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How the Confocal Laser Scanning Microscope entered Biological Research

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Abstract

A history of the early development of the confocal laser scanning microscope in the MRC Laboratory of Molecular Biology in Cambridge is presented. The rapid uptake of this technology is explained by the wide use of fluorescence in the 80s. The key innovations were the scanning of the light beam over the specimen rather than *vice-versa* and a high magnification at the level of the detector, allowing the use of a macroscopic iris. These were followed by an achromatic all-reflective relay system, a non-confocal transmission detector and novel software for control and basic image processing. This design was commercialized successfully and has been produced and developed over 17 years, surviving challenges from alternative technologies, including solid-state scanning systems. Lessons are pointed out from the unusual nature of the original funding and research environment. Attention is drawn to the slow adoption of the instrument in diagnostic medicine, despite promising applications.

1. The prototype

The Bedouin Tent, a small enclosure made of World-War II blackout material, stood within the mechanical workshops of the MRC Laboratory of Molecular Biology in Cambridge in 1986. Inside, against a background of blue laser speckle, a cluttered arrangement of improvised optical mounts and a pulsating green image of a nematode worm on an oscilloscope screen were visible. From time to time, an engineer, Michael Fordham, would emerge with some small piece of metal, perform a machining operation on it and rejoin the scientists in the rather sweaty space inside the tent. Within two years, this apparatus had evolved into a commercial product, the laser scanning confocal microscope, which almost every biological laboratory was seeking to purchase. Now, seventeen years later, the annual sales of such microscopes probably exceeds $150 million in value: every biomedical institution has them and their use is taken for granted in countless scientific papers.

2. Fluorescence as key method

The reason for the rapid uptake of the invention in the 1980s was that it improved the image in a familiar and widely-used instrument: the fluorescence microscope. Although this type of microscope had been invented in 1904 and fluorescently-labelled antibodies were introduced in 1941 (Kasten,89) the instrument and its reagents did not come together really effectively until the 70s. This was perhaps because of an erroneous belief that antibodies were useful only for studying infection. It came as a surprise that antibodies to normal proteins such as actin and tubulin could be raised (e.g. Lazarides & Weber, 1974). As soon as this became generally appreciated, fluorescent labels attached to antibodies were applied to cells and revealed the elaborate architecture of the cytoskeleton, provoking discussion and amazement in the scientific community in the late 70s and early 80s. In the same period, fluorescent stains came into use that responded to important intracellular parameters such as the calcium ion concentration (Tsien et al., 1985). Suddenly, microscope suppliers were inundated with orders for fluorescence microscopes and imaging started to increase in fields previously dominated by biochemistry or electrophysiology.

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A high proportion, perhaps half, of all published work in cell biology at that time came to rely on fluorescence microscopy. By 1984, the chief limitation of the method had become obvious: the out-of-focus parts of a thick specimen give rise to a uniform glow, against which fine detail cannot be distinguished. Many researchers got around this by restricting their studies to thin and flat cells, but both of us were interested in cell division, and all vertebrate cells, even the highly flattened ones which occur in endothelium, tend to round up during division. Some way had to be found to improve the microscope to get a clearer view.

3. Confocal Optics

White decided to investigate the confocal microscope, which had been invented by Minsky in 1955 (see Minsky,1988). The word 'confocal' seems to have been first used by Brakenhoff and others in 1979 to mean a microscope in which the illumination is confined to a diffraction-limited spot in the specimen and the detection is similarly confined by placing an aperture in front of the detector in a position optically conjugate to the focussed spot. The result of this arrangement is that the response of the instrument to a fluorescent point object falls off approximately according to an inverse fourth-power rule with distance from the plane of focus. This produces an 'optical sectioning' effect, in which the glare from out-of-focus regions is almost completely eliminated. Brakenhoff et al. (1979) had demonstrated this experimentally with microscope objectives of the highest available numerical aperture, of the type used in cell biology, and also verified the prediction that the resolution (as measured by the full-width at half maximum intensity of the point spread function) is improved relative to the non-confocal microscope by a factor of the square root of 2 (1.414). (In practical microscopes, it is the optical sectioning effect that is more important than the resolution improvement.) The underlying physics was understood (Wilson and Sheppard, 1984): why, then, had the method not been applied earlier to biological fluorescence?

