Confocal imaging for 3-D digital microscopy

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Optical serial sectioning based on the depth-discriminating ability of confocal laser scanning can be combined with digital image processing to realize fast and easy-to-use 3-D microscopy. A great advantage as compared with traditional methods, e.g., using a microtome, is that the specimen is left undamaged. An account is given of an instrument designed for this purpose and of feasibility studies that have been carried out to assess the usefulness of the method in fluorescence microscopy.

I. Introduction

An ordinary light microscope is not well suited for studying the 3-D structure of a specimen. It presents the operator with a 2-D image consisting of a superposition of in-focus and out-of-focus regions of the specimen. Stereomicroscopes are useful in some applications requiring only low magnifications, but are not useful in high-resolution microscopy. In spite of these problems, researchers have for a long time used microscopes for studying very complicated depth structures. One method of doing this is to photograph the specimen at a number of different focus settings. By manually tracing only the focused parts of each photograph, it is possible to get information on the 3-D structure of the specimen. This may be difficult, however, if the specimen does not contain very clear borders. The problem can be avoided by using a computer to process digitized photographs in such a way that the out-of-focus information is filtered out. While the feasibility of computer processing has been demonstrated in practical applications, a number of difficulties are encountered; e.g., the optical transfer function of the microscope must be known for different amounts of defocusing, and faint in-focus regions may be difficult to record in the presence of strong out-of-focus regions.

Another method to realize 3-D microscopy is to actually cut the specimen into a large number of thin sections, which are then studied under a microscope one at a time. While circumventing a number of problems associated with the previous methods, this method creates a number of new problems. The cutting process is time-consuming and may deform the specimen. It is impossible to study living specimens, and the reconstruction of the 3-D structure from the individual sections may require considerable computer processing to align the recorded sections.

II. Confocal Scanning Microscopy

A promising method for 3-D studies of specimens is confocal microscopy. The principle of confocal imaging, and the properties of the recorded images have been described in a patent, a number of articles, and a textbook. The basic principle is that the specimen is illuminated one point at a time, and the detector only registers light emanating from the illuminated point (Fig. 1). An image can be scanned either by moving the specimen or the light beam. A number of confocal scanning microscopes have been described in the literature. In one design a large number of points are illuminated simultaneously, making it possible to build a direct-view confocal microscope which allows the operator to view a depth section in real time through the eyepieces without the need for electronic recording.

The point-illumination and point-detection scheme results in imaging properties that are different from those of an ordinary microscope. One such difference is that the resolution limit is improved. Another is that a very pronounced depth discrimination is obtained (compare Fig. 1). This means that different depth layers can be studied much more clearly, since virtually no out-of-focus information is superimposed on the image. The depth discrimination can also be used to study surface structures.

Confocal scanning offers a unique possibility for 3-D microscopy, since a recording of the entire structure of a specimen can be made by scanning a number of confocal images, refocusing the microscope between successive images. The result is a stack of images representing the 3-D structure of the specimen.
Fig. 1. Simplified ray path of a confocal microscope with incident light illumination. A focused laser beam provides intense illumination of a small specimen volume located in the focal plane of the microscope, A. Reflected or fluorescent light from A is transmitted through the detector aperture, which effectively blocks light from out-of-focus planes, e.g., B. By scanning either the laser beam or the specimen, an image can be recorded that represents a thin section located at A. Repeated scanning, using different focus settings on the microscope, results in a stack of images representing the 3-D structure of the specimen.

Recording, optical serial sectioning, can be done quickly, and there is no need for processing the pictures to align them with respect to each other. The method also makes it possible to record thin sections of living specimens.

New perspectives are opened by combining confocal scanning with digital recording and image processing. The images resulting from optical serial sectioning can also be dealt with in this way, resulting in a digitally recorded data volume. It is then possible to make projections of this volume (Sec. V) to display the specimens from different viewing angles. Moreover, the possibility to perform photometry in three dimensions is opened.

