CHAPTER

1 INTRODUCTION

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1.1 BRIEF HISTORY OF MICROSCOPY DEVELOPMENT FROM MICRO- TO NANOSCALE

Biomedical optical imaging has been playing a crucial role in the advancement of biology and medicine since the inception of the optical microscope. The invention of the first compound microscope is generally accepted to be in the 1590s, although there is still a controversy over the inventors (Van Helden *et al.*, 2010). In 1625, the term "microscope" was first coined by Giovanni Faber to describe Galileo's instrument developed in 1609. A few decades later, Robert Hooke published his microscopic observations in 1665, wherein he observed the porous structure of cork and famously described the pores as cells, a term that is still widely used in science to date (Hooke, 1665). The pioneering work by Antoni (or Antonie) van Leeuwenhoek heralded the beginning of microbiology (Wollman *et al.*, 2015).

Over the following centuries, various innovations were made in both light delivery and detection to further improve the performance of conventional microscopes (Davidson and Abramowitz, 2002). The invention of lasers in 1960 led to rapid growth in scanning microscope technologies (Bertolotti, 2004). Two of the most widely used scanning microscopes are the laser-based confocal microscope, which was realized in 1969 by Davidovits *et al.* (1969), and the two-photon microscope, which was invented in 1990 by Denk *et al.* (1990). These advanced microscopes suppress the unwanted background signals found in conventional compound microscopes and allow for better imaging depth and spatial resolution.

However, even for these advanced microscopes, the final resolving power is still restricted by the diffraction limit, which was discovered by Ernst Abbe in 1873 (Stelzer, 2002). The diffraction limit originates from the fact that photons propagate as waves. Based on the Huygens-Fresnel principle (Baker and Copson, 2003), each point in the wavefront acts as a point source that emits waves that interfere with each other, degrading the final resolving power of the light's focus. The diffraction limit states that microscopes cannot be used to observe objects that are smaller than half of the wavelength, or approximately 200 nm for blue light (Neice, 2010). This resolution cannot resolve virus, protein,

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or cellular processes, many of which are carried out at the nanoscale (Huang *et al.*, 2010). While the diffraction limit can be bypassed via near-field imaging, where light propagation distance is less than the optical wavelength, this technique requires a very close working distance (within tens of nanometers), making biological imaging difficult (Huang *et al.*, 2010). Super-resolved fluorescence microscopy was thus developed to bring optical microscopy into the nanoscale dimension with a reasonable working distance. Pioneers in this field, such as Eric Betzig, Stefan Hell, and William Moerner, all received the Nobel Prize in Chemistry in 2014 (Betzig *et al.*, 2014). Table 1.1 lists select key events in microscopy developments in black font, along with enabling discovery/invention in red font.

1.2 BIOMEDICAL OPTICAL IMAGING IN MACROSCALE

While the super-resolution imaging expands the capability of optical imaging to probe nanoscale phenomena, various macroscale optical imaging techniques provide structural, anatomical, and functional imaging at the tissue or organ level. Many of these techniques operate in the diffusion regime that utilizes near-infrared light penetrating several centimeters below the biological tissue surface due to relatively low optical absorption (Jacques, 2013). This near-infrared window discovered by Jöbsis (Jöbsis, 1977; and Jöbsis-vanderVliet, 1999) opened the door for non-invasive or minimally invasive biomedical spectroscopy and imaging with optics. However, in this regime, light propagation is dominated by scattering. Modalities that image at the light diffusion regime include diffuse optical tomography (DOT) (Arridge, 1999; and Durduran *et al.*, 2010), photoacoustic tomography (PAT) (Xia *et al.*, 2014), and fluorescence tomography (FT) (Stuker *et al.*, 2011). DOT and fluorescence tomography are purely optical imaging techniques. Due to optical scattering, they have poor spatial resolution in the order of millimeters or centimeters in deep tissue. In contrast, PAT employs a hybrid approach that converts optical waves into acoustic waves. Compared to DOT and fluorescence tomography, PAT possesses a better spatial resolution because acoustic scattering in tissue is much weaker than light scattering. The downside of the optical to acoustic conversion is reduced detection sensitivity because optical detectors are much more sensitive than acoustic detectors (Winkler *et al.*, 2013).

