Principles and Practice

Imaging Cellular and Molecular Biological Functions

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Considerations for Routine Imaging

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Entering the Portal: Understanding the Digital Image Recorded Through a Microscope

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Abstract The primary considerations in imaging living cells in the microscope with a digital camera are detector sensitivity (signal-to-noise), the required speed of image acquisition, and specimen viability. The relatively high light intensities and long exposure times that are typically employed in recording images of fixed cells and tissues (where photobleaching is the major consideration) must be strictly avoided when working with living cells. In virtually all cases, live-cell microscopy represents a compromise between achieving the best possible image quality and preserving the health of the cells. Rather than unnecessarily oversampling time points and exposing the cells to excessive levels of illumination, the spatial and temporal resolutions set by the experiment should be limited to match the goals of the investigation. This chapter describes the fundamentals of digital image acquisition, spatial resolution, contrast, brightness, bit depth, dynamic range, and CCD architecture, as well as performance measures, image display and storage, and imaging modes in optical microscopy.

1.1 Introduction

For the most of the twentieth century, a photosensitive chemical emulsion spread on film was used to reproduce images from the optical microscope. It has only been in the past decade that improvements in electronic camera and computer technology have made digital imaging faster, cheaper, and far more accurate to use than conventional photography. A wide range of new and exciting techniques have subsequently been developed that enable researchers to probe deeper into tissues, observe extremely rapid biological processes in living cells, and obtain quantitative information about spatial and temporal events on a level approaching the single molecule.

The imaging device is one of the most critical components in optical microscopy because it determines at what level fine specimen detail may be detected, the relevant structures resolved, and/or the dynamics of a process visualized and recorded. The range of light-detection methods and the wide variety of imaging devices

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currently available to the microscopist make the equipment selection process difficult and often confusing. This discussion is intended to aid in understanding the basics of light detection, the fundamental properties of digital images, and the criteria relevant to selecting a suitable detector for specific applications.

1.2 Historical Perspective

Recording images with the microscope dates back to the earliest days of microscopy. The first single-lens instruments, developed by Dutch scientists Antoni van Leeuwenhoek and Jan Swammerdam in the late 1600s, were used by these pioneering investigators to produce highly detailed drawings of blood, microorganisms, and other minute specimens (Ruestow 1996). English scientist Robert Hooke engineered one of the first compound microscopes and used it to write *Micrographia*, his hallmark volume on microscopy and imaging published in 1665 (Jardine 2004). The microscopes developed during this period were incapable of projecting images, and observation was limited to close visualization of specimens through the eyepiece. True photographic images were first obtained with the microscope in 1835 when William Henry Fox Talbot applied a chemical emulsion process to capture *photomicrographs* at low magnification (Delly et al. 2007). Between 1830 and 1840 there was an explosive growth in the application of photographic emulsions to recording microscopic images. For the next 150 years, the art and science of capturing images through the microscope with photographic emulsions coevolved with advancements in film technology. During the late 1800s and early 1900s (Bradbury 1967), Carl Zeiss and Ernst Abbe perfected the manufacture of specialized optical glass and applied the new technology to many optical instruments, including compound microscopes.

The dynamic imaging of biological activity was introduced in 1909 by French doctorial student Jean Comandon (Gastou and Comandon 1909), who presented one of the earliest time-lapse videos of syphilis-producing spirochetes. Comandon's technique enabled movie production of the microscopic world. Between 1970 and 1980 researchers coupled tube-based video cameras with microscopes to produce time-lapse image sequences and real-time videos (Inoue and Spring 1997). In the 1990s the tube camera gave way to solid-state technology and the area-array charge-coupled device (CCD), heralding a new era in photomicrography (Inoue and Spring 1997; Murphy 2001). Current terminology referring to the capture of electronic images with the microscope is *digital* or *electronic imaging*.

1.3 Digital Image Acquisition: Analog to Digital Conversion

Regardless of whether light focused on a specimen ultimately impacts on the human retina, a film emulsion, a phosphorescent screen, or the photodiode array of a CCD, an *analog* image is produced (see Inoue and Spring 1997 for a comprehensive

explanation). These images can contain a wide spectrum of intensities and colors. Images of this type are referred to as *continuous tone* because the various tonal shades and hues blend together without disruption, to generate a diffraction-limited reproduction of the original specimen. Continuous tone images accurately record image data by using a sequence of electrical signal fluctuations that vary continuously throughout the image.

An analog image must first be converted into a computer-readable or *digital* format before being processed or displayed by a computer. This applies to all images regardless of their origin and complexity. The analog image is digitized in the *analog to digital (A/D) converter* (Fig. 1.1). The continuous analog output of the camera is transformed into a sequence of discrete integers representing the binary code interpreted by computers. The analog image is divided into individual brightness values through two operational processes: *sampling* and *quantization* (Fig. 1.1b, c).

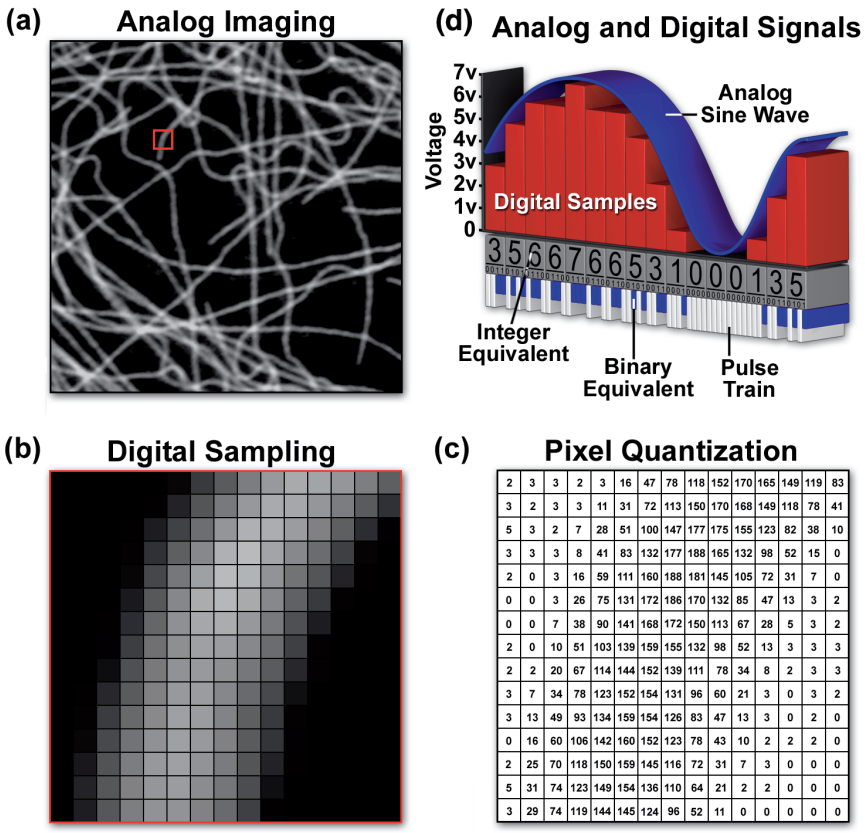

Fig. 1.1 Analog and digital Images. **a** The fluorescence image of human α-tubulin labeled with enhanced green fluorescent protein (EGFP). **b** Sampling of a small portion of **a** – the area with a red rectangle. **c** Quantization of pixel values. **d** The entire process