Scanning Electron Microscopy

Volume 4
Number 1 *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*

Article 9

1985

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Science of Biological Specimen Preparation (pp. 85-94)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507, U.S.A.

TANDEM SCANNING REFLECTED LIGHT MICROSCOPY

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Abstract

Most attempts at optical microscopy of bulk living objects have failed because of reflections from optical components and the object surface, and overlapping of unsharp out-of-focus images with the one (weak) sharp in-focus image. The optical signal in the image plane thus consists of a strong d.c. component, a weak noise and a still weaker signal. Most of the first two components can be suppressed by scanning in concordance with both the illuminating and the image-forming rays. In the first scan the focussed-on-object plane is illuminated only in several very fine discrete spots. In the second scan only that light is permitted to reach the image plane which has been reflected in the illuminated "points" of the object plane. Both scans are performed by a single rotating aperture disc and the whole field is covered within 0.05 sec. Because the total number of scanning lines is about 10,000 in the field of the microscope objective - giving a line period for the strongest objectives of 10nm - the resolution is not impaired by this scanning. On the contrary, it follows from theory that the resolution is better because the contrast is also improved.

The following living biological objects have been successfully observed: Eye tissues - cornea (epithelium, stroma, endothelium), lens, all layers of the retina: nerves and nerve fibres: brain cells in whole brain: muscle fibres and nerve endings in striated muscle, heart muscle cells (in juvenile mice, through epicardium): stratum corneum of human skin, frog skin cells: spermatozoa: blood cells: cartilage, bone and dental tissues: plant cells. Fossil teeth and bones, protozoa and bacteria in flint, insects in amber, and fossil plant tissues have also been successfully studied.

KEYWORDS: Vital microscopy, confocal, tandem scanning reflected light microscopy, method, applications

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Introduction

Optical microscopy will always have to play an essential role in biological structural and behavioural studies. In the context of this meeting, we note that light microscopy (LM) is the only approach which can be used to monitor events both in live cells, and in cells as they undergo fixation and further processing prior to other methodologies, including LM, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and microanalysis.

As regards the subdivision of LM approaches, we should remark that most require the subdivision of the material to be studied: animals or organs have to be partitioned into separated cells or cell layers, or cut into slices, to permit the use of LM in studying live tissues. There are few organisms which we wish to study in which the size is small enough and the tissues transparent enough to permit study of parts in the intact whole using transmitted light. The purpose of the work reviewed in this paper was, therefore, to develop the means of studying live tissues in animals using **reflected** light microscopy.

Ordinary reflected light microscopy (RLM)

If one attempts to use reflected light - epi-illumination - in looking at animal tissues, one will be very disappointed with the outcome. Even with the best contrivances, such as the darkfield Ultropak objectives of Leitz, one will normally see only a structureless, pink blur. The best results will be obtained in fluorescence, since the object is then self-luminous. However, this will generally imply that the cells of interest have already been interfered with in some way in the process of causing them to become fluorescent (since there are but few auto-fluorescent phenomena of interest to us) and our main aim was to be able to see as much as possible with as little interference as possible.

It was therefore necessary to find means of overcoming the disappointment of RLM - which the first two authors did by inventing the apparatus named the "tandem scanning reflected light microscope" (TSRLM or TSM). To understand how it works, and what are its advantages, we shall first consider the problems that beset the RLM.

One can resolve little and with poor contrast, because a very large proportional volume of the specimen is illuminated, and the "signal" giving rise to the image derives in part from the whole of this volume. Thus reflections from out-of-focus layers contribute too much unusable unwanted information: in the case of the immediate sub-surface layers, the very strong reflection from the surface of the specimen overwhelms the small signal contributed by the features of interest. In every case, however, myriad reflections from interfaces within the specimen drown the small reflections from the layer of interest. The weak reflections from the layer of interest are rendered even more insignificant by the DC noise of the reflections at all the optical surfaces inside the microscope.

The contrast can be improved in only one way, namely to prevent the unwanted information contributing to the image, which can only be done by limiting the illumination to the layer to be studied. One has also to suppress all the light which has not been reflected in this layer which is to be observed. Both steps can be achieved simply for extremely small objects, by using a high aperture immersion objective and limiting the illuminated area to a patch constituting only a very small fraction, e.g. 1/1000th of the width of the field of view. However, if we do this, we cannot see the feature in context.

A whole image can be obtained by **scanning,** but it is not sufficient to scan only the illumination, as is done in all SEM modes, STEM, and the original "flying spot microscopy" of Young and Roberts (1951). This is because only the central area of the light patch (pixel) in the image contains information which directly derives from the purposefully illuminated object plane, but the parts surrounding the patch also contain rays reflected from object planes above and below the focussed on layer, and from lens, prism or mirror surfaces. Further, if we **scan,** then the peripheral portions of many scanning lines will sum together to give a mixture of light carrying virtually no information - which overrides the signal which is required. It is therefore absolutely necessary to get rid of the outside portions of the images of the scanning patches.

Thus to come to the point, we have to scan twice: once in the illumination, and the second time in the image. These scans must be matched to each other, and exactly synchronised so that they appertain to the same point(s) in the object, i.e., they must be scanned in tandem.

There are many possible approaches we can use to achieve this aim. Here we will describe only that used in our microscope, because we suppose that it has very important advantages: namely, those of real time image formation, and the lack of need for complicated electronic equipment and computers.

Tandem scanning reflected light microscope (TSRLM) - see Figure 1.

