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Edited by Krishnarao Appasani , Raghu Kiran Appasani
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Single-Molecule Science

Single-molecule science (SMS) has emerged from developing, using, and combining technologies such as super-resolution microscopy, atomic force microscopy, and optical and magnetic tweezers, alongside sophisticated computational and modeling techniques. This comprehensive, edited volume brings together authoritative overviews of these methods from a biological perspective, and highlights how they can be used to observe and track individual molecules and monitor molecular interactions in living cells. Pioneers in this fast-moving field cover topics such as single-molecule optical maps, nanomachines, and protein folding and dynamics. A particular emphasis is also given to mapping DNA molecules for diagnostic purposes, and the study of gene expression. With numerous illustrations, this book reveals how SMS has presented us with a new way of understanding life processes. It is a must-have for researchers and graduate students, as well as those working in industry, primarily in the areas of biophysics, biological imaging, genomics, and structural biology.

KRISHNARAO APPASANI is an award-winning scientist and the founder and chief executive officer of GeneExpression Systems, a global conference-producing organization focusing on biomedical and physical sciences. He is also an elected life fellow of the Royal Society of Biologists, London, UK.

RAGHU KIRAN APPASANI is a psychiatrist, and neuroscientist. He is the recipient of the Leonard Tow Humanism in Medicine Award. He is also the founder and chief executive officer of The MINDS Foundation.

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Single-Molecule Science

FROM SUPER-RESOLUTION MICROSCOPY TO DNA MAPPING AND DIAGNOSTICS

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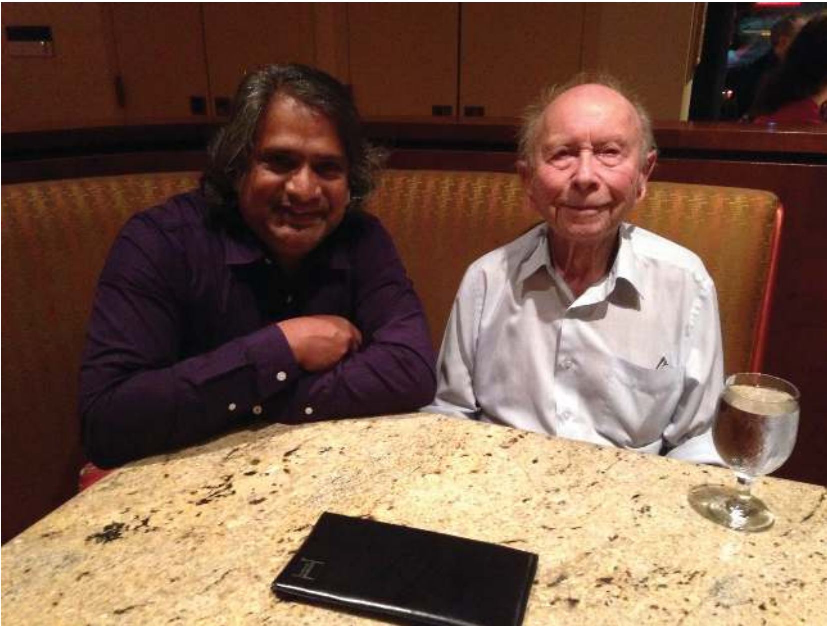
Dedicated to

To my late friend ***Arthur Beck Pardee*** *(1921–2019), an American-born legendary biochemist/molecular biologist/cancer biologist, who is famous for his part in the PaJoMo experiment in late 1950s, which greatly helped in the discovery of messenger RNA; discovered the restriction point in early 1970s in which a cell commits itself to a certain cell cycle; did a great deal of work in understanding tumor growth and regulation during 1980s; and codiscovered differential display methodology in early 1990s.*



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Foreword

Single-Molecule Science: From Super-Resolution Microscopy to DNA Mapping and Diagnostics provides a detailed overview of the enormous developments seen in the field since its beginnings in the 1970s. I am thankful to Krishnarao Appasani and Raghu Kiran Appasani for making the extraordinary effort to prepare this timely volume on the rapidly expanding molecular imaging techniques used to probe biology. In the introductory chapter, the editors summarize how the field has developed from single-molecule detection to the integration of super-resolution techniques and how this has impacted many fields across the basic science.

