Second Edition

Light and Video Microscopy

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Light and Video Microscopy

Dedication

**This book is dedicated to
my colorful wife Amy,
the light of my life,
and to
Zachary and Beth,
who shine as kids**

Preface to the Second Edition

When power leads men towards arrogance, poetry reminds him of his limitations.
When power narrows the areas of man's concern, poetry reminds him of the richness and diversity of his existence.
When power corrupts, poetry cleanses.

John F. Kennedy
Amherst College October 26, 1963.

In the first sentence on the first page of the first illustrated book on microscopy, Robert Hooke (1665) wrote:

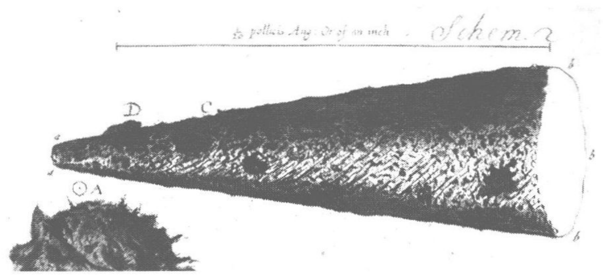
As in Geometry, the most natural way of beginning is from a Mathematical point; so is the same method in Observations and Natural history the most genuine, simple, and instructive. We must first endevour to make letters, and draw single strokes true, before we venture to write whole Sentences, or to draw large Pictures. And in Physical Enquiries, we must endevour to follow Nature in the more plain and easie ways she treads in the most simple and uncompounded bodies, to trace her steps, and to be acquainted with her manner of walking there, before we venture our selves into the multitude of meanders she has in bodies of a more complicated nature; left, being unable to distinguish and judge our way, we quickly lose both Nature our guide, and our selves too, and are left to wander in the labyrinth of groundless opinions; wanting both judgment, that light, and experience, that clew, which should direct our proceedings.

We will begin these our Inquiries therefore with the Observations of Bodies of the most simple nature first, and so gradually proceed to those of a more compounded one. In prosecution of which method, we shall begin with a Physical point; of which the Point of a Needle is commonly reckon'd for one; and is indeed, for the most part, made so sharp, that the naked eye cannot distinguish and parts of it . . . .But if view'd with a very good Microscope, we may find that the top of a Needle . . . appears a broad, blunt, and very irregular end . . . .

There are two lessons here that are just as important today as they were in 1665:

Start at beginning.
A point is not a point.

In this edition of *Light and Video Microscopy*, I continue to present the basics of what is known about light and its role in image formation in the light microscope from the perspective of "how we know what we know." One thing



"The Image we have here exhibited in the first Figure, was the top of a small and very sharp Needle, whose point a a nevertheless appear'd through the *Microscope* above a quarter of an inch broad, not round nor flat, but *irregular* and *uneven*; so that it seem'd to have been big enough to have afforded a hundred armed Mites room enough to be rang'd by each other without endangering the breaking one anothers necks, by being thrust off on either side." From Hooke, 1665.

that we know is that as a consequence of the wave properties of light, a single material point in the specimen is inflated to an ellipsoid of light in the image. The ellipsoid of light in the image has a major axis of about 400 nm and minor axes of about 200 nm. The inflation limits the resolving power of the light microscope. However, new forms of superresolution microscopy discussed in Chapter 12 of this edition allow one to use one's knowledge of light and the interaction of light with matter to remove the light that is out of place and put it back where it would have belonged if light were actually a mathematical point.

There are two lessons here that were not known in 1665:

We are a long way from the beginning.
A point even though it strays can become a point again.

My predecessor in teaching light microscopy at Cornell was Simon Henry Gage, the author of seventeen editions of *The Microscope*. Gage (1941) believed:

"(1) To most minds, and certainly to those having any grade of originality, there is a great satisfaction in understanding principles; and it is only when the principles are firmly grasped that there is complete mastery of instruments, and full certainty and facility in using them. The same is true of the methods of preparing objects for microscopic study, and the interpretation of their appearances when seen under the microscope . . . . (2) Need of

abundant practical work to go with the theoretical part has been shown by all human experience. In all the crafts and in all the fine arts mastery comes only with almost endless effort and repetition, the most common example being the attainment of facility in music . . . .It is also a part of human experience that in successfully going through the manipulations necessary to demonstrate principles, there is acquired not only skill in experiment, but an added grasp of the principles involved."

