HANDBOOK OF MICROSCOPY FOR NANOTECHNOLOGY
Dedicated to Professor John M. Cowley, our graduate study advisor, in memory of his outstanding contribution to science and education
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Science and technology ever seek to build structures of progressively smaller size. This effort at miniaturization has finally reached the point where structures and materials can be built through “atom-by-atom” engineering. Typical chemical bonds separate atoms by a fraction of a nanometer ($10^{-9}$ m), and the term nanotechnology has been coined for this emerging area of development. By manipulating the arrangements and bonding of atoms, materials can be designed with a far vaster range of physical, chemical and biological properties than has been previously conceived. But how to characterize the relationship between starting composition, which can be controlled, with the resulting structure and properties of a nanoscale-designed material that has superior and unique performance? Microscopy is essential to the development of nanotechnology, serving as its eyes and hands.

The rationale for editing this Handbook now has never been more compelling. Among many pioneers in the field of nanotechnology, Dr. Heinrich Rohrer and Dr. Gerd Binnig, inventors of the scanning tunneling microscope, along with Professor Ernst Ruska, inventor of the world’s first electron microscope, were awarded the Nobel Prize in Physics in 1986, for their invaluable contribution to the field of microscopy. Today, as the growth of nanotechnology is thriving around the world, microscopy will continue to increase its importance as the most powerful engine for discovery and fundamental understanding of nanoscale phenomena and structures.

This Handbook comprehensively covers the state-of-the-art in techniques to observe, characterize, measure and manipulate materials on the nanometer scale. Topics
described range from confocal optical microscopy, scanning near-field optical microscopy, various scanning probe microscopies, ion and electron microscopy, electron energy loss and X-ray spectroscopy, and electron beam lithography, etc. These are tremendously important topics for students and researchers in the field of nanotechnology. Our aim is to provide the readers a practical running start, with only enough theory to understand how best to use a particular technique and the situations in which it is best applied. The emphasis is working knowledge on the full range of modern techniques, their particular advantages, and the ways in which they fit into the big picture of nanotechnology by each furthering the development of particular nanotechnological materials.

Each topic has been authored by world-leading scientist(s), to whom we are grateful for their contribution. Our deepest appreciation goes to Professor John M. Cowley, who advised our graduate study. More than a great scientist, educator and pioneer in electron microscopy, diffraction and crystallography, he was a humble and kind man to whom we are very much indebted.

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I. OPTICAL MICROSCOPY, SCANNING PROBE MICROSCOPY, ION MICROSCOPY AND NANOFABRICATION
1. INTRODUCTION

Microscopy is the characterization of objects smaller than what can be seen with the naked human eye, and from its inception, optical microscopy has played a seminal role in the development of science. In the 1660s, Robert Hooke first resolved cork cells and thereby discovered the cellular nature of life [1]. Robert Brown’s 1827 observation of the seemingly random movement of pollen grains [2] led to the understanding of the motion that still bears his name, and ultimately to the formulation of statistical mechanics. The contributions of optical microscopy continue into the present, even as the systems of interest approach nanometer size. What makes optical microscopy so useful is the relatively low energy of visible light: in general, it does not irreversibly alter the electronic or atomic structure of the matter with which it interacts, allowing observation of natural processes in situ. Moreover, light is cheap, abundant, and can be manipulated with common and relatively inexpensive laboratory hardware.

In an optical microscope, illuminating photons are sent into the sample. They interact with atoms in the sample, and are re-emitted and captured by a detection system. The detected light is then used to reconstruct a map of the sample. An ideal microscope would detect each photon from the sample, and measure with infinite precision the three-dimensional position from which it came, when it arrived, and all of its properties (energy, polarization, phase). An exact three-dimensional map of the sample could then be created with perfect fidelity. Unfortunately, these quantities can be known only to
a certain finite precision, due to limitations in both engineering and fundamental physics.

One common high-school application of optical microscopy is to look at small objects, for example the underside of a geranium leaf. Micron-scale structure is easily revealed in the top layer of plant cells. But structure much smaller than a micron (such as individual macromolecules in the plant cell) cannot be seen, and looking deep into the sample (e.g. tens of cell layers) leads only to a nearly featureless blur. Clearly this is a far cry from the ideal microscope above.

