1

Super-Resolution Microscopy (SRM): Brief Introduction

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1.1 Optical Microscopy

There is a well-known saying "Seeing is believing." Undoubtedly, visualization is one of the most trusted means of perceiving an object. Hence, bioimaging using various optical microscopy techniques constitutes the foundation of biological research.

To improve the performance of microscopes, scientists have realized significant technological advancements over the last few centuries to achieve the present epoch of biological research. The quest for improving the performance of microscopes resulted in the discovery of newer optical microscopic methods, since its first invention. In the sixteenth and seventeenth centuries, the earliest functional microscopes were introduced to overcome the limitation of the human eyes, which revolutionized the study of the natural world from a new perspective, even though a low magnification of the object was achieved. Therefore, to appreciate the current quest for developing more advanced optical systems, it is important to first briefly review the history of the evolution of the development of microscopes.

1.1.1 History and Background

Direct observation of objects using the unaided eyes was the only means of perceiving the outside world until the emergence of the first optical microscope. Initially, lenses were significant components of any microscopic system and performed a function similar to that of the lens of the eye. Since the invention of the first compound microscope in the late sixteenth century, which consisted of an eyepiece, objective lens, wooden tube covered in fish skin, and cardboard, [1] it has become increasingly popular. In 1663, Robert Hooke initially employed his self-made compound microscope to observe microscopic objects, and 2 years later, Hooke obtained microscopic images of many familiar objects using his microscope. The term "cell" was first coined by him, which was later recognized as the basic building block of every living organism [2, 3]. In the 1670s, Antonie van Leeuwenhoek pioneered

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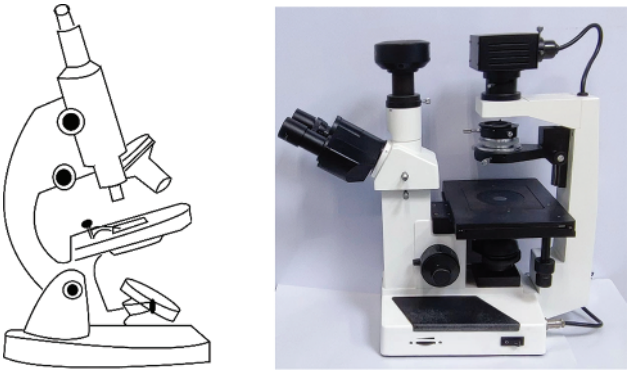

Figure 1.1 Images of antiquated (a) and modern (b) optical microscopes.

hand-made stamp-sized microscopes to observe freshwater microorganisms at a magnification of approximately 300. In 1674, with the assistance of the simple microscope, Leeuwenhoek effectively launched a new area of research— microbiology. Nevertheless, single-lens microscopes remained popular until the 1850s [4]. Figure 1.1 depicts images of an antiquated (left) and a modern (right) optical microscope.

The resolution of a simple microscope is limited owing to the constraints of using a single lens, which is improved in a compound microscope with an objective lens and an eyepiece. The focal length, numerical aperture (NA), and field of view (FOV) are the common features of an objective lens. In a compound microscope, the objective lens possesses a short focal length and is therefore placed close to the specimen. It enables the formation of a real image in the front focal plane of the second lens, whereas the eyepiece can form a magnified virtual image at the observing end. The total magnification of a microscope is the product of the magnification associated with the objective and eyepiece lenses. In addition, a compound microscope can be constructed using a converging lens, a body tube, and illumination sources. Typically, the aim of the converging lens is to focus the image of the light source onto a sample. However, in an alternative setup, the source image is focused onto the condenser so that it could ultimately be focused onto the entrance pupil of the microscope objective lens. This is known as Köhler illumination, which typically offers the advantage of averaging the nonuniformities of the source in the imaging process (Figure 1.2) [5].

