

Chapter 1

A Brief History of the Microscope and its Significance in the Advancement of Biology and Medicine

This chapter provides a historical foundation of the field of microscopy and outlines the significant discoveries in the fields of biology and medicine that are linked to the microscope. Microscopes, which are devices to image those objects that are invisible to the naked eye, were transformed from interesting instruments used by hobbyists to serious scientific instruments used to explore and understand the microscopic world. Because the technique of fluorescence microscopy is a major, if not the most widely used, application of both confocal microscopy and multiphoton excitation microscopy, I present a series of key developments of fluorescence microscopy. Microscopy began with the observation of live specimens and continues its growth with technical developments in the fields of intravital microscopy, endoscopy, and *in vivo* microscopy. In this chapter, I cite and discuss many of the advances in both biology and medicine that critically depended on the development of the optical microscope. These sections provide a framework for the book and support the premise that technical advances in microscopy have led to both the generation of new knowledge and understanding as well as advances in diagnostic and clinical medicine, which has ultimately resulted in an improvement of the human condition.

1.1 Timeline of Optical Microscope Development

The invention of the microscope (ca.1600) and its improvements over a period of 400 years has resulted in great advances in our understanding of the microscopic world as well as extremely important advances in biology and medicine. The optical microscope, a device that in many cases was used as an interesting toy, became a key instrument in basic science and clinical research: It gives the observer a view of inner space, that is, the world that cannot be observed with the naked eye because of insufficient resolution, such as atoms, molecules, viruses, cells, tissues, and microorganisms.

The reader may ask, why were the numerous early advances made in the design and manufacture of telescopes not rapidly transferred to the microscope? A partial answer is that telescopes were the domain of physicists and mathematicians, whereas the design, construction, and use of the early optical microscopes were left to laypersons, those whom today we call hobbyists. As we shall see, there were bril-

Chapter 2

The Optical Microscope: Its Principles, Components, and Limitations

2.1 What Is an Optical Microscope?

How does a slide projector differ from a microscope? A slide projector magnifies the image on the slide; hence, it projects a small image into a larger image on a screen. A slide projector does not increase the resolution of the object.

A microscope also provides a magnified image for the observer, although its most important function is to increase the resolution! With a microscope, we can observe microscopic specimens that would not be visible and resolve details that were unresolved to the naked eye. But unless there is sufficient contrast, no details can be observed. So, optical microscopy depends on both sufficient resolution and sufficient contrast.

2.2 Image Fidelity: Mapping the Object into the Image

As in all imaging systems, the optical microscope maps an object into an image. An ideal system would make this mapping with the highest fidelity between the object and the image. Even so, the finite aperture of the lens as well as many forms of optical aberrations place fundamental limits on the fidelity of this mapping. The aim of microscope design, manufacture, and practice is to minimize the aberrations, maximize the resolution, and approach the highest fidelity possible.

What are the requirements for **spatial and temporal resolution** in optical microscopy? Spatial resolution denotes the ability of the microscope to resolve or separate adjacent points on the object. Microscopic observations may only involve the detection or absence of a particle, or may require the full three-dimensional structure of a thick, highly scattering specimen such as the eye or skin. The microscope should be capable of resolving the highest spatial frequencies that are required to form an image that is appropriate to the questions posed by the observer.

In order to map the object into the image with high fidelity, it is necessary to map the intensities and the spatial frequencies of the object. **Spatial frequency** is the frequency in space for a recurring pattern, given in units of line pairs/mm. The **Nyquist theorem**, which is valid for both spatial and temporal frequencies, defines how to sample the object. The theorem states that the sampling must be performed at a minimum of two times the highest spatial frequency in the object to accurately reproduce the object in the image.

If the imaging system does not meet the Nyquist criterion, then there is aliasing in the image. **Aliasing** is the phenomenon that occurs when periodic structures in

Chapter 6

Early Antecedents of Confocal Microscopy

6.1 The Problem with Thick Specimens in Light Microscopy

It was evident to users of the light microscope that there were still unsolved problems with thick, highly scattering specimens. The use of the fluorescent light microscope together with fluorescent, thick specimens was difficult; moreover, light from above and below the focal plane contributed to a blurring of the image and a general loss of contrast. These problems were also evident during *in vivo* microscopy of embryos, tissues, and organs. On the other hand, these problems did not exist for very thin, fluorescent specimens.

