New Horizons for Light Microscopy

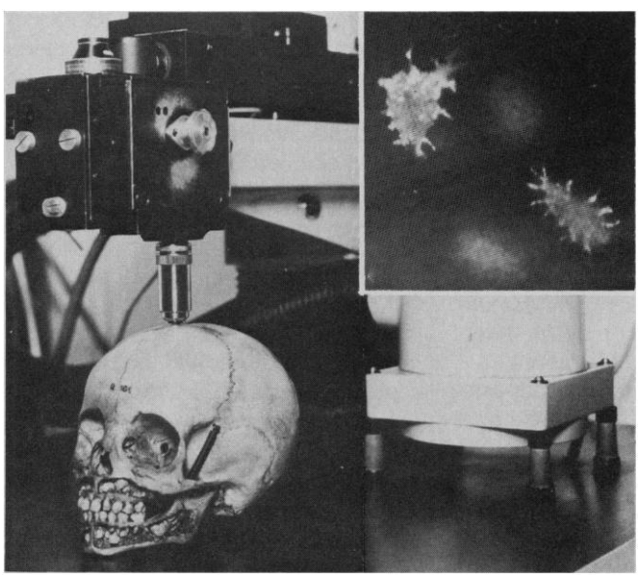
A combination of ingenious inventions, both new and old, has culminated in stereo images at the limit of resolution

It is now possible to record stereo images at the limit of resolution of conventional light microscopy, as a result of work by Alan Boyde of University College, London. The achievement marks one of the most important advances in light microscopy for a century. And yet, like all good inventions, this one is simple in concept, practical in implementation, and far reaching in its implications.

The realization of Boyde's technique, which is reported on page 1270 of this issue, depended squarely upon the prior development of a type of microscope that in itself will revolutionize light microscopy of hard and soft material, either living or dead. This machine, known as the tandem scanning reflected light microscope (TSRLM), of which there are just four in existence, is the ingenious product of Czechoslovakian scientists, conceived and brought to fruition in the midst of extremely difficult circumstances. With this new microscope one can look with high resolution at "slices" within a translucent object, without actually having to take a knife to it to cut a section. The benefits of being able to do this with living tissue or a priceless museum specimen are obvious.

The TSRLM is in fact not so new, the idea behind it having been patented almost 20 years ago and described in *Science* (*1*) shortly thereafter. But, perhaps because researchers simply failed to recognize its power, or perhaps because its inventors, Mojmír Petráň and Milan Hadravský of the Charles University of Prague, were isolated from much of the rest of the scientific world by tense political circumstances, the TSRLM remained no more than a dream and two rather crude Rube Goldberg prototype affairs in a modestly equipped laboratory in Plzeň for nigh on two decades.

It was Boyde, an anatomist by trade and an inventor by inclination, who rescued the machine from obscurity by dint of 10 years of collaboration with Petráň and other researchers in Czechoslovakia. Appropriate, then, that Boyde should be the one to push this remarkable machine that one extra step to achieve the light microscopist's dream: high resolution stereo images.



Skull in view

The "black box" above the skull contains the optics and the spinning disk; the eyepiece is at the top (front). Viewed here (inset) are two optically sectioned osteocyte lacunae, measuring about 20 micrometers in length. "Shadows" of others out of the immediate, focused-on plane can be seen.

Petráň invented the TSRLM because he wanted to do a specific job: to look at living nerve tissue with reflected light. He was interested, for instance, in monitoring morphological changes that might accompany electrophysiological activity. Light microscopists are familiar with the result of trying to do this kind of task by conventional means: "In most cases nothing other than a general pink hue can be seen, in some places decorated with brilliant reflections from the wet object surface," Petráň wrote recently (*2*). A lot of imaginative effort has gone into improving on this aesthetically pleasing but technically devoid result, including special tissue preparations and ingenious variations on conventional microscopy. For the most part, the results have always had shortcomings of one sort or another.

The problem of not being able to see enough in these blurred images is, paradoxically, that one sees too much. Reflections from levels in an illuminated object that are not in the focused-on plane swamp the desired image: hence the pink blur. The trick, Petráň realized, is to focus the illuminating light on a very thin plane in the object and to ensure that only light reflected from that plane reaches the eyepiece. This concept, which has come to be known as confocal microscopy, allows the viewer to see clearly just one single, very thin plane within the object. And by focusing up and down, the viewer sees the specimen in three dimensions, literally by generating a series of optical sections through it.