For modern microscopes, the objective lens enables a standard magnification ranging from 2 to 100 folds. In the imaging process, the objective lens collects rays from each target point, which are imaged at the front focal plane of the eyepiece. The common rules of ray tracing are used in image formation. Without consideration of aberration, geometric rays from each object point form a point image. However, in the presence of aberrations, each fine object point is replaced by a blur spot. The eyepiece is designed to observe the relayed image at a distance that is

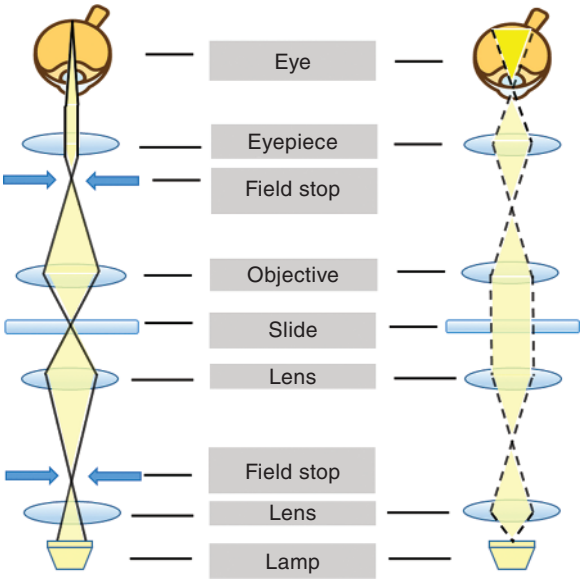

Figure 1.2 Schematic diagram of Köhler illumination. Source: Adapted from Education in Microscopy and Digital Imaging; http://zeiss-campus.magnet.fsu.edu/articles/basics/kohler.html.

convenient for the viewer. In this system, the brightness of an image is determined by the size of the lens aperture and the pupil of the eye. By adjusting the focal length, the resultant magnification of the objective can be modulated according to the requirements for viewing the object through the eyepiece with a suitable resolution. The light source imaged in the focal plane suffers from diffraction and other effects in the imaging system, which complicates the image formation process in the microscope. The German physicist Ernst Abbe is regarded as the founder of the modern theory of image formation using a microscope. In 1873, Abbe investigated microscopes in which objects in the focal plane were illuminated by focusing light from a condenser. The convergent light can be thought of as a collection of many plane waves, propagating in specific directions, which form the incident illumination *via* superimposition. Each of these effective plane waves is diffracted by features in the object plane, which implies that the smaller the objective features, the larger the diffraction angle [6].

1.2 Specialized Optical Microscopes

Gradual improvements in optical microscopy with different adaptations have been espoused for specific purposes. In 1958, the most successful microscope was invented by the British doctor John McArthur, and since then, different "McArthur" microscopes with slight modifications have been manufactured by different manufacturers. To meet the needs of professional researchers, different specialized microscopes (e.g. inverted, phase-contrast, and confocal) have been subsequently developed for modern optical applications. In this chapter, the inverted and confocal microscopes will be primarily discussed, given that these two categories of microscopes form the basis of super-resolution microscopy (SRM).

1.2.1 Inverted Microscopes

In 1850, the first inverted microscope was invented by John Lawrence Smith at Tulane University. Inverted microscopes are specifically designed for various life science, material science, and industrial applications, wherein images are mounted upside down, unlike an upright microscope. In inverted microscopes, the light source and condenser are positioned at the topmost part and point downward toward the stage, the objective lens is set below the stage pointing upward, and the eyepieces are angled upward to facilitate the observation of specimens. Inverted microscopes are crucial for various types of biological and medical research, considering their vast application in cell biology and biomedicine [7, 8].

1.2.2 Confocal Microscopes

In the 1950s, Marvin Minsky [9, 10] first conceived the idea of eliminating out-of-focus light to improve the image quality of a microscope so that only light in the focal plane can reach the detector. Based on this principle, the confocal microscope was constructed, wherein a pinhole was inserted in the image plane to restrict out-of-focus light from reaching the detector (Figure 1.3). A focused spot of illumination is generated in confocal microscopy via spatial filtering, which must be raster scanned across the sample to generate an image. The confocal microscope is equipped with pinhole apparatuses at the illuminator and the detector sides in a conjugate image plane in front of the detector, which is called "confocal." The construction of a confocal microscope includes the positioning of a pinhole in front of a light source (zirconium arc lamp) to generate a point of light, which is focused on the sample by an objective lens. A second objective lens is used to focus the illuminated specimen onto the second pinhole put in front of the detector. In this arrangement, the out-of-focus rays from the illuminated sample are