In addition to these techniques, there are several other optical imaging techniques providing different types of optical contrast based on different light-tissue interactions. Here we highlight two select optical imaging techniques: optical coherence tomography (OCT) is based on backscattering arising from gradients and discontinuities in the refractive index, and Raman imaging is based on inelastic Raman scattering from biomolecular constituents of tissue.

Interestingly, the time between the initial discovery of the concept and the wide adoption of the technique in the biomedical field varies (Table 1.1, blue font). For example, the concept of photoacoustics was demonstrated in 1880 by Alexander Bell, but it was not until the 1990s that the *in vivo* applications were demonstrated and finally evolved into well-accepted biomedical imaging techniques at the turn of the 21st century. On the other hand, optical coherence tomography has seen a relatively short time between the concept proposal in 1971 (Duguay, 1971; and Duguay and Mattick, 1971), demonstration

Table 1.1

A timeline of the key events in the development of biomedical optical imaging techniques. Events related to microscopy and spectroscopic imaging technique development are in black and blue font, respectively. Enabling discoveries and inventions are listed in red font. OCT (optical coherence tomography); DOT (diffuse optical tomography); STED (stimulated emission depletion); PALM (photoactivated localization microscopy); STORM (stochastic optical reconstruction microscopy); fPALM (fluorescence PALM).

Year	Events
1625	"Microscope" coined by Giovanni Faber (Wollman *et al.*, 2015)
1665	Robert Hooke published "Micrographia" and coined the term "cells" (Hooke, 1665; Wollman *et al.*, 2015)
1670s–1680s	Antoni van Leeuwenhoek (1632–1723) pioneered biological research (Wollman *et al.*, 2015)
1845	Fluorescence discovered by Fredrik W. Herschel (Herschel, 1845; Renz, 2013)
1873	Ernst Abbe on the diffraction limit (Wollman *et al.*, 2015)
1880	Alexander Bell demonstrated speech transmission with light (Manohar and Razansky, 2016)
1928	Raman effect discovered by Chandrasekhara V. Raman (Smith *et al.*, 2016)
1931	Two-photon excitation theory by Göppert-Mayer (So *et al.*, 2000; Sheppard, 2020)
1953	First commercial Raman spectrometer (Stamm and Salzman, 1953; Smith *et al.*, 2016)
1955	First confocal scanning microscope built by Marvin Minsky (Minsky, 1988; Paddock and Eliceiri, 2014)
1960	Invention of laser (Bertolotti, 2004)
1969	Implementation of laser-based confocal microscope (Davidovits and Egger, 1969; Sheppard, 2003)
1971	OCT concept proposed by Michel Duguay (Duguay, 1971; Duguay and Mattick, 1971; Fujimoto and Swanson, 2016)
1974	First commercial pulse oximeter (Severinghaus, 2007; Huppert, 2013)
1977	*In vivo* monitoring with near-infrared light by Frans F. Jöbsis (Jöbsis, 1977; Jöbsis-vanderVliet, 1999; Huppert, 2013)
1980s	Development of time-domain diffuse optical techniques (Chance *et al.*, 1988; Delpy *et al.*, 1988; Huppert, 2013)
1990	Invention of two-photon microscopy (Denk *et al.*, 1990; So *et al.*, 2000)
1990	First confocal Raman microscopy (Puppels *et al.*, 1990; Smith *et al.*, 2016)
1991	OCT of biological system demonstrated (Huang *et al.*, 1991; Fujimoto and Swanson, 2016)
1994	Concept of STED first proposed (Hell and Wichmann, 1994; Royal Swedish Academy of Sciences, 2014)
1994	First laser photoacoustic images (Manohar and Razansky, 2016)
1990s	DOT theory, experiments and *in vivo* imaging (Arridge and Schweiger, 1993; O'Leary *et al.*, 1995; Pogue *et al.*, 1995; Gibson and Dehghani, 2009)
2000	Experimental proof-of-principle of STED (Klar *et al.*, 2000; Royal Swedish Academy of Sciences, 2014)
2006	Implementation of single-fluorophore based super-resolution microscopies (PALM/STORM/fPALM) (Betzig *et al.*, 2006; Royal Swedish Academy of Sciences, 2014)
2014	Nobel prize for super-resolution microscopy (Betzig *et al.*, 2014; Vangindertael *et al.*, 2018)

