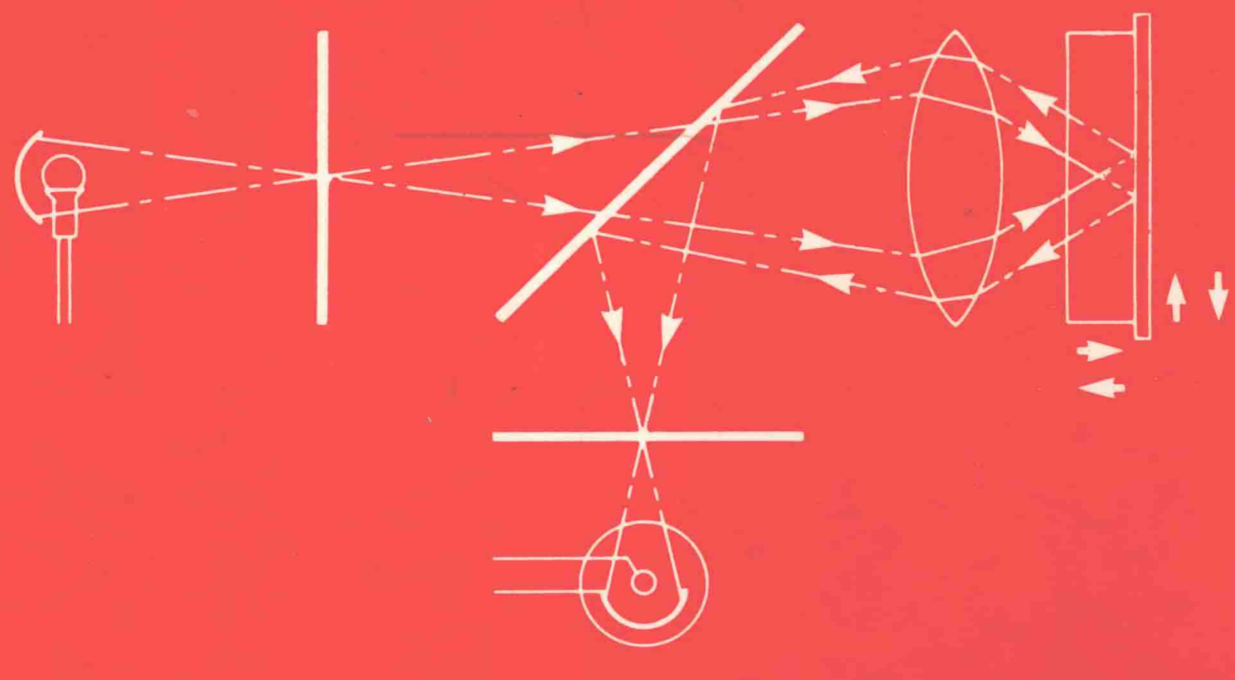
HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY



Edited by
James B. Pawley

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University of Wisconsin–Madison
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Preface

In 1987 the Electron Microscopy Society of America (EMSA) under the leadership of J. P. Revel (Cal Tech) initiated a major program to present a discussion of recent advances in light microscopy as part of the annual meeting. The result was three special LM sessions at the Milwaukee meeting in August 1988: The LM Forum, organized by me, and Symposia on Confocal LM, organized by G. Schatten (Madison), and on Integrated Acoustic/LM/EM organized by C. Rieder (Albany). In addition, there was an optical micro-analysis session emphasizing Raman techniques, organized by the Microbeam Analysis Society, for a total of 40 invited and 30 contributed papers on optical techniques.

Following this successful meeting, discussions among the participants revealed support for a slightly more focussed approach at the next meeting. The benefits of confocal techniques were now felt to be widely appreciated and it seemed time to really evaluate the actual performance of the various instruments and to compare this with theoretical benchmarks and so produce a consensus on where major improvements were likely to be possible in the future. It was felt important to shift from the assertion that "Confocal Works" to the matter of how to make it work better.

Because of the rapid pace of development in the field, we recognized that we were unlikely to be able to be totally definitive on all matters affecting the confocal microscope, but we also felt that the field would benefit from access to a good list of questions and as many answers as time permitted. To do this, it was decided to try to elicit a series of talks in which each one covered a single instrumentational feature unique to confocal microscopy (particularly biological confocal microscopy). The initial list included 12 topics ranging from laser and conventional sources through scanning systems, objective lenses, chromophors, "the pinhole", photon detectors and 3D data display, as well as three overviews on the genesis of the confocal approach, its fundamental limitations and its quantitative capabilities.