I hope this book continues in the tradition of *The Microscope* in helping you to not only use, but to understand and appreciate the relation between the real object and the image formed by the light microscope.

Moving from a point to a line, which is what we use to trace the path that the corpuscles of light take from the object to the image, we get to the word verse. The English word "verse" was originally used to indicate a line or lines of a psalm and later to indicate lines of poetry. Its root is the Latin root *vers*, which means "to turn" and which also gave rise to the word *vertere*, which also means "to turn," just as a farmer turns from one line to another while plowing. Related words, some of which are used in optics, include diverse (turned different ways), inverse (turned upside down), reverse (turned back), converse (turned about), transverse (turned across), adverse (turned against), perverse (turned away from what is right), and universe (turn into one).

I found a lost verse in The Rare & Manuscript Collections of Kroch Library, where I read Hooke's *Micrographia*. They also had a presentation copy of the seventeenth edition of *The Microscope* signed by the author, Simon Henry Gage, and given to the chemical microscopist Émile M. Chamot. Pasted into the book I found the following lost poem by Louis Ginsberg (Wayne, 2013d):

Microscope

With bated breath and buoyant hope,
Man bends above the microscope;
The question, pulsing deep in dark,
Splinters to many a question mark.

He looks upon a point to check
The tiny, faint and finite speck;
And yet the more he stares and broods,
It swells into infinitudes.

The more he peers into the middle
Of particles that shape the riddle,
The lens, for all that he can see,
But magnifies the mystery . . .

The microscope provides a natural interface between poetry and the sciences.

I thank Allan Witztum, my friend, colleague and fellow student of David Bierhorst, for sharing his appreciation for the interface between the humanities and the sciences.

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Preface to the First Edition

I am very lucky. I am sitting in the rare book room of the library waiting for Robert Hooke's (1665) *Micrographia*, Matthias Schleiden's (1849) *Principles of Scientific Botany*, and Hermann Schacht's (1853) *The Microscope*. I am thankful for the microscopists and librarians at Cornell University, both living and dead, who have nurtured a continuous link between the past and the present. By doing so, they have built a strong foundation for the future.

Robert Hooke (1665) begins the *Micrographia* by stating that "... the science of nature has already too long made only a work of the brain and the fancy: It is now high time that it should return to the plainness and soundness of observations on material and obvious things." Today, too many casual microscope users do not think about the relationship between the image and reality and are content to push a button, capture an image, enhance the image with Adobe Photoshop, and submit it for publication. However, the sentence that followed the one just quoted indicates that the microscope was not to be used in place of the brain, but in addition to the brain. Hooke (1665) wrote, "It is said of great empires, that the best way to preserve them from decay, is to bring them back to the first principles, and arts, on which they did begin." To understand how a microscope forms an image of a specimen still requires the brain, and today I am privileged to be able to present the work of so many people who have struggled and are struggling to understand the relationship between the image and reality, and to develop instruments that, when used thoughtfully, can make a picture that is worth a thousand words.

Matthias Schleiden (1849), the botanist who inspired Carl Zeiss to build microscopes, wrote about the importance of the mind of the observer:

It is supposed that nothing more is requisite for microscopical investigation than a good instrument and an object, and that it is only necessary to keep the eye over the eye-piece, in order to be au fait. Link expresses this opinion in the preface to his phytotomical plates: 'I have generally left altogether the observation to my artist, Herr Schmidt, and the unprejudiced mind of this observer, who is totally unacquainted with any of the theories of botany, guarantees the correctness of the drawings.' The result of such absurdity is, that Link's phytotomical plates are perfectly useless; and, in spite of his celebrated name, we are compelled to warn every beginner from using them.... Link might just as well have asked a child about the apparent distance of the moon, expecting a correct opinion on account of the child's unprejudiced views. Just as we only gradually learn to see with the naked eye in our infancy, and often experience unavoidable illusions, such as that connected with the rising moon, so we must first gradually learn to see through the medium of the microscope.... We can only succeed gradually in bringing a clear conception before our mind....