III. Equipment

At our institute a confocal scanning microscope aimed at digital 3-D microscopy has been built. The instrument has been used in a number of feasibility studies, some of which will be reported in this article. The instrument (Figs. 2 and 3) uses the beam scanning technique, i.e., a laser beam from an argon
laser performs scanning in two perpendicular directions while the specimen remains stationary. Incident light illumination is used, and the reflected or fluorescent light from the specimen is detected by a photomultiplier tube. A Zeiss Universal microscope is used in the scanner, the only modification being that a stepping motor has been connected to the fine focusing mechanism, allowing a 2-mm computer-controlled focusing range. In fact, the scanner can be regarded as a microscope accessory that can be used instead of a camera unit.

Prior to scanning, the operator can view the specimen using conventional microscopic techniques to find an interesting region. Scanning with the laser beam is performed by two mirrors located above the eyepiece of the photographic tube. The fast line scan mirror is driven by a galvanometric scanner (General Scanning, Watertown, MA) and the slow frame scan mirror is driven by a stepping motor. The scanned specimen area corresponds to 13 x 13 mm in the image plane of the microscope objective. This area can be scanned with a maximum resolution of 1024 x 1024 pixels (picture elements), each pixel containing 10 bits of data. The useful number of bits depends, of course, on the signal-to-noise ratio of the detector signal, which varies widely between different types of specimen. Usually only the eight most significant bits are used.

To illuminate the specimen, an Ar-ion laser (Coherent Innova 70-4) is used. The maximum output power of this laser, 4 W, is much higher than the power needed for scanning (a few milliwatts). The reason for using such a powerful laser is that it offers a wider range of wavelengths (458-514 nm). In many applications, however, a small air-cooled argon laser or a He-Cd laser would be sufficient. For reflected light scanning, the beam splitter (compare Fig. 1) is of the ordinary 50/50 type, splitting all wavelengths equally. When scanning fluorescent specimens a dichroic beam splitter, reflecting the excitation light while transmitting the fluorescent light, is used. To facilitate the change between different beam splitters, these are mounted on a wheel. Another wheel, located in front of the photomultiplier tube, holds a number of detector apertures of different sizes. While the aperture size should ideally be as small as possible to get high resolution (see Sec. IV), a certain minimum area is necessary to get enough light for the detector. Since the optimum trade-off between resolution and signal quality depends on the type of specimen, and the number of pixels in the digital image, we feel that a system allowing a quick change of aperture size is advantageous.

To control scanning and data collection a 16-bit microprocessor, Intel 80186, is used. Connected to the microprocessor is an image memory (Matrox MIP-1024) that can hold 1 Mbyte of data. During scanning, which takes ~5s for a 256 x 256 image, the image gradually appears on a TV screen. When the scanning of an image is complete, data are transferred to a host computer (Perkin-Elmer 3210) via an IEEE 488 data bus (Fig. 2). The transfer speed of the bus is 25 kbyte/s.

Computer processing of the digital images will be described in Sec. V.

IV. Resolution

The optical resolution both along and perpendicular to the optical axis is of fundamental importance when selecting the pixel spacing in each depth section, as well as the spacing between successive sections. As in ordinary microscopy, the resolution is determined by the wavelength, the numerical aperture of the objective, and the coherence properties of the light. Although a laser is usually used for illuminating the specimen in confocal microscopy, imaging may be incoherent. This is the case, e.g., in fluorescence microscopy, since the coherence of the laser light is not preserved in the fluorescent light. The focal plane resolution (two-point resolution) for an ordinary microscope and incoherent imaging is given by

$$R_f = \frac{0.61\lambda}{\text{N.A.}}$$  (1)

where N.A. is the numerical aperture. In confocal microscopy, the resolution is improved by a factor of 1.32 for incoherent imaging, and, therefore, the resolution limit is given by

$$R_f = \frac{0.46\lambda}{\text{N.A.}}$$  (2)

A more important improvement, as far as 3-D microscopy is concerned, is that a confocal microscope has a very pronounced depth discrimination. A suitable way of studying this is to calculate the fall-off in the total light intensity in the image of a point object as the microscope is defocused. The light fall-off as a function of a normalized distance $u$ from the focal plane is plotted in Ref. 9, giving a 50% fall-off at $u \approx 4.4$ ($u$ is defined in Ref. 29, p. 437). For small numerical apertures $u$ can be approximated by

$$u = \frac{2\pi(N.A.)^2}{\lambda n}$$  (3)

where $n$ is the refractive index of the immersion medium, and $z$ is the distance along the optical axis. Combining these results it is possible to estimate the half-width (full width half-maximum) along the optical axis as

$$R_d = \frac{1.4n\lambda}{(N.A.)^2}$$  (4)

