Three-dimensional microscopy using a confocal laser scanning microscope

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In a scanning laser microscope detecting fluorescent light from the specimen, the depth-discriminating property of confocal scanning has been used to carry out optical slicing of a thick specimen. The recorded digital images constitute a three-dimensional raster covering a volume of the specimen. The specimen has been visualized in stereo and rotation by making look-through projections of the digital data in different directions. The contrast of the pictures has been enhanced by generating the gradient volume. This permits display of the border surfaces between regions instead of the regions themselves.

Three-dimensional reconstructions of biological specimens studied in a microscope have been performed previously.[1] A series of photographs was taken in which the focal setting of the microscope was changed between each recording. Each photograph contained both in-focus and out-of-focus information. The photographs were then digitized and processed by a computer to carry out the reconstruction. Massive calculations were necessary to exclude the out-of-focus contributions in each picture. A severe measurement problem arises when bright out-of-focus regions are superimposed upon faint in-focus regions of the specimen. Furthermore, the optical transfer function of the objective for various degress of defocusing has to be accurately known.

A different way of recording, viz., direct photoelectric recording combined with confocal scanning, can circumvent these problems. Confocal scanning microscopes, and their ability to exclude out-of-focus information, have been described in numerous articles.[2-10] It has also been demonstrated that confocal scanning can improve the resolution in stereoscopic imaging of object surfaces.[11] The depth-discrimination mechanism is illustrated in Fig. 1. For a numerical aperture of 1.0, the integrated light intensity is reduced to 50% at a distance of 0.7λ from the focal plane.[5]

Thus the discriminating power of the confocal scanning allows for a depth resolution of the same order of magnitude as the spatial resolution within the focal plane. Therefore it should be possible to use the recorded data directly to obtain three-dimensional (3D) images. The aim of the present study was to verify this

method of registration by using a biological specimen and to display the 3D structure of the specimen by employing digital projection methods.

The combination of these techniques opens the prospect of making 3D studies of microscopic specimens in a direct and efficient way, as opposed to the method of mechanically slicing the specimens before examination. It also opens the way to carry out microfluorometry in three dimensions. For accurate quantitative evaluation, however, it may be necessary to take into account absorption effects and contributions from out-of-focus regions of the specimen. These problems are beyond the scope of the present study, but it should be noted that the out-of-focus contributions are much
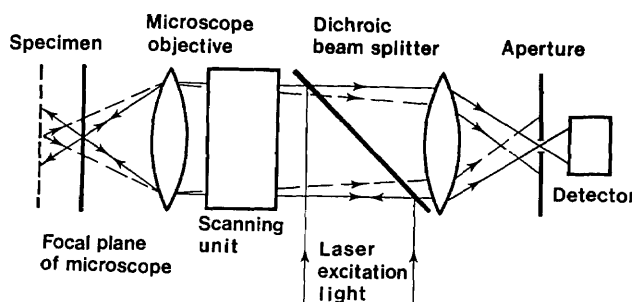

Fig. 1.   Ray paths in a confocal microscope scanner. Light from sections of the specimen located out of focus will largely fall outside the aperture and is thus strongly attenuated. This property makes it possible to slice the specimen optically into thin sections. By combining a number of such sections, one may obtain a 3D image of the specimen.
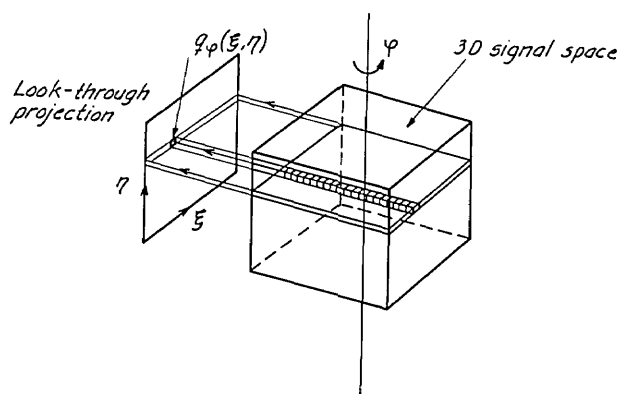
Fig. 2. Projection method used to produce stereo pairs from the image data obtained by scanning a number of pictures with different focus settings. The projection rays need not be parallel to the bottom plane, as in the figure. It is thus possible to choose any direction for the axis of rotation.

smaller when one uses data recorded with the present method rather than data obtained without employing confocal scanning.

