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Preface

Biological confocal microscopy is still a relatively young field. Most researchers in the field would date the modern era of biological confocal microscopy from the 1985 description of a particularly useful confocal design published by White and Amos in the Journal of Cell Biology. Since that time, the use of confocal microscopes by biologists has increased phenomenally, with new converts joining the ranks daily; many with little or no previous microscopy training. For this reason, in 2001 when we were asked to organize a 1 day session on "basic confocal microscopy" for attendees at the Southeastern Microscopy Society annual meeting in Clemson, SC, we decided to focus not only on the confocal microscope itself, but also on ancillary subjects that are critical for getting the most from confocal microscopy.

Our initial effort seemed to meet a growing need to train new students, technologists, and faculty wishing to use confocal microscopy in their research. Evidence for this need is that each year since 2001, we have been invited by several meeting organizers and microscopy core facility Directors to present our take on what is important to use confocal microscopy successfully for biological exploration. In 2005, we also began teaching a 5-day intensive, hands-on workshop at the University of South Carolina each year. As that course evolved, we invited various colleagues to help with the course. This book is a direct outgrowth of that course and follows the general structure of the didactic portion of the course. In line with the course philosophy, we have not attempted to cover each topic in depth. However, we have maintained a focus on basic information and we have endeavored to cover information that is important for designing, carrying out, and interpreting the results of basic confocal microscopy-based biological experiments completely. We were very fortunate that two of the other course instructors, Drs. Ralph Albrecht and Tom Trusk, have provided chapters for this volume and have embraced the overall philosophy of presenting a basic knowledge base in a complete but concise manner.

Although the forums have been different and the course lengths have varied anywhere from 1 to 5 days, we have always based the workshops on the original concept that there is a group of core issues that must be understood before one can efficiently get the best results from the use of a confocal microscope. The early chapters in this book address these core issues and it is not by accident that after an initial introductory chapter on confocal microscopy, the chapters describing the components of the confocal microscope and how to set the various operating

parameters correctly are located toward the end of the book. Without a well-designed research plan and properly prepared specimen, the data collected by the microscope will not be optimum. Thus, we have devoted Chaps. 2 and 3 to fluorescence and understanding the use of fluorescent microscopy, and Chaps. 4 and 5 to specimen preparation and labeling strategies. These chapters are essential since regardless of the quality of the confocal microscope, if the sample is not prepared properly, the data collected will not be optimal.

Most modern confocal microscope images are digital. Thus, many of the basic operating parameters for confocal microscopy involve setting up the analog to digital conversion of specimen information. It is essential that a confocal microscope operator have a thorough understanding of how digital images for scientific purposes should be collected and analyzed. For this reason, following the chapters on specimen preparation, Chaps. 6 and 7 discuss digital microscopy with respect to confocal imaging.

Although it might seem odd that a book on confocal microscopy contains only two chapters directly devoted to the actual operation of the confocal microscope, these chapters are packed with practical information and, taking advantage of the preliminary information presented in preceding chapters, they provide all that is necessary to begin doing confocal microscopy and optimizing the information obtained. After Chaps. 8 and 9, which discuss the types of confocal instruments and setting up proper operating parameters, the final set of chapters provide information on the 3D analysis and reconstruction of data sets and some ethical considerations in confocal imaging, and provide some resources that we have found useful in our own use of confocal microscopes. After mastering the basic information presented in this book, these resources are great guides for continuing your education into more advanced forms of confocal microscopy.

This book has benefited from our association with numerous colleagues who have challenged and informed us. In particular, numerous debates with one of the course instructors, Dr. John MacKenzie, Jr., have helped hone the information on digital image processing to the most important concepts. We are also grateful to Drs. K. Sam Wells, David Piston, and John Fuseler for stimulating and challenging conversations that have made us better microscopists. We also owe a huge debt to the many students over the years whose enthusiasm and questions have guided our decisions regarding what to include and exclude from the workshops and chapters in this book. We are also thankful to the many companies that have provided resources and applications experts who have significantly enhanced our hands-on workshops at the University of South Carolina.

Finally, we must thank our lab members and families for not only putting up with our obsession for microscopy but also encouraging us in our pursuits.

Columbia, SC Robert L. Price
Nashville, TN W. Gray (Jay) Jerome

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AOBS	Acousto-optical beam splitter
AOTF	Acousto-optical tunable filter
A to D	Analog-to-digital conversion
AVI	Audio-video interleave
BFP	Blue fluorescent protein
CCD camera	Charge-coupled device camera
CDRs	Complementarity-determining regions
CFP	Cyan fluorescent protein
CLAHE	Contrast limited adaptive histogram equalization
CMOS	Complementary metal oxide semiconductor
CMYK	Cyan, magenta, yellow, and black images
CSLM	Confocal scanning laser microscope
CTF	Contrast transfer function
Cy	Cyanine
DABCO	1,4-Diazabicyclo[2,2,2]octane
DIC	Differential interference contrast
DPI	Dots per inch
EMCCD	Electron-multiplied charge-coupled device
EGFP	Enhanced green fluorescent protein
FITC	Fluorescein isothiocyanate
FLIM	Fluorescent lifetime imaging
FRAP	Fluorescent recovery after photobleaching
FRET	Förster resonant energy transfer
FWHM	Full-width half maximum
GFP	Green fluorescent protein
HeNe	Helium–neon laser