In an ordinary microscope there is no light fall-off whatsoever when the specimen is defocused. Using Eqs. (2) and (4) it is possible to calculate the maximum resolution obtainable in confocal microscopy, using commercially available objectives (N.A. ≤ 1.4) and visible light ($\lambda \geq 400$ nm). The result, $R_d = 0.13$ and $R_d = 0.43 \mu m$, shows that a depth discrimination comparable to the focal plane resolution cannot be expected. Furthermore, while the focal-plane resolution is inversely proportional to the numerical aperture, the depth discrimination is inversely proportional to the square of the numerical aperture. Therefore, the depth discrimination drops considerably faster than
the focal-plane resolution when the numerical aperture becomes smaller, making it very important to use objectives with a large numerical aperture. A serious disadvantage with large aperture objectives, however, is that the working distance is very small, often below 100 μm. This proves to be a limiting factor when using confocal microscopy for 3-D studies of thick specimens. A numerical aperture of ~1.0, giving a working distance of 300–400 μm, is often a good compromise between depth discrimination and working distance. In this case the depth discrimination is 0.8 μm for λ = 400 nm.

The resolution limits given are not exact, since approximations have been made in the calculations.13 For example, the assumption that the numerical aperture is small is often not valid. Practical observations of the focal-plane resolution have shown, nevertheless, good agreement even for large numerical apertures.16 Equations (2) and (4) also assume that the detector aperture is infinitely small. According to theory, the focal-plane resolution is rather insensitive to aperture size. Even if the aperture is removed altogether, the two-point resolving power would only drop to a point where it is identical to that of a conventional microscope, i.e., Eq. (1). The real situation, however, is often complicated by light scatter in the specimen, which severely limits the image contrast if the detector aperture is too large. Therefore, the aperture should not be larger than the pixel spacing.

Depth discrimination, on the other hand, is sensitive to the size of the detector aperture. With a very large aperture, the scanning microscope behaves like an ordinary microscope in this respect, i.e., light from all depth layers can reach the detector. We have used aperture sizes ranging from one to one-quarter the diameter of the Airy disk with good results. An aperture size roughly equal to the Airy disk is sometimes the smallest that is practically useful in fluorescence microscopy, if a good signal quality is to be obtained. When studying the depth discrimination, using the interface between a fluorescein solution and a cover glass, it was not possible to detect any degradation when the larger apertures were used. For a numerical aperture of 1.3 and λ = 458 nm, the 10 to 90% transition in intensity took place over a vertical distance of 1.7 μm. We have not used apertures larger than the Airy disk, since this would reduce the lateral resolution (aperture size > pixel spacing). One could argue that, by simply using a higher laser power, it would always be possible to use a very small aperture. This is usually not the case in fluorescence microscopy, since most specimens are prone to fading if illuminated too strongly.

In our microscope, using N.A. = 1.3 and λ = 458 nm, the half width of the lateral spread function in the scanned images is ~0.2 μm (corresponding to a two-point resolution of ~0.25 μm), when using an aperture size of half of the Airy disk. To fully utilize this resolution the pixel spacing must be smaller than the resolution limit. But scanning the entire field of view of the microscope with such a small pixel spacing can yield prohibitively large amounts of data, especially when many depth sections are recorded. By scanning a smaller area, the optical resolution can, of course, be fully utilized. In a typical case we scan 200 depth sections, each having 256 × 256 pixels. The total amount of data, 13 Mbyte, is rather large but still manageable.

V. Computer Hardware and Software

Computer technology offers the possibility to store and access 3-D information in a fast and efficient way. The stack of digital images constitutes a 3-D matrix, where the values of the elements are related to the amount of light recorded from the corresponding volume element in the specimen. The sections of this stack can be displayed and evaluated one by one. Alternatively, it is possible to produce projections through the data volume24–26,30 (Fig. 4). The 3-D matrix stored in the computer may be processed in a number of ways. For example, it is possible to generalize the well-established techniques for dealing with images in two dimensions. Concepts like resampling, filtering, edge-detection, segmentation, etc. all have their counterparts in three dimensions.