The scanning is done by a modified (in fact, **highly derived**) Nipkow disc, which is the only scanning device currently rapid enough to permit real time imaging (figure 2). The modification of the classical Nipkow disc consists in using many holes at once - instead of one - and in using a central (circular) symmetry of the hole patterns. This allows us to use only one disc for both scans. One area of the disc scans the illumination, and the 180 deg opposite area scans the image. Both these areas must be at the same distance from the objective lens, which serves for both illumination and image formation. The two functions of the objective are separated by a beam splitting, semi-transparent mirror, which is **very** thin, so as not to double the image. The congruence of both patterns of holes formed by the objective in the focussed-on plane is accomplished by a mirror system, which converts central symmetry into congruency.

This mirror system (the beam splitter being a part of it) is shown in Figure 1. Since the holes are much smaller than the distance between them, it follows that the precision of manufacture of the pattern of holes in the disc, and of the adjustment of the mirrors, must be exquisite.

The practical realisation is such that the disc is a 20 micron thick copper foil perforated with holes arranged in 2 sets of Archimedean spirals. The total number of holes is several tens of thousands. The mean dimensions of the holes are a few tens of micrometres. The number of scanning lines which can be used may be of the order of 10,000. Thus we cannot observe the scanning lines in the image, because their separation is less than the resolution of the microscope.

On one side, the disc is illuminated by a strong source of light, so that a pattern of light spots is formed, the images of which are created in the focussed-on layer of the specimen, whose light is reflected by the features which are to be observed. Images of the instantaneously illuminated structures are formed by the objective on the other side of the scanning disc, so that only the light rays emanating from the sharp, focussed images of illuminated holes are allowed to pass the holes in the disc on the image side, and thus to enter the eyepiece.

The disc rotates at such a speed that it forms an image of the complete object field every 1/50 sec, to give an apparently stationary image.

Optical components used in the TSM

Objectives: Although any conventional LM objectives can be used, immersion objectives are to be preferred - with the refraction index of the immersion medium to be matched to that of the specimen to be observed.

We commonly use water immersion objectives for living animal and plant tissues, oil for bone, tooth and rock (fossil) samples, and glycerine for fluorescence, particularly if polymethyl-methacrylate (PMMA) embedded tissue is available.

The mechanical tubelength of the microscope is 165mm. Objectives designed for 160mm or 170mm tubelength can be used. For longer tubelength objectives, an appropriate prolonging nose piece must be used.

Eyepieces: Eyepieces have to be of the

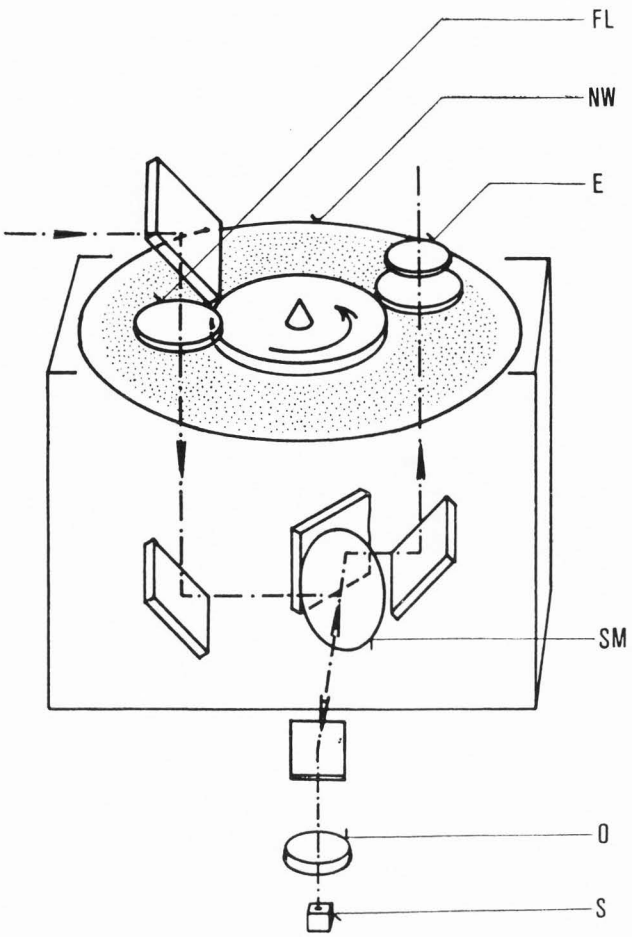


Fig. 1 Diagram of the optical layout
FL Field lens
NW Nipkow wheel
E Eyepiece
SM Semireflecting mirror (beam splitter)
O Objective
S Specimen

Ramsden type, that is, with the focal plane lying beyond the lenses, in the plane of the disc.

All other components (with the exception of the optics in the illuminating source) are front-surface, plane mirrors.

Beam splitter: The beam splitter must be extremely thin - =< 5µm - so as not to double the image, and not to introduce astigmatism. It is made of a mica membrane stretched on a silica supporting ring: appropriate interference coatings are used to give 50% (30% - 70%) transmission and reflection in the visible range. As regards the use of the TSM in the fluorescence mode, we should note that reflection for UV is <50% and the transmission >50%, thus enhancing its characteristics in this context.

Physical arrangement of recent TSMs

The resolving power of the TSM in the vertical, Z, direction is extremely high, i.e. the depth of focus is very narrow. This means

NOTE ON MICROGRAPHS

All the following photomicrographs were taken between 1974-1976 with an old aperture disc pattern with 12,000 holes in the active area of the disc. Rotation of this disc results in 300 curved scanning lines across the image. Most of the photographs were made using a 40X 0.75 water immersion objective (LOMO, USSR), a 10X "orthoscopic" eyepiece (MEOPTA, Prague) and a 25mm photographic camera lens (Biotar, Zeiss Jena). A 16mm negative film, ORWO NP27, was developed with a rapid metal-hydroquinone developer. All prints reproduced were copied to the same scale. For the 40X objective the scanning line interval represents 1.1µm in the object plane, for the 90X objective the interval is 0.5µm. All bars represent 20µm.

