Preface

The boundary between the fundamental themes of science (i.e., physics, biology, and chemistry) has blurred and new disciplines are taking shape as a mark of an era of modern science. Fluorescence microscopy and imaging has emerged as an important inter-disciplinary field that demands basic knowledge of physics, biology and imaging. Most of the fluorescence imaging applications are carried out by tagging the target molecule by fluorescent probes. These probes are easy to integrate with the desired biomolecule (proteins, lipids etc.) and fluoresces when excited, thereby pinpointing its spatial location. This forms the basis for understanding key biological mechanism at an unprecedented resolution and holds huge promise to many fields ranging from applied physics to medicine. The resolution is classically limited by the wavelength of the probing light and imaging optics, thereby fixing a lower bound to which the features can be resolved. With the advent of super-resolution technique, it is now possible to resolve molecules within few nanometers. As a result of these interesting developments, new fields have emerged such as, Nanobioimaging, Nanomedicine and lab-on-chip devices.

This interdisciplinary field of research requires complete knowledge of imaging optics and molecular physics. So, the best way to introduce the subject to the general audience is by introducing optical imaging concepts before pounding on the advanced imaging systems and their applications. Additionally, building up of molecular physics from molecular orbital theory seems logical for complete understanding of light-matter interaction at the geometrical focus. The two disciplines clearly overlap since light controls the states of molecules and conversely, molecular states control the emitted light. These two mechanisms together determine the essential ingredients for fluorescence imaging such as, molecular cross-section, Stoke shift, emission and absorption spectra, quantum yield, signal-to-noise ratio, Forster resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime. These properties form the basis of many fluorescence based devices.

The book provides an introduction to the fundamentals of fluorescence imaging and also includes advanced fluorescence imaging techniques. The term fluorescence imaging encompasses the following research areas and specialized techniques:

- Optical imaging that covers transmission of light in free space, through optical elements such as lens, pinholes and imaging systems.
- Field effects i.e., the electric field distribution of light at the focus of objective lens and cylindrical lens including the polarization and dipole-orientation effects.
- The detection geometry including, confocal, confocal-theta and widefield detection.
- Fluorescence techniques such as, phosphorescence, lifetime, FRET, FRAP and multiphoton excitation.
- The super-resolution imaging techniques such as, TIRF, STED, localization techniques (PALM, fPALM, STORM and GSDIM), structural illumination and point spread function (PSF) engineering.

Fluorescence has found several applications in microscopy, spectroscopy, cell biophysics, drug discovery, sensing and all types of optical imaging techniques including, multi-photon and multi-color imaging.

This book on fluorescence microscopy may serve as:

- An introductory text for students in Applied Physics, Biophysics and Biomedical Engineering.
- The flow of the chapters are maintained such that the book is good for self-study.
- The book will be valuable for professionals, scientists and engineers in both industry and academia.
- This book may be a guide to the experts in the broad field of bioimaging.

In general, the reader is assumed to have a little background in applied physics, biophysics or engineering but too much is not expected. The book starts at an introductory level and eventually links to the advanced topics in fluorescence imaging and super-resolution techniques. In this book, an introduction to fluorescence imaging is intended and the related concepts are borrowed from imaging optics and molecular physics. This book should not be considered as a complete text that encompasses all topics related to fluorescence. The book is a route to advanced fluorescence imaging beginning from our knowledge of basic sciences. Indeed some areas of fluorescence spectroscopy and special techniques are not at all included.

The book is organized into two parts: Basics and Fundamentals (Chaps. 1–5) and Advanced Imaging (Chaps. 6–11). The first part deals with basics of imaging optics and its applications. Advanced part takes care of several imaging techniques and related instrumentation that are developed in the last decade pointing towards far-field diffraction unlimited microscopy. One of the goals of this book is to provide the necessary fundamental basics (in Part I) and up to date account of few emerging microscopy techniques.

Chapters can be combined in various ways for representing short courses such as, Imaging Optics, Photophysics and, Advanced Fluorescence and Imaging.

Imaging Optics
1. Ray Optics, Wave Optics and Imaging System Design
2. Basics of Electromagnetic Theory for Fluorescence Microscopy
3. Electric Field Effects in Optical Microscopy Systems
4. Quantum Description of Radiation Field and Optical Microscopy

Photophysics
5. Molecular Physics of Fluorescent Markers
6. Basics of Fluorescence and Photophysics
7. General Fluorescence Imaging Techniques

Advanced Fluorescence Microscopy and Imaging
8. Multiphoton Fluorescence Microscopy
9. Super-resolution Fluorescence Microscopy
10. Image Reconstruction Methodologies for Fluorescence Microscopy
11. Future Perspective of Fluorescence Microscopy

A course on *Imaging Optics* may begin with the introduction to ray optics that gives the simplest theory of light. This includes total internal reflection that forms the basis of super-resolution technique such as TIRF microscopy. The wave behavior of light is explained by wave optics that takes care of the short comings of ray optics and satisfactorily explains diffraction and interference effects. These phenomenon forms the basis of Confocal and 4PI fluorescence microscopy. Then electromagnetic theory can be introduced to explain polarization effects. Electric field effects can be estimated at and near the geometrical focus using the vectorial theory of light, which manifests field-dipole effects for fluorescence microscopy. This is followed by the introduction of quantum theory of light to describe its quantum effects including squeezed light and photon antibunching that are extensively used precision optical microscopy.

A course on *Photophysics* may begin with the fundamental molecular theory describing the quantum state of the molecules, transition probabilities and molecular bonding. This explains molecular cross-section, triplet state lifetime, selection rules and related concepts required for understanding behavior of molecules in the presence of light. Then the general concepts of fluorescence microscopy are defined such as, phosphorescence, quenching, lifetime, absorption and emission spectra, energy transfer and quantum yield. The important aspect of photobleaching and its effects is also explained. The penultimate chapter in this section many interesting fluorophores currently used in biophysical research. Important fluorescence techniques such as, lifetime imaging, resonance energy transfer, second harmonic generation and fluorescence correlation spectroscopy are explained in the last chapter of this section.

A course on *Advanced Fluorescence Microscopy and Imaging* assumes the knowledge of multiphoton imaging including, two-photon cross-section, two-photon absorption, and intrinsic localization property. This can be followed by the detailed description of state-of-art super-resolution imaging techniques such as, STED, STORM, PALM, fPALM, Structured illumination and others. The penultimate chapter detail advanced techniques for image reconstruction in single- and multi-photon fluorescence microscopy such as, deconvolution, maximum likelihood and maximum a-posteriori methods. Final chapter discusses the future of fluorescence microscopy, its limitations and advantages over other imaging techniques.

Bangalore, India — Partha Pratim Mondal
Genova, Italy — Alberto Diaspro

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