Hermann Schacht (1853) emphasized that we should "see with intelligence" when he wrote,

But the possession of a microscope, and the perfection of such an instrument, are not sufficient. It is necessary to have an intimate acquaintance, not only with the management of the microscope, but also with the objects to be examined; above all things it is necessary to see with intelligence, and to learn to see with judgment. Seeing, as Schleiden very justly observes, is a difficult art; seeing with the microscope is yet more difficult.... Long and thorough practice with the microscope secures the observer from deceptions which arise, not from any fault in the instrument, but from a want of acquaintance with the microscope, and from a forgetfulness of the wide difference between common vision and vision through a microscope. Deceptions also arise from a neglect to distinguish between the natural appearance of the object under observation, and that which it assumes under the microscope.

Throughout the many editions of his book, *The Microscope*, Simon Henry Gage (1941) reminded his readers of the importance of the microscopist as well as the microscope (Kingsbury, 1944): "To most minds, and certainly to those having any grade of originality, there is a great satisfaction in understanding principles; and it is only when the principles are firmly grasped that there is complete mastery of instruments, and full certainty and facility in using them... . for the highest creative work from which arises real progress both in theory and in practice, a knowledge of principles is indispensable." He went on to say that an "image, whether it is made with or without the aid of the microscope, must always depend upon the character and training of the seeing and appreciating brain behind the eye."

This book is a written version of the microscopy course I teach at Cornell University. I introduce my students to the principles of light and microscopy through lecture–demonstrations and laboratories where they can put themselves in the shoes of the masters and be virtual witnesses to their original observations. In this way, they learn the strengths and limitations of the work, how first principles were uncovered, and, in some respects, feel the magic of discovery. I urge my students to learn through personal experience and to be skeptical of everything I say. I urge the reader to use this book as a guide to gain personal experience with the microscope. Please read it with a skeptical and critical mind and forgive my limitations.

Biologists often are disempowered when it comes to buying a microscope, and the more scared they are, the more likely it is that they will buy an expensive microscope, in essence, believing that having a prestigious brand name will make up for their lack of knowledge. So buying an expensive microscope when a less expensive one may be equally good or better may be more a sign of ignorance than a sign of wisdom and greatness. I wrote this book, describing microscopy from the very beginning, not only to teach people how to use a microscope and understand the relationship between the specimen and the image, but to empower people to buy a microscope based on its virtues, not on its name. You can see whether or not a microscope manufacturer is looking for a knowledgeable customer by searching the web sites to see if the manufacturer offers information necessary to make a wise choice or whether the manufacturer primarily is selling prestige. Of course, sometimes the prestigious microscope is the right one for your needs.

If you are ready to buy a microscope after reading this book, arrange for all the manufacturers to bring their microscopes to your laboratory and then observe your samples on each microscope. See for yourself: Which microscopes have the features you want? Which microscope gives you the best image? What is the cost/benefit relationship? I thank M. V. Parthasarathy for teaching me this way of buying a microscope.

Epistemology is the study of how we know what we know—that is, how reality is perceived, measured, and understood. Ontology is the study of the nature of what we know that we consider to be real. This book is about how a light microscope can be used to help you delve into the invisible world and obtain information about the microscopic world that is grounded in reality. The second book in this series, entitled, *Plant Cell Biology*, is about what we have learned about the nature of life from microscopical studies of the cell.

The interpretation of microscopic images depends on our understanding of the nature of light and its interactions with the specimen. Consequently, an understanding of the nature of light is the foundation of our knowledge of microscopic images. Appendix II provides my best guess about the nature of light from studying its interactions with matter with a microscope.

I thank David Bierhorst, Peter Webster, and especially Peter Hepler for introducing me to my life-long love of microscopy. The essence of my course comes from the microscopy course that Peter Hepler taught at the University of Massachusetts. Peter also stressed the importance of character in doing science. Right now, I am looking through the notes from that course. I was very lucky to have had Peter as a teacher. I also thank Dominick Paolillo, M. V. Parthasarathy, and George Conneman for making it possible for me to teach a microscopy course at Cornell and for being supportive every step of the way. I also thank the students and teaching assistants who shared in the mutual and never-ending journey to understand light, microscopy, and microscopic specimens. I have used the pictures that my students have taken in class to illustrate this book. Unfortunately, I no longer know who took which picture, so I can only give my thanks without giving them the credit they deserve. Lastly, I thank my family: mom and dad, Scott and Michelle, for making it possible for me to write this book.