A very neat idea. But how is it achieved? Initially, for Petráň, it involved the exploitation of a device patented in 1884, and resort to a mountain top. The first, a Nipkow disk, which had been invented as the first primitive attempt at television, provided the means for controlling the incident and reflected light beams; and the second was necessary to harness a light source of sufficient power and collimation, the sun, to illuminate the object and create a visible image in the eyepiece.

As used by Petráň, the disk is a device for reaching a compromise between two competing demands in seeking to produce a high-resolution image that is not overwhelmed by stray light: on one hand you want to restrict the amount of light in the system, while on the other you want to boost it all you can.

This conflict derives from the following. There are benefits to be gained by narrowing the field of illumination, because this reduces the blur from areas not focused upon. However, it goes without saying that the narrower the field, the more restricted you are in what you can see. Moreover, as the field narrows, it becomes surrounded by a strong halo, which at best is a nuisance and at worst can obscure the desired view.

Petráň uses a Nipkow disk, or rather

his very sophisticated development of it, as a device to generate a large number of tiny, optically crisp images, which are "assembled" as a single image of conventional size but focused on a very thin plane only. The assembly is achieved on a continuous basis, so the viewer sees the image in the eyepiece as he would in a conventional microscope. In this respect Petráň's TSRLM differs from a number of other more recently developed confocal microscopes that assemble the final image more slowly and on a television screen.

The original 1884 version of the Nipkow disk was a relatively simple design, having one row of holes arranged in one scroll of an Archimedean spiral occupying one rotation near the periphery of the disk. Each hole is therefore a slightly different distance from the center of the disk. So, when the disk is spinning and is illuminated by a discrete beam at one place near the periphery, light passes through a subset of the holes at any one time, each of which describes a unique line or arc in the projected "image."

In the earliest attempts at television, the image was reproduced by projection through a second, identical disk spinning in the same configuration and at the same speed. The key feature is that light that passes through a particular hole at a particular time in the first disk will be reproduced by light passing through the equivalent hole in the correct sequence in the second disk, hence the re-creation of the original image.

Petráň's modification of the Nipkow disk is extensive, and the result, says Boyde, should properly be called the Petráň-Hadravský disk. Perhaps the most important change is that the 100-millimeter-diameter disk in the TSRLM is in effect two disks combined as one. Instead of having just one spiral of holes, each of which occupies a unique position, the Petráň-Hadravský disk has pairs of spirals that begin on opposite sides of the disk, thus making it bilaterally symmetrical. This symmetry, in which diametrically opposite holes are on exactly identical radii, is central to the conceptual simplicity of the TSRLM, which works as follows.

The illuminating beam passes down through holes in one side of the disk, thus producing a series of light patches that will sweep across the specimen. These moving patches pass through the objective lens and are focused onto the desired plane in the specimen. Reflected light from the highly focused patches passes back through the objective and is projected onto the opposite side of the

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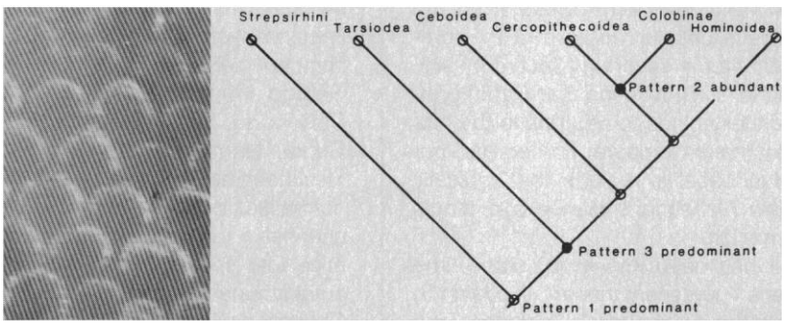
Prism Patterns Show Relations

Perhaps the most significant advantage of the TSRLM technique is that it is nondestructive: the worst assault on even the most valuable specimen is the application of a drop of water, which achieves the immersion optics. Alan Boyde and his colleague Lawrence Martin, at the time also at University College, London, but now at the State University of New York at Stony Brook, exploited this property and embarked on the most complete survey yet achieved of prism patterns in the enamel of primate teeth (*1*).

