Preface

The related techniques of freeze fracture and deep etching—techniques by which the ultrastructure of rapidly frozen biological specimens may be examined by transmission electron microscopy—have profoundly influenced our understanding of the functional organization of the cell. Freeze fracture is unique among electron microscopic methods in that it gives *en face* views of the internal organization of biological membranes, allowing study of the in-plane distribution of integral proteins spanning the lipid bilayer. The early freeze-fracture electron micrographs of Daniel Branton, Pedro Pinto da Silva, and others were decisive in the formulation of the fluid mosaic model of membrane structure, the consensus model on which our current concepts of membrane function are based. The demonstration by Stan Bullivant that the freeze-fracture process itself results in the formation of *en face* views of membranes, and the classical label-etch experiments conducted by Pedro Pinto da Silva, which demonstrated beyond doubt that freeze fracturing splits membranes down the center of the lipid bilayer, were critical to the correct interpretation of the images. Apart from providing the interpretative foundation for all subsequent applications, the classical label-etch experiments led to the development of freeze-fracture cytochemistry to correlate composition with structure, and the exploitation of etching—the removal of ice from the surface of the frozen specimen by vacuum sublimation—as a tool in its own right to investigate surface structure. The combination of ultrarapid freezing and deep-etching techniques, as developed by John Heuser, Tom Reese, and Dennis Landis, brought significant advances in our understanding of membrane fusion events and of the three-dimensional structure of the cytoskeleton. Indeed, ultrarapid freezing remains one of the primary tools available to the experimenter for the analysis of the transient dynamic events involved in membrane-mediated cellular functions, and has provided the cell biologist with, for example, a detailed picture of how exocytosis mediates processes as diverse as the discharge of defensive organelles in protozoa, and the release of neurotransmitters at the neuromuscular junctions of higher organisms. These are but a few of many examples that could be cited to illustrate the wide-ranging and enduring impact of *rapid freezing, freeze fracture, and deep etching* in the field of biomedical cell and membrane biology.

All these advances center on the use of rapid freezing as a means of stabilizing the specimen in the hydrated state, prior to further processing or direct observation in the electron microscope. Most specimens are too thick to be directly observed, and so must first be sectioned or replicated. Freezing of bulk specimens poses its own special problems, since heat loss from the interior is limited by the rate of heat conduction through the sample, however efficiently its surface is cooled. Slow cooling results in the growth of ice crystals which are large in comparison with subcellular components, and which consequently damage cellular structure. There are three practical solutions to this problem: to treat the specimen with a chemical cryoprotectant prior to freezing, as has traditionally been done with most freeze-fracture specimens; to apply ultrarapid freezing, either by enhancing the efficiency of cooling with liquid cryogens or by bringing the specimen into rapid

contact with a cold metal block, and then to study only the well-frozen surface layer; or to freeze the specimen while maintaining it at high pressure (hyperbaric freezing), thereby limiting the rate of nucleation and growth of ice crystals. Specimens frozen in these ways may be processed and examined by direct cryoultramicrotomy (frozen thin sectioning), by freeze substitution with plastic embedding at low temperature followed by conventional thin sectioning, or by the group of techniques centered on freeze fracture and deep etching which form the subject of this volume.

In this volume, the second in the series *Techniques in Modern Biomedical Microscopy*, we set out to cover the current state of the art in the related fields of rapid freezing, freeze fracture, and deep etching. The book is quite distinct from existing specialist texts, aiming, within a single integrated volume, both to take the reader through the principles of the techniques and to give detailed examples of their application in biomedical research. The book is thus neither a recipe manual of techniques nor a collection of specialist research reports which would all too rapidly become outdated. With the help of contributors selected for their special expertise, recent developments are presented within the context of the field as a whole. The principles of the basic techniques are explained at the outset, providing the platform from which recent developments in methodology, together with their advantages and limitations, can be followed in detail. Biomedical techniques stand or fall by whether they can effectively help us learn more about the fundamentals of life, and, in this respect, the contributions of rapid freezing, freeze fracture, and deep etching are unrivalled. A major emphasis is therefore placed on illustrating how, and for what purposes, these techniques may be used. This not only brings home their true value, but also provides a deeper understanding of the techniques themselves, thereby assisting those who wish to assess the potential applicability of the techniques in their own studies. Of course, no technique alone can provide all the answers. Thus, wherever appropriate, the subject matter is presented within the perspective of other low-temperature and microscopical methods and within the theme of multidisciplinary biomedical research.

An overriding message which shines through all the contributions is the remarkable versatility of rapid freezing, freeze fracture, and deep etching. This versatility stems from constant and progressive adaptations of the existing methodologies to meet new needs, and has sustained the value of the techniques for more than three decades through to the present day.