in biological systems in 1991 (Huang *et al.*, 1991), and commercialization in the 2000s (Fujimoto and Swanson, 2016).

1.3 IMAGING DEPTH AND RESOLUTION

Generally, there is a trade-off between imaging resolution and depth among imaging techniques used in biomedical applications, especially in terms of *in vivo* imaging. Figure 1.1 illustrates this relationship for optical imaging modalities featured in this book. Optical imaging modalities in a diffusion regime have deep tissue penetration (e.g., several centimeters) but are limited in the imaging resolution (i.e.,

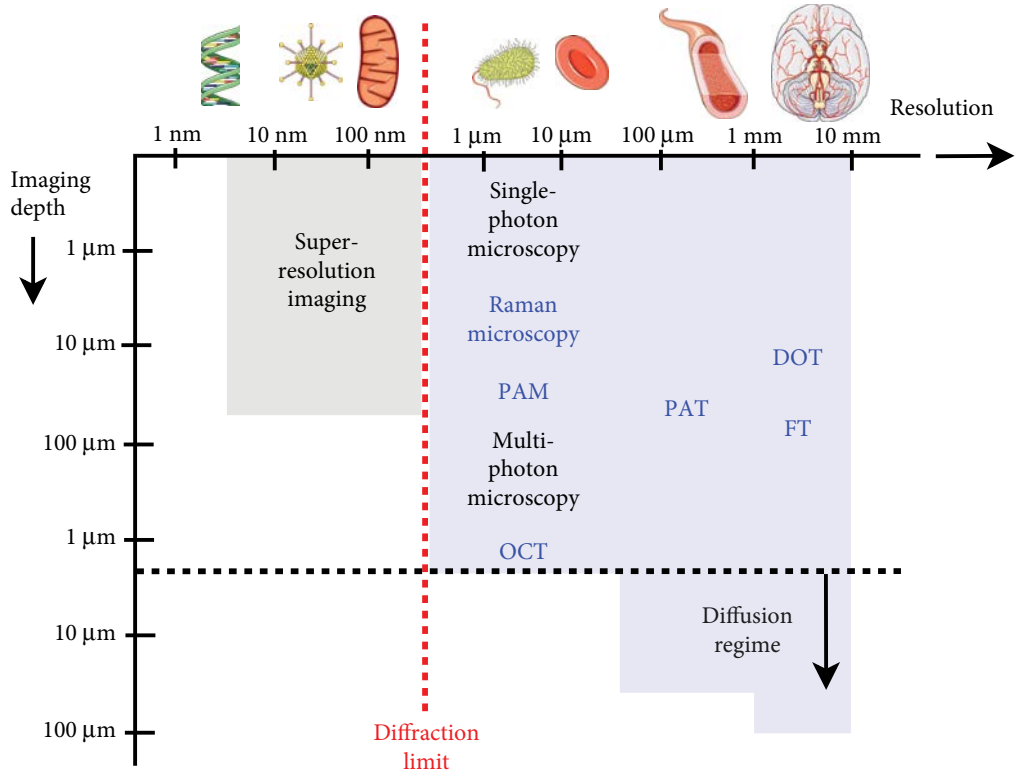

FIG. 1.1
Imaging resolution and depth of optical imaging techniques featured in the book. PAM (photoacoustic microscopy); OCT (optical coherence tomography); PAT (photoacoustic tomography); DOT (diffuse optical tomography); FT (fluorescence tomography). Illustrations of biological structures are adapted from Servier Medical Art, https://smart.servier.com.