The explanation lies in the fact that the confocal microscope views only a single point in the specimen at one time, so special apparatus is needed to build up a 2-dimensional image by scanning the illuminated spot over the specimen. Such apparatus tended to be found only in physics labs and it was not clear whether such complexity would be justified with biological specimens. A long series of confocal microscopes, stretching from Minsky's prototype to the SOM system supplied commercially in the 80s by Lasersharp Ltd., and including the systems of Brakenhoff and of Wijnaents van Resandt and Stelzer (1985), relied on moving the entire specimen and its supporting stage in two directions raster-fashion relative to stationary optics. These microscopes were too slow and vibration-sensitive to be convenient for biology. Nevertheless, Cox (1984)managed to obtain confocal images of cells stained with fluorescein, but the image improvements over conventional epifluorescence were unconvincing. With

the advantage of hindsight it seems probable that the particular specimens chosen may have lacked fine structure.

An alternative approach to confocal microscopy was adopted by Petran, who illuminated the specimen with many spots of light by placing a spinning Nipkow disk in an image plane in the epicondensor (Petran et al. 1968). The fluorescent emission from each spot was focussed upon and directed through an individual corresponding confocal aperture placed diametrically opposite on the disk. This was, in effect, many thousands of confocal optical systems working in parallel. The disk was spun to produce a continuous image on a camera target. Although the Petran apparatus and its derivatives worked well for reflection images (Boyde et al. 1983) the Nipkow disk attenuated the illumination so badly that fluorescence could not be detected except in the brightest specimens, so the fast framing rate intrinsic in this design could not be exploited usefully. If the holes were made bigger to improve transmission, more cross-talk occurred and the confocal performance became degraded.

To study fluorescence with the early stage-scanning confocal systems required great patience. It was difficult to search for the correct focus, as well as the right region of the specimen, so the image, which was constructed with sometimes painful slowness, would often turn out to be blank or unsuitable. The first biologically-convincing results were obtained by Brakenhoff and colleagues (1985) who obtained axial series of images of nuclei in which the chromatin, stained with a fluorescent dye, was shown with unprecedented clarity. Their paper, with a title appropriate to its status as a great advance in methodology, was initially rejected by Nature on the grounds that the journal did not publish methods. Under a new title, describing it merely as a study of chromatin in a particular type of cell, it reached A. Klug in the LMB, who asked White to review it. It was an encouraging sign that we were on the right track. Klug suggested that White should visit Wilson and Sheppard in Oxford, but he did not go, since he had realized that what was lacking was not physical theory (already the subject of a textbook by the Oxford group) but a new practical implementation with the specimen stationary in a scanning beam.