IgA	Immunoglobulin class A
IgD	Immunoglobulin class D
IgE	Immunoglobulin class E
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IR	Infrared
JPEG	Joint photographic experts group
LASER	Light amplification by stimulated emission of radiation
LED	Light-emitting diode
LM	Light microscopy
LUTs	Look-up tables
MPEG	Moving picture experts group
MSDS	Material safety data sheet
NAD(H)	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NA	Numerical aperture
NPG	*n*-propyl gallate
PEG	Polyethylene glycol
PerCP	Peridinin-chlorophyll protein
PDF	Portable document format
PMT	Photomultiplier tube
PPD	*p*-Phenylenediamine
PSF	Point-spread function
PPI	Pixels per inch
RESEL	Resolvable element
RFP	Red fluorescent protein
RGB	Red, green, and blue images
RGBA	Red, green, blue, alpha images
ROI	Region of interest
RSOM	Real-time scanning optical microscope
scFv	Single-chain variable fragment
TEM	Transmission electron microscopy
Tf	Transferrin
TIF(F)	Tagged image file format
TRITC	Tetramethylrhodamine-isothiocyanate
TSRLM	Tandem scanning reflected light microscope
UV	Ultraviolet
VaLaP	Vaseline, lanolin, and petroleum jelly
V_H	Variable heavy chain
V_L	Variable light chain
WGA	Wheat germ agglutinin
YFP	Yellow fluorescent protein

Contents

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Chapter 1
Introduction and Historical Perspective

Robert L. Price and W. Gray (Jay) Jerome

Keywords Dichroic • Gamma • Refractive index mismatch • Resolution • Signal-to-noise ratio

1.1 Why an Introductory Text on Confocal Microscopy?

During our combined 35 plus years of operating confocal microscopes and managing core microscopy facilities, and through teaching our Basic Confocal Microscopy Workshop at several venues, we have found that students and technicians who are novice users of confocal microscopes are often instructed by their mentors to go to the confocal system and collect some images. Often the implied message is that it should be easy and quick since it is only a microscope. Unfortunately, all too often the advisor of the student or supervisor of the technician does not have a full understanding of the complexity of a confocal microscope. Unless these novice users are in a situation where others have the time and knowledge to properly train them, their initial efforts often amount to an exercise in futility because key parameters are not properly considered. This leads to specimens that are not prepared properly and a lack of understanding of how to operate the confocal microscope in a way that maintains the fidelity of the specimen information. In too many instances, this lack of user training is exacerbated further because there is little or no daily oversight of the setup and maintenance of the microscope. In this combined scenario, neither the experimental preparation nor the microscopes are capable of producing the highest quality information.

Good confocal microscopy is obviously dependent upon proper specimen preparation and the correct setup of various microscope parameters. However, even if an excellent confocal image is collected there is often a poor understanding of how to

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properly enhance and analyze two-dimensional (2-D) and 3-D confocal images. There is an abundance of good image processing and analysis software available to the user. However, these robust programs also provide the capability of inadvertently degrading the image information. A lack of understanding of basic digital imaging and image processing theory frequently results in improper image processing in 2-D programs, such as Image J (NIH freeware), Photoshop (Adobe Systems, Inc., San Jose, CA), Metamorph (Molecular Devices, Sunnyvale, CA), or others and in more advanced 3-D volumetric programs such as AMIRA (Visage Imaging, Carlsbad, CA) or Voxblast (VayTek, Inc., Fairfield, IA).

The goal of this book is to provide beginning and intermediate users of confocal microscopes a resource that can be used to address many of the frequently asked questions concerning confocal imaging and to provide a strong foundation for maximizing the data obtained from experiments involving confocal microscopy. While most of the information is directly relevant to single photon scanning laser systems, much of the information also applies to spinning disk and multiphoton confocal systems. In several chapters, specific comparisons of the technology that differentiates these systems will be made and advantages and disadvantages of each will be presented. The information presented will also provide the background information necessary when moving forward to complex imaging protocols such as Forster (or Fluorescent) resonant energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), fluorescent lifetime imaging (FLIM), and other advanced techniques.

1.2 Historical Perspective

It has long been recognized by microscopists that as the thickness of the specimen increases, light emerging from scattering objects above and below the focal plane of the microscope degrade the quality of the image. This occurs primarily because of reduced image contrast. The loss of contrast is caused by impinging light produced from the out-of-focus planes. Like turning on the lights in a movie theater, this stray light reduces the signal-to-noise (S/N) ratio and obscures important image details. The various factors affecting the axial resolution (ability to distinguish two small objects as separate and distinct along the axial axis) were explored by Berek in 1927. In Berek's analysis, the three key elements affecting image quality were (1) spreading of the light beam emerging from objects in the specimen, (2) the magnification of the image, and (3) the sensitivity of the detection system. For Berek, the detection system was the observer's eye. However, in the modern age of microscopy the eye has been replaced with more sensitive detectors. With regard to Berek's item 2, microscopists have always worked with the highest magnification required for maintaining image data fidelity. This leaves the spread of out-of-focus light into the image plane as the last of Berek's parameters that needs to be minimized to obtain good axial resolution. Obviously, if one could limit the projection of out-of-focus light onto the image then a significant gain in resolution should be achieved.

The removal of the obscuring out-of-focus light is precisely what the confocal microscope is designed to do and the subsequent gain in axial resolution remains the biggest advantage of confocal microscopy. However, as described in subsequent chapters, several other advantages accrue from the confocal design, including increases in lateral resolution.

The first confocal microscope is generally credited to Marvin Minsky (1988). In his 1957 patent application, Minsky described a microscope in which the typical widefield illumination arrangement is replaced with one in which a point source is focused to a small spot within the specimen. Light arising from the illuminated spot is focused by the objective lens to a small spot at the image plane. Thus, a point source of light is in conjugate focus (confocal) at the specimen and at the image plane (Fig. 1.1a). Placing a small pinhole aperture made of an opaque material at the image plane permits only the light coming from the focal point of the specimen to pass to the detector. In contrast, light coming from above and below the plane of focus will not be in focus at the image plane and will be rejected by the opaque material surrounding the pinhole. This confocal setup can also be achieved in an epi-illumination setup (Fig. 1.1b). The confocal arrangement dramatically improves contrast by removing the out-of-focus light originating above and below the focal plane. The arrangements diagramed in Fig. 1.1 are not the only possible designs. Since its inception, various other designs have been introduced for creating the required confocality of focus at the specimen and image planes.