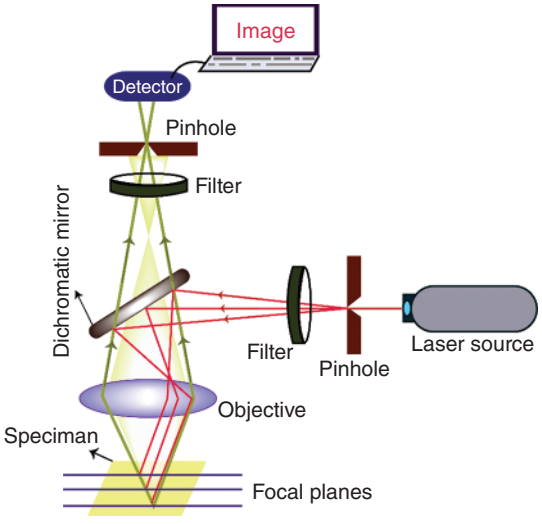

Figure 1.3 Schematic representation of a confocal microscope.

successfully removed using this "double focusing" system since they cannot reach the detector (photomultiplier, PMT, or avalanche photodiode, APD).

Therefore, a confocal microscope only detects the clear structures in the focal point, and the out-of-focus light is filtered. Since only the light from the focal point contributes to forming the final image in the confocal imaging system, this system can facilitate the three-dimensional visualization of fine structures of biological specimens. Essentially, the commonly used laser scanning confocal microscope (LSCM) can acquire two-dimensional images at different focal depths, wherein the focal point of the laser is scanned across a specimen to obtain two-dimensional optical sections. Three-dimensional images can be constructed by overlapping a series of two-dimensional images at different focal depths of the specimen (called a z-series). Moreover, a confocal microscope can be adapted with a two-photon or multi-photon optical system using argon and krypton/argon lasers or synthetic titanium–sapphire lasers to enable single- or multi-color imaging of specimens, either stained with fluorescent probes or exhibiting auto-fluorescence. It is important to note that multi-color imaging methods are useful for the study of the inter-organelle dynamics of living cells.

Although confocal optical microscopy is frequently utilized to study biological samples, given that it is highly effective for distinguishing the morphology of objects, it cannot be applied in the study of the morphology of gels. It can be directly used to observe and study amorphous and crystalline materials with structural diversity. In a confocal microscope, a tunable laser is usually used as the excitation source to excite fluorescent dyes with different spectral characteristics and to identify different labeled intracellular targets. To separate and detect the emission light, generally, specific filters and dichroic mirrors are included in the optical system.

For scientific researchers with a background in biology, chemistry, and materials science, commercial confocal laser fluorescence microscopes are among the most common and useful means of studying a diverse array of objects. In recent years, confocal fluorescence microscopy has been successfully used to gain insights into intracellular functions, such as intracellular localization, interactions, and subcellular events, occurring in live systems with excellent spatial resolution. However, even the best-performing conventional confocal microscope that uses an objective lens to collect the fluorescence signal can only achieve a resolution of c. 200 nm at best using visible light. Therefore, the focus of research on the development of advanced microscopes has shifted toward the development of new strategies to overcome the optical diffraction limit.

1.3 Optical Diffraction Limit

Without any external assistance, the human eye can only identify objects with a size larger than 0.1 mm. The evolution of the optical microscope has resulted in the improvement of the resolving power to directly observe specimens at the nanoscale. However, in 1873, Ernst Abbe predicted that a conventional optical microscope cannot achieve a resolution better than ~200 nm in the lateral plane

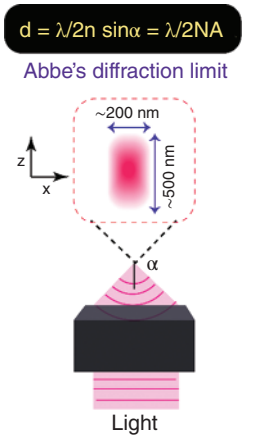

Figure 1.4 The schematic of Abbe's diffraction limit.

(x–y plane), which was defined as the optical diffraction limit [11]. Since then, for more than 100 years, the optical diffraction limit remained a major obstacle. Essentially, Abbe revealed that the physical limit of resolution is proportional to the sine of half the angular aperture. Therefore, the lens plays a key role in Abbe's experiment (Figure 1.4). Lenses are an integral part of optical systems. Depending on their function, lenses may exhibit a characteristic semi-angular aperture (α) for a wavelength of illuminating light (λ) and a refractive index of the medium (n). Abbe used the term numerical aperture, which is calculated using the formula NA = $n\sin\alpha$. The spatial resolution (d) can be interpreted as the minimum distance between two target objects that can be distinguished as separate entities in the x, y, and z direction (alongside the optical axis) image plane and can be denoted as