Real biological specimens have internal structures that vary with depth and position. Prior to the use of three-dimensional computer reconstructions, in order to obtain a valid understanding of the heterogeneous specimen, it was necessary to use the light microscope to image many focal planes from the top to the lower surface, and then to reconstruct either a mental three-dimensional visualization of the specimen, or use computer techniques to make this visualization. This was the technique used by Ramón y Cajal in his seminal microscopic studies of the vertebrate nervous system.

The laser was invented by Theodore Maiman in 1960. Two years earlier, Arthur Schawlow, Charles Townes, and, independently, Alexander Prokhorov showed that it was possible to amplify stimulated emission in the optical and infrared regions of the spectrum.

The Minsky patent for his confocal microscope was issued in 1961. Prior to these milestones, there were many technical innovations that aimed to increase the resolution of the light microscope. The laser is not a requirement for the confocal microscope, since usable light sources include the sun, white light arc lamps, and a 12V halogen lamp. We now discuss some of these innovations and their role in the development of light microscopy.

6.2 Some Early Attempts to Solve These Problems

A series of creative technical innovations in the field of light microscopy resulted in technical improvements and a deepened theoretical understanding of confocal light microscopy. The basic advances will be briefly discussed and classified into common groupings: advances in fluorescence microscopy and in light sources and point scanning. It is interesting that there were both parallel developments and

reinvention of technical advances in disparate fields, and these processes continued to occur throughout the development of confocal and multiphoton excitation microscopy. Inventions were patented, patents were contested, and the process continues into the present.

The prerequisites for the developments of confocal and multiphoton excitation microscopy were the technical advances of the fluorescence microscope. In particular, the work of Brumberg demonstrated how to use light from above or a vertical illuminator together with a beamsplitter (dichroic) and filters in the filter block to separate the excitation light path from the emission light path to the eyepiece or detector (Brumberg, 1959). *In vivo* fluorescence microscopy must use the technique of epi-fluorescence or vertical illumination, since the standard technique of transmission fluorescence microscopy is not appropriate for *in vivo* microscopy. Brumberg also pointed out that the fluorescence signal is very weak compared with the reflected light signal; therefore, it is critical to separate the two signals in order to be able to detect the very weak fluorescence.

Furthermore, Brumberg's *in vivo* microscope with vertical illumination had several types of microscope objectives. Some objectives were sharply pointed, designed to penetrate tissues and organs. Others were constructed with a flat surface that applanates the surface of the tissue or organ during *in vivo* microscopy. Since the tip of the applanating microscope objective was on the surface of the specimen, it was necessary to devise a technique to vary the focal plane within the specimen. Brumberg also solved this technical problem.

Brumberg developed a method to shift the focal plane of the microscope objective while the objective was stationary with respect to the organ or tissue under observation. He constructed a movable lens within the microscope tube that would be translated along the optical axis to change the position of the focal plane of the objective. He further stated that changing the tube length induces optical aberrations with a given objective; however, small displacements (up to 10 mm) would not exceed the permissible limits of aberrations. Brumberg's microscope has been rediscovered many times in recent years and incorporated into the microscopes designed by others.

Brumberg also discussed the problem of glare in the field of view during *in vivo* microscopy of thick specimens. He noted that the source of the glare was fluorescence of the layer of tissue above and below the focal plane. He reported that this glare reduced image contrast and makes the image less distinct. Brumberg's solution he was to use an annular ring in the microscope objective to achieve dark-field illumination, together with a two-color light filter mounted in the pupil of the microscope objective. There was some loss of brightness, but this technique achieved a gain in image contrast.

The second set of technical advances came in the form of scanned image microscopy. First, two very different types of illumination must be explained: Bright-field (wide-field) illumination and point scanning. To explain these terms, more definitions are needed. **Bright-field microscopy** involves direct light passing through the objective aperture and illuminating the background against which the

Chapter 9

The Components of a Confocal Microscope

Many components of confocal microscopes have been described in the previous chapters. In this chapter I will briefly consolidate the key points and provide some additional critical details.

The selection of components should be optimized for the type of specimen to be observed and for the imaging mode, i.e., reflected light or fluorescence. The clinical applications of confocal microscopy to ophthalmology and dermatology are predominantly based on reflected light. However, the biological applications of confocal microscopy are chiefly based on fluorescence techniques. These applications include imaging specific proteins based on green fluorescent protein (GFP) fluorescence, fluorescent indicator dyes that respond to ion concentration and transmembrane voltage differences, fluorescent labeled antibodies to image specific proteins, and fluorescent membrane dyes.