Of course, a single point within a specimen does not provide much information about the specimen. In order to acquire full details across the lateral focal plane of the specimen, the spot must be scanned across the image and the image information collected sequentially. In Minsky's original design, the scanning was produced by translating the specimen laterally. This method was slow and prone to vibration, both of which presented problems for biological work. A notable advance for the use of point scanning instruments in biology was made in the 1980s with the development of the ability to raster the illumination across the specimen rather than translating the stage. This allowed for faster scan rates without the introduction of vibration. The publication of images of biological samples using the beam-scanning instrument (White et al. 1987) spurred an extreme interest in confocal microscopy for biological research.

Arguably, the development of beam scanning along with concurrent advancements in laser technology, fluorescent labels, lens design, and computer processing really set the stage for the rapid deployment of laser scanning confocal microscopy as a key tool for cell biological research. However, laser scanning instruments are not the only mechanism for implementing confocal microscopy. A parallel development occurred based on Paul Nipkow's (1884) invention of a method for converting an optical image into an electrical signal that could be transmitted over a cable. Nipkow's technique converted the 2-D image information into a 1-D serial signal by scanning the image using a spinning wheel with precisely placed rectangular holes. The holes were arranged in a spiral pattern around the wheel such that when the wheel was spun the small areas being sampled changed. The moving holes filled in the gaps between the initially sampled regions. In 1967, Egger and Petráň

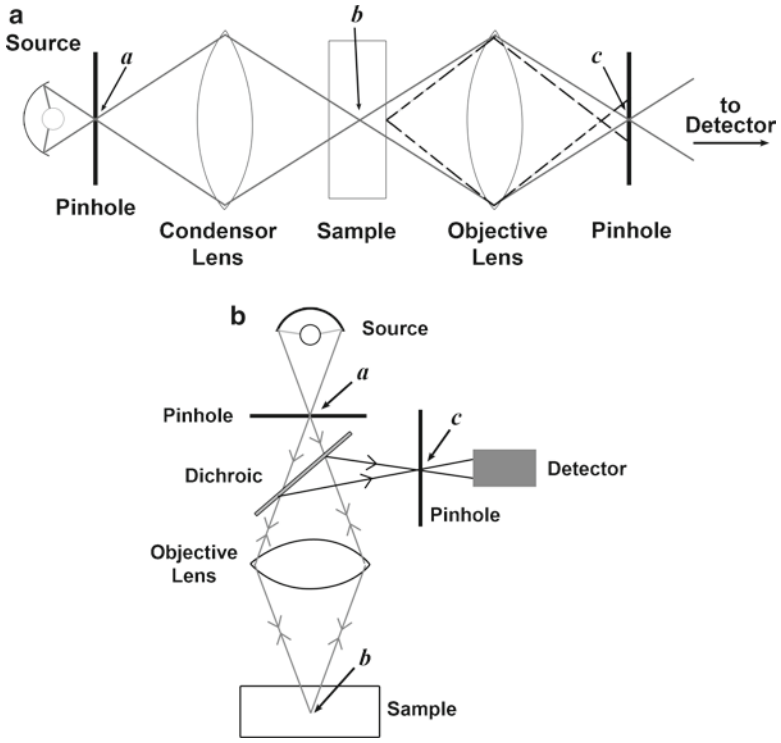

Fig. 1.1 Optical train for confocal microscope in conventional (**a**) and epi-illumination setups (**b**). The light path of the confocal beam is represented by the *gray lines*. In the conventional arrangement, light from the photon source is focused onto the entrance pinhole (***a***). This pinhole provides a bright focused point source. Light from this point source is collected by the condenser lens and focused to a spot (***b***) within the sample. The light emerging from the focused spot within the specimen is collected by the objective lens and focused at a second (exit) pinhole (***c***). Points a, b, and c are in conjugate focus (confocal). The path of light emerging outside of the focal point b is represented by the *dotted black lines* and arrives at the exit pinhole out of focus. Thus, most of this light is rejected and not transmitted to the detector. In an epi-illumination setup (**b**), the objective lens acts as both the condenser and objective lens. Light returning from the specimen is diverted by the dichroic (dichromatic beam splitter) and this diverted light (*dark gray lines*) is focused on the exit pinhole (*dark gray lines*). As with the conventional arrangement, light from above or below the focal point in the specimen arrives at the pinhole out of focus (not depicted) and so is rejected. Conventional wide field fluorescence systems lack the pinhole so all out-of-focus light becomes a component of the final image as shown in Fig. 1.3

(Petrán et al. 1968) modified the design of the Nipkow disk by including multiple spirals in a single wheel. They then used the spinning disk to provide both illuminating and imaging pinholes for a confocal microscope.

As with point scanning microscopes, over the years several different arrangements have been designed for spinning-disk confocal microscopes. Figure 1.2 illustrates one such arrangement for an epi-illumination system. In this design, light is passed through the pinholes, directed onto the specimen and the image light

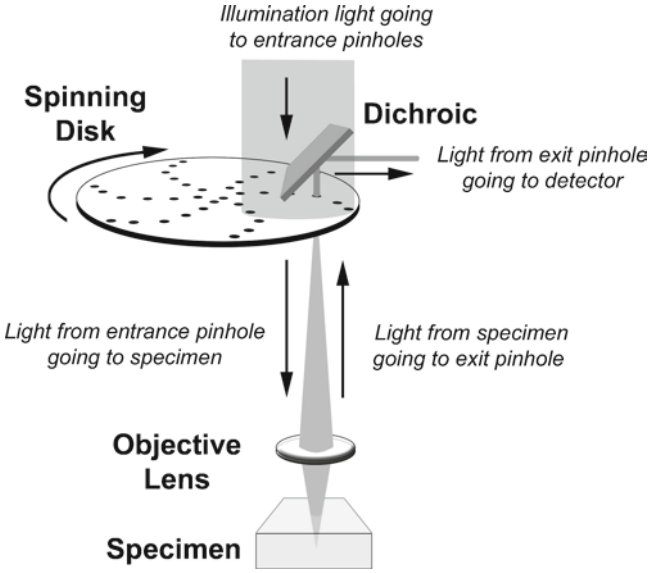

Fig. 1.2 Design of an epi-illumination spinning-disk confocal microscope. Although multiple areas of the specimen will be illuminated at once, to simplify the diagram only light from one pinhole is depicted. As in Fig. 1.1 only focused light reaches the detector. Since light emitted from all pinholes reaches the detector simultaneously image collection is rapid, but resolution and often overall signal is compromised in spinning-disk systems as discussed in Chap. 8