$$d_{x,y} = \lambda/2n\sin\alpha = \lambda/2\mathrm{NA}, \tag{1.1}$$

$$d_z = 2\lambda/(n\sin\alpha)^2 = 2\lambda/(\mathrm{NA})^{2.} \tag{1.2}$$

Generally, the excitation wavelength (λ) is chosen in the range of c. 380–740 nm, which is visible to the human eye. Practically, the illumination light cannot be focused to an infinitesimal spot, and the refractive index is different when using a glass lens in air ($n = 1$), water ($n = 1.33$), or immersion oil ($n = 1.52$). In traditional optical microscopy, the best lateral spatial resolution in the x–y plane is calculated to be 200–250 nm, where objects at a vicinity (distance less than 200 nm) cannot be discriminated.

1.4 Super-Resolution Microscopy: Overcoming the Diffraction Limit

It is theoretically impossible to break the optical diffraction limit, which restricts the best attainable resolution in a conventional microscope. This diffraction barrier can be circumvented to achieve image resolution beyond the diffraction limit via extra-information analysis in the image forming and reconstructing stages,

which constitute the basic concept of super-resolution microscopy. The term super-resolution microscopy was first used by Giuliano Toraldo di Francia in his academic publications in the early 1950s. Approximately 10 years later, Charles W. McCutchen proposed the theoretical basis for building a super-resolution optical system to distinguish spatial details beyond the diffraction limit. Since then, different theoretical methods based on special illumination patterns and mathematical algorithms have been devised to complement the super-resolution concept.

Existing optical techniques with complex hardware and software setups are not suitable for integration with super-resolution methods for substantially overcoming the diffraction barrier since these systems are based on the use of lenses and visible light. Most of the early theoretical methods only facilitated marginal circumvention of the diffraction limit. In recent years, imaging resolution has been considered to be part of information theory, rather than a fundamental limit wherein an image can be produced using analytical processes for a time series depending on localization precision and shape of the illumination using new detection modalities. In 1994, Stefan W. Hell and others first proposed the theory of stimulated emission depletion microscopy as an advanced super-resolution imaging technique that was practically achieved in 2007 [12–14]. Thereafter, several super-resolution imaging techniques have been developed to overcome the optical diffraction limit. In several SRM techniques, the theoretical spatial resolution can be unlimited, despite the utilization of lenses and visible light. However, improving the spatial resolution in SRM techniques has become more reliant on the choice of optical probes and sample preparation than on the optical methods. For their pioneering contribution to the development of super-resolution fluorescence microscopy, the 2014 Nobel Prize in Chemistry was awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner.

In a biological system, owing to the limitation of spatial resolution, a large number of fundamental processes pertaining to chemical biology that occur inside cells at a scale of tens to hundreds of nanometers and over a timescale of a few milliseconds can be observed using conventional microscopy. Therefore, the development of new fluorescence imaging approaches that are capable of breaking the diffraction barrier to resolve the complex structure and functional details of biological systems is in high demand. Therefore, Abbe's diffraction limit can be theoretically and experimentally overcome under special conditions, which introduced a new era of microscopy: super-resolution microscopy/optical nanoscopy. Thus, an optical microscope could be designed to facilitate imaging, even at the nanoscale level.

1.5 Near-Field Scanning Optical Microscopy

In conventional optical microscopes, the optimal spatial resolution is limited mainly by the wavelength of the incident light and the NA of the objective lens. In 1928, Edward H. Synge first proposed the concept of near-field scanning optical microscopy (NSOM), also usually called scanning near-field optical microscopy (SNOM). This technique is capable of circumventing the diffraction limit to achieve ultra-high resolution. NSOM has been successfully employed to obtain ultra-high

spatial resolution images and was developed based on atomic force microscopy (AFM) and optical fluorescence microscopy. Technically, the efficacy of NSOM is largely dependent on the size of the aperture tip [15]. Additionally, several technical issues must be addressed during the acquisition of images in NSOM. To acquire super-high spatial resolution images through NSOM, it is important to ensure an extremely short narrow-aperture probe to sample distance (almost 0), a longer scanning time, and low incident light intensity. The fabricated aperture size should be smaller than the wavelength of light, especially for scanning optically flat samples with feature sizes that are smaller than the aperture size. As a result, the evanescent light in the near-field region is not diffraction-limited, which allows for nanometer-scale spatial resolution.