Conceptually, the book is divided into three sections. The first covers the fundamentals, starting with two introductory chapters aimed at those new to the field. In the first and second chapters, we explain the basic principles and practice of the techniques of freeze fracture and deep etching, and of rapid freezing. The more advanced contributions by subject specialists which follow, both within the first section, and in the subsequent sections of the book, are developed from this background. Chapter 3 builds on Chapter 1 by providing a survey of the equipment available for freeze fracture, and Chapter 4 presents a detailed review on artifacts.

The emphasis of the second section is on advanced techniques, each chapter exploring a separate topical theme in depth, together with illustrative applications. Chapter 5 focuses on hyperbaric freezing, a technique currently attracting substantial interest because, in contrast to ultrarapid freezing, it potentially permits cryopreservation throughout the depth of bulk tissue samples. As noted earlier, ultrarapid freezing techniques have revolutionized the study of dynamic cellular processes, and Chapter 6 is devoted to reviewing this topic in detail. History has shown us how new technologies have constantly changed the face of freeze fracture. That this is continuing today is illustrated by the integration of confocal microscopy with freeze fracture to permit the precise fracturing of selected cells identified within heterogeneous tissues, described in Chapter 7. Chapter 8 continues the theme of technical versatility and its cell biological applications with a detailed review of approaches to visualization of the inside and outside surfaces of the plasma membrane using cell monolayers. A significant success story of the 1980s was the development of a range of practicable methods in freeze-fracture cytochemistry, and Chapter 9 gives a comprehensive explanatory

guide to the development of these techniques from their inception. The theme of cytochemistry is further elaborated with Chapters 10 and 11, which respectively detail the complementary use of scanning electron microscopy with one established cytochemical method, fracture-label, and the potential pitfalls of using lipid perturbing agents for lipid cytochemistry, a once popular technique which all too often continues to trap the unwary today.

In the third section, the basic theme of advanced techniques and their applications is continued, but with a switch in focus. Here, we present a series of applications, selected to illustrate the scope of the techniques in advancing our understanding in a range of fundamental areas of biomedical research. As the underlying principle of freeze fracture centers on the properties of frozen lipids, the technique has quite naturally made major contributions to the study of membrane lipids and model membranes, which are reviewed in Chapter 12. Of the entire field of cell biology, neurobiology is arguably one that has been most transformed by application of rapid freezing, freeze fracture, and deep etching, and this story is told in Chapter 13. Chapter 14 illustrates how ultrarapid freezing and deep etching have advanced our understanding of the dynamic processes of exocytosis and remodeling of the extracellular matrix, expanding on topics introduced in Chapter 6. The theme of the extracellular matrix is continued in the following two chapters, emphasizing that the techniques now have major roles to play in structural research outside the area of biological membranes. Chapter 15 deals with the visualization of isolated molecules of the extracellular matrix, specifically those of basement membranes, and provides a lesson in how our understanding of complex biological structures can be enhanced by studies of the individual molecules of which they are composed. The section is concluded with two chapters on the theme of medical cell biology, the first dealing with coronary heart disease, extending yet further the topic of extracellular matrix, and the second focusing on mechanisms of viral infection in relation to the cell biology of membrane biogenesis and trafficking. Taken as a whole, the chapters exemplify how the impact of the techniques extends from viruses and protozoa to man, from membranes to the non-membranous components of tissues and cells, from individual molecules isolated in the test tube to complex molecular assemblies existing in nature, and from basic biology to medicine.

A major potential strength of a multi-author volume such as this is the authoritative coverage that individual experts can provide; a potential weakness is that coherence of the text as a whole may all too easily be marred by repetition, inconsistencies, and apparent contradictions. Not only can these be a source of irritation to the informed reader, but they can also utterly confuse a newcomer to the field. To produce an integrated text, of value to the novice and expert alike, we have edited the chapters with a firm hand, and we wish to thank all our contributors for their forbearance in cutting, altering, and tailoring their manuscripts to fit our concept of the volume as a whole. At the same time, we are of the firm view that, if authors are to communicate their own expertise effectively, their style and freedom must not be unduly cramped by overbearing editorial interference. There are instances, for example, where variable but potentially confusing uses of terms are well established. Standardization should not, in such instances, be pushed to the limit; instead, we have opted to explain the different senses in which terms are used. Because each chapter is intended to be complete in itself, some topics inevitably recur in different parts of the book. We see this as a positive asset, and, by introducing appropriate explanations and cross-references, we have sought to integrate the information in a complementary manner. A degree of overlap has quite deliberately been retained between the chapters of the first section, the intention being to introduce topics in rudimentary form in the first two chapters, so that they may be taken up again and expanded within the context of more specialized chapters that follow. In this way, we hope to have served the interests both of those who are already *cognoscenti* and wish to go straight to the chapter of interest, and of newcomers to the field.

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