Most of the shortcomings of these figures were caused by the film size and graininess, but some are due to imperfections in the aperture disc. Images obtained with new designs of disc (see e.g. Fig. 2) will be published soon.

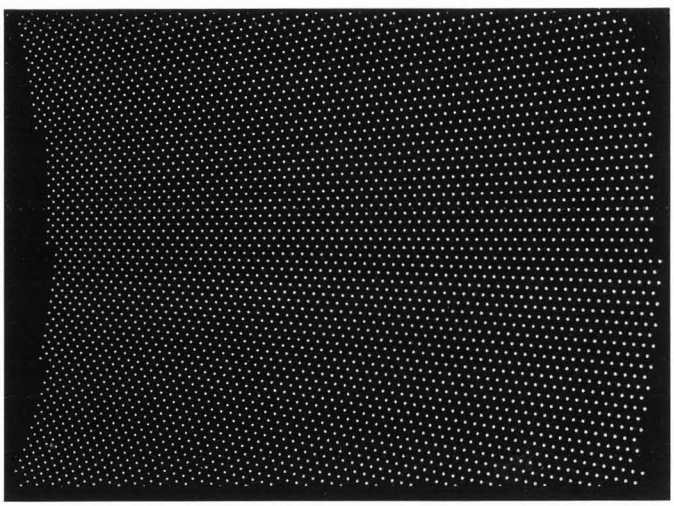


Fig. 2 A sector of a Petran-Hadravsky aperture disc. (Hole sizes and shapes are not accurately reproduced in this photograph.)

that it is better to make the finest adjustment of focus by moving the objective only. In this case, the coarse focussing can be very rigid and long, without great demands on measurement of the vertical movement and its smoothness - these can be provided by the fine focussing mechanism.

The illuminating source is best attached firmly to the microscope proper, with both moving together.

The design of the microscope stand can then be tailored for its intended use. For example, for very large specimens - for microscopic examination in living animals or humans - we have mounted the microscope on a carriage sliding on a one meter high column, which rests on a broad, ground-flat cast iron plate, on which large

specimens are simply rested. Small specimens can be mounted on especially constructed object stages placed on this plinth. These can be moved either by gears provided on the stage, or the stage can be made to glide on the plinth. The movement is then similar to that provided in Zeiss-Oberkochen microscope tables, or in Zeiss-Jena micromanipulators.

Photographic cameras, TV cameras or image intensifiers are coupled to the arm which carries the microscope proper so that they move with it.

Coarse **focussing** can be achieved either manually or by using an electric motor, with variable speed control, to drive the microscope up and down for large distances.

If it is envisaged to work only with smaller (perhaps pre-prepared) specimens, the microscope stand can be designed to have the size familiar in the longer range of conventional microscope. The microscope proper is still rigidly connected to the optical bench of the illuminating source, and is fixed to a rigid, sturdy support. Specimens are placed on a stage providing the coarse focussing, as well as fine X and Y movements. Fine focussing can still be provided by the vertical movement of the objective only.

Illumination Practical experience has shown that the most convenient light sources are high pressure mercury vapour lamps of the type HBO-100 or HBO-200, which are equally suited for visible, ultra-violet and infra-red illumination. Xenon discharge lamps and metal halide discharge lamps can also be used.

We have at present rejected the use of laser illumination because: a) the available power is rather too low to provide the simultaneous illumination of so many holes in the scanning disc - it has to be remembered that the illuminating light is spread evenly over the 18mm diameter circle of the disc which is used, and the disc is as little as 1% transmissive - 99% of the available light is cut off by the illuminating side of the disc, and b) because of the safety hazard, and/or safety regulations, c) speckle noise and interference, d) monochromatic light limits the observation of natural colours and fluorescence.

Preparation of the TSM specimen

As we have previously intimated, the very purpose of the TSM is to enable us to avoid specimen preparation procedures. This can, of course, only be true conditionally - we have to obtain access within a limited distance of the layer of interest. Minimally, therefore, the skin and other tissue layers have to be reflected (in the anatomical-surgical sense). Thin layers of intervening tissues do not create an obstacle.

We can, for example, observe heart muscle cells in small animals through the epicardium, we can see through human skin, through the stratum corneum down to the basal, germinative layer. In intact eyes, one can see to the corneal endothelium and the lens fibres. In isolated retinae, one can focus through all the layers. The limits are imposed by the transparency of the specimen and by the free working distance of the objective. Unfortunately, most water immersion objectives (which we use with most live tissues) are designed for use with a cover glass, imposing a further limit on working distance.

Specimen preparation basically amounts to opening up to near the tissue layer to be studied, bathing in the appropriate saline or culture medium, and placement under the TSM. To facilitate the easy observation of large specimens, we have constructed the recent models of the TSM so that the microscope is offered down to the specimen, rather than a small specimen being moved up under the microscope.

The TSM image of unstained biological objects

The TSM image of unstained biological objects (figures 3-10) is superficially similar to phase contrast and interference contrast images, the contrast being in most cases just a little stronger. However, the signal stems from other properties of the structures in the specimen, namely from refractive index differences at boundaries between constituents and not from the **product** of these differences and the optical path length, as in phase and interference contrasts. In many cases (e.g., retina) the contrast is as strong as in silver impregnated specimens. Of course, this contrast has the opposite sense, since this is a reflection microscope.