Another approach is to perform data compression based on vector representation.31 The underlying as-

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Fig. 4. (a) Values of volume elements along projection lines (here parallel to one of the coordinate axes) are added to generate a projected image. (b) For many objects the amount of information that needs to be stored can be considerably reduced, e.g., if the object consists of a shell. This can be done by retaining only the interesting volume elements in vector form. Figures 5–7 have been handled in this way.
We have selected three different applications for presentation, two physiological applications, and one from botany. In all cases fluorescence microscopy with epi-illumination was used. The first application relates to the study of neurons, which often display a very complicated 3-D structure. In a joint project between our institute and the Karolinska Institute in Stockholm, Sweden, we have undertaken to make a study of the structure of neurons. The neurons studied include motoneurons, lateral interneurons, edge cells, and dorsal cells in the spinal cord of lamprey (Ichthyomyzon unicuspis). The neurons were stained by intracellular injection of the fluorescent dye Lucifer Yellow, fixed in formaldehyde, dehydrated in ethanol, and cleared in methyl salicylate. Confocal microscopy has proved useful in studying specimens of this kind (Fig. 5). By displaying a number of projections through the recorded data volume in rapid succession on a TV screen, a good understanding of the general 3-D structure can be obtained. A more detailed study of the entire tree of neuronal processes, which often extends for several millimeters and in which the individual processes may have a thickness of <1 μm, is difficult because of the enormous amount of data that must be handled. Even when using vector representation, it is impractical with our present computer equipment. However, a detailed study of part of the dendritic tree, e.g., tracing individual processes and calculating their entire length, seems feasible. A difficulty that often arises in the study of neurons is that the specimen thickness, often several hundred microns, makes it impossible to scan the deepest part of the specimen using an objective with a large numerical aperture.

Although projections have been successfully used for displaying, e.g., neurons, there are a number of cases where the original data consisting of a number of sections are more useful. If this is the case, display and evaluation can be performed more quickly since much computer processing can be avoided. One application where the original sections have proved useful is the study of plant seeds. In cooperation between our institute and the University of Gothenburg, Sweden, a new species of Neuwiedia (an orchid) has been studied (Fig. 6). The traditional way to study ovules of plants is to embed the ovule in paraffin, section it with a microtome, and stain the sections to make their structure visible in transmitted light microscopy. Because the ovules are fragile, thinner sections than 8 μm are difficult to obtain. By using confocal microscopy, serial sections with a thickness of ~1 μm can easily be recorded from whole ovules. Furthermore, it turned out that the autofluorescence from the ovule wall, and the nuclei located in the ovule, was so strong that staining the specimen was not necessary. As a result, ovules could be studied much more quickly and accurately by confocal microscopy than by using traditional methods. Since data are in digital form, it was also easy to perform quantitative measurements of 3-D positions of nuclei, the size of the ovule, etc.

The last application reported is a study of lung tissue, which was started only recently, but has shown
promising results. The study is made in cooperation with The Memorial Hospital, Brown University, Rhode Island, U.S.A. Again it seems that data in the form of scanned optical sections of lung tissue can be used directly for evaluation. As in the previous case, the traditional way to study the specimen has been to physically section it with a microtome. By fluorescent
staining of the tissue before it is embedded in epoxy, it has proved possible to use confocal microscopy to register optical sections with high contrast (Fig. 7). From a stack of such sections interesting parameters concerning the 3-D structure of lung tissue can be measured.

VII. Conclusions

Feasibility studies have shown the potential of confocal microscopy scanning as a tool in 3-D microscopy. Since the recorded volume data are in digital form, they are directly accessible for computer processing and evaluation. This opens up a whole new field of possibilities for display and evaluation. However, the amount of data to be processed is often very large, requiring powerful computer hardware and efficient software. We have found that fluorescent specimens are often well suited for 3-D scanning. Objectives with large numerical apertures should be used, but the small working distance of very large aperture objectives (>1.0) can sometimes create problems.

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