PHOIBOS is a confocal microscope scanner developed at the Royal Institute of Technology, Stockholm.[12] It scans specimens by using incident-light illumination and detecting the fluorescent or reflected light from the specimen. In the present study only the fluorescent light was detected. The confocal scanning method permits accurate photometric point measurements in a raster format. Furthermore, fluorescein fading is kept at a minimum because of the point illumination. The excitation light is provided by an argon-ion laser, and the power used is approximately 10 mW. The specimen remains stationary during scanning, and the focused laser beam is moved in two perpendicular directions by rotating mirrors. The microscope is a Reichert Fluorpan equipped with a Zeiss Planapo 40/1.0 objective. To change the focal setting of the microscope, its stage is moved vertically by a computer-controlled stepping motor. A microprocessor controls the scanning and data storage as well as data transfer to a host computer. The pictures scanned by PHOIBOS consist of 256×256 or 512×512 pixels with 256 gray levels.

The specimen in question is approximately 200 μm thick and contains a neuron cell from a lamprey, stained with the fluorescein Lucifer Yellow.[13] The excitation-laser light has a wavelength of 458 nm, and the fluorescein emission has a maximum at 540 nm. A dichroic beam splitter is used to reject the excitation light reflected by the specimen (Fig. 1). Each pixel represents an area of 1.25 μm $\times$ 1.25 μm. By stepping the focus wheel in 2-μm steps, 70 sections of 256×256
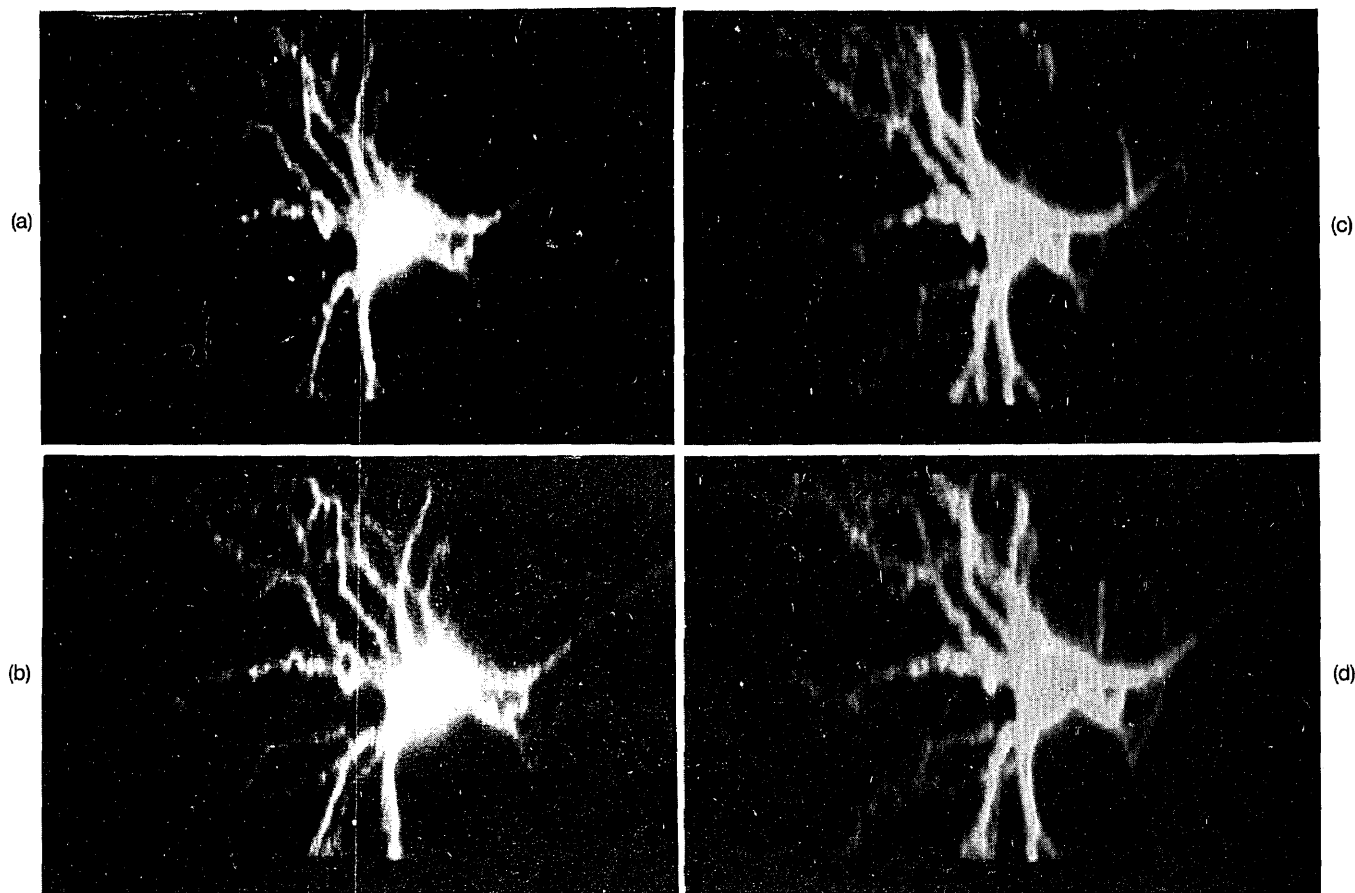

