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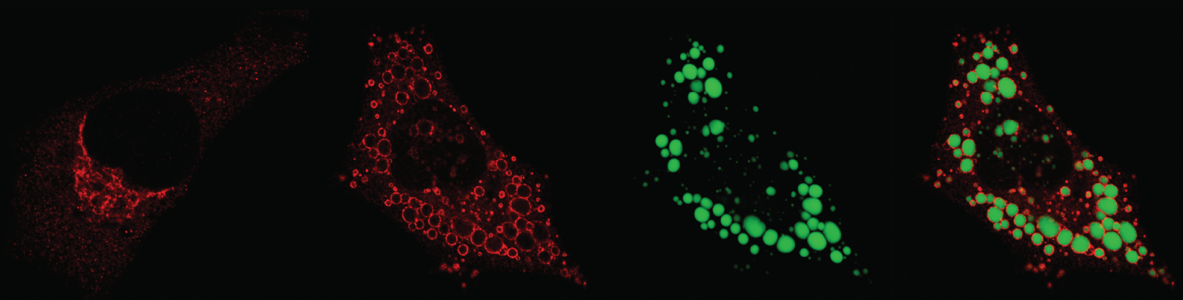
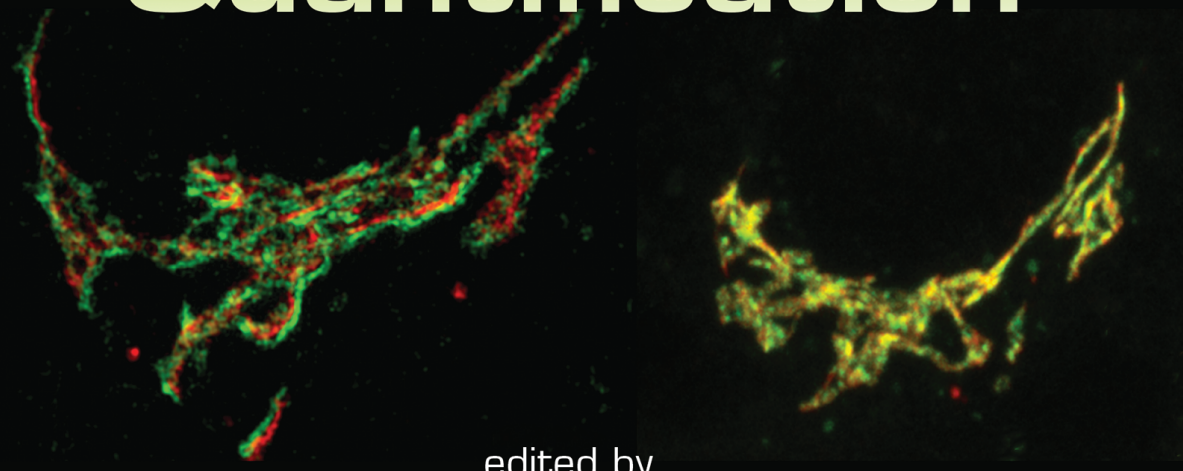


Fluorescence Imaging and Biological Quantification



edited by
Raquel Seruca
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Fluorescence Imaging and Biological Quantification

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The ideal scientist is enchanted by the scientific poetics of nature. As scientists, we dedicate this book to everyone who gets fascinated by Science and Art.

Contents

Preface

Huge numbers of biomedical images are generated each year in routine and research labs. In this book, we describe new developments and solutions to analyze and quantify fluorescence images, tagging DNA, RNA, and proteins in single cells as well as in cell populations. This book is a collaborative effort by large group of scientists working in complementary disciplines as biology, biochemistry, microscopy, physics, and engineering.

In Section I, we present different microscopic techniques that allow the production of high-quality 2D and 3D images as confocal microscopy to more quantitative methodologies, namely imaging flow cytometry and atomic force microscopy (AFM). This includes chapters that define strategies to circumvent limitations of fluorescent nanoparticles and include novel strategies to track, quantify, and map these signals. Multiple fluorochromes and fluorochrome dyes are currently available allowing single or multiple complex visualization of molecular events. In every chapter, the advantages and limitations of every microscopic approach will be discussed and the future technical developments in each scientific area will be addressed.

