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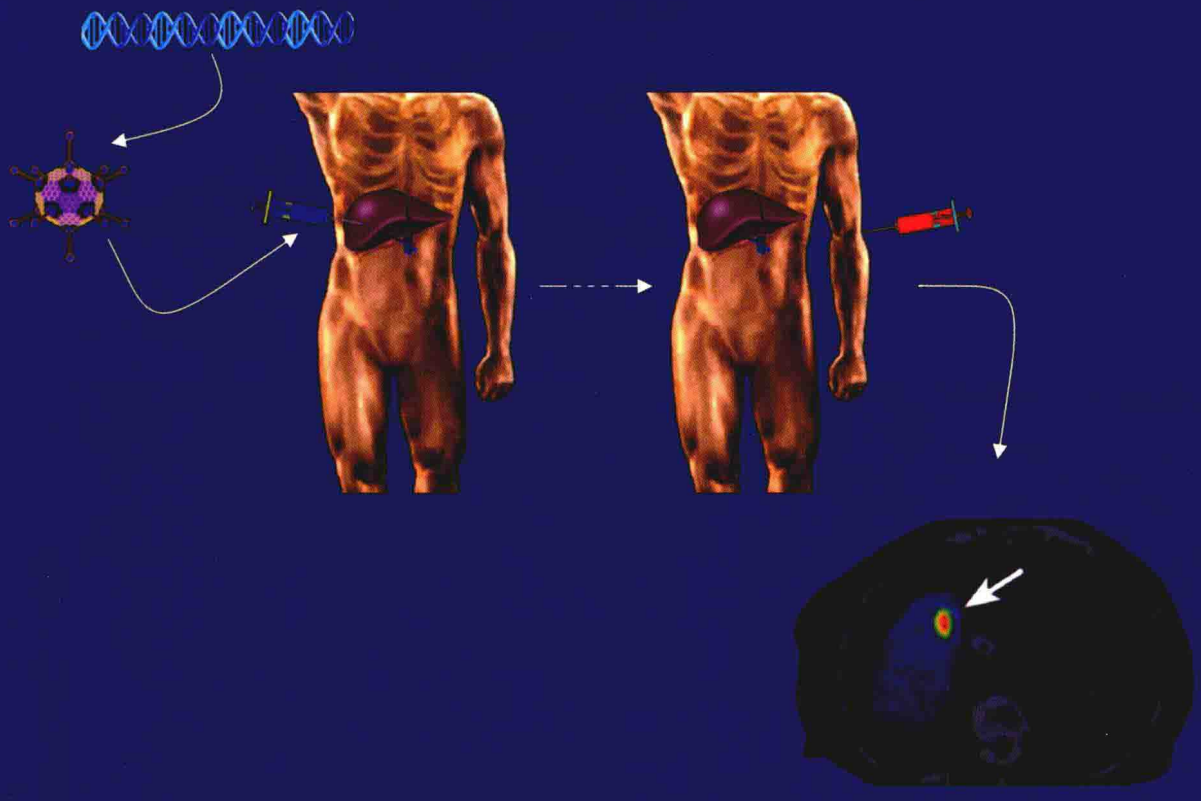
MOLECULAR IMAGING WITH REPORTER GENES

Edited by

Sanjiv Sam Gambhir · Shahriar S. Yaghoubi



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Molecular Imaging with Reporter Genes

Reporter genes have been used for several decades to study intracellular molecular events, such as regulation of gene expression *in vitro*. However, it was little more than a decade ago that a new class of reporter genes was developed for imaging intracellular and intercellular molecular events within living subjects. Studying molecular biology and medicine in the intact and natural environment within living research subjects will enable better translation of knowledge for use in humans. In fact, reporter gene imaging is now possible in humans. This book focuses on this group of imaging reporter genes, starting with detailed descriptions of all reporter genes from different imaging modalities, including optical, MRI, and radionuclide-based imaging. This is followed by a chapter describing all instrumentation used in molecular imaging. Key scientists in the field then explain various applications of the reporter gene imaging technologies.