When I provided my summary comments to conclude one of the big conferences on single molecule spectroscopy and super-resolution techniques around six years ago in Berlin, it was already evident then that multiple super-resolution techniques (SRT) were being used in parallel, and it looked like this would continue into the near future with developments such as stimulated emission depletion (STED), *d*STORM, photoactivated localization microscopy (PALM), and more. SRT had already had a big impact, but it looked as if the big unsolved biological questions were not yet cracked. However, it was obvious to everybody that this was coming. The reviews presented in this book assert that this prediction was correct.

Among the most valuable achievements strengthening biological understanding are combinations between SRT and cryo-electron microscopy (Cryo-EM) techniques. With the amazing developments both techniques are taking, combined 3D reconstruction will have a large impact in the future. The same holds true for the combination SRTs, such as STED, and fluorescence fluctuation analysis at single-molecule resolution, which, like fluorescence correlation spectroscopy (FCS), have contributed strongly to the understanding of cell membranes. Another strong development in the field of structural biology emerged from the combined progress of single-molecule fluorescence resonance energy transfer (FRET) techniques. We have now seen site-specific labeling techniques linked to synthetic biology (with DNA and RNA synthesis) entering into the world of high resolution structural biology, particularly in combination with

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molecular dynamics. Amazing combinations of simulation, graphical modeling, and of single-molecule FRET experiments have in recent years led to fantastic contributions to improve our understanding of molecular crowding and of intrinsically unfolded proteins.

Biosensors play an increasing role in many fields, and for basic and applied biology they are indispensable. Exciting new developments are happening in the exploitation of plasmonics, e.g., single gold nanorods used for background free imaging in cells or metal-induced FRET on surfaces. Chemical design strategies for small molecules, peptides, and proteins are lined up for integration with very bright tracers such as quantum dots to resolve difficult to resolve signals such as voltage changes in membranes. In general, it looks like the increased funding of basic neuroscience research, due to the pressing burden of Alzheimer's, Parkinson's, and other neurodegenerative diseases, with several big initiatives happening around the world has stimulated science in SRT, and biosensing, with more scientists combining these techniques.

It is noteworthy that during the last few years SRT and single-molecule techniques have been getting more accessible and cheaper, with some instruments achievable for as little as $20,000. Also, SRT for standard wide field microscopes were developed, such as super-resolution optical fluctuation imaging (SOFI); other techniques have the highest probability of making an impact in high-content screening. Multicolor applications matured during recent years, and for this purpose brighter and more photostable dyes were developed. From a physical and engineering development period, the field has now moved toward addressing the chemical challenges needed to advance the science.

With so much progress made in the development and application of SRT and single-molecule techniques (SMT) as presented in this book, there is an area of translation science for which SMT held great promise, namely pharmaceutical drug screening, often referred to as high-throughput screening (HTS). Unfortunately, after years of investment into the most sophisticated integrated screening devices ever built, SMT are, to my knowledge, not broadly used for HTS these days. Despite the label "free affinity selection techniques," fluorescence is still the most used detection techniques for HTS. When fluorescence-based assays are applied for drug screening, the 384 well plate format is the preferred design, as it provides a good compromise between limited costs, due to low screening volumes of 20–50 μL, and relatively high throughput. However, interferences from surface interactions, fluorescent compounds, light-scattering particles, and inner-filter effects dramatically increase with assay volumes below 5 μl. After more than 20 years of technical development, groups in Sweden, Germany, and the US finally achieved the detection of single fluorescent molecules in a microscope. At the same time, combinatorial chemistry and function genomics were on the rise, methodologies that were assumed to dramatically increase the number of compounds and the number of drug targets. It was an obvious step to link these developments to more miniaturized screening technology. Fluorescence fluctuation analysis at single-molecule resolution was translated into the development of arguably the most integrated, most