passes back through conjugate pinholes in the disk as it spins. By including sufficient numbers of pinholes and spinning the disk at a suitable speed, a real-time confocal image of the specimen can be obtained that can be viewed by eye or collected directly by a detector. One of the key benefits of this type of confocal microscope compared to laser scanning instruments is that spinning disks allow much faster image acquisition times. Further information on the design and use of spinning disk confocal systems is given in Chap. 8.

The Minsky and Petrán microscopes define the two principal implementations of confocal microscopy; the sequential scan (point scan) and spinning-disk (multipoint scan, area scan) microscopes, respectively. As one might imagine, however, variations on these two schemes have been designed to overcome specific limitations of each for specific applications. A nice review of some of these implementations is provided by Shinya Inoué (2006). Of course, the full power of imaging a thin plane within a specimen is best exploited by scanning multiple thin planes in succession and reconstructing a high-resolution 3-D map of the specimen by stacking the 2-D images. As described in Chaps. 6–10, key advances in digital imaging and improved computer power over the last two decades now provide a convenient method of capturing, storing, and displaying sequentially acquired image information in both 2-D and 3-D formats.

1.3 Is the Confocal Hype Legitimate?

Why has confocal microscopy revolutionized the way many laboratories image their samples? The simple answer is that the use of specific wavelengths of light, typically emitted from a laser, and the use of pinholes to eliminate out-of-focus light as described above, has significantly increased our ability to resolve and co-localize small structures and molecules in high contrast images. An example of this is shown in Fig. 1.3. Widefield images (Fig. 1.3a) contain large amounts of out-of-focus light that significantly deteriorates image resolution and contrast making it difficult to observe specific structures and detail. A confocal image (Fig. 1.3b) from the same region of the same sample clearly shows increased resolution and contrast making it much easier to discern the structures present in the section of heart muscle shown.

With the development of rapid computing capabilities and high density media for storage, confocal imaging technology grew rapidly. These advancements made it possible to collect a large number of optical sections through a sample and to rapidly reconstruct them into a high resolution high contrast projection of the sample where all detail was in focus (Fig. 1.4). Further advances in imaging software have made the use of 3-D data sets an important element in studying most biological systems. Many of these advances are discussed in subsequent chapters of this book. However, both confocal imaging hardware and digital imaging software technologies are advancing at a very rapid pace making it essential that researchers stay vigilant in determining how confocal imaging may benefit their individual research programs.

The answer to the above question about confocal hype is obviously a resounding yes. Even though commercially available systems have been available for less than 30 years, and well equipped confocal systems often cost $500K or more and can be expensive to maintain, the thousands of publications that utilize confocal imaging and the large range of applications from biological to material samples imaged clearly indicates that confocal microscopy has revolutionized the way many laboratories perform their research. Recent advances including spectral imaging, new fluorochromes and lasers, and increased imaging speed and resolution all indicate that confocal imaging will continue to be an important component of the imaging sciences in many fields of investigation.

1.4 The Ten Commandments of Confocal Imaging

As part of our Basic Confocal Microscopy Workshop we often have students create a list of Confocal Commandments, which are comprised of statements we make that might be considered unequivocal in nature. The following is a list of some of these commandments that we have collected over the years that need to be considered by all undertaking the task of learning and using confocal microscopy as a research tool. These commandments establish some general guidelines to consider

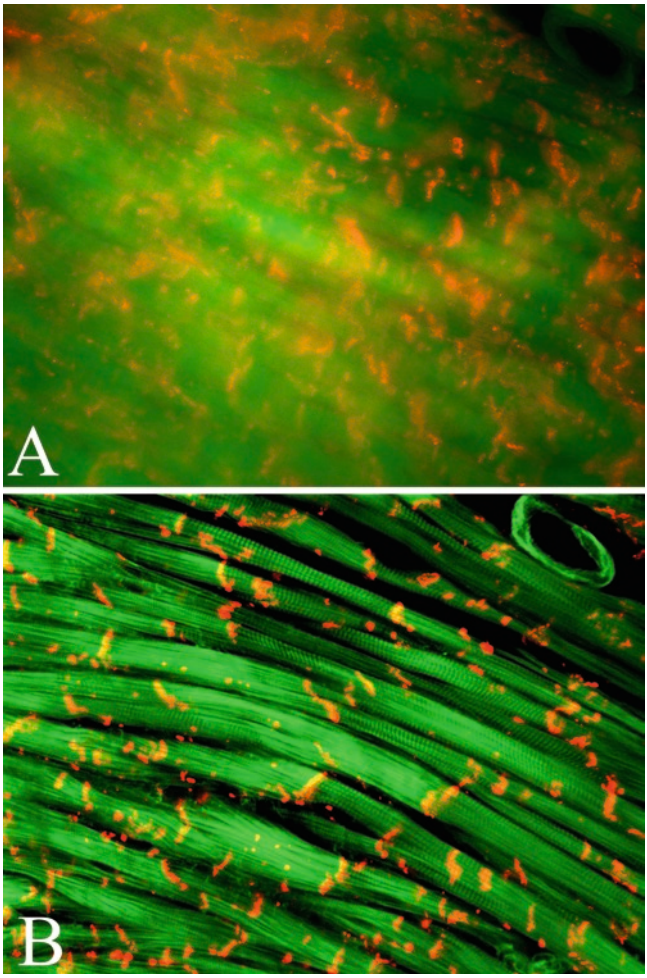

Fig. 1.3 Widefield fluorescent (*top*) and single photon confocal scanning laser microscope (CSLM) (*bottom*) images taken from a 100-μm thick vibratome section of mouse heart that has been stained for f-actin (*green*) and connexin 43 (*red*). In the widefield image out-of-focus light that contributes to the formation of the image significantly decreases the resolution and contrast of the image. Use of the pinhole in the confocal image to remove the out-of-focus light results in an image of much higher contrast and resolution as shown by the striated pattern of the myocyte sarcomeres and distinct cell: cell junctions labeled by the connexin 43 antibody