Microscopes with improved resolution fall into two broad categories, near-field and far-field. Near-field techniques rely on scanning a nanoscale optical probe only nanometers above the surface of interest. Spatial resolution is then physically limited only by the lateral size of the tip of the probe, and information can only be gathered from the surface. This technique is the subject of another chapter in this text. In far-field microscopy, a macroscopic lens (typically with mm-scale lens elements) collects photons from a sample hundreds of microns away. Standard microscopes, like the one used in high-school, are of this type. The light detected often comes from deep within the sample, not just from the surface. Moreover, there are often enough photons to allow collection times sufficiently brief to watch a sample change in real time, here defined to be the video rate of about 25 full frames per second.

But all far-field techniques encounter the fundamental physical diffraction limit, a restriction on the maximum spatial resolution. In the present parlance, the precision with which the location of the volume generating a given detected photon (here termed the illumination volume) can be determined is roughly the same size as the wavelength of that photon [3]. Visible light has a wavelength of roughly a half micron, an order of magnitude greater than the feature size of interest to nanotechnology.

At first blush, then, the idea that far-field optical microscopy can contribute much to nanotechnology may appear absurd. However, a number of techniques have been developed to improve the precision with which the spatial position of an illumination volume can be determined. The most prevalent of these is confocal microscopy, the main subject of this chapter, where the use of a pinhole can dramatically improve the ability to see small objects. Other techniques have the potential for further improvements, but none so far has been applied widely to systems relevant to nanotechnology.

Several terms are commonly used to describe improvements in “seeing” small objects. Resolution, or resolving power, is the ability to characterize the distribution of sample inhomogeneities, for example distinguishing the internal structure of cells in Hooke’s cork or the geranium leaf. Resolution is ultimately restricted by the diffraction limit: no optical technique, including confocal, will ever permit resolution of single atoms in a crystal lattice with angstrom-scale structure. On the hand, localization is the determination of the spatial position of an object, and this is possible even when the object is far smaller than the wavelength. Localization can be of an object itself, if there is sufficient optical contrast with the surrounding area, or of a fluorescent tag attached to the object. The former is generally more common in the investigation of nanoscale materials, where in many instances (e.g. quantum dots) the nanomaterials are themselves fluorescent. The latter is common in biology, where the confocal...
microscope is often used to localize single-molecule fluorescent probes attached to cellular substructures. But in many of these systems, the tags can be imaged without confocality, such as in thin cells where three-dimensional sectioning is unneeded, or when the tags are spaced out by microns or more.

Precise localization is of tremendous utility when the length scale relevant to the question at hand is greater than the wavelength being used to probe the sample, even if the sample itself has structure on a smaller length scale. For example, Brown observed micron-scale movements of pollen grains to develop his ideas on motion, while the nanoscale (i.e. molecular) structure of the pollen was entirely irrelevant to the question he was asking. The pollen served as ideal zero-dimensional markers that he could observe; their position as a function of time, not their structure, was ultimately important. In many instances, the confocal plays a similar role, where fluorescent objects serve as probes of other systems. By asking the right questions, the diffraction limit only represents a barrier to imaging resolution, not a barrier to gathering information and answering a properly formulated scientific question.

Ultimately, the confocal is not a fancy optical microscope that through special tricks allows resolution of nanoscale objects. Rather, the confocal makes the greatest contribution to nanotechnology with rapid, non-destructive three-dimensional nanoscale localization of the sample area generating a given detected photon, and the analysis (spectroscopy) of that photon. This localization property of the confocal allows real-time spectroscopy of individual nanoscale objects, instead of ensemble averages. As such, the confocal plays a singularly important role in the investigation of structure and dynamics of systems relevant to nanotechnology, complementing the other techniques described in this volume.

This review begins with a qualitative overview (no equations) of confocal microscopy, with a brief discussion of recent advances to improve resolution and localization. Following that is a survey of recent applications of confocal microscopy to systems of interest to nanotechnology.