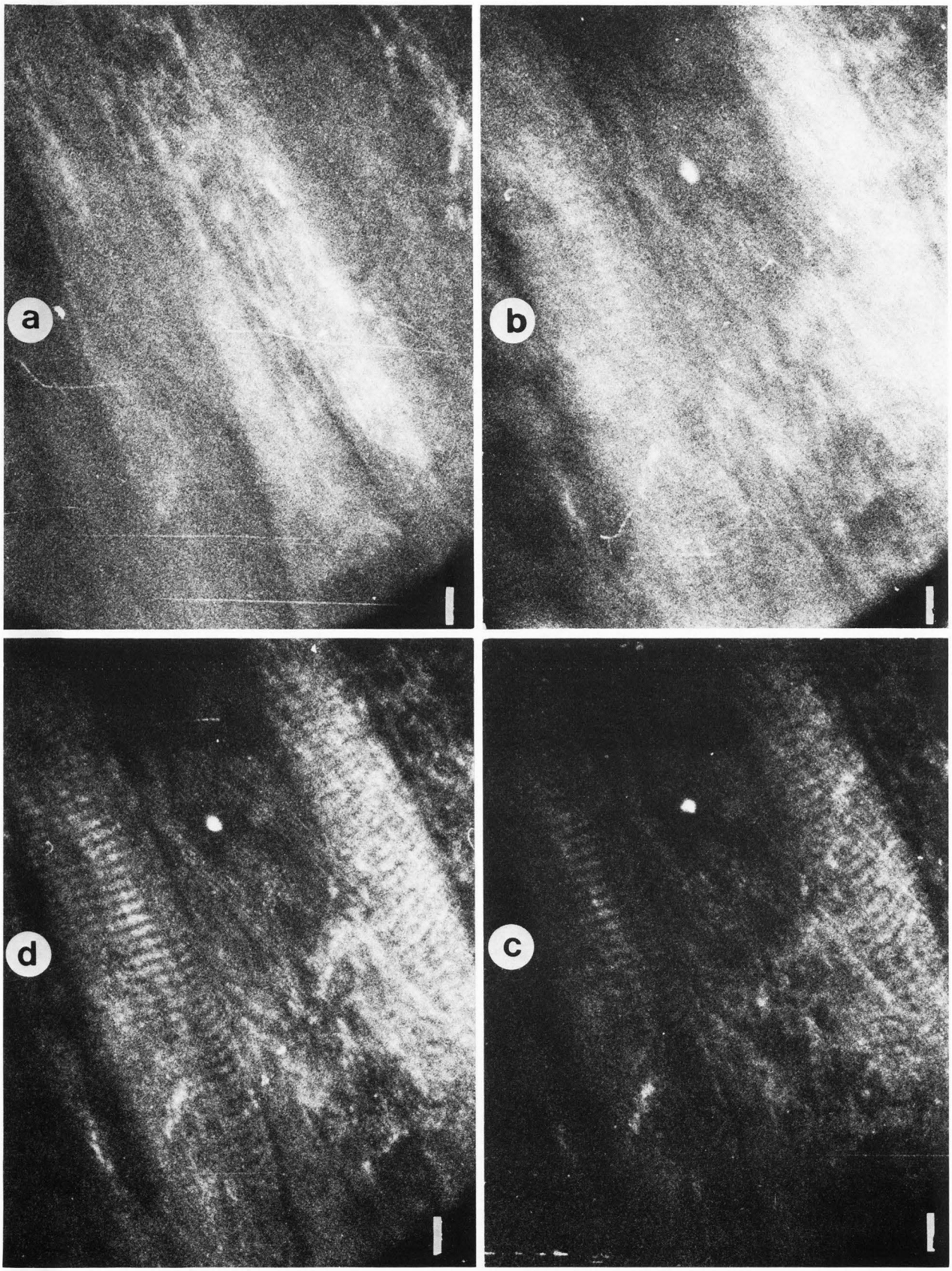
It must be stated, however, that the images are usually not like anything we are accustomed to after training in histology using fixed and stained, thin tissue sections. Surprisingly, several structures which usually have high contrast in conventional histological preparations have a low contrast in the TSM and can easily be overlooked - for example, nuclei in striated muscle fibres. For specialists within a particular field, the images are usually interpreted without much difficulty after a few hours' practical experience.

Generally, it can be stated that all structures known from classical histology can also be seen in this microscope, and without any fixation, staining or other preparative treatment.

Applications in Plzen

So far, the TSM has been used in studies of interrelationships between morphological and physiological events in: isolated retinal preparations of the chick (figs 9 & 10); striated muscle fibres and their nervous apparatus (figs 4-6); heart muscle cells; peripheral nerve (fig 6); cells and fibers in the central nervous system; intact eyes - conjunctiva, through cornea (fig 8) to lens; skin and skin adnexa; microfossils in flint and fossilized plants.