4. Making confocal fly

The idea of scanning the beam and illuminating the specimen in a microscope with a 'flying spot' was not new. It dated from the work of Young and Roberts (1952), who used a scanned spot on the surface of an oscilloscope tube as the illuminant in an image plane of a microscope and a photocell positioned to capture all the light that entered the eyepiece as the detector. This pioneering system gave images very similar to a conventional wide-field microscope and caused as little interest as Minsky's contemporaneous invention of the confocal microscope. Later, many optomechanical microscanning designs and commercial models were produced. The commonest application was microdensitometry, where the transmittance of each pixel was converted by a simple
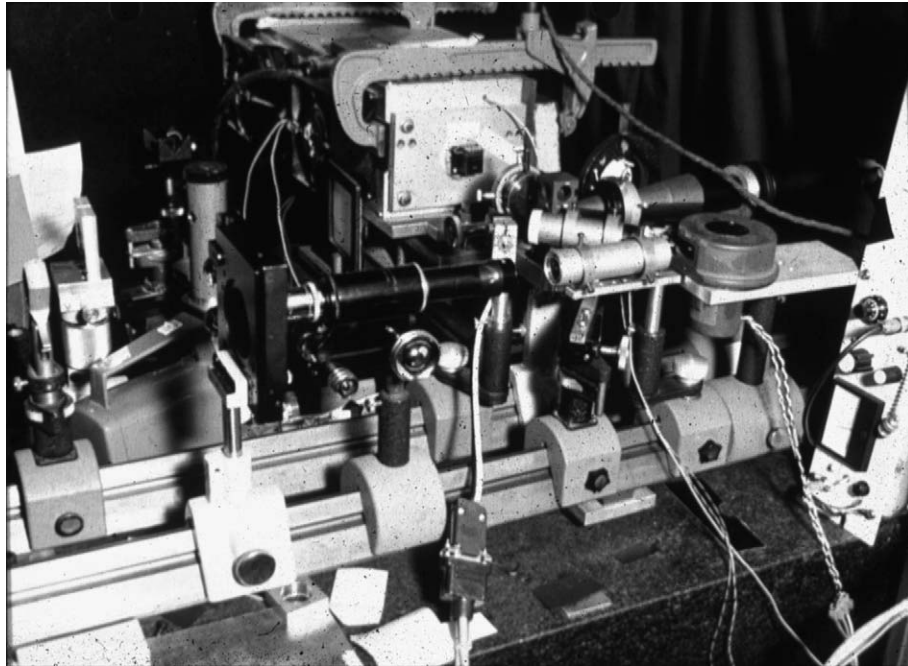
Fig. 1. The prototype confocal laser scanning microscope in 1986. The optical axis of the microscope is horizontal, with a telescope linking the scanning eyepiece to a 25-facet polygon mirror on the right. This prototype was capable of scanning speeds in excess of 4000 lines per second (unidirectional).

analogue computing circuit into a number proportional to the quantity of absorbing material which could then be summed over any desired area.

The scanning requirements for a flying spot microscope were simple and known: the beam must fill the back aperture of the objective lens at all times and must *rotate* around a point in this aperture plane, producing a corresponding *translation* of the focussed spot in the plane of focus. The simplest and optically best solution is to have a single reflector in the back aperture and tilt it in two orthogonal directions. This is mechanically difficult to achieve at high speed. White adopted the scheme shown in Figs 1 and 2, in which the two tilts are achieved by separate mirrors: a slow oscillating mirror and a fast polygonal mirror. In order to make both scans appear to rotate about the same origin, the two were linked by a unit-power refractive telescope which imaged the first scan mirror on the second. This scheme was chosen in preference to other scanning systems because it gave a relatively high scanning rate, equivalent to four video frames of 512 lines each per second.

5. Enlarging the confocal aperture

In the first experiments, White found that it was not necessary to focus the emitted light on to a detector aperture of a few tens of micrometers in diameter, as had been done in all previous confocal microscopes: he obtained good optical sectioning with an aperture of approximately one millimetre in diameter, provided it was placed sufficiently distant from the scanning optics. In his 1985 patent application (see White, 1987) he described the emission path as an 'infinity optical system' which was a good approximation to the truth.

In fact, the optics were adjusted to produce an image, of the order of 80 times the magnification of the microscope objective, at a large but finite distance of one or two metres. This arrangement, with a large pre-aperture magnification, was totally novel for a confocal microscope and of great practical importance, for it allowed the confocal aperture to be constructed as an iris diaphragm. The iris could be opened and closed to achieve different compromises between signal strength and confocal stringency. When the system came to be manufactured, this design feature allowed the optical path to be aligned without micromanipulation; it permitted multiple optical channels to be co-aligned with ordinary engineering tolerances; it allowed optimal circular apertures to be used and it made the aperture immune to blockage by microscopic particles of dust, which had bedevilled earlier systems. The value of a continuously-variable aperture had been realized by Brakenhoff at the start of his fluorescence work, and he had designed a piezo-operated micro-aperture for this purpose.