Fig. 3. Two stereo pairs produced by the method of Fig. 2. The projection angle between the two images of each pair is 8° Note that the dendrites are almost perfectly oriented in a plane. The axon leaves this plane in a perpendicular direction. (a) and (b) were obtained from the original data volume, (c) and (d) from the gradient volume.

pixels were digitized and processed to obtain a 3D representation of the specimen. This scanning was rather coarse compared with the maximum resolution obtainable with a confocal scanning microscope; a resolution of better than 0.2 μm in a plane perpendicular to the optical axis, and a depth discrimination of approximately 0.5 μm, should be possible.[5,6,9] The time required for scanning 70 sections of a specimen is approximately 15 min.

3D data in their original form as a stack of slices can be displayed and perceived slice by slice by using an ordinary display system. However, better perception effects were obtainable by utilizing the capabilities of the PICAP II image-processing system.[14] One of the salient features of PICAP II is a fast digital disk memory with 300-Mbyte storage capacity and 10-Mbyte/sec data rate, which allows us to select and access image data at a video rate.

The 70 × 256 × 256 pixels, or *voxels*, as they should be called from now on, were processed on PICAP II as follows.

First, the original volume was expanded to 140 × 256 × 256, resulting in approximately cubic voxels. Next, simulated projection rays entered the volume, and the accumulated voxel values, ρ, were computed for each ray (see Fig. 2). To create the illusion of a look-through projection, we computed the quantities $\exp(-\gamma\rho)$ as if the projection rays were attenuated in an absorbing medium, where the voxel values represent the absorption factor at each point. γ is a parameter that can be selected to achieve a favorable contrast.

For display purposes an inverted picture is preferable. Therefore we define the pixel value $q(\xi,\eta)$ as

$$q = 1 - \exp(-\gamma\rho). \tag{1}$$

Several parallel projections from different consecutive angles θ were computed. Two projections from somewhat different angles constitute a stereo pair (see Fig. 3). By displaying a number of consecutive stereo pairs, we can create the effect of a rotating object on the screen, which unfortunately is impossible to convey to readers of this Letter. It is possible to give the user full control over rotation with the help of a keyboard or a joystick. We have found interactively controlled rotation combined with stereo viewing to be a powerful tool for 3D perception.

Transformations of the original data volume give us enhanced visibility and perception effects. Figures 3(c) and 3(d) show a stereo pair of look-through projections from a transformed data volume. This second volume is obtained by computing the approximate 3D gradient for each point in the original data. Consequently we can now observe the surface between the neuron and the background plus possible discontinuities of the fluorescence intensity inside the neuron itself.

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