As new fluorescent probes are developed, there may be a need to change the light source to match their absorption bands. Similarly, new dichroic mirrors and filters may be required. New types of detectors are facilitating new techniques; multi-grid photomultipliers in conjunction with fluorescence dispersion devices, and the quantum efficiency and SNR of two-dimensional detectors is improving along with their spectral range. Manufacturers are responding to their users by developing microscope objectives that are optimized for specific applications of confocal microscopy, e.g., neurobiology and developmental biology.

9.1 Light Sources

Light is defined as the visible form of electromagnetic radiation. In the light microscope it is important to make use of these specific characteristics of electromagnetic radiation: wavelength, polarization, and coherence. When choosing a light source one must evaluate broadband or white light sources versus narrowband sources; noise and stability; and the cost of cooling, replacement, and maintenance.

Again, the first question to ask is: What type or types of specimen will be imaged? Is the microscope to be dedicated to imaging integrated circuits? Is it to be used in the clinic to image *in vivo* human skin or the living human eye? What types of fluorescent probes are used with the specimen? What are the absorption bands of the fluorescent probes that will be studied?

White light sources are necessary for real-color direct-view Nipkow disk-based confocal microscopes. Broadband light sources are obtained from arc lamps to-

gether with sets of filters. Nonlaser light sources have a lower degree of coherence as compared with lasers. It is desirable to have a low-coherence source of light for reflection confocal microscopy, to reduce interference and speckle in the specimen; high-coherence lasers may cause fringes to develop in the image if there are multiple reflections. When necessary, there are methods available to break the coherence of a laser light source, e.g., vibrating a fiber optic that transmits the laser light.

A variety of light sources can be used with various types of confocal microscopes. Several Nipkow disk-based confocal microscopes use arc lamps as the light source. A halogen lamp is the light source in the scanning slit clinical confocal microscope. Light-emitting diodes are a good alternative light source for Nipkow disk and scanning-slit confocal microscopes; they are stable, efficient, and have long lifetimes.

Light sources for confocal microscopes can be divided into spatially coherent or spatially incoherent groups. LSCMs are spatially coherent. In contrast, many of the clinical confocal microscopes used in the clinic, for example, the scanning-slit confocal microscope used in the ophthalmology clinic uses halogen lamps as a spatially incoherent light source. With spatially incoherent illumination, the phase relations between fields at nearby points are statistically random.

Spatially coherent light sources have the important property that the phase difference between any two points is constant with time. Examples of spatially coherent light sources are lasers and arc lamps with a small aperture that acts as a spatial filter. There is another important term: **temporal coherence**. A laser with a single frequency (actually a very narrow range of frequencies) would have a high temporal coherence. That term implies that there exists a definite phase relationship between the fields at a given point after a time delay of T . Usually practical lasers show this definite phase relationship for a fixed time, called the coherence time.

Lasers usually have a single wavelength or they can be tuned to output a few discrete wavelengths. Another possibility is to use two or three different lasers to provide a wider selection of wavelengths. Most modern LSCMs use one or more lasers as the light source. The output from a laser is extremely bright, monochromatic (a single color, with a very narrow range of frequencies), coherent, and highly collimated. The output is typically also linearly polarized; this can be exploited in differential interference contrast microscopy, polarized light microscopy, and studies of fluorescence polarization anisotropy. A high-NA microscope objective can focus the laser beam to a diffraction-limited point or volume of light that is extremely intense.

The laser beam may be expanded in diameter before it is coupled to the confocal microscope. Typically, the diameter of the laser beam is adjusted to overfill the back aperture of the microscope objective. When the laser operates in the TEM_{00} mode, it forms a Gaussian beam; in that case an illumination pinhole aperture is not needed, because the light appears to originate from a point source at infinity. The laser beam may be expanded. With a high-NA microscope objective, a diffraction-limited spot is formed at the focus.

sorption cross section. In a two-photon excitation process, the number of photons absorbed per molecule of fluorophore per pulse scales with the average incident laser power squared. This is the source of the experimental verification of two-photon excitation processes; hence, on a log-log plot of detected intensity versus laser power, the slope of the plot is 2. The authors also state that the two-photon excitation fluorescence can be increased by increasing the pulse repetition frequency until saturation of the excited state is achieved.

Another key feature of the patent is the description of a non-descanned detection of the fluorescence intensity derived from the two-photon absorption process. Since the fluorescence signal depends on the square of the excitation intensity, there is an optical sectioning effect through the specimen even in the absence of a pinhole used as a spatial filter in front of the detector. Therefore, the detector can be a large-area detector such as a PMT. This avoids many of the problems associated with conventional LSCMs. With the publication of the *Science* paper and the patent from the Webb group in 1991, the reality of two-photon excitation microscopy began.