6. Electronics and control in the prototype

White's first prototype (Figs. 1 and 2) used the time-base of an oscilloscope to supply a sawtooth voltage for the slow (frame) scan, while the angular velocity of the polygon determined the line rate. Because polygon mirrors cannot be made with sufficiently accurate inter-facet angles, a photodiode was used to detect the laser beam at the start of each sweep and trigger the sweep of the display. Initially, the noisy images were integrated by long-exposure photographs. After some early promising results, a digital image capture and integration system was purchased.
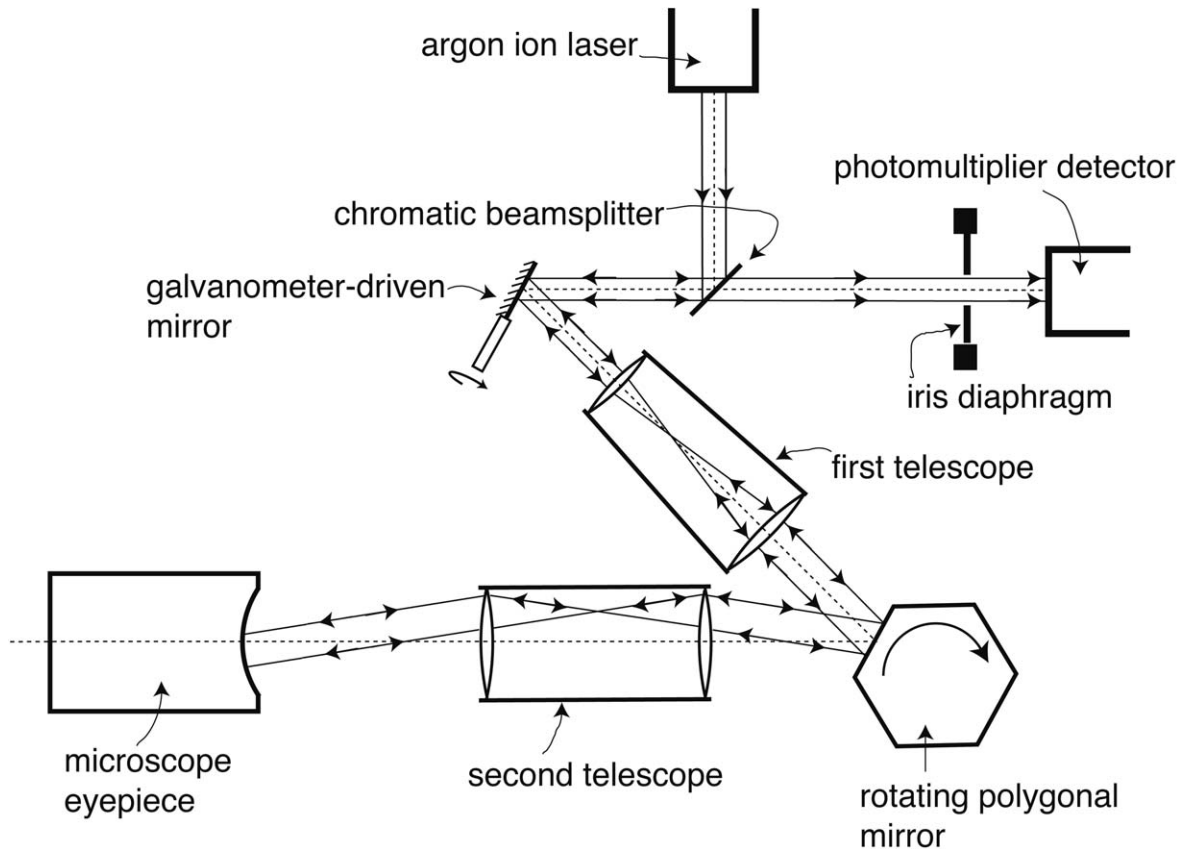
Fig. 2. Schematic layout of the prototype confocal laser scanning microscope. The prototype was designed as an adjunct to a conventional microscope, of which only the eyepiece is shown. A galvanometer-driven mirror was used for the frame scan and the polygon for the faster line scanning. Note the key innovation: the use of a variable iris as the confocal aperture, positioned distant from the scanning optics, so that confocal performance could be obtained even though the aperture had millimetre dimensions. (redrawn from White's UK patent application filed in 1986).