In NSOM, the spatial resolution is markedly improved by 10–20 folds compared to conventional optical microscopes. The aperture is kept sufficiently close to the specimen so that the shear force can be detected as feedback. The tip experiences a constant force while horizontally raster scanning the surface of the sample. Generally, a piezoelectric device is used to induce the oscillation of the probe to allow the tip to oscillate. The shear force produces a reduced amplitude or oscillation frequency when the tip approaches the sample to generate a feedback signal that is used for the reconstruction of topographic images. The acquired images have a very high resolution of up to 10 nm. Since 1992, NSOM has evolved as a scientific tool and represents one of the promising techniques for overcoming Abbe's diffraction barrier.

NSOM is particularly useful in nano-science research, especially for the study of a broad range of materials with high spatial resolution. However, significant improvements are necessary to expand the scope of this instrument, especially in terms of more sensitive feedback. Additionally, NSOM has some crucial limitations in terms of scanning depth and acquisition time, apart from fundamental issues related to the study of soft materials, which need to be addressed in the future.

1.6 Far-Field Super-Resolution Microscopy

Traditional optical microscopic techniques, such as wide-field optical microscopy and confocal laser scanning microscopy, are examples of far-field optical microscopy techniques. They are advantageous compared to conventional optical microscopy in terms of ease of implementation and their broad applications. However, the resolution of far-field optical microscopy is limited by the diffraction of light. Conventional microscopy does not allow ultra-fine objects to be resolved beyond the optical diffraction limit. Traditional optical microscopy can generate images with a spatial resolution limited by the wavelength of the incident light and the NAs of the objective lens. For an optical microscope, reducing the wavelength of light and increasing the NA are important in increasing the resolution. However, irrespective of the type of lens that is used, an optical microscope cannot resolve objects smaller than half the wavelength of light (visible light: 400–700 nm).

In conventional fluorescence microscopy, fluorescent signals are collected by the objective lens of a microscope. For a microscope working in air with an NA of c. 1, the maximum resolution is approximately 0.61 λ, although some resolution improvements can be achieved by imaging in an immersion media of high refractive index. Thus, owing to the limitation of spatial resolution, various fundamental processes pertaining to chemical biology that occur inside living cells in the range of tens to hundreds of nanometers and within a timescale of a few milliseconds are difficult to resolve using conventional optical microscopy. Moreover, new imaging procedures that allow complex structures in biological cells, with a size less than 200 nm, to be resolved are in high demand.

To improve spatial resolution, several super-resolution imaging techniques have been developed in recent years, and the contribution to this area was recognized with the 2014 Nobel Prize to Eric Betzig, Stefan W. Hell, and William E. Moerner. The most recently developed prominent super-resolution microscopy techniques mainly involve point spread function engineering, single-molecule localization, and moiré fringe effect-based frequency modulation. Among the frequently used SRM techniques that typically facilitate far-field observations are stimulated emission depletion microscopy (STED), reversible saturable optical fluorescence transitions (RESOLFT), photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), structured illumination microscopy (SIM), points accumulation for imaging in nanoscale topography (PAINT), super-resolution optical fluctuation imaging microscopy (SOFI), and the newly developed minimal photon fluxes (MINFLUX) super-resolution microscopy [16–19]. The prevalent utilization of these far-field SRM techniques can be ascribed to their comparatively low complexity, ease of implementation, and a wider FOV, compared to the NSOM.

1.7 Fluorescent Probes for Super-Resolution Microscopy

Fluorescent probes play key roles in super-resolution microscopy to achieve super-resolution imaging and are of significant interest to researchers in chemistry, biochemistry, and related areas. The fluorescent probes for STED microscopy must exhibit a good fluorescence quantum yield and brightness, in addition to very high photostability to withstand the high STED laser illumination and resist photobleaching. However, for PALM/STORM microscopy, fluorescent probes should be reversibly or irreversibly photo-switchable so that a super-resolution image can be reconstructed from the collected thousands of frames of raw data images. Similarly, in SOFI, the fluorescence intensity of the probes should fluctuate rapidly under laser illumination, and super-resolution images are then reconstructed from the raw data using specific algorithms. To date, many outstanding fluorescent probes, including fluorescent proteins, organic fluorescent dyes, and nanoparticles, have been developed that enabled the visualization of subcellular features in

unprecedented detail using various super-resolution imaging techniques. However, several label-free fluorescent super-resolution microscopy techniques have also been recently developed.