2. THE CONFOCAL MICROSCOPE

2.1. Principles of Confocal Microscopy

Several texts comprehensively review the confocal microscope, how it works, and the practical issues surrounding microscope construction and resolution limitations [4–7]. This section is a brief qualitative overview to confer a conceptual understanding of what a confocal is, namely how it differs from a regular optical microscope, and why those differences are important for gaining information from structures relevant to nanotechnology. All of the applications of confocal microscopy described here rely on fluorescence. That is, the incoming beam with photons of a given wavelength hits the sample, and interactions between illumination photons and sample atoms generates new photons of a lower wavelength, which are then detected. The difference in the two wavelengths must be large enough to allow separation of illumination and detection beams by mirrors, called dichrosics, that reflect light of one color and pass that of another. In practice, the separation is usually tens of nanometers or more.
The noun “confocal” is shorthand for *confocal scanning optical microscope*. Parsing in reverse, *optical microscope* indicates that visible radiation is used, and confocals are often based on, or built directly as an attachment to, optical microscopes with existing technology. Unlike traditional widefield optical microscopes, where the whole sample is illuminated at the same time, in confocal a beam of laser light is *scanned* relative to the sample, and the only light detected is emitted by the interaction between the illuminating beam and a small sample illumination volume at the focus of the microscope objective; due to the diffraction limit, the linear extent of this volume is approximately the wavelength of light. In a confocal, light coming back from the illumination volume is focused down to a another diffraction-limited spot, which is surrounded by a narrow pinhole. The pinhole spatially filters out light originating from parts of the sample *not* in the illumination volume. Because it is positioned at a point conjugate to the focal point in the sample, the pinhole is said to be *confocal* to it, and the pinhole allows only the light from the focused spot (that is, the illumination volume) to reach the detector.

A schematic of a typical confocal is given in figure 1. Light from a laser beam is reflected by a dichroic and focused onto a spot on the sample in the \(x-y\) plane by the microscope objective. The optic axis is along the \(z\) direction. Light from the sample, at a lower wavelength, comes back up from the illumination volume via the objective, passes through the dichroic, and is focused onto a point, surrounded by a pinhole, that is confocal with the objective’s focal point on the sample. The detected light then
passes to the detector. The laser beam illuminates parts of the sample covering a range of depths, which in an ordinary microscope contribute to the detected signal, and blur the image out; this is the reason that, tens of cell layers deep, the image of the geranium is blurry. In the confocal, however, the pinhole blocks all light originating from points not at the focus of the microscope objective, so that only the light from the illumination volume is detected; this effect is also known as optical sectioning. Translating the sample relative to a fixed laser beam, or moving the laser beam relative to a fixed sample, allows the point-by-point construction of the full three-dimensional map of the sample itself, with resolution limited by the size of the excitation volume, itself limited by the diffraction limit of the illuminating light.

2.2. Instrumentation

The different implementations of a confocal microscope differ primarily in how the illumination volume is moved throughout the sample. The simplest method from an optical standpoint is to keep the optics fixed, and translate the sample (figure 2a); modern piezo stages give precision and repeatability of several nanometers. Ideal from an image quality standpoint, as the optical path can be highly optimized and specific aberrations and distortions removed, sample translation is also the slowest; moving the piezo requires milliseconds, precluding the full-frame imaging at 25 frames/sec needed to achieve real-time speeds.

For higher speeds, the beam itself must be moved. Two galvanometer-driven mirrors can be used to scan the laser beam in \( x \) and \( y \) at up to a kilohertz, while maintaining beam quality (figure 2b). While not quite fast enough to achieve real-time full-frame imaging, commercial confocal microscopes based on galvanometers can reach about ten full images a second, each with about a million pixels. Beam scanning is usually accomplished much like that of a television, by first quickly scanning a line horizontally, then shifting the beam (at the end of each horizontal scan) in the vertical direction, scanning another horizontal line, and so on. Replacing the galvanometer mirror that scans horizontally with an acousto-optical device (AOD) significantly increases speed (the galvanometer is fast enough to keep up with the vertical motion). However, the AOD severely degrades the quality of the beam, and image quality correspondingly suffers. AOD-based confocals are primarily useful where gathering data at high speed is more important than achieving high resolution, as is the case in dynamical situations with relatively large (i.e. greater than micron-sized) objects.