As each viewing can be completed in just a couple of minutes, Boyde and Martin were able to include representatives of most families of extant primates and many families of extinct primates, most of which have never previously been studied. Optical sections through enamel reveal the arrangement of so-called prisms, the configuration of which depends on various features of the activity of the cells, ameloblasts, that secrete the mineral. There are three basic forms: pattern 1, which appears as circles; pattern 2, in which the horseshoe cross sections are stacked directly one above the other; and pattern 3, in which the gaps in the horseshoe shape in one row open above the spaces between prisms in the row below.

Boyde and Martin showed that the depth at which the viewing is done, and the position on the tooth structure itself, can affect the chances of



Prism patterns and their relationships in primates

At left is a pattern 3 seen in the tooth of an extinct hominid (Australopithecus boisei), *in which the horseshoe-shaped prism boundaries measure about 6 microns. At right is the change in prism pattern through the primate family tree.*

finding the "characteristic" pattern, because other patterns often appear too, albeit sometimes in small quantities. The ability to survey thoroughly an individual tooth is therefore important, especially as the details of the distribution of the different types of prism pattern itself hold important information.

The basic condition in mammals is pattern 1, which is also found in prosimians, excluding the tarsiers (the Strepsirhini). Pattern 3 then turns up with the common ancestor of the anthropoids and tarsiers. The New World Monkeys (Ceboidea) have a complex configuration, with both patterns 1 and 3 figuring. Pattern 2 appears with the Old World Monkeys, which should be a helpful diagnositic character in early fossils from this group. Pattern 3 is the predominant type in apes and humans, with interchanges in the pattern revealing useful information about developmental sequence and phylogeny (*2*). The approach is clearly useful in providing taxonomic data that are complementary to other characters, and can with certainty exclude some possibilities, as Boyde and Martin did with a putative fossil gorilla that turned out to be a nonprimate.—**ROGER LEWIN**

References

1. A. Boyde and L. Martin, "A non-destructive survey of prism packing patterns in primate enamels," *Tooth Enamel* **4**, 417 (1984).
2. L. Martin, "Significance of enamel thickness in hominoid evolution," *Nature* **314**, 260 (1985).

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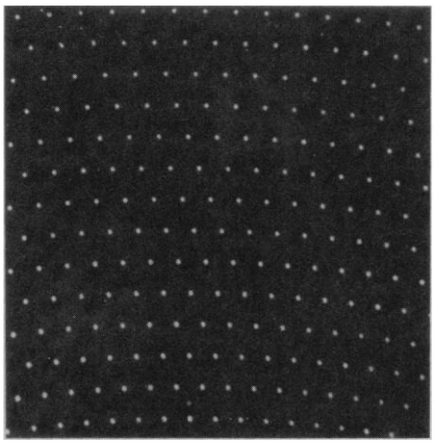
disk, with mirrors and beam-splitters performing the variously required optical tricks.

Now, although some of the reflected light will have come from above or below the focused-on plane in the specimen, its passage to the eyepiece of the microscope is effectively blocked by the disk. This occurs because only light reflected from the focused-on plane will be brought to a focus at the holes and can therefore pass through and into the eyepiece. Reflected light from other planes will be brought to a focus either below or above the holes, and will therefore strike the solid part of the disk.

Remember, the microscope is aligned so that light that passes through a particular hole on one side of the disk as it spins will return through its exact equivalent hole on the opposite side of the disk after being reflected on the focused-on plane. This horizontal and vertical control of tiny illuminated patches is the basis of the complete image that the viewer sees.

The image projected into the eyepiece is of course made up of a series of lines or arcs described as the patches scan the objective field. In one current version of the Petráň-Hadravský disk there are some 48,000 holes in ten pairs of spirals, only a few hundred of which are illuminated at any one time by the 18-millimeter-diameter incident beam. But, as each line in the image is scanned several times per revolution, and as the disk is rotating 100 times a second, the time taken to form a quiet, stable image is just one 20th of a second. As a result the TSRLM produces images in real time and real color, and can be used to follow relatively rapid changes in, for instance, living tissue. These abilities surpass those of most alternative confocal microscope systems.