This is the first comprehensive book on all aspects of reporter gene imaging, detailing what is known in the field and future goals for research. Reporter gene imaging plays a role in basic biomedical science research, in the development of novel therapeutic agents and will soon be an important part of clinical molecular imaging. Therefore, this book will benefit biomedical scientists of all fields in both academia and pharmaceutical/biotechnology industries, assist regulators in forming better policies for assessing safety and efficacy of novel therapeutic agents, and inform physicians of a new molecular imaging tool that will in the near future have clinical applications.

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Preface

Multimodality molecular imaging is a combination of imaging strategies that are playing an increasing role in all biological, biomedical, and clinical fields. Molecular imaging can be used to study a whole variety of molecular events in cells, tissues, organs, and the whole body of living organisms. This includes detecting and measuring the levels of mRNA, proteins, enzymes, and protein-protein interactions. Additionally, molecular imaging can be used to detect intracellular metabolic events, the presence and quantity of specific cells within tissues, and changes in cell characteristics through time. Adding to the power of molecular imaging is the fact that many of these techniques can be applied non-invasively in living subjects, allowing repetitive interrogation of molecular events within intact systems.

Reporter genes are among the most powerful tools in molecular imaging. They were originally introduced several decades ago for studying biochemical events *in vitro* including cell/tissue lysates. Later, their use advanced to optical imaging of molecular events within intact cultured cells using microscopes. It was in the early 1990s that imaging reporter genes of several types were developed for non-invasive molecular imaging in *living* subjects. Imaging reporter genes are general tools for imaging gene expression, protein function, protein-protein interactions, and a variety of other molecular events, repetitively and usually non-invasively within living organisms, including humans. Besides their applications in biological research, they have many biomedical applications, including disease diagnosis and optimization of therapeutics.

This is the first book dedicated to teaching all aspects of multimodality molecular imaging of reporter genes. Imaging reporter genes are now available for optical, magnetic resonance, and radionuclide-based imaging systems. We have gathered international experts in the field to detail reporter gene systems of all imaging modalities (Chapters 1–4). In Chapters 5–6, leading scientists describe available techniques for enhancing the power of imaging reporter genes through multimodality imaging and gene expression amplification strategies. There is also a dedicated chapter on instrumentation for reporter gene imaging in animal models and humans (Chapter 7). Chapters 8–12 were written by molecular imaging experts who have worked extensively to develop many of the current applications of imaging reporter genes. Finally, the book concludes with Chapter 13, which discusses the current progress using imaging reporter genes in cell and gene therapy clinical trials.

This book should help investigators in all biomedical fields to learn about imaging techniques based on reporter genes that are essential for the study of molecular events within intact living systems. We also hope that this knowledge will lead to the development of new applications for imaging reporter genes. Graduate and medical students in all biological and medical fields will also benefit from learning about the technologies described in this book. Investigators in biotechnology and pharmaceutical firms will be able to use the technologies described in this book in their search to optimize novel therapeutics. Finally, clinical investigators, especially in regenerative medicine, cell therapy, and gene therapy will gain tremendous benefits from the knowledge of imaging reporter genes and their applications in clinical therapeutics.