Another major approach to increasing beam-scanning speed is to split the main laser beam into thousands of smaller laser beams, parallelizing the illumination (figure 2c). Each individual mini-beam then needs only to be moved a small amount in order for the total collection of beams to image an entire frame. This typically involves a Nipkow disk, where thousands of tiny microlenses are mounted in an otherwise opaque disk. These focus down to thousands of points, surrounded by thousands of tiny pinholes created in another disk. The laser light is then split and focused, and then the multiple tiny beams are focused onto the sample with a single objective lens. Light from the multiple illumination volumes comes back up first via the objective and then through
Figure 2. Confocal microscope instrumentation. (a) stage-scanning, in which the optical train remains fixed and the stage is moved. (b) beam scanning, with two moveable mirrors that move the beam itself. (c) Nipkow disk, where rotating disks of microlens and pinholes parallelize the illumination beam.
1. Confocal Scanning Optical Microscopy and Nanotechnology

the pinholes, then goes to the camera detector, where the thousands of mini-beams are simultaneously imaged. By spinning the disk and arranging the holes in a spiral pattern, full coverage of the frame can be achieved. The main advantage of this technique is that image quality can remain high (no AOD, for instance), and speed can be increased simply by spinning the disk faster. From an engineering standpoint, Nipkow disks are durable and easy to fabricate with existing technology; their major drawback is a total lack of flexibility: Nipkow disk systems are usually optimized for only one magnification, and after fabrication, the size of the pinholes cannot be changed to accommodate different conditions.

2. Techniques for Improving Imaging of Nanoscale Materials

2.3.1. 4-Pi Confocal

The biggest recent development in confocal microscopy has been the use of two objectives, focused on the same point, to collect light. The name 4Pi microscopy has been applied to this general technique, and is meant to evoke the idea that all of the light is collected from a sample simultaneously (i.e. the 4 pi steradians of a complete sphere); in reality, while most of the light is collected by the two objectives, they cannot image the whole sphere [8]. A full discussion of the principles and advances in 4Pi confocal microscopy is beyond the scope of this article (see [7], [8]); only a brief qualitative discussion to convey the underlying ideas behind the superior resolution of 4Pi confocal is included here.

A regular confocal rejects light coming from parts of the sample outside of the illumination volume by means of spatial filtering through a pinhole, but even if it is made arbitrarily small, the pinhole cannot localize the light coming from the sample to better than within the typical size of this region (i.e. the wavelength) because of diffraction. In addition, there is still a small contribution to the detected signal from light outside of the focal point, though that contribution decreases with greater distance from the focal point. Limitations to resolution therefore come from a combination of the finite size of the excitation volume in the sample, and the imperfect discrimination of the pinhole itself, both fundamental physical constraints inherent to the design of a confocal microscope; they cannot be overcome simply with better implementation of the same ideas. 4Pi confocal relies on coherent illumination or detection from both objectives simultaneously, effectively doubling amount of light involved and creating an interference pattern between the two beams. This allows a dramatic increase in axial resolution, often around five-fold, though lateral resolution is unchanged.

From an instrumentation standpoint, there are three different types of 4Pi confocal microscopes, A, B and C (figure 3). In type-A 4Pi confocal, illumination beams are sent through both objectives and interfere in the sample; the light coming out of only one objective is used for detection. This is the earliest, and simplest, system, and has thus far been most widely used. In type-B, illumination occurs through just one objective, but detection of interfering light from the sample comes through both objective lenses, [9] and thus its theoretical optical properties are identical to that of type-A 4Pi [10]. In
Figure 3. 4Pi Confocal configurations. (a) 4Pi-A configuration, with two illumination paths, but only one detection path. (b) 4Pi-B configuration, with only one illumination path, but two detection paths. (c) 4Pi-C configuration, with two illumination and two detection paths. (See color plate 2.)
type-C, both illumination and detection are of interfering light in the sample volume, through both objectives, [8] permitting even greater resolution [10].