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expensive, miniaturized target-based screening devices, called Mark-II and Mark-III by Evotec in a consortium that was first joined by Novartis, followed by GSK and finally Pfizer. The use of single-molecule detection (SMD), reduced the screening volume to ~ 0.25 femtoliter, the size of an *E.coli* bacterium, which allowed HTscreens to run with as little as 800 nl/well. In fluorescence fluctuation detection, the raw data acquired consist of a multichannel-scaler (MCS) trace, which is obtained by counting the number of detected photons in consecutive windows of constant size. This MCS can then be analyzed in different ways. For HTS, several methods for evaluated. FCS was first. In FCS, the correlation function of the MCS trace is computed. It decays with time constants characteristic of the molecular process causing the fluorescence change (e.g., diffusion of the fluorescent molecules through the detection volume). In fluorescence intensity distribution analysis (FIDA), a histogram of the signal amplitudes is built from the MCS trace. FIDA distinguishes the species in a sample according to their different fitted values of specific molecular brightness. More sophisticated variations of FIDA, such as 2D-FIDA, fluorescence intensity multiple distributions analysis (FIMDA), and fluorescence intensity and lifetime distribution analysis (FILDA) were developed over time and combined different fluorescent detection modes, in addition to fluctuation techniques; fluorescence lifetime methods, such as fluorescence lifetime analysis (FLA) and time-resolved fluorescence anisotropy analysis (TRA), were also adapted to confocal devices (cFLA and cTRA).

During the early years of assay development, the involved groups mainly adapted existing assays that had already been developed for standard fluorescence screening. In a period of around five years, this led to the development of brighter and more photostable dyes. FCS was the pioneering method in fluctuation analysis, and since its development (from adapting autocorrelation that had been invented for noise reduction in electronics), it has been established as an extremely successful method to research many different types of mechanistic analyses. However, FCS was effectively eliminated from pharmaceutical drug screening on account of the dependence of the translational diffusion time of a labeled reagent on the third root of its molecular weight over volume. Often, protein domains applied for a screen that binds to a labeled ligand, such as another protein, a peptide, or an oligo(ribo)nucleotide, which weigh 20–50 kDa. The difference in molecular weight is much too small to allow assays with sufficiently high Z′-values for HTS. One method, however, which fulfilled the expectations in "nanoscreening" – as fluorescence fluctuation analysis at single-molecule resolution was called – was FIDA, or photon counting histogram (PCH). This offered a variety of options for intensity-based techniques: 1D-FIDA, for detecting simple fluorescence emission changes; 2C-2D-FIDA, which allows two-color coincidences to be measured; and 2D-FIDA-anisotropy. The latter, which in essence comprises nothing more than ensemble average anisotropy measured with single-molecule sensitivity, was by far the most successful technique for HTScreens on the Evotec Mark-III instrument. The RNA-protein interaction assay described in PMID:17632515 was run with 848,384 compounds screened

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at ~ 800 nL per well, with 196 primary hits identified. Typically, on the Evotec Scarina Mark III, assay volumes were 0.9–1.6 μL volume/well in 2,080 well plates, 2.8–8 μL volume/well in 1,536 well plates, with throughputs of > 100,000 compounds/day run in non-confocal mode and around 70,000 cFLA run in the confocal detection mode. With such exceptional performance data, a reader new to the HTS field might ask: "Are all fluorescence-based screening campaigns now run on such exceptional instrumentation?" The disappointing answer is no: this technique has disappeared, and there are a several reasons for this. Firstly, a Mark-III device was a several million-dollar investment, and for running screens on this device, specialized teams needed to develop single-molecule assays, and the costs for maintaining these integrated pipetting, fluidics, and detection instruments are very high. One of the main arguments for the development of nanoscreening was the scope of running primary HTScreens not only with much higher throughput and at reduced costs, but also with strongly increased quality. "Quality" refers to the elimination of screening artifacts and increased mechanistic understanding of the mode of action of hit compounds. When it became clear that the outcome of a screen performed with 2D-FIDA-anisotropy detection was largely the same as that of an ensemble average assay, management in pharmaceutical companies made the unfortunate decision to cease nanoscreening.

And so, one of the most exciting periods in the development of technology for drug discovery had come to an end. Could this waste of investment and multidisciplinary effort have been avoided? While the insurgence of nanoscreening had seen success in designing, developing, and deploying screening hardware and software to pharma and a limited number of academic customers, an important, originally planned step was never attempted: the creation of a global analysis software capable of analyzing the major parameters that fluorescence has to offer as a result of the diffusion of a single molecular entity through the confocal focus of a microscope. It might be speculated that such a global analysis of translational and rotational diffusion, fluorescence intensity, fluorescence lifetime, colocalization, and cross-correlation might have delivered decision criteria for judging if a primary screening hit is an artifact, and if not, justified the decision to take it forward into a validation process. With all the new developments in SRT and SMT that have happened since then, and with such strong contributions to fundamental science being published on a daily basis, it can only be hoped that the next wave of integration of these methods into translational science will happen soon or is even happening already. The world needs new and better drugs, and not only to battle pandemics.