when using a confocal microscope, preparing a specimen, and handling digital images, which are all integral and equal parts of operating a confocal microscope. In fact, how we process and present the images we collect is every bit as important as how we do the initial data collection. The various chapters in this book expand on the basic principles that lead to these commandments.

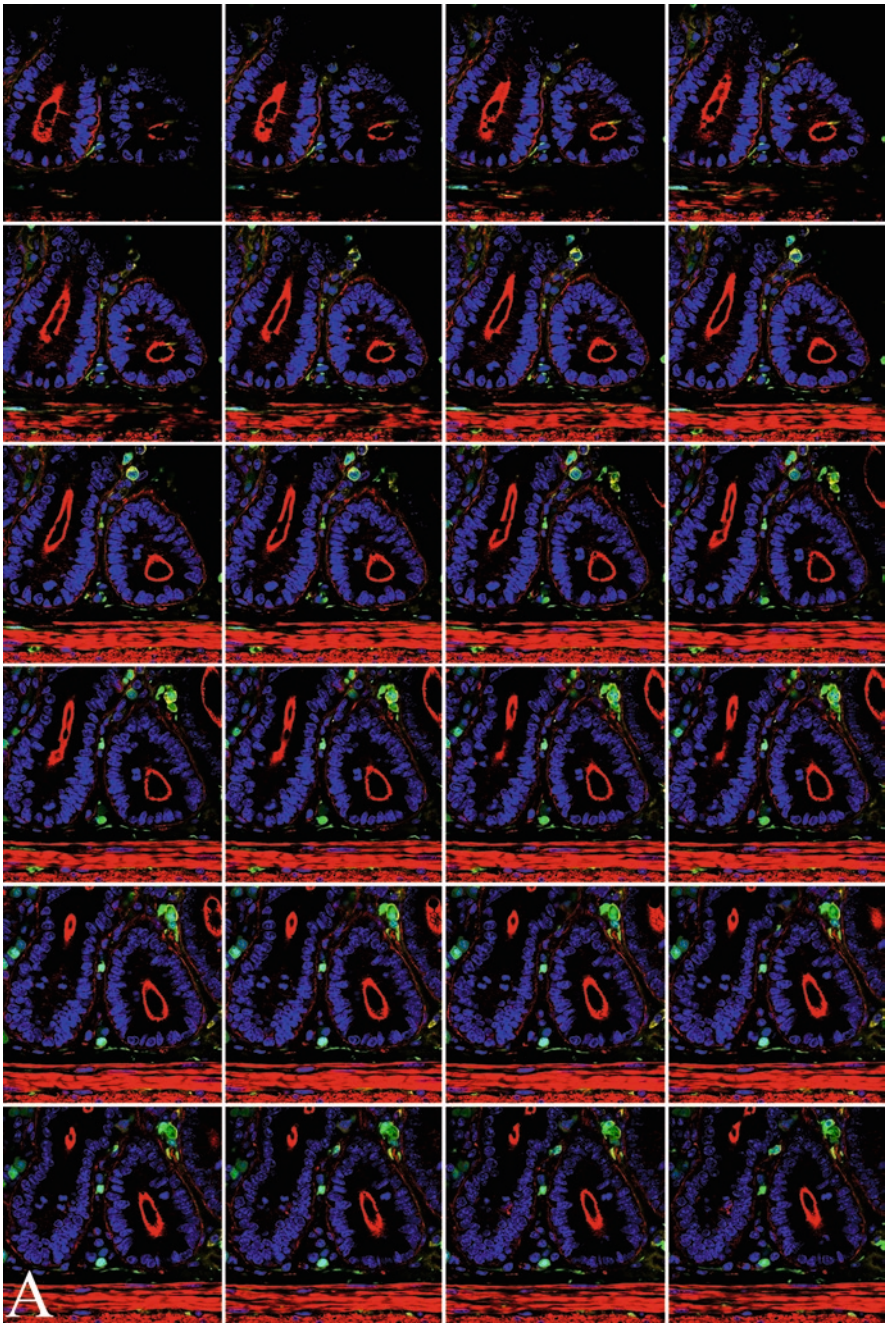

Fig. 1.4 Confocal optical sections (Z-series) through a section of intestine stained with multiple fluorescent dyes. Images were collected at 1 μm intervals through a 50-μm thick section of tissue and every other section (2 μm intervals) are shown in (**a**). All sections were then projected into a single composite image as shown in (**b**). The procedures for collection and projection of data sets are discussed in later chapters. *Blue* – DAPI stain for nuclei, *red* – f-actin stain, *green* – green fluorescent protein, *yellow* – mRNA stabilizing protein