Section II compiles new imaging and computer-based technologies to access the inner machinery of living cells and shows how different methodologies contribute to advance on the understanding of highly dynamic biochemical processes occurring at cell, tissue, and organism level. We focused on a wide variety of biological questions related to signaling events and networks, formation of protein complexes, maintenance of cellular homeostasis by lysosomes, circadian rhythms, cell cycle, membrane trafficking, behavior of cancer-cell populations, and macrophages dynamics. Overall, we aim to demonstrate in this book how fluorescence microscopy and images can be mathematically processed to allow depiction of molecular events and pathways underlying cell function, tissue morphology and mechanics, and individual physiology.

Raquel Seruca, Jasjit S. Suri and João M. Sanches

Acknowledgments

We thank all coauthors for their contributions to the book and, in particular, to Paula Sampaio who provided an historical view of the topic.

Editors

Raquel Seruca (RS) received her PhD in Medicine from the faculty of Medicine from the University of Porto, Portugal in 1995. RS developed her PhD project in the field of genetics of gastric cancer.

Presently, RS is vice president of Institute of Molecular Pathology and Immunology at the University of Porto, Portugal (Ipatimup) and coordinator of the cancer research program at i3s, both located in Porto.

At i3s, Seruca is the group leader of Epithelial Interactions in Cancer (EPIC). As group leader, RS has been able to create a truly multidisciplinary environment, stimulating collaborations and interactions between surgeons, oncologists, pathologists, biologists, biochemists, and bioengineers.

The long-term goal of the group is to uncover how epithelial cell–cell and cell–matrix junctions, as well as the surrounding microenvironment, can influence gastric, breast, and colorectal cancer progression. The focus of the group is to unravel the role of E- and P-cadherin in epithelial homeostasis and cancer development.

Seruca's lab is a reference center of the International Gastric Cancer Linkage Consortium, responsible for the functional studies of E-cadherin mutations in hereditary gastric cancer. Using this disease model, the group has made significant contributions on the role of E-cadherin and associated signaling in cancer-cell migration, survival, and invasion.

Seruca has more than 200 publications in international peer-reviewed journals, with an h-index of 52.

Seruca collaborates with several research groups worldwide, serves as a reviewer for top journals in the area of cancer, and is invited to speak and chair sessions at major conferences dedicated to cancer research. Further, she is involved in the evaluation of several international grants and institutes.

In 2009, Seruca received the distinction Ordem do Infante D. Henrique from the Portuguese Presidency (Presidência da República) for her scientific merit. More recently, in 2014, she got the gold medal of the Porto City for her contribution for science internationalization.

Dr. Jasjit S. Suri, PhD, MBA, fellow of AIMBE is an innovator, visionary, scientist, and an internationally known world leader. Dr. Jasjit S. Suri received the Director General's gold medal in 1980 and the fellow of American Institute of Medical and Biological Engineering (AIMBE), awarded by National Academy of Sciences, Washington, DC in 2004. He has published more than 550, which includes journals, book chapters, and proceeding articles having an H-index 46, coauthored more than 40 books, 100 innovations, and trademarks. He is currently chairman of Global Biomedical Technologies, Inc., Roseville, California, company dedicated to cancer imaging and biomedical devices. Dr. Suri is also in the board of AtheroPoint, Roseville, California, a company dedicated to atherosclerosis imaging for early screening for stroke and cardiovascular monitoring. He has held positions as a chairman of IEEE Denver section and advisor board member to health care industries and several schools in the United States and abroad.