Fig. 3 Frog, sartorius muscle. Focus intervals 2µm.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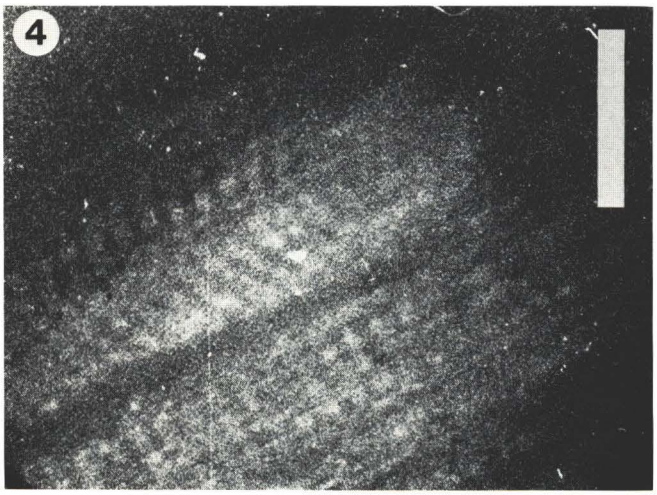
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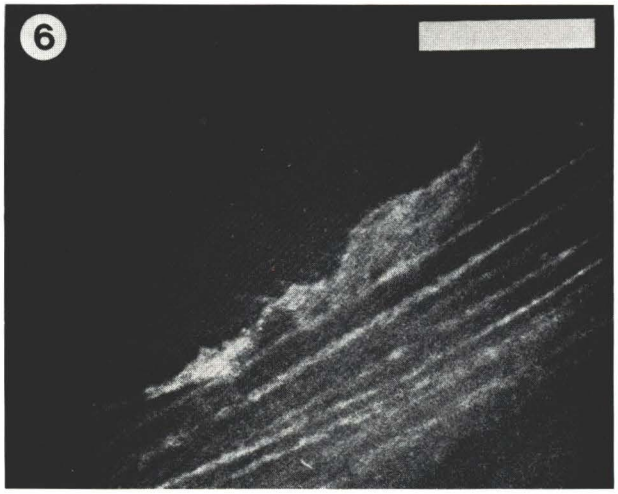
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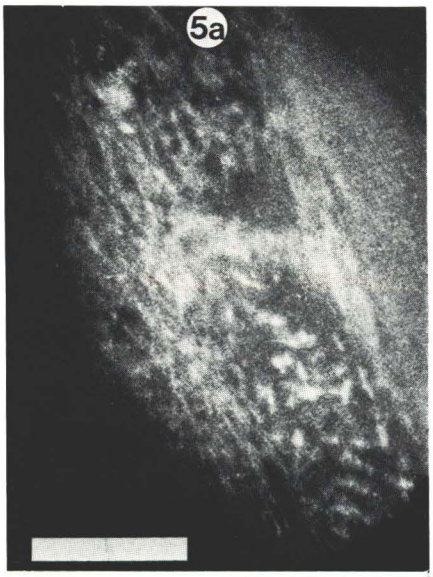
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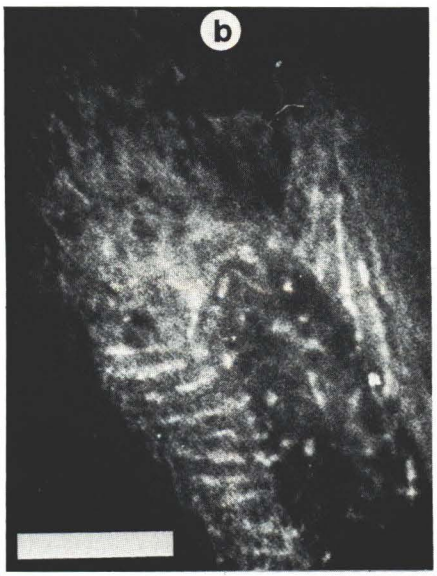
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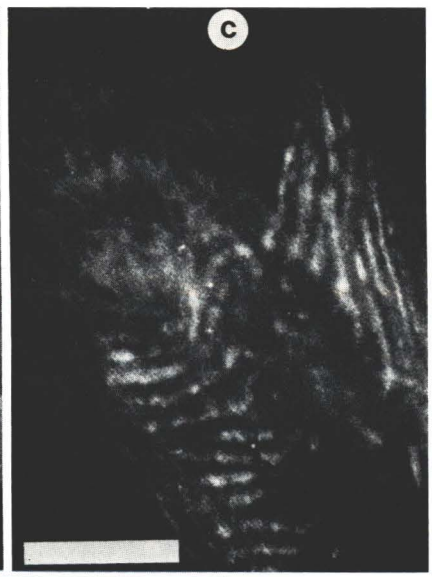
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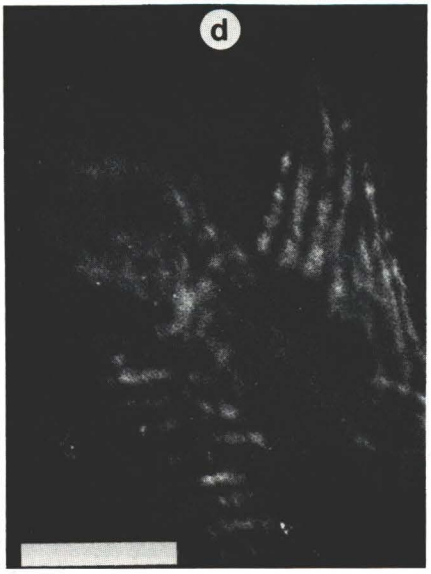
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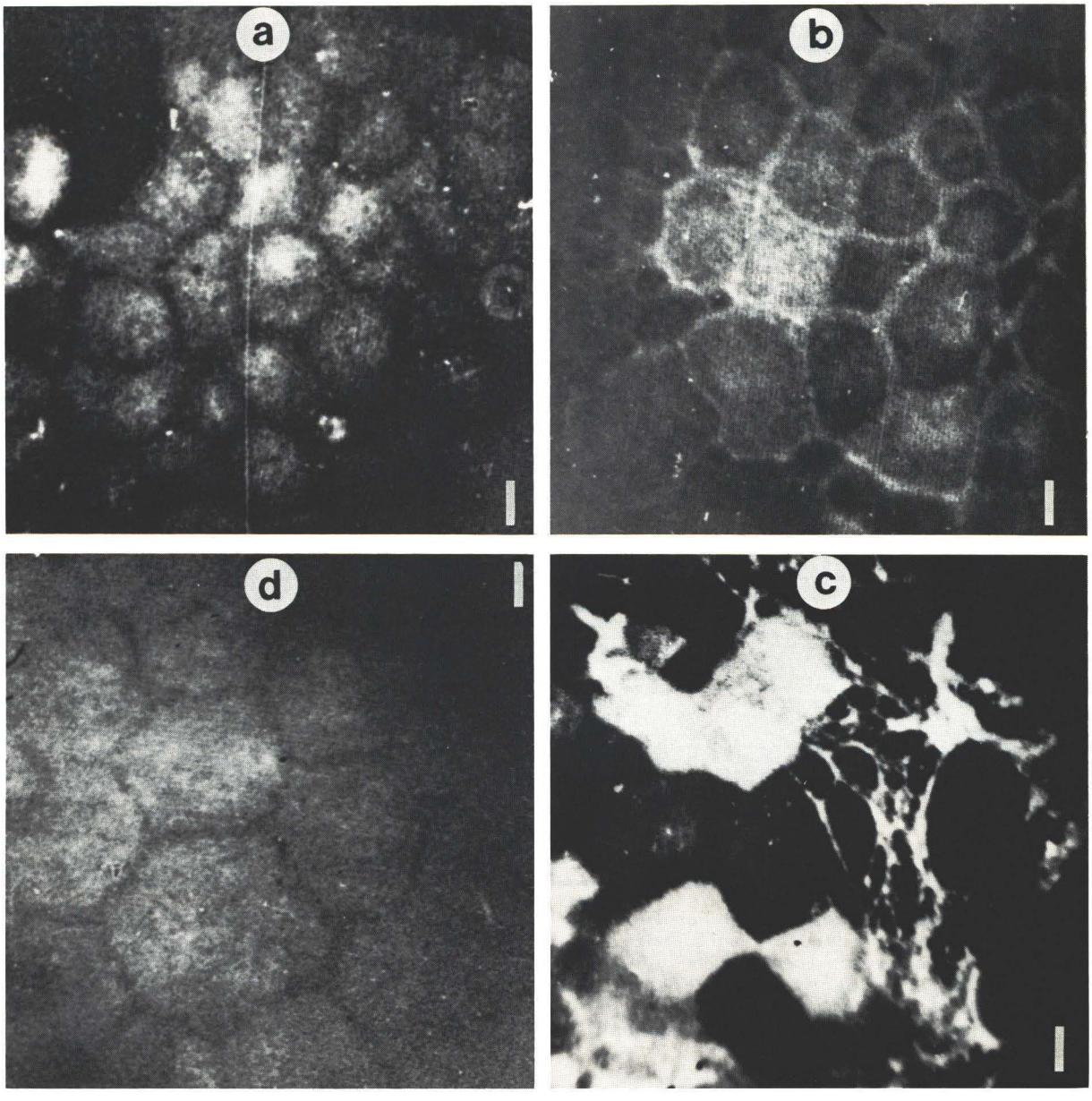