In parallel with these developments at EMSA, Drs. J. Wooley and S. Pierce of the Instrumentation and Instrument Development Program at the National Science Foundation had followed a similar path. The Instrumentation and Instrument Development Program is responsible for supporting the purchase of major items of multi-user instrumentation for the conduct of basic research in the life sciences, particularly that which is supported by the NSF Divisions of Behavioral and Neural sciences, Cellular Biosciences, Molecular Biosciences, and BIOTIC Systems and Resources. For the past five years, the Program had emphasized three areas of activity: 1) New instruments that either extend current sensitivity or resolution, or provide new techniques for detection, quantification or observation of biological phenomena. 2) New computer software to enhance current or new instrumentation, and 3) Sponsored workshops in emerging areas of instrumentation or instrument development. They believed that it was clear that confocal microscopy and other new microscopical instrumentation was going to drive important scientific discoveries across wide areas of physiology, cellular biology and neurobiology. They had been looking for a forum in which they could advance the state of the art of confocal microscopy, alert manufacturers to the limitations of current instruments, and catalyze progress toward new directions in confocal instrument development.

These goals were so close to those of the EMSA project that the two groups decided to join forces with EMSA to provide the organization and the venue for a Confocal Workshop and NSF to provide the financial support for the speakers expenses and for the publication of extended abstracts.

The abstracts were initially envisioned as each being about 10–15 pages of camera-ready manuscript but, because of the generous and enthusiastic response of the many leaders of the confocal LM community who agreed to participate, the manuscripts actually submitted were up to fifty pages in length. In addition, scissions and additions increased the list of the topics covered to a total of 19, plus an annotated bibliography.

As the aim of the volume was to discuss the instrument rather than to describe specific applications, the biological emphasis emerges in most chapters as the need to use photons efficiently at every stage of the imaging process and thereby reduce the effects of bleaching and photo-damage to the specimen. In this context, several chapters in this volume emphasize for the first time limitations imposed by everything from fluorescence saturation and sub-optimal signal digitization to specimen preparation.

On a more general level, chapters were added on related instrumentation and on the often unrecognized limitations imposed by the process of pixelating the data contained in digitally recorded images. Several months of frenzied activity got the final mock-ups to the printer in early July.

The nineteen papers were presented at a two day workshop on August 8–9, 1989 at the EMSA Meeting in Houston, TX where the first, soft-cover edition of the Handbook was distributed at that time under the convenient but largely fanciful imprimatur of the IMR Press.

The response was so enthusiastic that it was decided to produce a second, hard-cover edition with an established publisher. This would permit wider distribution and would allow us to correct the errors associated with the short preparation time of the first edition. In addition, extra paragraphs and figures were added to fill gaps in the original or to take note of recent developments.

Taken as a whole, I believe these papers constitute the most complete consideration on the topic available at this time. I am sure that all of the other authors join me in the hope that it will prove to be a catalyst in the development of yet better instrumentation and techniques in the field of biological confocal microscopy. Indeed, improvements evident in the design of the Biorad MRC-600 and of the Leitz CLSM show some evidence of this trend.

Many people have contributed to the production of this volume starting with Drs. Pierce and Wooley and all of the authors.

In addition, I should like to single out R. and C. Moen and K. Hamele for their editorial assistance and C. Thomas, C. Ewing, K. Morgan and P. Henderson for help in retyping some of the manuscripts, A. Freidman and L. Moberly of University of Wisconsin-Madison Publications and W. Kasdorf and N. MacMiller of Impressions for their patience with the typesetting. Special thanks are also due to G. Benham of the Biorad Corporation, and V. Argiro of Vital Images who, when rising costs threatened to delay publication of the first edition, stepped in to fund the printing of the colored cover. This gesture is noted here because, due to a printing mix-up, no mention of the source of the cover images or of the support was included in that edition.

My heartfelt thanks to you all.