7. Proof of performance

We realized that the instrument could have general application and set about to try it on a wide variety of specimens. In each case, we took conventional fluorescence images first and then found the same regions under the prototype confocal system in the Bedouin Tent. In every case, the detail in the image was improved dramatically. First came a nematode embryo and HeLa cell mitoses with anti-tubulin staining, then mitotic sea-urchin embryos and interphase HeLa . We stained polytene nuclei of *Drosophila* with chromomycin and used the same stain on a chick embryo to demonstrate how well the system worked on a large specimen. One of the most effective demonstrations of confocal optical sectioning was the result with immunofluorescent staining of a plasmacytoma cell (Fig 3). We sent off these results in a paper to The Journal of Cell Biology: the paper was accepted (White et al., 1987) and one of the editors sent a request to purchase the instrument as soon as possible.

8. Problems and improvements

Although the prototype worked well, and our colleagues were beginning to bring specimens to us for imaging, there were some problems. The polygon was noisy and the setting of the line-sweep triggering of the polygon was critical. Also there was chromatic aberration, which manifested itself as chromatic differences in magnification when green and red fluorescent dyes were used. We knew also that the polygon did not produce the ideal purely-rotary movement of the scanning beam and the scan angle was fixed (precluding the possibility of optically zooming the image). We decided to dispense with the polygon and substitute a pair of oscillating galvanometer mirrors. Amos hit upon the arrangement of concave mirrors shown in Fig. 4 as a substitute for the 1:1 refractive telescope which was causing our chromatic problems. At first sight, this seems unpromising, at least with simple spherical surfaces, since the mirrors are used off-axis and would be expected to introduce serious astigmatism. Amos noticed that when one examines one's own eye with a spherical mirror of 50mm focal length, astigmatism does not become visible until quite high angles of view are reached. The test is quite relevant, since the beam diameter and angles can be made identical to those required to scan through an eyepiece into a microscope. Later tests showed that astigmatism in the simple scanning arrangement of Fig. 4 does not limit the resolution in microscope imaging. We made an adjustable scanning model and quickly found that there was a
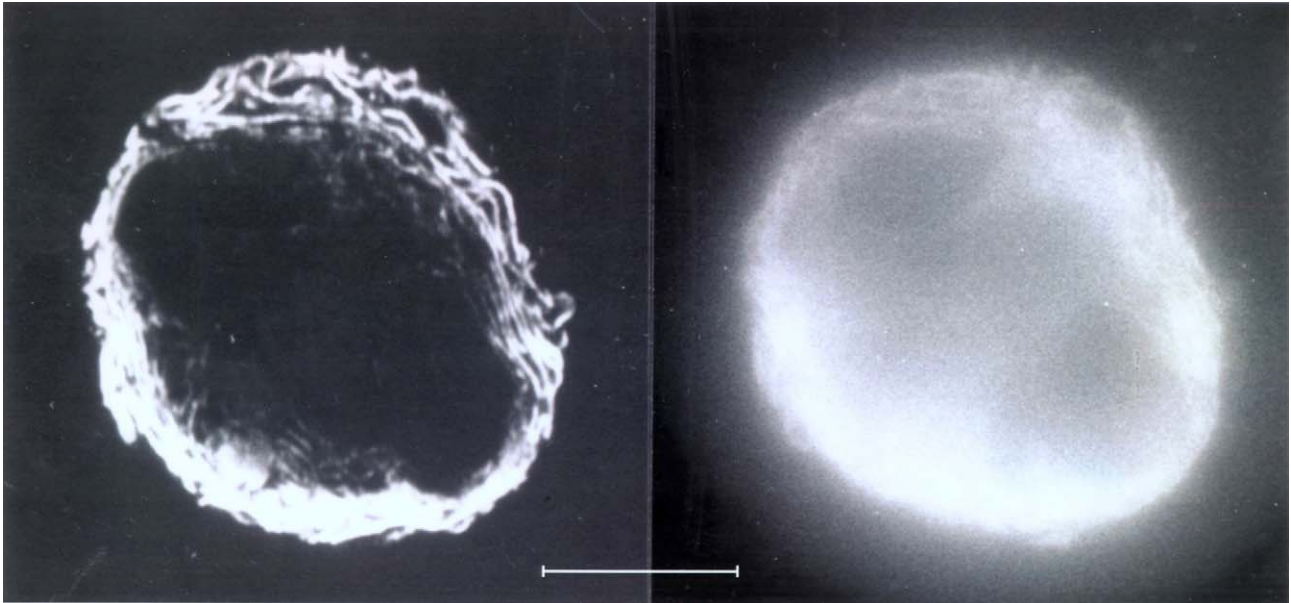
Fig. 3. An early comparison of a conventional epi-fluorescence image with a confocal one obtained with the polygon scanner. The specimen is a plasmacytoma cell labelled with anti-endoplasmin, which binds chiefly to cisternae of the endoplasmic reticulum. In the conventional image it is not possible to establish whether the central nucleus lacks endoplasmin and the individual cisternae are unclear. Scale bar = 10 um. (Specimen prepared by Gordon Koch. Image previously published in the Journal of Cell Biology, 105, p44 (1987)).