August 18, 2020

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Preface

The scientist only imposes two things, namely truth and sincerity, imposes them upon himself and upon other scientists.

Erwin Rudolf Josef Alexander Schrödinger, 1933 Nobel Prize–winning Austrian physicist (1887–1961), who discovered the principles of quantum mechanics

As the famous Austrian-British philosopher of science Karl R. Popper (1902–1994) believed, “before we can find the answers, we need the power to ask new questions, in other words, we need new technology.” A key example of such a technological advance is the recent development of the new field of *single-molecule science or single-molecule biology*. Single-molecule science is a new high-tech frontier that has emerged, which integrates disciplines such as cell biology, biophysics, and physiology. It also incorporates chemistry, physics, mathematics, and engineering, as well as approaches from biotechnology, nanotechnology, and nanofabrication. One can now observe how live cells divide, molecule by molecule, which has opened up the possibility of visualizing whole new worlds. Single-molecule techniques, among the most exciting tools available in biology today, offer powerful new ways to elucidate biological function, both in terms of revealing mechanisms of action on a molecular level as well as tracking the behavior of molecules in living cells.

The first experiment on optical single-molecule detection was carried out by Hirschfeld in 1976 to study multiply labeled antibody molecules. Later two German biophysicists, Erwin Neher and Bert Sakmann, demonstrated the detection of “currents from single ion channels in membranes,” for which they received the Nobel Prize in 1991. Subsequently, Steven Chu, a physicist (while at Bell Laboratories, presently at Stanford University) who won the Nobel Prize in 1997, developed a method to trap and manipulate individual atoms. Since then, many scientists, including William Moerner (Stanford University, USA), Eric Betzig (Janelia Farms, Howard Hughes Medical Institute, USA), Stefan Hell (Max Planck Institute for Biophysical Chemistry, Germany), Carlos Bustamante (University of California at Berkeley, USA), Xiaoliang Sunney Xie (Harvard University, USA and presently at Peking University, P.R. China), Andreas Engel

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(University of Basel, Switzerland), Xiaowei Zhuang (Harvard University, USA), Shimon Weiss (University of California Los Angeles, USA), and others, have worked to further develop the field of single-molecule biology, focusing particularly on the study of nucleic acids (DNA, RNA), enzymes (ribozymes), protein folding, molecular motors, cell signaling, chromatin dynamics, gene expression, and real-time visualization of ribosome movements. In fact, in 2014, Moerner, Betzig, and Hell received the Nobel Prize in chemistry, "for their work on developing super-resolved fluorescence microscopy."

Over the last two decades, the scientific community has witnessed several breakthroughs in the field of *single-molecule science*. Monitoring the action of biomolecules live, including molecular motors, enzymes, ribosomes, proteins, and nucleic acids, can be challenging for both biochemists and molecular biologists. It is clear that the biochemistry of DNA transcription, DNA/RNA replication, and protein translation are very well understood. In contrast, how the molecular events take place in the cell and how one can measure and visualize these molecular motions in dynamic state are less understood.