João M. Sanches (JS) received the EE, MSc, and PhD degrees from the Instituto Superior Técnico (IST) and Universidade Técnica de Lisboa (UTL), Lisbon, Portugal in 1991, 1996, and 2003, respectively and the habilitation (agregação) in 2013 by the Universidade de Lisboa (UL), Lisboa, Portugal in biomedical engineering. JS is associate professor at the Department of Bioengineering (DBE) at the IST and he is from the coordination board of the biomedical engineering master and doctoral programs. Before he was at the Department of Electrical and Computer Engineering (DEEC) where has taught in the area of signal processing, systems, and control.

JS is a senior researcher and member of the board of directors of the Institute for Systems and Robotics (ISR–IST). His work has been focused in biomedical engineering (BME), namely, in biological and medical image processing and statistical signal processing of physiological and behavioral data. Currently, JS aims to develop new tools and

methodological strategies to quantify and map molecular and morphometric pathologic cancer biomarkers.

As a group leader, Professor Sanches has been able to create a truly multidisciplinary and stimulating environment where researchers from top institutions from the biology and medicine areas work close together with engineers and computer scientists. Approximately 150 international publications (ORCID and Google Scholar) and several patents were already produced in the scope of this collaborative work.

He is senior member of the IEEE Engineering in Medicine and Biology Society (EMBS) since 2011 and member of the bioimaging or bio-imaging and Signal Processing Technical Committee (BISP-TC) of the IEEE Signal Processing Society.

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Introduction

Brief Historical view

Microscopy, fluorescence, and imaging

Two millenniums ago, Romans discovered that glass could be used to enlarge objects, but the first instruments capable to make visible structures not visible by the naked eye, only appeared in the past 400 years. Microscopes made possible to observe microorganisms, blood cells, sperm, and small details in plants and animals for the first time. Anton van Leeuwenhoek and Robert Hooke represented their pioneer observations by hand drawings and quantified some of their observations. As an example, Leeuwenhoek estimated the number of *animalcules* (bacteria and protozoa) in drops of water and determined the size and shape of *red corpuscles* (red blood cells).

Improvements in optics and theoretical studies of image formation and optics carried out in the second half of nineteenth century founded the grounds for the modern optical microscopy we experience today. Nowadays, scientists have a broad range of imaging methods available to study biological systems. The most classical transmission microscopy contrast techniques such as brightfield, phase-contrast, differential interference contrast, polarization, and dark-field are based on the alterations of light induced by interaction with the specimen. Transmission microscopy is a routinely choice approach for morphological analysis and for live-cell imaging to study cell shape, cell cycle, or migration of cell in culture. However, it is limited, as it does not allow to differential and simultaneously label molecules with high sensitivity and in a quantitative way. Fluorescence microscopy surpasses these problems and allows exploring the cells and tissues at more molecular and subcellular levels. In fluorescence microscopy, the specimens are stained by fluorochrome dyes, quantum dots, or express chimeric fluorescently tagged proteins. These fluorophores get into an excited state after absorbing energy, as a photon, at specific wavelengths. This high-energy state is transient and first, the excited state electrons go to lower energy levels by vibrational relaxation, after which the molecule returns to ground state by emission

of a photon of lower energy than excitation. Multiple fluorochromes with very sensitive emission profiles are commercially available to label different cellular components allowing their independent visualization with high sensitivity as well as their spatial and temporal correlation.

The main constrain of fluorescence is blurring as fluorophores are self-luminous. So, out-of-focus light from different focal planes mix with the in-focus signals originating blurred images in widefield fluorescence microscopy (WFM). Confocal fluorescence microscopy overcomes this problem, by using a spatial filter, known as pinhole, at the detection level that suppresses the light from out-of-focus areas. This leads to the formation of a high contrasted image, optical section, that have reduced contribution of out-of-focus light. A specimen can then be optically sectioned and those images can be used to build a 3D reconstruction of the specimen.