particular value for the input angle a at which the scan lines became straight. This scanning system cured the problem of chromatic aberration and has been patented (Amos, 1991) and used in all the confocal point-scanning systems manufactured by Bio-Rad ever since.

Initial approaches to Zeiss, Leica and Cambridge Instruments to develop the instrument proved unfruitful. At this point, we set out to redesign the instrument into a shoe-box-shaped case, in which the long emission light-path was folded and divided into two optical channels. The Medical
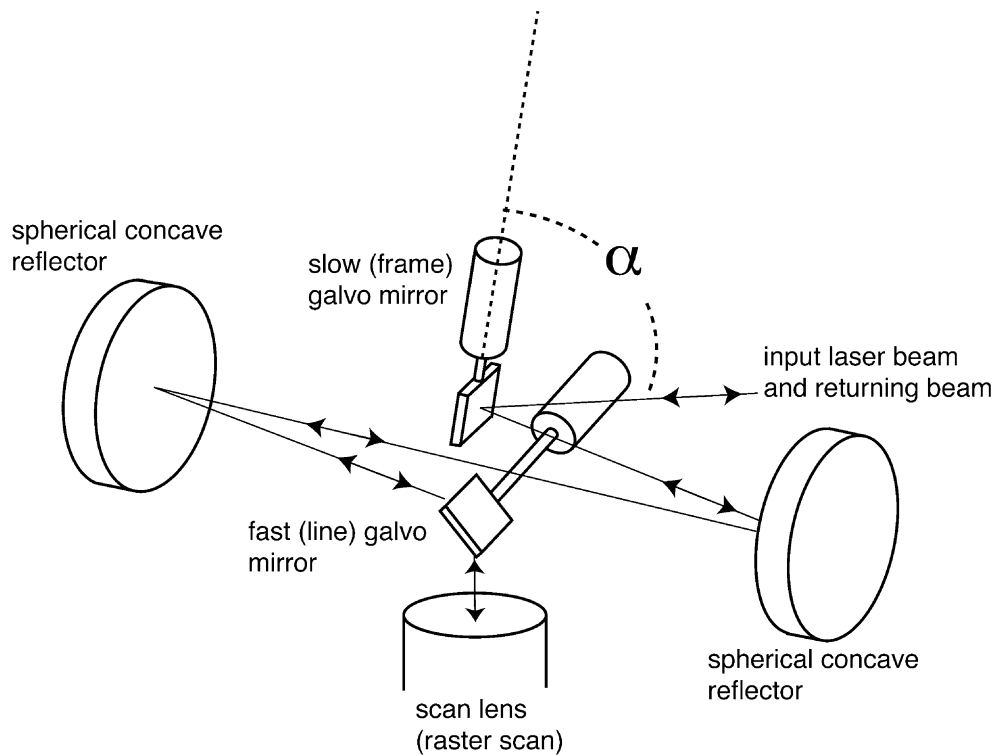

Fig. 4. The all-reflecting scanning system introduced as a replacement for the telescope relays shown in Fig. 2. The polygon is replaced by a second galvanometer-driven oscillating mirror. By careful adjustment of the angle α, it was possible to eliminate curvature of the scan lines.