Contents

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PART

I

Types of Imaging Reporter Genes

1 Fluorescent Reporter Proteins

Robert E. Campbell and Michael W. Davidson

INTRODUCTION

For more than a decade the growing class of fluorescent proteins (FPs) defined as homologues of *Aequorea victoria* green FP (avGFP), which are capable of forming an intrinsic chromophore, has almost single-handedly launched and fueled a new era in cell biology. These powerful research tools provide investigators with a means of fusing a genetically encoded optical probe to any one of a practically unlimited variety of protein targets to examine living systems using fluorescence microscopy and related methodology (see Figure 1.1; for recent reviews, see references [1–4]). The diverse array of practical applications for FPs ranges from targeted markers for organelles and other subcellular structures, to protein fusions designed to monitor mobility and dynamics, to reporters of transcriptional regulation (Figure 1.2). FPs have also opened the door to creating highly specific biosensors for live-cell imaging of numerous intracellular phenomena, including pH and ion concentration fluctuations, protein kinase activity, apoptosis, voltage, cyclic nucleotide signaling, and tracing neuronal pathways [5–9]. In addition, by applying selected promoters and targeting signals, FP biosensors can be introduced into an intact organism and directed to specific tissues, cell types, and subcellular compartments to enable monitoring a variety of physiological processes using fluorescence resonance energy transfer (FRET) techniques.

If FPs are the "fuel" for the live-cell imaging revolution, the "engines" are the technical advances in widefield fluorescence and confocal microscopes. Some notable advances include low light level digital charge coupled device (CCD) cameras as well as spinning-disk and swept-field instruments. As of today, avGFP and its color-shifted variants, in conjunction with sophisticated imaging equipment, have demonstrated invaluable service in many thousands of live-cell imaging experiments. One of the most important features of FPs is that they are minimally invasive for living cells, especially compared to many traditional synthetic fluorophores (that are often toxic or photoreactive). The relatively low or nonexistent toxicity of FPs (when expressed at low levels relative to endogenous proteins) permits visualization and recording of time-lapse image sequences for extended periods of time [10, 11]. As we will discuss in this chapter, continued advances in FP engineering technology have enabled the fine-tuning of critical fluorescent imaging parameters, including brightness, spectral profiles, photostability, maturation time, and pH insensitivity, to provide a stream of new and advanced probes for optical microscopy. These structural and functional enhancements have stimulated a wide variety of investigations into protein dynamics and function using FP chimeras imaged at low light intensities for many hours to extract valuable biochemical information.

Today we take the exceptional and revolutionary utility of FPs for granted, and it may be hard for some researchers to imagine research without them. It is therefore somewhat surprising that more than 30 years had to pass between the first scientific report of the isolation of avGFP [12] and its first application as a tool for biological imaging [13]. The first report of fluorescence in the bioluminescent hydrozoan jellyfish species *Aequorea victoria* was recorded more than 60 years ago [14] and a protein extract was independently demonstrated by two investigators to be responsible for this "green" fluorescence in the 1960s and 1970s [12, 15]. It took several more decades to identify the responsible protein, clone the gene encoding the protein, and elucidate the primary amino acid structure [16]. In light of the time span between the original discovery and cloning of avGFP, it is rather remarkable that only 2 years later, an image revealing the fluorescent sensory neurons of the nematode highlighted with the same jellyfish protein was featured on the cover of the journal *Science* [13]. This landmark event unambiguously demonstrated the utility of avGFP as a genetic marker in cells evolutionarily far removed from hydrozoans and ushered in a new