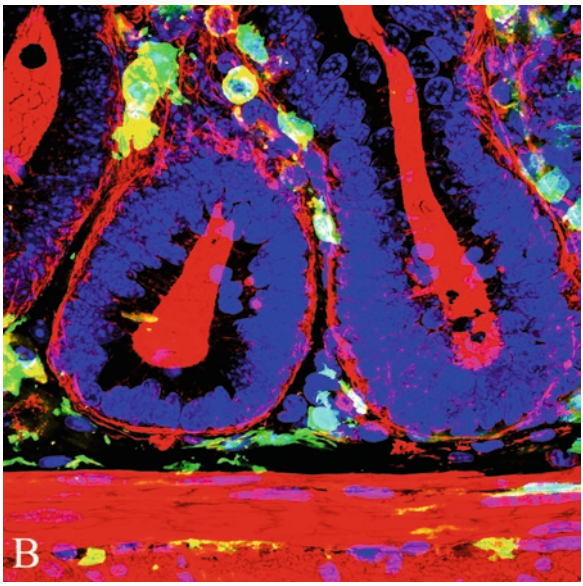

Fig. 1.4 (continued)

Our Ten Commandments of Confocal Imaging are:

1.4.1 The Perfect Microscope, and the Perfect Microscopist, Does Not Exist

As we discuss in great detail, physical factors in the design of microscopes result in image aberrations that affect the amount of light that can be collected and limits the resolution of images. Although these defects can be minimized by selection of optimal microscope components, they cannot be totally eliminated. Even with the best microscope optics available, the physical nature of light and refractive index mismatch as the light passes through the several interfaces in the optical path of the microscope and specimen will result in image defects. These defects result in the loss of signal and resolution.

Even with optimal image quality, the human element of understanding image collection and data interpretation is often a limiting factor in getting the most out of a microscope. North (2006), in a feature article for the *Journal of Cell Biology*, noted that all data are subject to interpretation and that in microscopy a great number of errors are introduced in complete innocence. A common example is the frequent interpretation that the appearance of the yellow color in a sample stained with green and red emitting fluorophores indicates co-localization. However, many factors may affect this interpretation. Without a thorough understanding of sample preparation, optics, imaging parameters, and data analysis, an incorrect conclusion of co-localization may be reached in complete innocence. Several reasons why yellow in an image generated from a sample stained with green and red fluorophores may not represent true co-localization will be discussed in subsequent chapters.

1.4.2 Confocal Microscopy Is More Than a Confocal Microscope

To effectively use a confocal microscope, investigators must have an understanding of specimen fixation and processing, antigen–antibody interactions, fluorescence theory, microscope optics and hardware components, and the handling of digital images for both image enhancement and analysis protocols. Each of these topics will be addressed in subsequent commandments and discussed in detail throughout the text.

The fact that performing confocal microscopy is much more than operating a microscope is illustrated by the sequence of the following chapters. It is essential that information on specimen preparation, fluorescence theory, and the basics of digital imaging be provided prior to material on confocal instrumentation if users are to understand the operation of a confocal microscope and be able to get the optimum amount of information from their samples.

1.4.3 During Specimen Processing the Integrity of the Specimen Must Be Maintained as Much as Possible

The integrity of the specimen includes the 3-D architecture. A major advantage of confocal imaging when compared to widefield epifluorescence imaging is the acquisition of high-resolution, high contrast images which can be obtained through the *Z*-axis of a sample, and the capability of software programs to reconstruct the 3-D nature of cells and tissues (Fig. 1.4).

Biological confocal microscopy often involves antigen staining to localize specific molecules and structures. It is essential that specimen fixation and subsequent processing maintain, as much as possible, the antigenicity of a specimen and the in vivo localization of cell and tissue antigens, analytes, structural components, etc. This may require extensive adjustment of protocols involving time, temperature, pH, and concentrations of fixatives and primary and secondary antibody solutions. Chapter 5 addresses antigen–antibody interactions, labeling strategies, and potential problems that may arise during staining of samples with various fluorochromes.

Once successful processing protocols are developed it is also essential that specimens be mounted properly to maintain the 3-D architecture of the sample. Chapter 4 presents information on various aspects of specimen preparation including the use of various fixatives, buffers, mounting media, and strategies for mounting specimens to insure maintenance of the 3-D architecture of the specimen.

1.4.4 Photons Are Your Friends and Signal-to-Noise Ratio Is King

Many factors including microscope optics and fluorochrome characteristics tend to reduce the number of photons available for formation of an image. While we are

trying to maximize the number of photons (signal) collected, microscope hardware such as detectors and electronics introduce electronic noise that may result in a poor S/N ratio. As a result, the operator must always be aware of the S/N ratio in an image in an effort to establish operating parameters that maximize image quality while minimizing specimen damage. Several chapters discuss various aspects of fluorochrome and system properties that affect the S/N ratio and provide suggestions on how to maximize the signal for optimal image quality.

1.4.5 Quantification of Fluorescence in a Confocal Micrograph Is a Challenge and at Best Is Only Semiquantitative

This is perhaps one of the most important commandments when dealing with today's competitive research environment and the need for quantitative data that is essential for funding opportunities and high impact publications. Even though a large percentage of researchers using confocal microscopes report quantitative results from their studies, one must use caution when inferring numerical data from images collected with a confocal microscope. Pawley (2000) posed the question "does a fluorescent micrograph reveal the actual location and number of labeled molecules in a cell or tissue" to members of his well-known *3D Microscopy of Living Cells* course. Based on responses collected in the course he published "The 39 Steps: A Cautionary Tale of Quantitative 3-D Fluorescence Microscopy" in *BioTechniques*. Table 1.1 is an abbreviated list of some of the factors that microscopists using confocal systems must be aware of during every imaging session. The conclusion of Pawley's paper is that "all you can really be sure of measuring with most laser-scanning confocal microscopes in the fluorescence mode is some feature of the number of photons collected at a particular time." Throughout the following chapters, we will discuss many of the issues that limit the effectiveness of confocal microscopes as a quantitative research tool and provide tips and suggestions for specimen preparation, imaging parameters, and handling digital images so that as much data as possible can be collected from each image data set.