James Pawley
Editor
12/89

Contents

Chapter 1

Foundations of Confocal Scanned Imaging in Light Microscopy

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Seldom has the introduction of a new instrument generated as instant an excitement among biologists as the laser-scanning confocal microscope. With the new microscope one can slice incredibly clean, thin optical sections out of thick fluorescent specimens; view specimens in planes running parallel to the line of sight; penetrate deep into light-scattering tissues; gain impressive 3-dimensional views at very high resolution; and improve the precision of microphotometry.

While the instruments that engendered such excitement mostly became commercially available in 1987, the optical and electronic theory and the technology that led to this sudden emergence had been brewing for several decades. The development of this microscope stems from several roots, including light microscopy, confocal imaging, video and scanning microscopy, and coherent or laser-illuminated optics (see historic overview in Table 1). In this chapter, I will review these developments as they relate to the principles and use of the confocal microscope, and then end with some general remarks regarding the new microscope.

LIGHT MICROSCOPY

Lateral Resolution

The foundation of light microscopy was established, a century ago, by Ernst Abbe (1873, 1884). He demonstrated how the diffraction of light by the specimen and by the objective lens determined image resolution, defined the conditions needed to design a lens whose resolution was diffraction limited (rather than limited by chromatic and spherical aberrations), and established the role of objective lens and condenser numerical apertures on image resolution (Equation 1). Thus,

$$d = 1.22 \times \lambda_o / (NA_{obj} + NA_{cond}) \qquad (1)$$

where d is the minimum distance that the diffraction images of two points in the specimen can approach each other laterally before they merge and can no longer be resolved as two separate points (in accordance with Rayleigh's criterion for visually resolving two nearly equally bright points). d is expressed as distance (within the focused plane) in the specimen space; λ_o is the wavelength of light in vacuum; and NA_{obj} and NA_{cond} the numerical apertures (NA)s of the objective and condenser lenses respectively. The NA is the product of 'the sine of the half angle of the cone of light either acceptable by the objective lens or emerging from the condenser lens' and 'the refractive indexes of the imbibition medium between the specimen and the objective or condenser lens, respectively.' [The impact of the quality and NA of the condenser lens on image resolution are considered from a more precise, theoretical standpoint by Zernicke and Hopkins (see Born and Wolf 1980). Their derivations lead to a more complex relationship which is somewhat at variance with Equation (1) or with the alternate Sparrow criterion.]

Using the Rayleigh criterion for resolution, the value for d equals the radius of the Airy disk, namely the radius of the first minimum of a unit diffraction image. The unit diffraction image is the diffraction pattern (produced in the image plane under the particular conditions of observations) of an infinitely small point in the specimen space.

In addition to the wavelength and NA of the objective and condenser lenses, three additional factors or conditions affect the unit diffraction image and image resolution in the microscope. The first factor is the degree of coherence of the light waves. Equation (1) assumes that one is dealing with points (or periodic objects) in the specimen that emit or scatter light waves whose coherence varies with the condenser NA. For objects that are illuminated fully coherently (a condition that pertains when NA_{cond} approaches 0, namely when the condenser iris is closed down to a pinhole), the minimum resolvable distance becomes 2d; in other words, the resolution decreases by a factor of two compared to the case when adjoining specimen points are illuminated incoherently. As the condenser iris is opened up and NA_{cond} becomes larger, the illumination becomes progressively less coherent. [Note, however, that laser beams tend to illuminate objects coherently even when the condenser iris is not closed down (see "LASERS AND MICROSCOPY").]

The second factor is the field size. Equation (1) holds true only when the field of view is not extremely small. When the field of view is extremely small, as in confocal microscopy, the resolution can in fact be greater than when the field of view is not limited. We shall return to this point later.

The third, and equally important, condition is that the resolution criterion applies only to objective lenses used under conditions in which the image is free from significant aberrations. This implies several things: a well-corrected, clean objective lens is used within the wavelengths of light and diameter of field for which the lens was designed (in many cases in conjunction with specific oculars); the refractive index, dispersion, and thickness of the immersion media and cover slip are those specified for the particular objective lens; the correct tube length and ancillary optics are used and the optics are axially aligned; the full complement of image-forming rays and light waves leaving the objective aperture converge to the image plane without obstruction; the condenser aperture is homogeneously and fully illuminated; and the condenser is properly focused (see Chapters 6, 7, 8, 9, and 11; also Inoué, 1986, Chapter 5).

