Preface

Fluorescence microscopy is an established tool for a variety of applications in biology and biomedical research. Recent advances leading to improved contrast and high sensitivity allow for the detection of signals at the single-molecule level. In conjunction with this platform, fluorescence lifetime imaging microscopy (FLIM) provides another dimension of contrast and sensitivity and also offers the additional benefit of independence from fluorophore concentration and excitation intensity. Moreover, the fluorescence lifetime is often sensitive to the physical and chemical environment of the fluorophore; as such, it is an excellent reporter of conformational changes and variations of the molecular surroundings of biological molecules.

These unique advantages of lifetime-resolved fluorescence measurements extend the information that is obtained from measuring only the intensity. The rationale for performing lifetime-resolved measurements in an imaging environment is to acquire, at every pixel of a fluorescence image, the critical information provided by dynamic fluorescence measurements, which has been available for decades from single-channel (cuvette) fluorescence dynamics measurements. The lifetime-resolved fluorescence parameters coupled with the spatial dimension provide valuable insight into the functioning of complex biological systems.

The primary objective of a FLIM investigation is usually quite different from that of single-channel measurements. One is still interested in determining the lifetime-resolved information as accurately, reproducibly, and robustly as possible; however, in FLIM, the structure/morphology of some object (e.g., a structure in a biological cell) under physiological conditions is often the investigation's target of major concern. Thus, the scientific questions asked are analogous to those for normal intensity fluorescence imaging; that is, one is interested in correlating the spectroscopic information with different locations in the imaged object. When lifetime-resolved fluorescence is acquired in addition to the intensity, the identification and quantitative differentiation of fluorophores are considerably improved.

For instance, in the case of FRET, if the lifetime of a donor fluorophore is known in the absence of an acceptor, it is relatively easy for FLIM to differentiate locations in an image with dissimilar lifetime decays; faster lifetimes indicate increased efficiency of energy transfer. Because lifetimes in FLIM are independent of the concentration, complicated control experiments and multiple wavelengths, which may be difficult to align in the image, are not required. FLIM is also an excellent way to discriminate objects that have similar

emission wavelengths but different lifetimes; thus, the image contrast is improved. A common application of FLIM is the elimination of background fluorescence, such as intrinsic fluorescence, or unbound fluorophores, where the bound and unbound fluorophores exhibit different lifetimes. FLIM can be used for many quantitative determinations of ion concentrations, pH, oxygen content, protein–protein interactions, cell motility, and cancer diagnosis. All of these applications are discussed in the various chapters of this book.

It is difficult to say when and where the first FLIM images were observed. The "dawn of FLIM" took place in a few research laboratories with ready expertise in time-resolved fluorescence, fast electronics, and, usually, a strong interest in solving biological problems. The original developments made use of instrumentation already available in cuvette-based spectrofluorometers to acquire the fluorescent decay. The lifetime data were analyzed using on-hand fitting methods.

Once the power and broad applicability of FLIM became evident to the general scientific community, biological investigators' interest in exploring lifetime measurements developed quickly. Both time- and frequency-domain methodologies were rapidly improved and extended in many laboratories. Thanks to the development of various technologies, including optics, electronics, detectors, and the new discovery of visible fluorescent proteins, this development took place at a rapid pace. Not surprisingly, after the introduction of various commercial units, which simplified data acquisition and analysis and provided biological laboratories with ready-made instrumentation, publications covering FLIM microscopy have grown rapidly since 2000.

This book presents the fundamentals of FLIM so that a wider audience can appreciate the rapid advances and increasing applications reported in the literature. In this sense, the goal is pedagogical: In addition to reviewing the latest developments, applications, and approaches to data analysis, we want to convey the exciting future of FLIM and indicate the present state of the art in FLIM imaging as described in the instrumentation section. No measurement method is perfect; the authors have strived to present pros and cons of different methods and to give some indication of where improvements are necessary and desired. Each chapter critically compares FLIM measurements to other techniques.

The book also describes ancillary techniques related to the direct determination of lifetimes, including imaging fluorescence anisotropy for the study of molecular rotations. Moreover, in addition to discussions related directly to FLIM, we also address the fundamentals of dynamic fluorescence measurements and the basic pathways of de-excitation available to electronically excited molecules. An awareness of the diversity of pathways available to an excited fluorophore will assist potential users in recognizing the value of FLIM measurements, as well as inspire innovative experiments using lifetime-resolved imaging.

As time passes, more of the sophisticated methods used in photophysics and photochemistry, as well as new instrumentation, are being incorporated into FLIM. Novel features that apply exclusively to FLIM are being developed, including sophisticated image analysis. Our purpose has been to showcase the broad application of fluorescence lifetime-resolved imaging in biology. We include different aspects of FLIM data acquisition and applications, as well as discussions of FLIM data processing, in a separate section on data processing.

The discussion sections in all the chapters clearly show the challenges for implementing FLIM for various applications. Certain chapters discuss limits on the number of photons required for highly accurate lifetime determinations, as well as the accuracy with which multiple, closely associated lifetime components can reliably be determined. Highly accurate determinations of fluorescence lifetimes are sometimes necessary for answering certain specific, detailed questions concerning some molecular mechanisms. On the other hand, the change in lifetime-related parameters and their location in a cell are of primary concern for many investigations. Such considerations are important for the user when he or she is selecting the most advantageous method of FLIM to use for a particular application. These aspects are discussed in the various chapters. We hope that this book will be useful for experts in FLIM as well as for newcomers to this field.

We realize that the field of FLIM has grown rapidly in the recent past and that it is impossible to do justice to all those who have contributed unique FLIM applications, instrumentation, and data analysis. We acknowledge our indebtedness to all the FLIM enthusiasts who have made this such an exciting field. Most importantly, we wish to thank all the authors who have contributed chapters to this book and have strived to present the fundamentals so that novices can implement FLIM in their research and laboratories, as well as appreciate the uniqueness and usefulness of FLIM in their own research. It has been a great honor to work with them.

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