Fig. 7 Chicken eye. Cornea. (a) Epithelium, surface layer. (b) Deeper epithelial layer. (c) Boundary between epithelium and stroma. (d) Corneal endothelium observed through cornea in intact eye.

Fig. 4 Mouse diaphragm. Water immersion 90/1.15. Line period 0.5μm.

Fig. 5 As Fig. 4, Muscle spindle. Focus steps 2μm.

Fig. 6 Mouse. A branch of sciatic nerve in the popliteal fossa, showing an aberrant ganglion cell in this nerve branch. Water 90/1.15.

Applications at UCL, London

The TSM has been used mostly in studies of the mineralised tissues, bone, dentine, cement, enamel and calcifying cartilage, and their associated cells (see **Suggested Further Reading**). Its usefulness in studying brain, muscle, ocular tissues, skin, and hair have also been proven.

Conclusions

In comparison with other methods of intravital microscopy, we know of no present rival. We have been able to examine any living tissue which has interested us, and to do this with whole animal or whole organ preparations. It is

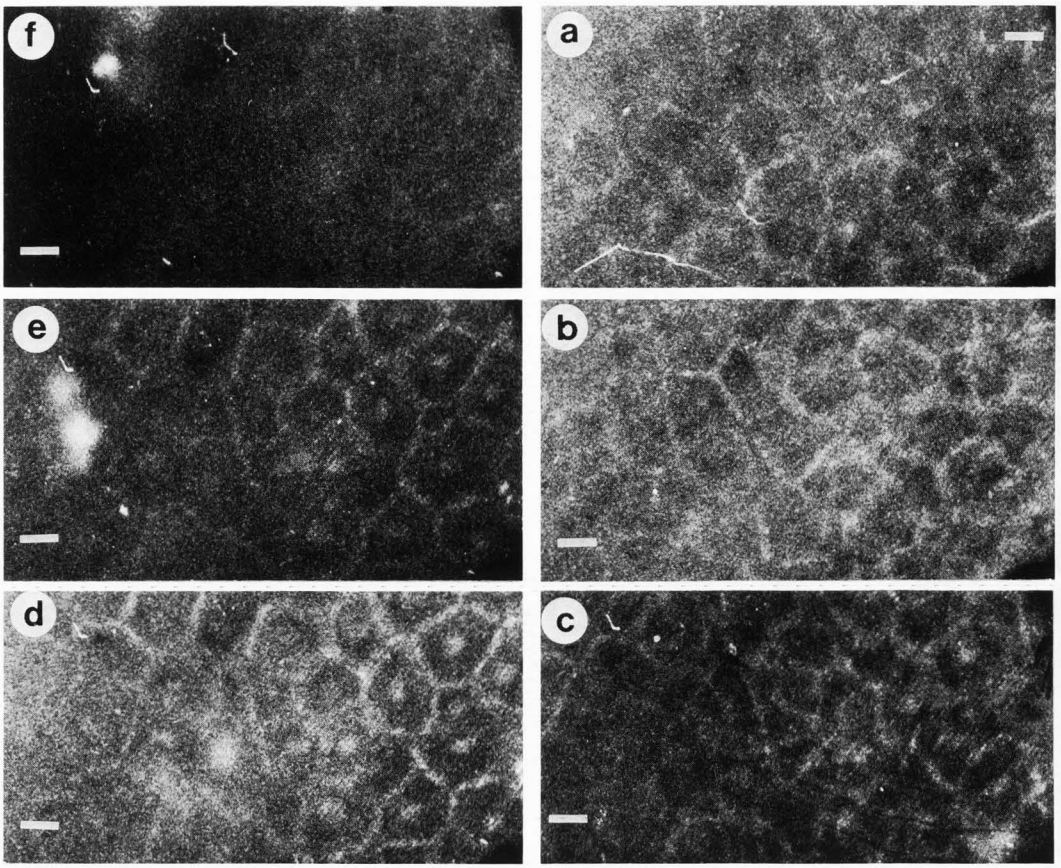

Fig. 8 Chicken eye. Corneal epithelium. Steps 2μm.