TABLE 1 HISTORIC OVERVIEW

Confocal Microscopy	Microscopy	Video (Microscopy)
	Abbe (1873, 1884)[a]	Nipkow (1884)
	Berek (1927)[d]	
	Zernicke (1935)[a,c]	Zworykin (1934)
	Gabor (1968)[a]	
	H.H. Hopkins (1951)[a]	Flory (1951)
	Linfoot & Wolf (1953) 3-D diffraction by annul. apert.[a,d]	Young/Roberts (1951) Flying spot[c]
	Tolardo di Francia (1955) Limited field[b]	Montgomery et al (1956) Flying spot UV[c]
	Nomarski (1955)[c]	
	Linfoot & Wolf (1956) 3-D diffraction pattern[a,d]	
	Ingelstam (1956) Resolution and info. theory[a]	
Minsky Patent (1957) Insight[a,b,c,d] Stage scanning[f]	Kubota & Inoué (1959)[a]	
	Smith & Osterberg (1961)[a]	
	Harris (1964)[a,b]	Freed & Engle (1962) Flying spot[c]
	Ellis (1966) Holomicrography[c,d]	
Petráň et al (1968) Tandem scanning[d]		
Davidovits & Egger (1971) Laser illumination, Lens scan[d]	Hoffman & Gross (1975) Modulation contrast[c]	
Sheppard & Choudhury (1977) Theory[a,b,d]		
Sheppard et al (1978) Stage scanning	Ellis (1978) Single sideband edge enhancement microscopy[c]	
Brakenhoff et al (1979) Specimen scan[e] (1985)		Castleman (1979) Digital image processing[a,c,e]
Koester (1980) Scanning mirror[d]	Quate (1980) Acoustic microscopy[a,c]	
		Inoué (1981)[a,c]
		Allen et al (1981a,b)[a,c]
		Fuchs et al (1982)[f]
		Agard & Sedat (1983)[a,c,d]
Boyde (1985a) Nipkow type[d,e]		
Cox & Sheppard (1983) Digital recording[d,e]		
Åslund et al (1983) 2-mirror laser scanning[d]		
Hamilton et al (1984) Differential phase[c,d]		
Wilson & Sheppard (1984) Extended depth of field[c,d,e,f]		Sher & Barry (1985)[f]
Carlsson et al (1985) Laser scan Stacks of confocal images[d,e]		Inoué (1986) Overview, how to[a,c,e,f]
Wijnaendts van Resandt et al (1985) x-z view[d,e]	Ellis (1985) Light scrambler[d]	Fay et al (1985)[a,c,d]
Suzuki-Horikawa (1986) Video rate laser scan, Acousto optical modulator, No exit pinhole	Cox & Sheppard (1986)[b]	Castleman (1987)[a]
Xiao & Kino (1987) Nipkow type[d]		
McCarthy & Walker (1988) Nipkow type[d]		
Amos et al (1987)[c,d,e]	Ellis (1988) Scanned aperture phase contrast[a,c,d]	

a—Diffraction theory; b—Superresolution; c—Contrast modes; d—Optical sectioning/depth of field; e—Stereo; f—3-D in objective space

Axial Resolution

We now turn to the axial (z-axis) resolution, measured along the optical axis of the microscope, i.e., perpendicular to the plane of focus.

In the case of lateral resolution, i.e., the resolution in the plane of focus, we defined resolution in terms of the minimum distance that the diffraction images of two point sources in the specimen could approach each other and still visually be distinguished as two. Using the Rayleigh criterion, this minimum distance equaled the radius of the first minimum of the unit diffraction image.

Similarly, axial resolution can be defined using two criteria, either the minimum distance that the diffraction images of two points can approach each other along the axis of the microscope and still be seen as two, or by the radius of the first minimum of the diffraction image of an infinitely small point object. I shall now expand on the latter point.

The precise distribution of energy in the image-forming light above and below focus, especially for high NA objective lenses, cannot be deduced by geometric ray tracing, but must be derived from wave optics. The wave optical studies of Linfoot and Wolf (1956) show that the image of a point source produced by a diffraction-limited optical system (such as a properly constructed and used microscope) is not only periodic around the point of focus in the focal plane, but is also periodic above and below the focal plane along the axis of the microscope. [Such 3-dimensional diffraction images (including those produced in the presence of lens aberrations) are presented photographically by Cagnet et al. (1962). The intensity distribution calculated by Linfoot and Wolf for an aberration-free system is reproduced in Born and Wolf (1980); also in Inoué (1986, Fig. 5–21). The 3-dimensional pattern of a point source formed by a lens possessing an annular aperture was calculated by Linfoot and Wolf (1953).]