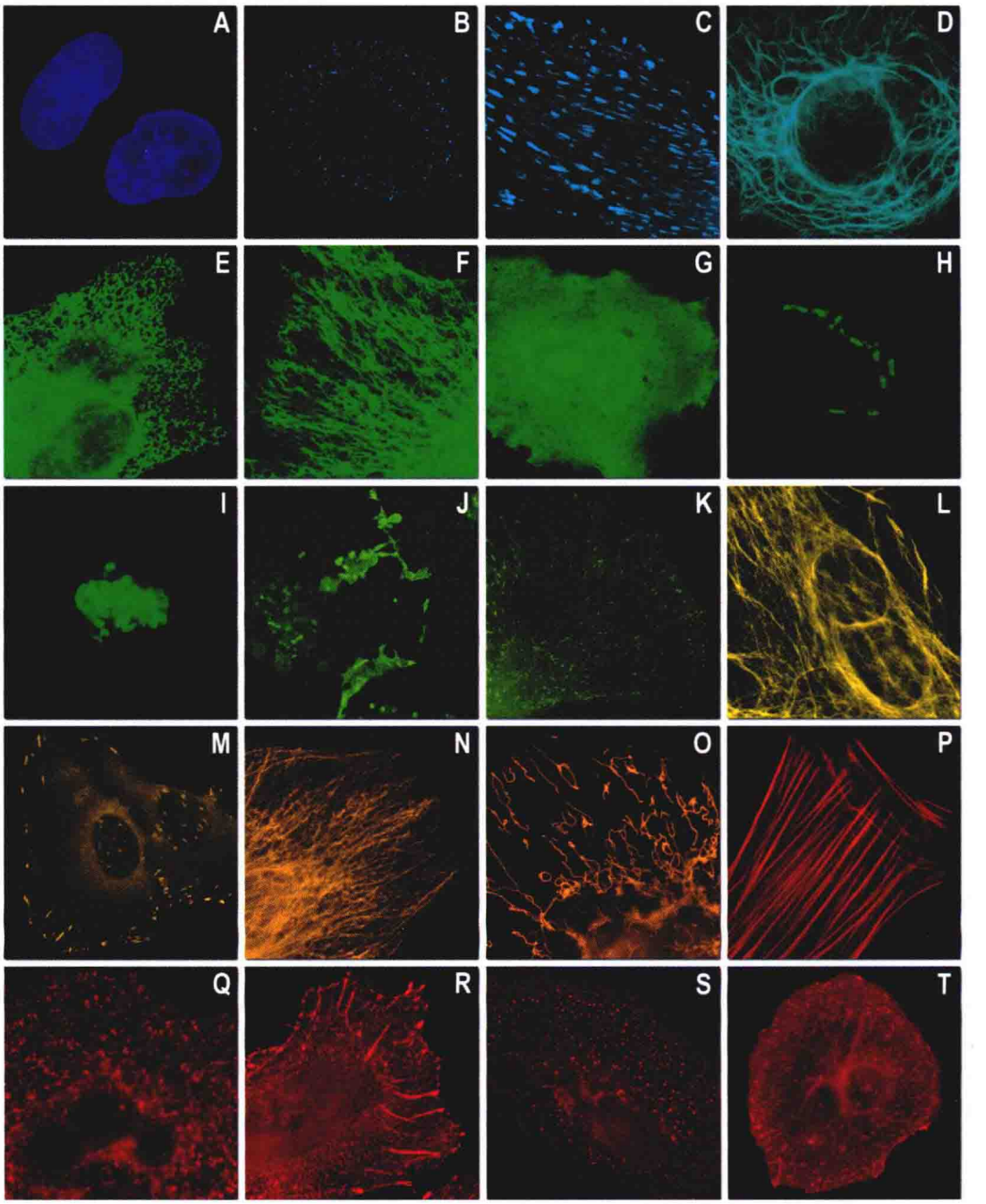

Figure 1.1. Subcellular localization of selected FP fusions (listed in Table 1.1) with targeting proteins imaged in widefield fluorescence. Images are pseudocolored to match the FP emission profile. The FP fusion terminus and number of linker amino acids is indicated after the name of the targeted organelle or fusion protein. The fusion protein and host cell line is given in parentheses (**A**) EBFP2-lamin-B1-N-10 (human lamin B1; nuclear envelope; HeLa); (**B**) ECFP-peroxisomes-C-2 (peroximal targeting signal 1; PTS1; HeLa); (**C**) mCerulean-vinculin-C-23 (human; focal adhesions; Fox Lung); (**D**) mTFP1-keratin-N-17 (human cytokeratin 18; intermediate filaments; HeLa); (**E**) EGFP-endoplasmic reticulum-N-3 (calreticulin signal sequence and KDEL retention sequence; HeLa); (**F**) mEmerald-vimentin-N-7 (human vimentin; intermediate filaments; HeLa); (**G**) mAzami Green-N1 (cloning vector; whole cell fluorescence; HeLa); (**H**) Superfolder avGFP-Golgi-N-7 (N-terminal 81 amino acids of human β-1,4-glactosyltransferase; Golgi complex; HeLa); (**I**) mT-Sapphire-H2B-N-6 (human histone H2B; metaphase; HeLa); (**J**) mVenus-Cx43-N-7 (rat α-1 connexin-43; gap junctions; HeLa); (**K**) YPet-EB3-N-7 (human microtubule-associated protein; RP/EB family; Fox Lung); (**L**) mKusabira Orange-vimentin-N-7 (human; intermediate filaments; Opossum Kidney); (**M**) tdTomato-paxillin-N-22 (chicken; focal adhesions; Fox Lung); (**N**) TagRFP-tubulin-C-6 (human α-tubulin; microtubules; HeLa); (**O**) DsRed2-mitochondria-N-7 (human cytochrome C oxidase subunit VIII; mitochondria; HeLa); (**P**) mStrawberry-actin-C-7 (human β-actin; filamentous actin; Fox Lung); (**Q**) mRFP1-lysosomes-C-20 (rat lysosomal membrane glycoprotein 1; HeLa); (**R**) mCherry-α-actinin-N-19 (human nonmuscle; cytoskeleton; HeLa); (**S**) mKate-clathrin light chain-C-15 (human; clathrin vesicles; HeLa); (**T**) mPlum-farnesyl-C-5 (20-amino acid farnesylation signal from c-Ha-Ras; plasma membrane; HeLa).