difficult to document the data which has led to this enthusiasm using recorded images, mainly because one cannot ascertain the influence of the third dimension in image interpretation. Furthermore, live tissues move, and we cannot make the exposure time (for photographic recording) short enough. It is much better to see the live image of the live object on the real microscope in real time.

Some investment in image intensifying TV cameras can greatly improve this situation regarding the permanent recording of transient events. Video image enhancement plays as important a role in the TSM as in conventional LM, allowing much lower contrast features to be seen with ease, and to be recorded on videotape.

As regards comparisons with other confocal scanning optical microscopes (Wilson and Sheppard, 1984) we feel that the TSM has handsome advantages. These other microscopes use a single optical beam, which is itself scanned mechanically, or the specimen is scanned mechanically. In the latter case, real tissue samples - of the type which would be of interest to the majority of the readers of these proceedings - cannot be effectively studied, since the whole, floppy specimen has to vibrate continuously. In both of these cases, the frame time is minimally several seconds, so that images have to be read from a TV monitor as in SEM, STEM or XRMA. This means that moving samples cannot be followed, and that the acquisition of three-dimensional information becomes painfully long: perhaps 30 mins to acquire the stack of images of one location, which is obviously too long in a dynamic, biological situation. This has to be compared with the steady, continuous image in the TSM, and the immediacy of the changing image as one focusses up and down.

Future developments

We are convinced that some of the major channels for the development of the TSM technique in the near future will be: 1. the evaluation of different designs of objectives, to ascertain their suitability for different purposes and applications compared with routine LM. Other features of the objective may be more important, such as good anti-reflective coatings, good correction for chromatic aberration. For fluorescence microscopy, the coincidence of conjugate planes for the wavelengths of the exciting and excited radiation has to be taken into account. 2. the proper choice of the different trade-offs to make the most suitable scanning

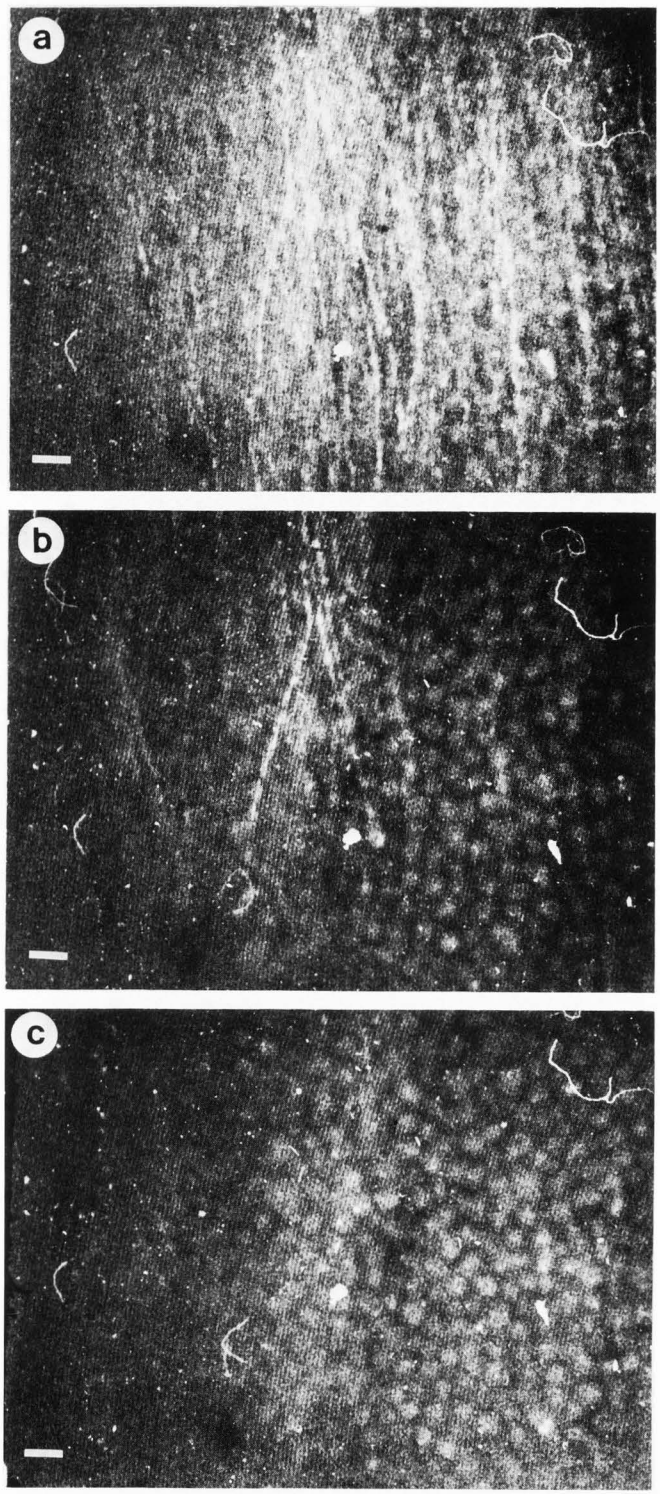

Fig. 9 Chicken retina: corpus vitreum removed, the dish thus formed filled with saline into which the objective was immersed. Steps 2μm. (a) Layer of fibers. (b) Boundary. (c) Layer of ganglion cells.

disc. The size of the holes and their mutual separation can not be calculated, but can only really be chosen by practical experiment. In this regard, our scanning disc has been improved substantially in recent years, but the development is not finished yet. It may be that different samples will require different designs to produce optimal contrast and brightness.