Near the plane of focus, the axial magnification of the microscope rises as the square of the lateral magnification, and the distance from the center of the diffraction image to the first minimum along the microscope axis turns out to be approximately twice as far as it is to the first minimum in the plane of focus (again both translated into dimensions measured in the specimen space). The central zone of the unit diffraction image is thus stretched along the z-axis of the microscope like an American football, whose major radius is twice as long as its minor radius.

For an incoherent source or scatterer, the cross section through the middle of the football (transverse to its axis of elongation) yields the familiar Airy disk. Therefore, the minimum distance that one can resolve axially is twice as large as the minimum distance that one can resolve transversely. In other words, the axial resolving power is approximately one half of the lateral resolving power.

The axial resolution of the microscope also gives rise to its "axial setting accuracy." The axial setting accuracy is defined as the distance that one has to shift the fine focus of the microscope before the image of an infinitely thin object changes perceptibly. According to Françon (1961), the axial setting accuracy (2ζ) is given by:

$$2\zeta = \lambda / \{4n \times \sin^2(u/2)\} \qquad (2)$$

where, λ is the wavelength of light, n the refractive index of the immersion medium, and u the half angle of the cone of light that is captured by the objective lens. Françon points out that measurements of the axial setting accuracy is influenced by several factors, and that a value as small as 2ζ is seldom achieved in practice.

Regardless of whose equation is chosen for calculating the axial resolution, it is important to note that the axial resolution (and the related axial-setting accuracy and the shallowness of depth of field) rises with the square of the NA, in contrast to the lateral resolution which rises with the first power of the NA [cf. Eqs. 2, 1].

Depth of Field

The depth of field of a microscope is the depth of the image (measured along the microscope axis translated into distances in the specimen space) that appears to be sharply in focus at one setting of the fine focus adjustment. In bright field microscopy, this depth should be approximately equal to the axial resolution, at least in theory. The actual depth of field has been measured, and the contribution of various factors that affect the measurement have been explored by Berek (1927).

According to Berek, the depth of field is affected by several factors, including (a) the geometric spreading, above and below the plane of focus, of the light beam that arose from a single point in the specimen; (b) accommodation of the observer's eye; and (c) the final magnification of the image. The second factor becomes irrelevant when the image is not viewed directly through the ocular but is instead focused on a thin detector (in the absence of an auto-focusing device). The third factor should also disappear once the total magnification is raised sufficiently so that the unit diffraction image becomes significantly larger than the resolution unit of the detector (see e.g., Castleman, 1987; Hansen in Inoué, 1986).

Many other authors have calculated the depth of field, but unlike Equation (1) that specifies the lateral resolution, no equation is generally accepted as specifying the depth of field. One reason that several equations have been proposed for the depth of field is that different criteria have been used for what is "in focus."

In conventional fluorescence and dark field microscopy, the light that makes up each point of the image spreads in a solid cone that can reach a significant distance above and below focus (as seen in the point spread functions for these modes of microscopy; see e.g., Streibl, 1985, and also Chapters 8, 9, 10, 11, 13 and 14). The spreading cone of light blurs the focused image of the specimen. Also, fluorescent (or light-scattering) objects that are out of focus can inject unwanted light and further reduce the contrast of the specimen region in focus.

For these reasons, the depth of field may be difficult to measure or even to define precisely in fluorescence and dark field microscopy. Or, one could say that the apparent depth of field is very much greater than the axial resolution when objects that are not infinitely thin are observed in conventional fluorescence and dark-field microscopy.

The unwanted light that expands the apparent depth of field is exactly what confocal imaging eliminates. Thus we can view only those fluorescent and light-scattering objects that lie within the depth that is given by the axial resolution of the microscope and attain the desired shallow depth of field.

As mentioned earlier, the lateral resolution of a microscope is a function of the size of the field (observed at any one instant). Tolardo di Francia (1955) suggested, and Ingelstam (1956) argued on the basis of information theory that one gains lateral