1.8 Image Analysis Algorithms

The robustness of an optical system and the aptness of fluorescent probes are the two key factors that fundamentally determine the performance of a specific super-resolution imaging technique. However, even if the steps for data collection are properly followed, super-resolution imaging can be hindered by the inappropriate analysis of image data. In particular, during the image reconstruction stage, post-processing algorithms are critical to data analysis, which is important in the extraction of useful information from the images. Different super-resolution imaging methods require specific algorithms to appropriately analyze the raw image data. In point-spread-function–based super-resolution imaging techniques such as STED microscopy, simple deconvolution algorithms are typically used to process the raw data. However, the efficacy of single-molecule localization microscopy (SMLM) techniques is highly dependent on post-processing algorithms. Therefore, different algorithms have been developed to reconstruct super-resolution images with a gradual improvement in image quality. Although initial single-molecule localization algorithms used for PALM/STORM were appropriate for reconstruction of fine structures in fixed cells, the quest for new algorithms that apply to densely labeled sample analysis and the requirement of fewer frames during image reconstruction are pertinent to the improvement of the temporal resolution of live cells in super-resolution imaging experiments. In recent years, different algorithms have been developed to accomplish robust live-cell PALM/STORM imaging, which has been complemented by the advancement of new fluorescent probes. Different reconstruction algorithms, including compressive sensing, multiple Gaussian fitting, fast and unbiased localization reconstruction (Falcon), and neural network–based reconstruction methods, have been developed for the reconstruction of SMLM images with enhanced temporal/spatial resolution. The technical complexity of SIM, in addition to its sensitivity to optical aberrations and background fluorescence, means that it is challenging to use common image reconstruction algorithms for this technique, especially for thick samples. To address this issue, different new progressive image reconstruction algorithms, such as spot-scanning SIM, have been designed that can enhance spatial resolution without compromising SNR and sensitivity and facilitate 3D imaging of thick samples. Moreover, the newly developed spot-scanning SIM technique is readily compatible with many other microscopy techniques, such as multi-photon fluorescence microscopy and fluorescence lifetime microscopy. Apart from the traditional analytical image algorithms, artificial intelligence algorithms, such as deep learning and metrics learning neural networks, have become increasingly popular and are widely applied in image optimization, especially in super-resolution microscopy.

1.9 Applications

The exploration of biological events using the rapidly developing super-resolution microscopy techniques has revealed new areas of study in biological research. A detailed understanding of the biological events that occur in cellular organelles, which is extremely significant for understanding the origin of critical physiological disorders, is now possible using SRM. In particular, the study of ultra-small subcellular organelles, such as mitochondria, lysosome, and endoplasmic reticulum, at the nanoscale using SRM methods is instrumental to the discovery of important insights in cell biology, which can facilitate the discovery of new drugs. Super-resolution imaging techniques, which are being applied to both live and fixed cell imaging to obtain intra-cellular details with unprecedented high resolution, are contributing to the study of fundamental processes in cell biology such as inter-organelle communication, in a subcellular context.

1.10 Outline of the Content of Succeeding Chapters

The preceding discussions present a brief account of the systematic evolution of microscopy techniques toward the visualization of specimens with high resolution. From confocal microscopy to SRM, there have been notable improvements that have benefited research in biology pertaining to the discovery of new subcellular pathways. In particular, SRM facilitates the study of intracellular dynamics at nanoscale resolution, which has the potential to augment the advancement of biomedical research in terms of finding a cure for complex chronic ailments. However, unlike confocal microscopy, SRM has started to flourish, and significant effort has been invested in this area not only to improve the performance of SRM methods but also to expand their practical application in real-time sample analysis. In this regard, it is important to provide a detailed account of the status of these SRM techniques in modern biological research.

Therefore, in the succeeding chapters, we will present a chronological overview of the technical details and the working principle of different SRM methods, along with the scope of further technological advancements. Moreover, these chapters will highlight the effort in developing new algorithms and computational methods toward the enhancement of image quality. A chapter is dedicated to highlighting the progress in designing newer fluorescent probes, including FPs, organic probes, metal complexes, and nanoparticles, for various SRM methods, in addition to their specific pros and cons. These chapters have been comprehensively written to satisfy not only a general readership but also to answer specific queries of the advanced scientific audience.

Acknowledgment

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