Single-Molecule Science: From Super-Resolution Microscopy, Molecular Imaging to DNA Mapping and Diagnostics is intended for those working in the fields of genetic engineering, molecular imaging, molecular agriculture, stem cell biology, biotechnology, genetics, genomics, pharmacogenomics, and molecular medicine. There are a number of books already available covering single-molecule sciences, biophysics, or techniques. They include P. Selvin and T. Ha, (2008), *Single Molecule Techniques: A Laboratory Manual,* Cold Spring Harbor Laboratory Press; A. Knight (2009), *Single Molecule Biology,* Elsevier's Academic Press; P. Hinterdorf and A. van Oijen (2009), *Handbook of Single Molecule Biophysics,* Springer Business Media Press; A. Graslund, R. Rigler, and J. Widengren (2009), *Single-Molecule Fluorescence Spectroscopy in Chemistry, Physics and Biology: A Nobel Symposium,* Springer Series in Chemical Physics; T. Komatsuzaki, M. Kawakami, S. Takahashi, H. Yang, and R. Silbey (2011), *Single Molecule Biophysics: Experiment and Theory,* John Wiley Press; S. Lindstrom and H. Andersson-Svahn (2012), *Single Cell Analysis: Methods and Protocols,* Springer Business Media Press; M. Leake (2013), *Single Molecule Biophysics,* Cambridge University Press; D. Makarov (2015), *Single Molecule Science: Physical Principles and Models,* CRC Press; J. P. Robinson and A. Cossarizza (2017), *Single Cell Analysis: Contemporary Research and Clinical Applications,* Springer Press; and A. Kapanidis and M. Heilemann (2018), *Single-Molecule Fluorescence Spectroscopy of Molecular Machines*, World Scientific Press.

Most of these works focus primarily on methods and lab protocols, except the recent books by Makarov and by Kapanidis and Heilemann, which discuss details of the field from single-molecule biophysics/science and its technological breakthrough perspectives. This present book differs in that it is the first text completely devoted to combining super-resolution microscopy and molecular imaging in developing single-molecule optical maps and applications in protein folding. Special emphasis is given to highlight studies of DNA mapping for

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diagnostics purposes using atomic force microscopy, and single-molecule detection methods in the study of gene expression.

This volume explores the advent of optical single-molecule spectroscopy, and how atomic force microscopy has empowered novel experiments on individual biomolecules, opening up new frontiers in molecule and cell biology and leading to new theoretical approaches and insights. Single-molecule experiments have provided a fresh perspective on questions such as how proteins fold to specific conformations from highly heterogeneous structures, how signal transductions take place on the molecular level, and how proteins behave in membranes and living cells. This volume is designed to further contribute to the rapid development of single-molecule research. This book is filled with cutting-edge research reported in a cohesive manner not found elsewhere in the literature, and this serves as the perfect supplement to any advanced graduate class devoted to the study of biochemical physics.

The goal of this book is to serve as a reference for graduate students, postdoctoral researchers, and primary investigators, as well as provide an explanatory analysis for executives and scientists in molecular medicine, molecular imaging, biotechnology, and pharmaceutical companies. Our hope is that this volume will serve as a prologue to the field for both newcomers and as a reference for those already active in the field. Most importantly, this book serves as a bridge between the basic science of microscopy and imaging and its diverse applications in areas such as agriculture and, biomedicine. The chapters listed in the present volume discuss insights that have been revealed about mechanisms, structures, or function by single-molecule techniques. Many topics are covered in this text, including enzymes, motor proteins, membrane channels, DNA, and other key molecules of current interest. An introduction by the editors provides a brief review of the key principles along with a historical overview. The final part of the introduction provides a discussion of future perspectives, including the latest applications and the relevance of single-molecule biology techniques to molecular diagnostics and drug discovery.

Many people have contributed to making our involvement in this project possible. We thank our teachers for their guidance and mentorship, as well as excellent teaching, which have helped us to bring about this educational enterprise. We are extremely grateful to all of the contributors, without whose commitment this project would not have been possible. Many people have had a hand in the preparation of this book. Each chapter has been passed back and forth between the authors for criticism and revision; hence each chapter represents a joint contribution. We thank our reviewers, who have made the hours spent putting together this volume worthwhile. We are indebted to the staff of Cambridge University Press, and in particular to Katrina Halliday for her generosity and efficiency throughout the editing of this book; she truly understands the urgency and need for this volume. We also extend our appreciation to Alexandra Serocka and Samuel Fearnley for their excellent cooperation during the development of this volume. We want to thank Professor Manfred Auer, an authority in the fields of drug screening and translational biology from

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This book is the tenth in the series of *Gene Expression and Regulation* that we have worked on and the fifth joint project between father and son. A portion of the royalties will be contributed to the Dr. Appasani Foundation (a nonprofit organization devoted to bringing social change through the education of youth in developing nations) and The MINDS Foundation (**M**ental **I**llness and **N**eurological **D**isorder**s**), which is committed to taking a grassroots approach to providing high-quality mental healthcare in rural India.

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