Acknowledgements

We gratefully acknowledge the help given and interest shown by the Pro-Rector of the Charles University, E. Klika; the President of the Royal Society, Sir Andrew Huxley FRS; the President of the CSSR Academy of Sciences, J.Riman and the Vice-President, J Zachar; the Dean of the Faculty of Medicine in Plzen, F. Macku; the Vice-Dean, P. Sobotka; and many other officials of the University, Faculty and the State Committee for Technical Investment and Development, especially Dr B. Cihlar of Sluzba Vyzkumu; also all our present and previous colleagues and collaborators, especially R. Galambos (La Jolla, CA), M.D. Egger (New Brunswick, NJ), M. Sallam-Sattar (Cairo), R. Kucera (Hodonin, CSSR) and J. Benes (Plzen), F. Franc and P. Vesely (Prague).

The UCL TSRLM project was supported by the MRC. AB gratefully acknowledges travel funds from the Royal Society, and the British Council in the early stages of this work.

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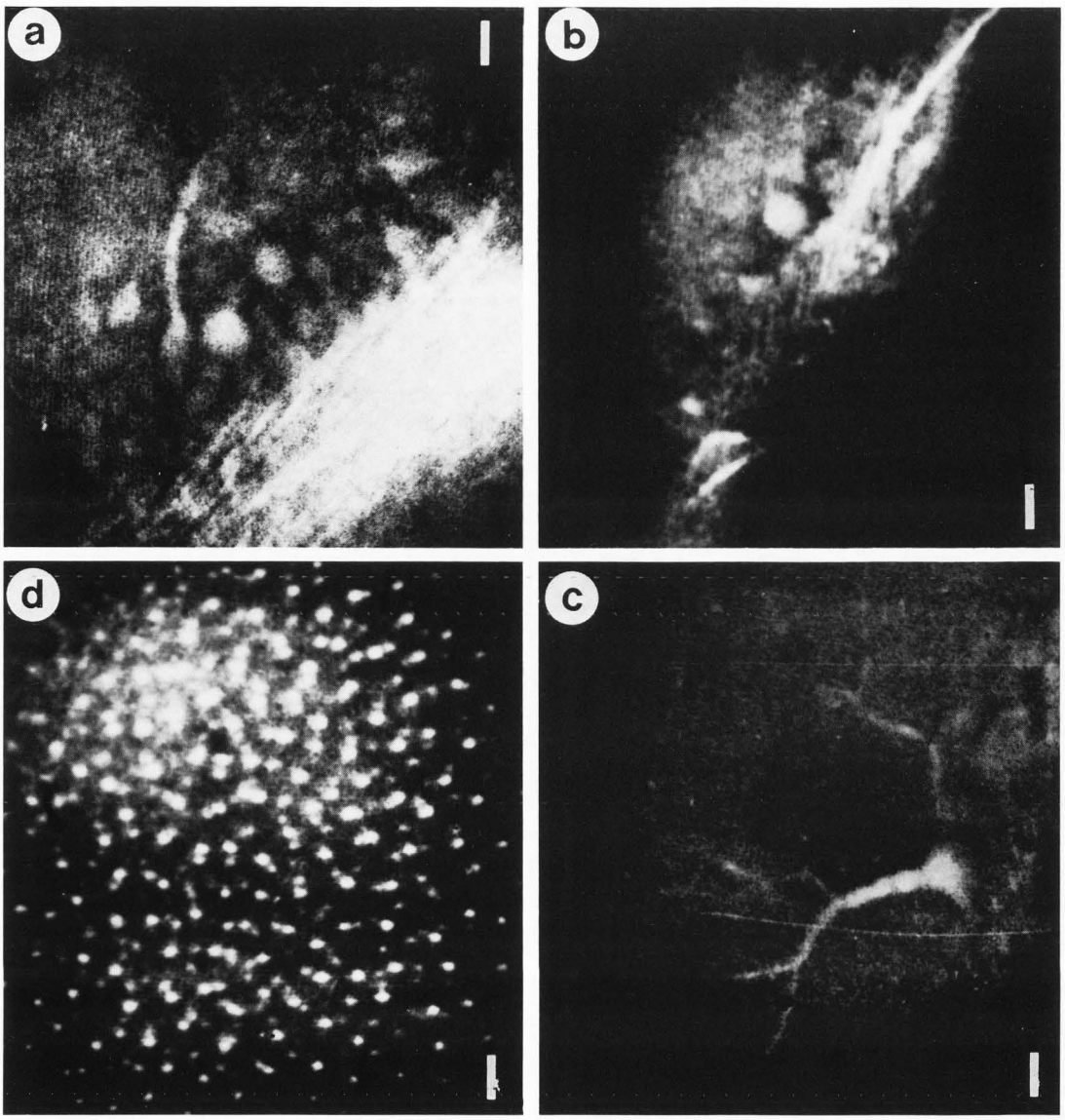

Fig. 10 Chicken retina as in Fig. 9. (a) Tilted object: showing in one corner a layer of fibers, and in the rest of the image a layer of ganglion cells with processes as observed through the overlying layer of fibers. (b) Periphery of retina. Connections between ganglion cells and nervous fibers. (c) Periphery of retina. A large, ramifying ganglion cell. (d) Strongly reflecting and refracting pigment in processes of rods and cones as seen through all overlying layers.

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