As Hermann Schacht wrote in 1853, “Like my predecessors, I shall have overlooked many things, and perhaps have entered into many superfluous particulars: but, as far as regards matters of importance, there will be found in this work everything which, after mature consideration, I have thought necessary.”

Randy Wayne

Contents

Chapter 1

The Relation Between the Object and the Image

And God said, "Let there be light," and there was light. God saw that the light was good, and he separated the light from the darkness.

Gen. 1:3-4

Since we acquire a significant amount of reliable information regarding the real world through our eyes, we often say, "seeing is believing." However, seeing involves a number of processes that take place in space and time as light travels from a real object to our eyes and then gets coded into electrical signals that travel through the optic nerve to the brain. In the brain, neural signals are processed by the visual cortex, and ultimately the brain projects its interpretation of the real object as a virtual image seen by the mind's eye. To ensure that "seeing is not deceiving" requires an understanding of light, optics, the interaction of light with matter, and how the brain functions to create and interpret the relationship between a real object and its image. According to Samuel Tolansky (1964), "There is often a failure in co-ordination between what we see and what we evaluate … .surprisingly enough, we shall find that most serious errors can creep even into scientific observations entirely because we are tricked by optical illusions into making quite faulty judgments." Simon Henry Gage (1941), author of seventeen editions of the classic textbook, *The Microscope,* reminds us that the "image, whether it is made with or without the aid of the microscope, must always depend upon the character and training of the seeing and appreciating brain behind the eye."

The light microscope, one of the most elegant instruments ever invented, is a device that permits us to study the interaction of light with matter at a resolution much greater than that of the unaided eye (Dobell, 1932; Wilson, 1995; Ruestow, 2004; Schickore, 2007; Ratcliff, 2009). Due to the constancy of the interaction of light with matter, we can peer into the would-be invisible world to discover the hidden properties of objects in that world (Appendix I). We can make transparent and invisible cells visible with a dark-field, phase-contrast, or differential interference microscope. We can use a polarizing microscope to reveal the orientation of macromolecules in a cell, and we can use it to determine the entropy and enthalpy of the polymerization process. We can use an interference microscope to weigh objects and to ascertain the mass of the cell's nucleus. We can use a fluorescence microscope to localize proteins in the cytoplasm, genes on a chromosome, and the free Ca^{2+} concentration and pH of the surrounding milieu. We can use a centrifuge microscope or a microscope with laser tweezers to measure the forces involved in cellular motility or to determine the elasticity and viscosity of the cytoplasm. We can use a laser Doppler microscope, which takes advantage of the Doppler effect produced by moving objects, to characterize the velocities of organelles moving through the cytoplasm. We can also use a variety of laser microscopes to visualize single molecules.

I wrote this book so that you can make the most of the light microscope when it comes to faithfully creating and correctly interpreting images. To this end, the goals of this book are to:

- Describe the nature of light.
- Describe the relationship between an object and its image.
- Describe how light interacts with matter to yield information about the structure, composition, and local environment of biological and other specimens.
- Describe how optical systems work so that you will be able to interpret the images obtained at high resolution and magnification.
- Give you the necessary procedures and tricks so that you can gain practical experience with the light microscope and become an excellent microscopist.

LUMINOUS AND NONLUMINOUS OBJECTS

All objects, which are perceived by our sense of sight, can be divided into two broad classes. One class of objects, known as luminous bodies, includes "hot" or incandescent sources such as the sun, the stars, torches,

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oil lamps, candles, coal and natural gas lamps, kerosene lamps, and electric light bulbs, and "cold" sources such as fireflies and glow worms that produce "living light" (Brewster, 1830; Hunt, 1850; Harvey, 1920, 1940). These luminous objects are visible to our eyes. The second class of objects is nonluminous. However, they can be made visible to our eyes when they are in the presence of a luminous body. Thus the sun makes the moon, Earth, and other planets visible to us, and a light bulb makes all the objects in a room or on a microscope slide visible to us. The nonluminous bodies become visible by scattering the light that comes from luminous bodies. A luminous or nonluminous body is visible to us only if there are sufficient differences in brightness or color between it and its surroundings. The difference in brightness or color between points in the image formed of an object on our retina is known as contrast.