Research Council's Laboratory of Molecular Biology agreed to fund the development of this commercial prototype. We used subcontractors to develop the image-processor and scan-generation system. The mechanical design and construction together with the detector amplifiers were developed in the LMB's workshops. We then contacted David Shotton (Oxford) who told Andrew Dixon of Bio-Rad about our work. Dixon recognised the advantages of our design immediately, and a commercial agreement was concluded quickly and easily with Bio-Rad. We offered to retain control of the pre-production design and the company wisely agreed to this. Richard Durbin, who had been White's research student, set about to design software to run the system. Soon he had written an attractive and powerful package: the SOM (scanning optical microscope) software. All the control settings could be seen on one CGA monitor and were executed by single keystrokes, while macros, which could be constructed easily (even by non-specialists in computing) from a simple macro language, were called and executed from the menu, or could be listed and edited. Image-analysis features were provided and the live display was particularly well designed, including a gallery format in which previous members of any image series (time or focus) were displayed alongside the live image. All this was released at a time when computer frame-stores were rare and costly, and caused almost as much excitement as the confocal optical system itself. The software and frame store were so good that some scientists used them for imaging with conventional cameras.

Finally, we decided to add a scanned (non-confocal) transmission image to the system. This had actually been done many years before and even phase-contrast images had been obtained from a flying-spot microscope (Mellors & Silver, 1951), but we did not know this. Amos tried to obtain a kind of oblique illumination contrast by picking up off-axis light that had passed through the condenser and passing it to the photomultiplier by means of a fibre bundle. During these attempts it became obvious that the best phase-contrast or differential interference contrast (DIC) image was obtained if as much as possible the laser light passing out of the microscope condenser towards the lamp was collected. The resolution was not impaired, and a more uniform field could be obtained, if an aperture-plane diffuser was placed between the condensor lens and the detector fibre bundle. The way this works is readily understood in the case of DIC (Amos, 2000): the generation of a phase contrast image, with the light passing first through the phase plate and then the annulus, is harder to explain. The ability to form a transmission image simultaneously and in precise registration with the epifluorescence confocal image provided another clear advantage for our system over conventional camera-based non-scanning microscopes.

9. The roar of the crowd

One of the first public showings of our prototype was at the Symposium of the International Society for Analytical Cytology in Cambridge, UK, in 1987. We presented images and a movie taken with the polygon scanner. Bio-Rad caused some indignation by contacting our US colleagues and informing them that they had been selected "to have an opportunity for an early purchase of the MRC 500" (as the first commercial model was called). The scientists found the presumption all the more galling because it was justified: the images were so much better that no research group could afford not to have the confocal technology. The instrument was received with great enthusiasm throughout the world, particularly in Japan and the Netherlands.

There were many examples in the early use of our instrument of striking results that could not have been obtained previously. One example was the full three-dimensional mapping of a specific neurone labelled with Di I in the cortex of a Xenopus tadpole over several days, during which the dendritic arborescence of the cell could be observed to grow (O'Rourke & Frazer, 1990). Another was the discovery of three-dimensional spiral waves of calcium release within Xenopus eggs (Lechleiter & Clapham, 1992).

We were, and continue to be, disappointed at the slow uptake of confocal technology into diagnostic pathology, in spite of our early publication of a detailed confocal image of a living kidney slice (White & Amos, 1987)) and further demonstrations on cervical smear and other materials (Boon, 1996). A few diagnosticians have realized that, as well as giving cellular morphological detail, the confocal microscope is superior to flow cytometry in that smaller biopsy samples may be sufficient. Smaller needles can be used, with consequently less trauma. This is of particular value in the diagnosis of infant leukaemias (personal communication of D. Van Velzen, then at the Royal Liverpool Children's Hospital).