1.4.6 Scientific Digital Imaging and Normal Digital Imaging (Family Photography) Are Not the Same

The greatest power of digital imaging is that exact copies of data can easily be made. This is excellent when archiving data and reverting to the original files when image processing does not result in the desired effect. However, while it may seem obvious that much of the processing we do on images collected with over-the-shelf digital cameras should not be done with scientific images, the innocence of the investigator again may be a problem. For example, when adjusting the contrast and brightness of a confocal image in programs, such as Photoshop, the gamma function

Table 1.1 List of some factors (adapted from Pawley 2000) that may affect the quality and quantification of confocal images

Microscope, specimen, or image component	Consideration that may affect quantitation
Laser unit	Alignment
	Instability with age
	Efficiency of optical coupling
Scanning system	Zoom magnification/Nyquist considerations
	Raster (pixel) size
	Distortions
	Environment (stray fields, vibrations)
Microscope objective characteristics	Numerical aperture
	Magnification
	Dipping/immersion lens
	Spherical/chromatic aberrations
	Cleanliness
Other optical components	Mirrors
	Excitation and emission filters
	Coverslips
	Immersion oil
Fluorochromes	Concentration
	Quantum efficiency
	Saturation state
	Loading
	Quenching
	Reaction rates
	Dye/dye interactions – FRET
Pinhole	Alignment
	Diameter
Detectors	Sensitivity
	Inherent noise
Digitization	Linearity – statistical noise

The relevance of these and other factors will be discussed in subsequent chapters with the goal of improving the confocal imaging experience of students, technologists, and principal investigators

should always be used rather than the contrast and brightness functions. Gamma corrections should also be performed only after the histogram stretch functions are completed. While rules such as this are not important in family photography applications, not applying them correctly to digital images collected for scientific applications has the potential to alter the appearance of the data.

As discussed extensively in Chaps. 6 and 11, it is essential that an original, unaltered file of the data is archived for reference. All changes in the image should be made only on a copy of the original file. There are specific guidelines that have been published by several groups, including the Microscopy Society of America (http://www.microscopy.org), that specifically state how scientific digital images should be handled. More information concerning these guidelines and the ethics of handling digital images generated for scientific studies are provided in Chaps. 6 and

9–11 on processing of confocal images and the ethics associated with the presentation of the images.

Most hardware used for the collection and display of digital images utilizes software that includes some form of image processing prior to rendering the image. Frequently, manufacturers do not make this information available resulting in images that are collected without a full understanding of how they have been processed by the hardware used in image capture. While this is typically not a problem in recreational photography, processing of scientific data by collection devices prior to saving the information should always be a concern. Whenever possible, when working with images collected as scientific data, a thorough understanding of how the images are collected and processed by the system hardware is desirable. Unfortunately, this information is sometimes difficult to obtain from the manufacturer of the equipment or even worse, considered proprietary and so never revealed. We strongly feel that equipment and software manufacturers owe it to the scientific community to make critical information that can affect image fidelity readily available.

1.4.7 Your Image Is Your Data. Garbage in Will Result in Garbage Out

One should always be detail oriented in sample preparation, image collection, and in handling digital images. The factors listed in Table 1.1 and by Pawley (2000) that affect quantitative confocal microscopy imaging are equally important in the acquisition of images for qualitative studies in which "pretty" pictures to demonstrate a scientific point are required. Without heeding each of the factors it is unlikely that publication quality confocal images will be generated or that data collection from images will be maximized.

1.4.8 The Resolution and Bit Depth Present in a Digital Image are a One-Way Street

After image capture, resolution can only get worse through processing of the image. While it may be possible through gamma and other types of filters to improve the esthetic appearance of an image, as seen in Chaps. 6, 7, and 10, once an image is collected with hardware available on a system, any structures that can be resolved in the image will be present. Increasing the number of pixels in a digital image will not improve the resolution but only result in the creation of pixels by interpolation. These pixels are created by an algorithm such as averaging neighboring pixel values and appear as the computer "believes" they should look. One may also argue that image processing through deconvolution may improve the resolution of the data set, but the limits of resolution have already been determined by the hardware present on the microscope and the physical properties of the light used to collect it.

Deconvolution to remove out-of-focus light and enhancement of images following image collection may improve image quality and enable one to further define the data present, but the limits of resolution were set during collection of the image.

1.4.9 The Joint Photographers Experts Group Image File Format Is EVIL, But Useful

This statement applies to any file format that compresses the data and does not allow full recovery of all of the information present in the original file. The Joint Photographers Experts Group (JPEG) format is the one encountered most often in imaging and so the one we chose to single out. As noted above, resolution is a one-way street and the original data should be stored as collected. Chapter 6 shows that saving files in the JPEG format results in significant loss of information, and especially damaging to scientific images, this loss is greatest along the edges. All original data should be stored in a lossless format such as a tagged image file (TIF) or a proprietary version of a TIF format as provided by the instrument manufacturer. JPEG and other compression formats may be used in situations where images either need to be shared electronically or inserted into formats for lectures, seminars, and posters. In these situations, resolution may be sacrificed in favor of smaller file sizes to make handling of images more reasonable. However, these compressed images should never be used as the primary source of data. File format options will be discussed in detail in subsequent chapters.

1.4.10 Storage Media Is Essentially Free, and Infinite

Even though confocal data sets frequently approach a gigabyte or more in size it is essential that all original data be saved and archived and that all subsequent image processing be performed on a copy of the data. While this may consume large numbers of CDs, DVDs, or other storage media, the cost of data storage is minimal compared to generating new experiments if the data is questioned and the original files are no longer available. As noted in Commandment Six, an advantage of digital imaging is that multiple exact copies of data can be easily made. With the ready availability of high density inexpensive storage media archival copies of each step made in collection of the original image and the subsequent processing of the data should be stored.

1.5 Summary

These Ten Commandments for confocal imaging provide a set of principles to guide users in a confocal microscopy laboratory. Other commandments have occasionally been added to the list during our Workshops, but if close attention is paid

to each of the above, and a detailed understanding of the importance of each is developed, users will have a strong understanding of confocal technology for use in their research.