OBJECT AND IMAGE

Each object is composed of many small and finite points composed of atoms or molecules. Ultimately, the image of each object is a point-by-point representation of that object upon our retina. Each point in the image should be a faithful representation of the brightness and color of the conjugate point in the object. Two points on different planes are conjugate if they represent identical spatial locations on the two planes. The object we see may itself be an intermediate image of a real object. The intermediate image of a real object observed with a microscope, telescope, or by looking at a photograph, movie, television screen, or computer monitor should also be a faithful point-by-point representation of the brightness and color of each conjugate point of the real object. While we only see brightness and color, the mind interprets the relative brightness and colors of the points of light on the retina and makes a judgment as to the size, shape, location, and position of the real object in its environment.

What we see, however, is not a perfect representation of the physical world. First, our eyes are not perfect, and our vision is limited by physical, genetic, and nutritional factors (Wald, 1967; Helmholtz, 2005). For example, we cannot see clearly things that are too far or too close, too dark or too bright, or things that emit radiation outside the visible range of wavelengths. Second, our vision is affected by physiological and psychological factors, and we can be easily fooled by our sense of sight (Goethe, 1840; Sully, 1881; Gregory, 1973; Békésy, 1967; Wade, 1998; Russ, 2004). Third, as Goethe learned when he studied the colors of the Italian landscape as they transformed from vibrant to muted and back again as the weather changed (Heisenberg, 1979), or as humankind learned upon the introduction of artificial illumination (Wickenden, 1910; Steinmetz, 1918; Otter, 2008), we must remember to take the source of illumination as well as the environment surrounding the object into consideration.

The architects of ancient Greece knew that the optical illusions that occur under certain circumstances, if not taken into consideration, would diminish the beauty of great buildings such as the Parthenon, which was built in honor of the virgin (parthenos) Athena (Penrose, 1851; Fletcher and Fletcher, 1905; Prokkola, 2011). For example, stylobates, or long horizontal foundations for the classical columns, and architraves, the horizontal beams above doorways, would appear to sag in the middle if they were made perfectly straight. Consequently, the architects used horizontal beams with convex tops to compensate for the optical illusion—the result being a perfectly square-looking structure. The columns of the Parthenon are famous, but they are not identical. The columns that are viewed against the bright Greek sky were made thicker than the columns backed by the inner temple or cella wall, since identical columns viewed against a bright background appear thinner than those viewed against a dark background. By compensating for the optical illusion, the columns appear identical and magnificent.

The sculptors of ancient Greece also knew about optical illusions, as evidenced by an apocryphal legend concerning two sculptors, Phidias, the teacher, and his student, Alkamenes (Anon, 1851). They were contenders in a contest to produce a sculpture of Athena that would stand upon a pedestal. Alkamenes sculpted a beautiful and well-proportioned figure of Athena, while Phidias, using his knowledge of geometry and optics, fashioned a grotesque and distorted figure. While the two sculptures were on the ground, the judges marveled at the one created by Alkamenes and laughed at the one created by Phidias. However, once the sculptures were put on top of the column, the perspective changed, and Phidias's sculpture assumed great beauty while Alkamenes sculpture looked distorted. Knowing that the angles subtended by each feature of the object become proportionally smaller as the height and distance of the feature increased, Phidias formed the facial features proportionately larger and the lower features proportionately smaller so that the sculpture of Athena would look normal and beautiful atop its final location. As Alexander Pope (1711) wrote in *An Essay on Criticism*,

Some Figures monstrous and mis-shap'd appear,
Consider'd singly, or beheld too near,
Which, but proportion'd to their Light, or Place,
Due Distance reconciles to Form and Grace.

Leon Battista Alberti, perhaps the original Renaissance man, restored the union of the arts and sciences that had been lost during the Middle Ages. He combined his love of nature with Euclidean geometry and