Webb and coworkers further stated that the microscope can be operated in sum or difference frequency mode. It is not necessary that the two photons that are absorbed in a two-photon excitation process be of the same wavelength. In the sum frequency case, an excitation transition requiring energy hc/λ_{ab} can be achieved using lasers with wavelengths, λ_a and λ_b , based on the following conservation equation:

$$\frac{1}{\lambda_{ab}} = \frac{1}{\lambda_a} + \frac{1}{\lambda_b}, \quad (11.1)$$

where h is Planck's constant, and c is the speed of light.

Both the *Science* paper and the patent had a great impact on scientists worldwide. As soon as a commercial version was available scientists scrambled to apply this new form of microscopy to live cell microscopy.

11.3 Comparison of Multiphoton Excitation Microscopy and Confocal Microscopy

In a confocal microscope, the confocal aperture or pinhole location is critical. Also, the position of the detector aperture must be precisely located within a plane that is conjugate with the image plane and therefore the focal plane, so that the images of the source and detector apertures are cofocused.

However, in a multiphoton excitation microscope the position of the detector is not critical. The fluorescence need not be descanned prior to detection and a detector pinhole is not necessary. A non-descanned wide-area detector can be placed anywhere, but preferably close to the microscope objective after the dichroic beamsplitter.

The advantages of these nonlinear microscopes include improved spatial resolution without the use of pinholes or slits for spatial filtering. Since the optical sectioning capability of the two-photon excitation microscope derives from the physics of the excitation process, there is no requirement for a spatial filter in front of the

detector as in confocal microscopy. Two-photon microscopy furthermore allows deeper penetration into thick, highly scattering tissues, and confines photobleaching and photodamage to the focal volume. The excitation wavelength is far removed from the fluorescence wavelength. This large separation in wavelengths makes the suppression of the laser light more efficient. Finally, a very important factor is increased cell and tissue viability with infrared illumination.

The main limitations of two-photon excitation microscopy are (1) it is only suitable for fluorescent imaging; reflected light imaging is not possible, and (2) it is not suitable for imaging highly pigmented cells and tissues that absorb near-infrared light.

Multiphoton excitation microscopy has the capacity to image deeper within highly scattering tissues such as *in vivo* human skin; its ability to image the elastin and collagen fibers within the tissue is a significant advantage.

The technique of two-photon excitation microscopy is ideal for deep-tissue imaging. This nonlinear microscopy technique has several notable characteristics: there is submicron resolution (0.3 μm lateral resolution; 0.8 μm axial resolution; a penetration depth that is tissue dependent (500 to 1000 μm); and has the capability of spectroscopic analysis of the specimen. The excitation wavelength is far removed from the fluorescence wavelength. This large difference in wavelength makes the suppression of the laser light more efficient.

The technique of single-photon confocal microscopy has the joint capability of either fluorescence confocal imaging or reflected light confocal imaging.

What have we learned about the fundamental divide between conventional (wide-field) fluorescence microscope on one hand, and confocal and multiphoton microscopes? The most important aspect is the property of optical sectioning in confocal and multiphoton microscopes; furthermore, it is the result of the PSF having a maximum at the focal plane. More specifically, the integrated signal intensity of the detection PSF is a constant as a function of depth in a conventional (wide-field) fluorescence microscope. On the contrary, and this is the most important statement about the origin of optical sectioning, for both confocal microscopes and multiphoton excitation microscopes the integrated intensities have a maximum at the focal plane! There is depth discrimination in both confocal and multiphoton excitation microscopes. As we shall see in the next paragraph, the widths at half height of the peaks are different.

Theoretical modeling of the point spread function for an ideal confocal microscope versus a multiphoton microscope using the same fluorescent molecule indicates a broadening in x , y , and z for the multiphoton microscope. That is because of the doubled wavelength for the multiphoton excitation microscope. This small difference in resolution can be eliminated by the use of a confocal aperture in front of the detector of the multiphoton excitation microscope. There is also a loss of signal with confocal detection. In the case of a real confocal microscope, the resolution is degraded by chromatic aberration, the use of a finite confocal aperture for efficient detection, and possibly imperfect alignment. Therefore, in practice, for imaging the same fluorescent molecule there may be no significant difference in resolution between a confocal microscope and a multiphoton excitation microscope.

When we compare the optical sectioning capabilities of both confocal and multiphoton microscopes we notice a difference; the optical sectioning capability of the multiphoton microscope is less than that for a confocal microscope. The explanation is as follows. The wavelength of the illumination in a multiphoton microscope is longer (usually by a factor of 2) than that used with a confocal microscope. While the integrated PSF for both the confocal microscope and the multiphoton microscope have peaks that occur at the focal plane, because of the longer wavelength of the illumination in the multiphoton microscope, its PSF peak is wider than the corresponding PSF peak for the confocal microscope.