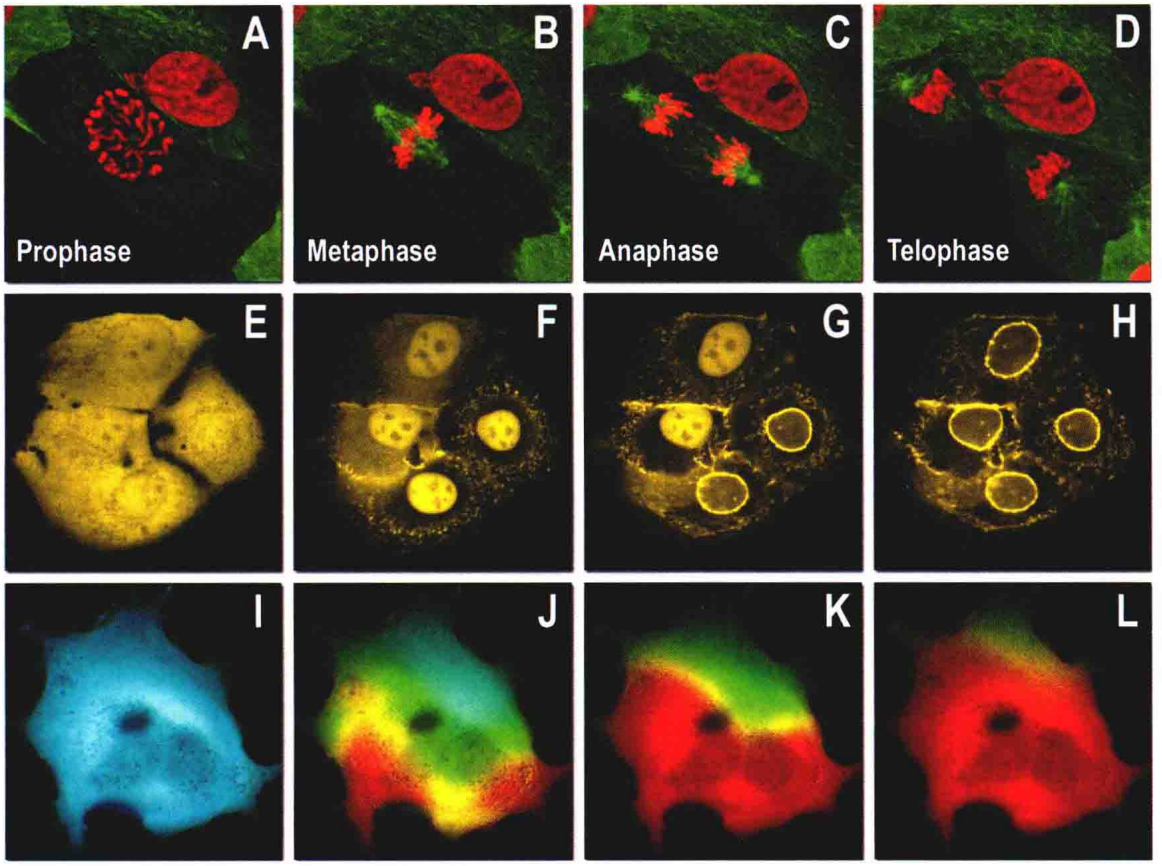

Figure 1.2. Fluorescent protein reporters in action imaged with spinning disk confocal and widefield microscopy. **A–D**: Observing mitosis in dual-labeled normal pig kidney (LLC-PK1 cell line) epithelial cells stably expressing mCherry-H2B-N-6 (histones) and mEmerald-EB3-N-7 (microtubule + end binding protein), (**A**) A cell in prophase (lower) is captured adjacent to a cell in interphase, t = 0; (**B**) The lower cell forms a spindle and enters metaphase. Note the EB3 patterns emanating from the spindle poles and traversing to the plane, t = 20 min; (**C**) During anaphase, the spindle poles translocate to opposite sides of the cell, pulling the condensed chromosomes along, t = 60 min; (**D**) The chromosomes begin to decondense during telophase as the daughter cells recover from cell division (midbody not visible). **E–H**: Spinning disk confocal images selected from a time-lapse series of human cervical adenocarcinoma (HeLa cell line) epithelial cells expressing mKusabira Orange-annexin (A4)-C-12 during ionomycin-induced translocation to the plasma and nuclear membranes, (**E**) A cluster of four cells exhibits expression of the chimera throughout the nucleus and cytoplasm t = 0, ionomycin (10 μM) added; (**F**) Shortly after addition of ionomycin, the annexin chimera begins to translocate to the plasma membrane, clearly revealing the nuclei, t = 3 min; (**G**) The annexin chimera migrates to the membrane in two of the nuclei, time = 5 min; (**H**) The nuclear membranes of all four nuclei display translocated annexin chimera, time = 7 min. (**I–L**) Widefield fluorescence calcium imaging in the cytosol of HeLa cells expressing the circularly permuted cameleon YC3.60; (**I**) Real color image of a single cell, t = 0, histamine (10 μM) added; (**J**) Pseudo-colored ratio image of the HeLa cell as a calcium wave initiates at the two loci on the membrane, t = 10 sec; (**K**) The calcium wave propagates through the cytoplasm, t = 10.5 sec; (**L**) The calcium wave reaches the distant portion of the cell, t = 11.0 sec.

era in biological fluorescence imaging. Through the mid-1990s, a number of genetic variants of the original avGFP nucleotide sequence were developed that featured enhanced green fluorescence (EGFP) [17] and altered fluorescence emission spectral profiles in the blue (BFP) [18, 19], cyan (CFP) [20], and yellow (YFP) [21] regions of the visible spectrum. Perhaps the single most significant advance following the initial cloning and early mutagenesis efforts on wild-type avGFP was the discovery of cyan, green, yellow, orange, and red-fluorescing avGFP homologues in nonbioluminescent reef corals and sea anemones [22]. This discovery not only provided a source of new FPs with new emission colors but also demonstrated that this protein motif can potentially occur in a wide range of classes and species.

FPs have now been discovered in organisms ranging from marine invertebrates to crustaceans and probably exist in many other species [23–26]. In fact, a protein known as nidogen [27], found tucked away in basement membrane of all mammals, has been characterized to have a domain consisting of an 11-stranded β-barrel remarkably similar to the three-dimensional structure of avGFP, despite having only 10% sequence homology.