of preparing nucleic acids for electron microscopy. The negatively charged DNA (or RNA) is mixed with a basic protein (usually cytochrome *c*) and spread via a ramp onto the surface of water or of a dilute salt solution. Alternatively, the surface film can be formed by diffusion (3). The nucleic acid molecules are held in a relaxed two-dimensional conformation in a surface-denatured film of protein, with polar groups tending to face the water and non-polar groups the air. The nucleic acid–protein complex is adsorbed to a support film on an EM grid, presumably by hydrophobic interaction with the non-polar groups. The adsorbed complex is usually treated with heavy metals to increase contrast before visualization with the EM. Modifications of this method allow single-stranded (ss) as well as double-stranded (ds) DNA to be prepared for electron microscopy. Various aspects of the technique and its applications have been reviewed (4–14). The methods described below give satisfactory results, but can be modified

to suite individual requirements with the quality of the preparation as the ultimate criterion.

2.2 General environment and precautions

Spreading should be performed in a clean, vibration-free area of the laboratory. Draughts cause swirling of the DNA–protein monolayer and should be prevented, using screens or a hood if necessary. Room temperature should be 20–25°C. Poor results are sometimes obtained during humid weather.

Some precautions concerning the cleanliness of the apparatus and reagents are important. The continuity of the DNA–protein monolayer is destroyed by surface-active agents such as detergent, oil and grease which must not contaminate equipment or solutions, directly or indirectly. For this reason, grease from the fingers must not be transferred to anything that comes into contact with the preparation. Fumes from organic solvents or oil vapour from pumps can also cause problems.

2.3 Equipment

For spreading methods the following items (see *Figure 1*) are required.

(i) A Teflon-coated or translucent plastic dish, to hold hypophase solution. We use the base or top of a Millipore filter box (9 × 9 × 2 cm) which is well rinsed after use with distilled water only and stored in a dust-free place. Clean Teflon-coated or plastic bars may be used to sweep the surface of the hypophase to clean it, to confine the area of spreading or to compress the DNA–protein film slightly.

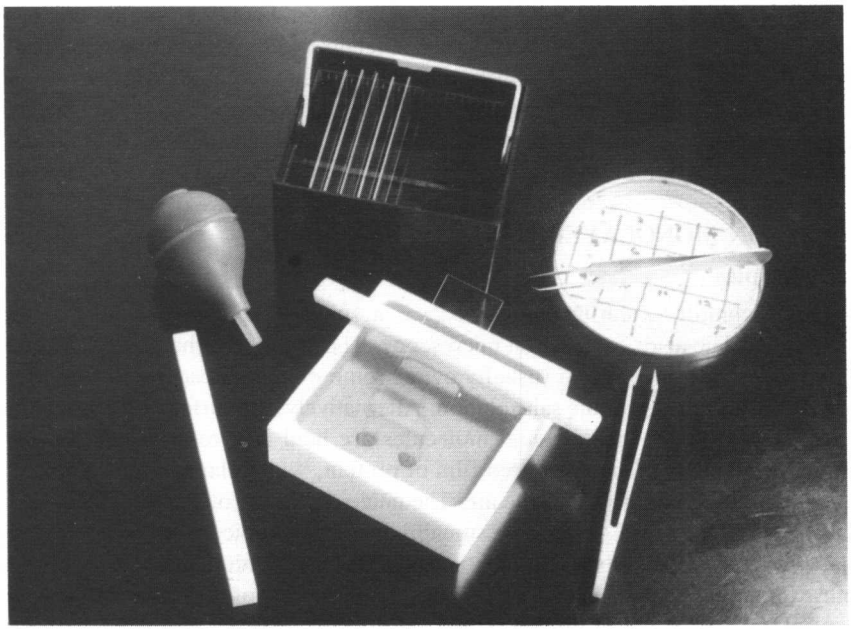

Figure 1. Equipment for spreading nucleic acids. Clockwise from top: cleaned slides stored under water in slide box, Petri dish with grids on filter paper and fine forceps, plastic forceps for handling slides, Teflon dish and bars, puffer for talcum powder.

Table 1. Preparation of collodion[a] support films.

1. Make a 3–4% (w/v) solution of collodion in *n*-amyl acetate and store in a tightly closed bottle which is placed in an opaque jar containing silica gel. Collodion can be obtained as strips or a solution in *n*-amyl acetate.
2. Place a stainless steel mesh or nickel grid-coating plate under the surface of distilled water in a dish that can be drained. A glass crystallization dish, 15 cm diameter and 3 cm deep, and painted black for better viewing, is suitable. Alternatively, a porcelain Buchner funnel of similar diameter with a screw clip on a plastic outlet hose can be used (*Figure 2*). The dish must be stable.
3. Place acetone-cleaned grids flat on the mesh so they do not overlap. Always orientate them in the same way, i.e. with the Cu side up (if using grids with a Rh or Pd coating on one side) or with the shiny or dull side up.
4. Take up a little of the collodion solution in a clean dry Pasteur pipette. Hold the tip 1–2 cm above the water, and allow a drop of the solution to fall on the surface where it spreads out to form a thin layer. Interference colours are seen as it dries to a thin silvery film. Remove the film with clean forceps, so that surface contaminants are discarded.
5. Form a fresh collodion film with another drop. If the film appears to be good, lower it onto the grids by slowly draining or pumping water from the dish. The film can be guided to some extent by touching the very edge, but tends to adhere to the forceps unless care is taken.
6. Pick up the mesh carrying the grids and the film with forceps and drain off excess water with a piece of filter paper. We dry and store the grids in a vacuum desiccator evacuated with a water pump. They are removed from the mesh as required once they are dry and can be used for 2–3 weeks after being made. Rinse the dish or funnel with distilled water and store covered with aluminium foil; clean the mesh with acetone.
7. Examine coated grids made from a new batch of collodion with the EM. If the film is too thick (film shows gold or higher interference colours, and gives poor contrast in the EM) dilute the collodion solution with more *n*-amyl acetate. If it is too thin (and tears when examined in the EM) add more collodion. Thin films can be stabilized by a light coating of carbon (*Table 2*) after specimen preparation and shadowing. Small holes in the film (which decrease its strength) indicate the presence of water in the collodion solution. Remove water from the collodion strips by heating overnight in an oven at 60–80°C, or by keeping them in a vacuum desiccator for several days. Water is removed from amyl acetate by treatment with anhydrous magnesium sulphate. Amyl acetate and collodion solutions can be stored over molecular sieves to keep them dehydrated.

[a]**CARE:** Collodion (nitrocellulose, Celloidin, Parlodion or Pyroxyl) is inflammable and explosive when dry.

(ii) Acid-cleaned glass microscope slides, extensively rinsed with distilled water and stored under distilled water in a slide storage box or staining trough. Water adheres well to clean slides. Handle them with plastic forceps.

(iii) Talcum or graphite powder; puffer or small paint brush.

For diffusion methods the following items are required.

(i) Petri dish.

(ii) Flat Teflon-coated surface or Parafilm.

For both spreading and diffusion methods the following items are required.

(i) Fine stainless steel watchmakers forceps (Dumont No. 5, standard or anti-capillary type). The tips should be sharpened occasionally on an oiled Arkansas stone to ensure that grids can be picked up from a flat surface and held securely. Clean forceps before use with acetone or ethanol to remove dirt and grease.

(ii) Copper EM grids coated with collodion (*Table 1* and *Figure 2*) or carbon film (*Table 2* and *Figure 3*). 200 or 300 mesh grids give good support to collodion