For both types of microscopes, the signal rapidly falls to zero outside the focal plane. Thus, with defocus the signal disappears. Remember that for a conventional (wide-field) fluorescence microscope the signal is constant with defocus. This property of depth discrimination is key to three-dimensional microscopy via optical sectioning of a thick specimen.

Confocal microscopes use spatial filtering to obtain optical sectioning (depth discrimination). A small pinhole will achieve good depth discrimination and background rejection.

Multiphoton excitation microscopes use the physics of the excitation process, in which the excitation is proportional to the square or cube of the intensity and only within the diffraction-limited focal volume of the microscope objective is there sufficient intensity for the process to occur, to obtain optical sectioning. Multiphoton excitation microscopes have the advantage of using non-descanned detection, therefore a two-dimensional solid state detector with a very high quantum efficiency (e.g., 90%) can be used to maximize the signal.

An important advantage of multiphoton excitation microscopy is that photobleaching is restricted to the focal volume (see Fig. 11.3). In a confocal mi-

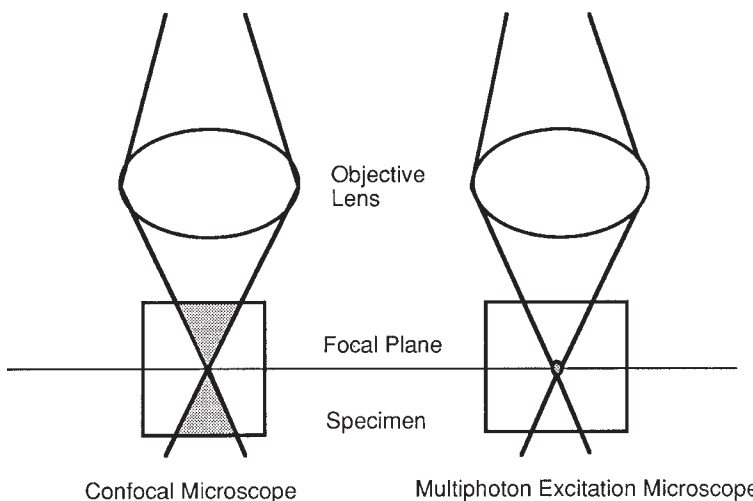


Figure 11.3 Schematic diagram comparing the photobleaching zones for a one-photon confocal microscope and a multiphoton microscope.

croscope, photobleaching occurs in all regions of the double, inverted cone of the excitation light from the microscope objective.

In the next section I describe and compare the theory and the components of multiphoton excitation microscopy. The emphasis is placed on those characteristics that optimize their use with multiphoton excitation microscopes.

11.4 Summary

- The theoretical basis for two-photon quantum transition (emission and absorption) in atoms was the subject of a doctoral thesis published in 1931 by Maria Göppert-Mayer.
- Göppert-Mayer showed that the probability for the two-photon absorption process is proportional to the square of the light intensity.
- The seminal work of Denk, Strickler, and Webb launched a new revolution in multiphoton excitation microscopy and its application to biology.
- An advantage of multiphoton excitation microscopy is improved spatial resolution without the use of pinholes or slits for spatial filtering as in confocal microscopy. The optical sectioning (depth discrimination) capability of two-photon excitation microscopes derives from the physics of the excitation process. Only in the focal volume is the intensity sufficient for observable two-photon absorption to occur.
- The use of infrared or near-infrared illumination in multiphoton excitation microscopy results in other important advantages, including greater sample penetration and increased cell and tissue viability. These factors are extremely important for live cell and tissue imaging over extended periods of time, and for applications of *in vivo* microscopy to optical biopsy.
- The main limitations of two-photon excitation microscopy are (1) it is only suitable for fluorescent imaging and not reflected light imaging; and (2) it is not suitable for imaging highly pigmented cells and tissues that absorb near-infrared light with resulting photodamage.
- The technique of two-photon excitation microscopy is ideal for deep-tissue imaging. This technique has several notable characteristics: there is submicron resolution (0.3 μm lateral resolution; 0.8 μm axial resolution); penetration depth is tissue dependent (500 to 1000 μm); and it has the capability of spectroscopically analyzing the specimen.
- In multiphoton excitation microscopy, photobleaching is restricted to the region of the focal volume. In one-photon confocal microscopy, photobleaching occurs in the volume of the double, inverted cone of the excitation light.