Imaging cell in culture is still the most common approach to study the biology of the cells; however, higher eukaryotes are multicellular organism where the cells are integrated in a 3D community, tissues. So, the ability to study the cells within its natural environment is essential to have an integrate vision of the biological processes, and confocal microscopy ability to generate optical sections is still limited to few dozens of micrometers deep into tissues due to scattering of the light by matter. Multiphoton microscopy use pulsed IR light that is less scattered by tissues allowing to image deep. Since, IR photon have less energy, fluorophores must absorb energy of two photons to get excited. The high probability of excitation occurs only at focal plane, with no out-of-focus light being generated. Emitted light can be only detected close to the objective and this increases the detection sensitivity experimented in multiphoton microscopy.

In the past decade, a new microscopy technique to image live embryos in toto had a great development. In light sheet microscopy, a plane of the sample is illuminated by a sheet of light and emitted light is detected on an objective at 90°. Combining the acquisition at multiple planes and angles permits to make a total 3D reconstruction of each sample. Due to light sheet microscopy low phototoxicity, it is instrumental for live imaging of embryo development. Actually, live-cell imaging is essential to know the rules of biological processes or cell fate decisions and had a great expansion with development of new probes, including fluorescent proteins, which could *illuminate* targeted cell components. This enabled observing and measuring dynamic cellular events at molecular level with high spatial and temporal resolution.

The chemical distribution within an unstained specimen can be also evaluated by Raman confocal microscopy, a spectroscopic approach that provides a specific fingerprint of molecules.

Recent developments let to create techniques based in switch on/off of fluorescents molecules (generically known as *super-resolution*

microscopy) that overcome the limit of resolution (~200 nm) of optical microscopy and approaching it to nanoscopy with resolution limits reaching already 10 nm. These techniques are *super-resolution* microscopy plus the combination of light microscopy with electron microscopy and atomic force microscopy will open a new vision of cell biology in future years.

As the pioneers already demonstrated, imaging is not only getting a nice picture of fine details. This is particularly evident with digital imaging that generates images, which are 2D matrices. So, the digital images can be analyzed for extraction of quantitative information in order to perform accurate evaluation of data and science of excellence.

Paula Sampaio

Section I

Advanced methods of analysis

Chapter 1 Confocal microscopy in the life sciences

Miguel Aroso and
M. Gomez-Lazaro

Contents

Introduction

Microscopy imaging is, in general, achieved by reflecting light off the specimen or by illumination of fluorescently labeled molecules (e.g., proteins). One of the main advantages of fluorescence microscopy is the increase in signal of the fluorophores against a dark background [1]. In widefield microscopy, the brightest and highest intensity of the incident light, from an incoherent mercury or xenon arc-discharge lamp, is at the focal point of the objective but there is illumination of other parts of the sample and as a result, different focal planes emits light resulting in high background, which might compromise the quality of the image [2]. This effect is more pronounced in thicker specimens (>2 μm), where out-of-focus fluorescence contributes to a higher background and to a degradation of most of the fine details. In this respect, the development of the laser scanning confocal microscopy (referred as confocal microscopy in this chapter) revolutionized the field of life sciences, since this

technology allows the generation of sharper images with significant lower background. The basis of confocal microscopy was developed by Marvin Minsky in 1955 and patented in 1957 [3]. However, further developments of Minsky's prototype were hampered by limitations in the illumination and in the imaging system. The first commercial confocal microscope arrived at the market 30 years later—the Bio-Rad MRC-500.

Modern confocal microscopes can be considered as completely integrated electronic systems [4], where the optical microscope plays a central role in a configuration that consists of one or more electronic detectors, a computer (for image display, processing, output, and storage), and several laser systems combined with wavelength selection devices and a beam-scanning assembly [5]. One of the most important components of the scanning unit is the pinhole aperture, which acts as a spatial filter and is positioned directly in front of the detector [6] (Figure 1.1).