millimeter range for DOT and fluorescence tomography). Even PAT, the modality with the best image resolution in a diffuse regime, has a resolution of a hundred microns. On the other hand, imaging depths of super-resolution or microscopic imaging are severely limited. For example, the imaging depth of 50 to 100 μm was reported for an *in vivo* mouse brain imaging with super-resolution structured illumination imaging (Turcotte *et al.*, 2019). Using the state-of-the-art multiphoton microscopy, one could potentially image up to 1.6 mm depth (Miller *et al.*, 2017). So far, no single imaging technique can image the whole body with sub-micron resolution within a reasonable time period. Instead, the spatial resolution of these techniques can be tuned to operate either in micro- or macroscale by modifying the instrument design. For example, photoacoustic tomography can be modified to photoacoustic microscopy (PAM) that operates in microscale.

1.4 ADVANTAGES OF OPTICAL IMAGING

Optical imaging techniques offer several advantages over other imaging techniques, especially for biomedical applications. Light, non-ionizing radiation, does not have a cumulative effect on tissue at subthermal levels typically used for imaging (Bigio and Fantini, 2016). This enables imaging of live specimen and monitoring of dynamic processes occurring in biological systems. Various interactions between light and tissue bring about different kinds of imaging contrast, thus providing unique functional or structural information not accessible by other imaging techniques. In addition, optical techniques are relatively inexpensive compared to certain imaging techniques and are relatively easy to use in general. These features, along with continuous innovations to overcome the limitations of the techniques, make optical imaging techniques the versatile choice of imaging for many biomedical scientists and researchers.

Since the invention of electron microscopy in 1931 (Freundlich, 1963) and its first usage in cell biology applications in the early 1940s (Winey *et al.*, 2014), it has provided unprecedented imaging resolution that enables valuable investigation of biological ultra-structures. However, the inability to image living cells, the requirement of complicated sample preparation, and the cost of both time and money spent on electron microscopy have swayed many scientists in favor of optical imaging over the years. With the advent of super-resolution microscopy, the imaging resolution gap has been narrowed remarkably between electron microscopy and light microscopy. Study of subcellular structures and dynamic cellular processes at nanometer scales are now possible.

In macroscale imaging, modalities often utilized in preclinical or clinical settings have their own share of limitations in terms of signal contrast, type of radiation, and cost. For example, magnetic resonance imaging (MRI), positron emission tomography (PET), or computed tomography (CT) provides excellent anatomical images, but the use of ionizing radiation (e.g., PET, CT) or high cost (e.g., MRI) prohibits imaging on a frequent basis or usage in populations such as neonates or infants. Optical imaging techniques that are inexpensive and ideal for non-invasive or minimally invasive applications

not only offer solutions for frequent monitoring or niche patient populations, but also provide unique complementary functional information. This unique information holds great potential to generate breakthroughs in translational biomedical research.

1.5 ORGANIZATION OF THE BOOK

In this book, optical imaging techniques with recent advances in biomedical research are organized by spatial scale (i.e., from nanoscale to macroscale) after the introduction of photon-tissue interactions in Chap. 2. Two different types of super-resolution imaging techniques are described in Chaps. 3 and 4, respectively. Chapters 5 and 6 are on the topic of single- and multiphoton fluorescence microscopy techniques. Microscopic techniques based on different optical contrast are covered in Chaps. 7 and 8. Optical coherence tomography is described in Chap. 9. Chapters 10–12 cover macroscale imaging techniques, including diffuse optical/correlation tomography, fluorescence tomography, and photoacoustic tomography. Each chapter is structured to provide principles and techniques behind the instrumentation, the image formation, and the data analysis. For all the imaging techniques, we highlight example biomedical applications and discuss future prospects.

In closing, we hope that this book provides informative guidance for researchers entering the field of biomedical optical imaging and for those who seek views of experts in different imaging techniques.

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