Resolution is best understood in the context of the axial optical transfer function (OTF), also called the z-response function. Qualitatively, the OTF shows the contribution to the detected light from different depths in the sample (i.e. points along the optical axis). An ideal microscope would have only light from a single point in the focal plane contributing to the detected signal; in that case, the OTF would be delta function at the focus of the microscope objective (figure 4a). In a regular confocal, instead of a single delta function, the effects of finite-sized illumination volume and imperfect pinhole discrimination combine to smear out the delta function into a nearly gaussian OTF (figure 4b); with 633-nm HeNe laser illumination, the OTF of a regular confocal has a full-width at half-maximum (FWHM) of 500 nm (theory and experiment) [10]. In 4Pi confocal microscopy, the counter propagating light waves of the same frequency and intensity that illuminate the sample create an interference pattern (a standing wave). Instead of a simply gaussian shape, the OTF now has one central peak and several so-called “side-lobes” (figure 4c,d). The main advantage is that this central peak has a far narrower FWHM, theoretically calculated to be 130 nm for type-A (and thus for the optically equivalent type-B) and 95 nm for type-C, and measured at 140 nm and 95 nm, respectively [10]. The width of the central peak is independent of the relative phase between the two illuminating wavefronts (i.e. constructive or destructive interference are equivalent), [11] but nevertheless comes at the cost of having prominent side-lobes. That is, there is now a greater contribution to the light detected through the pinhole from some points farther away along the optic axis from the focal point than from some points closer, which creates artifacts. Almost all of the more recent technological developments in the 4Pi area have focused on optical “tricks” to eliminate the effects of those side bands: spatially filtering illuminating light beams with specifically-placed dark rings [12, 13] or illuminating with two photons [14, 15] to cut off the light that contributes mainly to side lobes, and computational modeling of an ideal microscope to reconstruct an “ideal” image from real data in a process known as deconvolution [15–17]. Such techniques have yielded a confocal with an effective point-spread function with a width as small as 127 nm for a type-A 4Pi confocal, with no significant contribution from the side lobes (figure 4e), [12] allowing sub-10 nm distances between test objects to be measured with uncertainties less than a single nanometer [18].

Such high resolution may finally allow direct imaging of nanoscale structures, and Leica Microsystems has just introduced the first commercial 4Pi system, the TCS 4PI, in April 2004 (figure 5). Nonetheless, there still remain some limitations to current 4Pi technology. The number of optical elements to be aligned and controlled in a 4Pi setup is at least twice that of a regular confocal, and since the stage is usually scanned in a 4Pi setup, scanning speeds are much lower, requiring minutes to image a full frame. While fast enough to image stationary samples like fixed cells, [19] or even slow-moving live ones, [20] this is too slow to monitor most real-time dynamics at present, though scanning speed can be improved by using multiple beam scanning techniques in setups similar to the Nipkow disk, cutting imaging time down to seconds [21].
2.3.2. Other Optical Techniques to Increase Resolution

Several other far-field optical techniques have achieved high resolution without spatial filtering by means of a pinhole. As they are neither confocal techniques, nor have been widely applied to systems relevant to nanotechnology, they will receive only brief mention.

Removing the pinholes and illuminating with an incoherent (non-laser) source in the 4Pi-A, 4Pi-B and 4-Pi-C geometries results in a setups known as $I^3M$, $I^2M$, and
I\textsuperscript{\textregistered}M, respectively [22, 23]. Compared with 4Pi, these widefield techniques show an equivalent increase in axial resolution, though the lateral resolution is not as great. The main advantage is collection speed: light is collected from the entire imaging plane at once, as there is no beam to be scanned. The major drawback is the requirement for a large amount of computationally intense deconvolution to obtain images. Other techniques have used different geometries, objectives, mirrors, or multiple photons for illumination, but none thus far has achieved better resolution than 4Pi or I\textsuperscript{\textregistered}M, and have not been applied widely to systems of interest to nanotechnology; an excellent survey comparing the techniques is given in [24].

A couple of non-traditional optical techniques have also increased resolution in novel ways. Placing a solid hemispherical lens against the surface of the sample (figure 6a) can improve resolution to a few times better than can be achieved with only a regular objective, with light collection efficiency improved five-fold. Interestingly, these
Figure 6. Other components to increase resolution. (a) Solid immersion lens, placed up against the sample. (b) 2×1 optical coupler to interfere the light from the two fibers.

Improvements still persist even if the lens is slightly tilted, or there is a small air gap between the lens and the sample [25]. Also, common light detectors (PMT, APD, CCD) collect only intensity information, and can not measure phase directly. Interfering two beams, however, creates the a single output beam whose intensity is directly dependent on the phase difference of the two interfering beams. In practice, light can collected from two optical fibers (in place of the pinhole at the detector of the confocal), one along the optic axis, and one slightly displaced in the lateral direction. The signals from the two fibers are then interfered in a 2×1 optical fiber coupler (figure 6b), which creates a single output beam whose intensity is measured. This interferometric technique is sensitive to single nanometer displacements on millisecond timescales [26]. Though not strictly an optical technique, another way to increase localization precision is to use objects that emit several colors. By detecting the different colors in separate channels, then combining the position data from different colors, the final position of the objects can be determined to an accuracy of better than 10 nm; [27, 28]