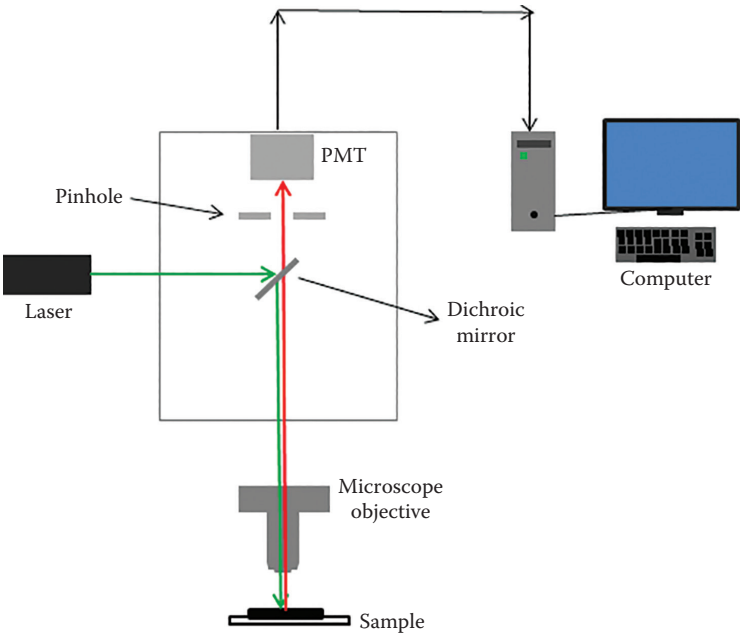

Figure 1.1 Schematic representation of a confocal microscope. A typical confocal microscope is composed of a laser as a source of excitation light (which can be different lasers with different laser lines or even a unique white laser), which will be used to scan the focused laser beam across the sample. The light is reflected off a dichroic mirror to direct the light to the sample. The objective of the microscope is used to focus the point illumination in the sample that will be scanned. The emitted light passes through the mirror and will be detected by the detector (usually a photomultiplier tube [PMT]) after passing through a pinhole that removes the out-of-focus light. The smaller the opening of the pinhole the higher amount of out-of-focus light is rejected. The photons arriving at the detector are processed by a computer for image display.

It is possible to adjust the pinhole aperture to exclude fluorescent signals from out-of-focus features positioned above and below the focal plane and control the optical section thickness [5,7]. Thus, the image obtained has less haze and better contrast and represents a thin cross section of the specimen [8–13]. It is also possible to acquire several optical sections from the specimen that later can be used to create 3D representations. Nevertheless, the reduction of the pinhole size leads to a reduction of the image intensity, as fewer photons can be captured. Thus, there is a need to have a bright and coherent excitation source (e.g., laser) and very sensitive photon detectors [14]. Those detectors should be highly sensitive and respond very quickly to a continuous flux of varying light intensity. The most common choice in many commercial confocal microscopes are the photomultiplier tubes (PMT) [15–17], which convert the fluorescent signals that pass through the pinhole into an analog electrical signal with a continuously varying voltage that corresponds to the intensity of the signal. Then, the analog signal is converted into pixels and the image information is displayed in the computer's monitor. The confocal image of a specimen is reconstructed, point-by-point, from emission photon signals and does not exist as a real image that can be observed through the microscope eyepieces [7].

Within this chapter, you will find advice on sample preparation, image acquisition, and preprocessing. It also includes the description of two common applications of confocal microscopy within the life science field (colocalization and fluorescence recovery after photobleaching) and a summary of commonly used fluorophores. Although confocal microscopy represents a popular technology, it has some limitations that will be revealed together with some advanced technological developments.

Experimental procedures

Sample processing: Needs and troubles

The observation of biological samples by confocal microscopy should, ideally, be carried out in living specimens. However, most of the times, it is not possible and previous sampling preparation is required. Common sample preparation for widefield and confocal microscopy relies on four main steps: (1) fixation to preserve cellular morphology and adherence of the specimen to the coverslip, (2) permeabilization to grant access of the labeling reagents to intracellular components, (3) labeling of the desired structures, and (4) mounting of the sample with addition of appropriate antifading reagent.

The fixation step must preserve the cellular organization, 3D structure, and antigenicity of the target when performing immunofluorescence. However, no fixation protocol is perfect and it should be chosen accordingly with the main objectives of the sample visualization. For example, if the samples will be analyzed by confocal microscopy