The scope of the TSRLM is to some extent limited by the optical nature of the specimen under the lens but mostly by the mechanics of high-magnification light microscopy. For instance, the resolution of the system at its limit is such that the image changes as one focuses through something less than 1 micrometer, which allows many crisp optical sections to be visualized through a specimen. But the highest power conventional lenses are currently designed to move through about 170 micrometers before they slam into the cover slip. This means that in tandem scanning microscopy, in which no cover slip is used, one may focus only 200 micrometers into the specimen before the lens makes contact with the surface. Petráň hopes that lens manufacturers will come up with designs that will permit a great range of movement of the objective, perhaps doubling the optical penetration that is possible at present.

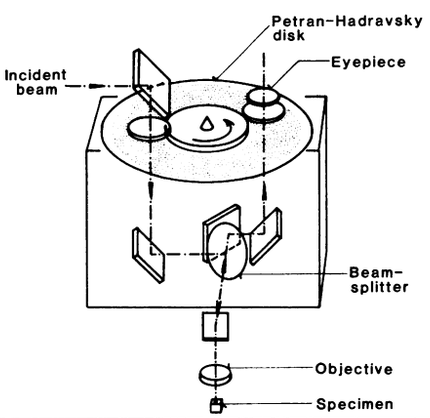


Holes in focus

A section of the Petráň-Hadravský disk in the UCL machine, in which there are 32,000 holes, each measuring 50 micrometers in diameter.

The ability to section optically a specimen at great speed compared with conventional procedures may be regarded by some practitioners as simply a rather intriguing but not very practical novelty. But, as Petráň points out, the real value of the system lies in "the fact that it is not possible, or not permissible, to obtain serial sections with many classes of objects, including museum specimens in general." Boyde puts it this way: "The advantage of the TSRLM . . . has been to permit us to see the previously unseeable."

Being a hard tissue anatomist, Boyde has been particularly interested in seeing how far the microscope will help in studying bone and tooth material, but he identifies many other potential applications too, including use in medicine, microelectronics, and botanical sciences (*3*). His most driving interest, however, is in stereoscopy: "As far as microscopy is concerned," he says, "I'm a stereo nut." For instance, Boyde was one of the first to recognize the potential value to biology of scanning electron microscopy, and it was this interest that, via a British Council lectureship, took him to Eastern Europe in 1970, when he first came across Petráň and the TSRLM.



Optics of the TSRLM

The incident beam is focused through a group of holes on the illumination side of the disk, passes through the objective, which focuses it within the specimen. Light reflected from the focused-on plane passes back through the objective, is focused on the holes in the detection side of the disk, and passes through to the eyepiece. Light from other planes is blocked by the disk.

Four years were to pass before Boyde came to appreciate the power of the tandem scanning microscope. "It seemed to be an interesting trick, but initially I really couldn't see how it could be applied to my work. Maybe this is the reason why the whole thing has been ignored by others too."

Once the scales had fallen for Boyde, he worked hard to maintain support for and collaboration with Petráň and his colleagues, and finally obtained a machine of his own late in 1983. This machine was initially used to study prism patterns in primate teeth (see page 1259).

Currently there are four microscopes in existence: two in Plzeň, Boyde's in London, and one in Zurich. Negotiations for a fifth are nearing completion, and this one is to be installed in the Department of Anatomical Sciences, SUNY, Stony Brook.

The fact that the microscope could slide through crisp three-dimensional space with such facility, and yet apparently lacked an easy method of recording the total image, tantalized Boyde for a while. Surely there must be a way of capturing the three-dimensional image, he thought. The solution occurred to him in a flash this summer. Simply open the shutter of a camera at the eyepiece as the objective is focused through the specimen, preferably by electric motor. Repeat the process, with the angle of attack appropriate for parallel projection. And there you have your stereo pair. Simple.

As Boyde notes, it is ironic that this major development of light microscopy, of recording tremendous depth of an image in high resolution, could be achieved only after the development first of a way of obtaining sharp images of minimal depth.—**ROGER LEWIN**

References

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2. M. Petráň *et al.*, "The tandem scanning reflecting light microscope," *Scanning* **7**, 97 (1985).
3. A. Boyde, "The tandem scanning reflected light microscope: Pre-Micro '84 applications at UCL," *Proc. R. Microsc. Soc.* **20**, 131 (1985).