In Chaps. 2–7, we present information on the topics of fluorescence, specimen preparation, and digital imaging which are essential for understanding confocal imaging. In subsequent chapters, we present information on various types of confocal instruments, the proper setup of operating parameters for confocal imaging, and appropriate techniques for enhancing and analyzing confocal images. Topics pertinent to the various commandments as well as some frequently asked questions such as:

1. Are these fluorescent markers co-localized?
2. Can I quantify the amount of labeled material present based on the fluorescence intensity which is present?
3. Can I measure the size or area of these structures based on a confocal data set?
4. How deep can I image into my sample?

Will be addressed. Hopefully, by learning the basic principles of confocal imaging the quality of the confocal imaging experience of many beginning and intermediate users of the technology will be improved.

References

Berek, M. 1927. Grundlagen der tiefenwahrnehmung im mikroskop. Marburg Sitzungs Ber 62:189–223

Eggar, M.D. and Petráň, M. 1967. New reflected light microscope for viewing unstained brain and ganglion cells. Science 157(786):305–307

Inoué, S. 2006. Foundations of Confocal Scanned Imaging in Light Microscopy. In Handbook of Biological Confocal Microscopy, Third Edition. Pawley, JP (ed). Springer. 985pp

Minsky, M 1957. U.S. Patent #3013467. Microscopy Apparatus

Minsky, M. 1988. Memoir on inventing the confocal scanning microscope. Scanning 10:128–138

Nipkow, P. 1884. German Patent no. 30,105. Germany

North, AJ. 2006. Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. J Cell Biol 172:9–18

Pawley, J. 2000. The 39 steps: A cautionary tale of quantitative 3-D fluorescence microscopy. Biotechniques 28:884–888

Petrán, M. Hadravsky, M., Egger, M.D., Galambos, R. 1968. Tandem-scanning reflected-light microscope. J. Opt.Soc. Am. 58: 661–664

White, JG, Amos, WB and Fordham, M. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. J Cell Biol 105:41–48

Chapter 2
The Theory of Fluorescence

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Keywords Fluorochrome • Green fluorescent protein • Photobleaching • Quantum yield • Stokes' shift

2.1 Introduction

Confocal microscopy can be performed in transmission or reflection mode for observing nonfluorescent material. However, for most biological work, the confocal microscope is used in conjunction with fluorescent samples. Fluorescence imaging provides a specific, high contrast signal that maximally exploits the ability of the confocal microscope to remove out-of-focus light. For this reason, it is imperative for confocal microscopists to have a basic knowledge of fluorescence theory and imaging. In this chapter, we review the fundamentals of fluorescence as applied to confocal imaging. In most cases, these basic principles are also applicable to widefield fluorescence microscopy.

2.2 General Principles

Fluorescence microscopy usually involves observing light within the visible range, although detection systems such as charge coupled device (CCD) cameras are available that will detect fluorescence in the ultraviolet (UV) and infrared (IR) range. Although UV and IR detectors have their uses, the current discussion will be limited to detecting visible light, since this is the range of wavelengths used most in biological confocal microscopy.

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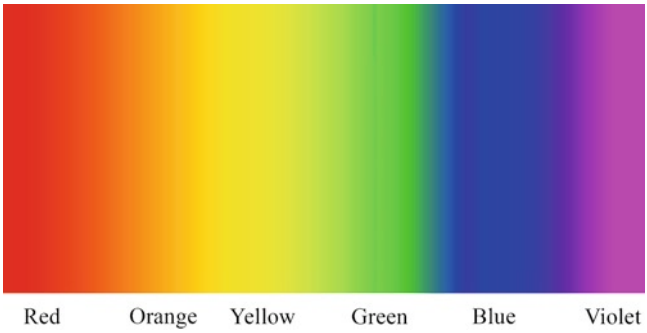

Fig. 2.1 The visible electromagnetic spectrum

Visible light is the portion of the electromagnetic spectrum that can be detected by the human eye. The color that the brain perceives is a function of the specific wavelength of the light; this encompasses electromagnetic wavelengths in the range from about 380 to 750 nm. Although the visible light spectrum is continuous, it can be somewhat arbitrarily divided into discrete colors (Fig. 2.1) according to wavelength. These divisions are violet (380–425 nm), indigo (425–450 nm), blue (450–495 nm), green (495–570 nm), yellow (570–590 nm), orange (590–620 nm), and red (620–750 nm). Indigo is not well differentiated from violet by human eyes, so wavelengths in the indigo range are often included in the violet category. Near-UV light (300–380 nm) is useful in fluorescence microscopy primarily as a source of excitation photons, and the near-infrared wavelengths (750–1,400 nm) are useful for multiphoton excitation (Chap. 8).

A fluorescent molecule (fluorochrome) is one that absorbs a photon of light of a particular wavelength and, after a brief interval, emits some of that energy in the form of a photon of a different wavelength. The delay in emission is called the fluorescence lifetime. Some of the absorbed energy is lost to nonradiation processes and so the emitted photon has less energy than the absorbed photon. Plank's law indicates that the radiation energy of a photon is inversely proportional to its wavelength. Since the emitted photon will have less energy, it will have a longer wavelength and thus a different color than that of the absorbed photon. The difference in wavelength between the absorbed and the emitted light is called the Stokes' shift. Although the shift will always be to a longer wavelength, the degree of shift is highly dependent upon the molecule being excited. Table 2.1 lists the excitation and emission maxima for some fluorochromes often used in confocal microscopy.

Energy absorption by the fluorochrome is the result of interactions between the oscillating electric field vector of the light wave and the electrons in the fluorochrome. For a given electron, a certain amount of energy is required for an electronic transition. Only energies close to this transition energy will be absorbed. Since energy and wavelength are related, this means that for each fluorochrome, there are only certain wavelengths capable of a productive interaction that generates fluorescence. This provides specificity to the fluorescence process.