10. Competition and development

Although we were unaware of it until quite late, a parallel development had been going on in the Institute of Physics of the University of Stockholm (Carlsson et al. 1985). This resulted in a commercial system, launched almost simultaneously with ours. Unlike us, the Swedish instrument was not restricted to 768 x 512 lines of resolution and gave larger image sizes than ours. However, the optical resolution was the same, the aperture was a fixed and minute pinhole and the scanning system was rather slower. This system competed with ours for many years but was eventually withdrawn. Another laser-scanning system was being developed at that time by Heidelberg Instruments. This system was designed so that the microscope could not be used conventionally, and never made an impact in biological work. However, this project seems to have aided the subsequent development of successful confocal microscopes by Leica. Other competition, which looked at first as it might render our system obsolete, was given by two other developments, both using solid-state scanning instead of oscillating mirrors. In the instrument of Draaier and Houpt (1988), the emitted beam

was descanned by a mirror in one direction and then passed, still scanning on a line, through a quasi-confocal aperture in the form of a slit. This gave results which, surprisingly, were optically isotropic. A commercial development of this survived for many years but did not gain wide market acceptance. Goldstein and others (1990) developed a highly original fast system based on an image dissector tube. In this device, which dates from the early days of television, the fluorescence image was converted into a coherent electron beam image which was descanned electronically. This system was not widely adopted, possibly because of problems with linearity.

The big microscope companies were not long in producing copies or developments of our system. Zeiss were the first, with a version of their flying spot microscope modified to make it capable of producing a confocal image. However, they did not understand the significance of White's variable iris and omitted it. Later, purchasers were quietly offered the opportunity of returning their microscopes to the factory and having an iris fitted. Although no details were published, we assume that this was a micro-iris comprised two pieces of metal with opposed V-shaped notches, thus forming a square aperture whose size could be varied by translating the metal pieces with piezo actuators, as developed earlier by Brakenhoff for Leitz.

Bio-Rad eventually produced an improved version, the MRC 600. Useful developments of the software were made by N.S. White in Oxford and Todd Brelje in the USA. The first substantial change in the scan head was a redesign by Amos, acting as consultant to the company in 1997/8. In this, the long light-path of the MRC 500/600 was collapsed into a few centimeters by the use of an achromatic Keplerian telescope. With other changes, this allowed the instrument to be reduced in size.

A most significant development from our apparatus was its use in 1990 as a basis for proving the possibility of two-photon-induced fluorescence imaging by Denk, Strickler and Webb (1990). Sheppard had demonstrated imaging by a non-linear optical process (second harmonic generation) and, in a far-sighted review (Sheppard 1980), had suggested that several methods, including pulsing the laser, might be used to lessen the heat-induced specimen damage. With our faster scan head and a laser that could produce peak powers more than a million times higher than ours, the Cornell group showed not only that fluorescence induced by two-photon absorption could be imaged but that this could be done without killing the cells.

11. Can lessons be learned from this development?

It will be obvious from the foregoing that we were not the only developers of the confocal laser scanning microscope but, without our efforts, it would perhaps have taken a few more years to reach an equally convenient and practical form. It may be worth pointing out that our work on this project was neither supported by the instrumentation industry nor by a peer-reviewed grant. We now know that instrument manufacturers cannot afford to develop really new technology of this kind: the work has to be carried out in research institutions. But most funding bodies forbid even the slightest deviation from a strictly defined biomedical project. Everyone agrees that instrumentation developers are crucial to new bioscience, but few realise that they exist in a no-man's land where, often, neither private nor public funding can be found, since public bodies tend to have an exaggerated view of the resources available to the industry. We had the opportunity to work with Durbin and Fordham under the benevolent protection of Aaron Klug and of the MRC LMB, in an environment that was privileged but above all flexible and informal, particularly in regard to access to high-quality workshops.

12. Why aren't we (and the MRC) rich?

We are often asked. The answer may be of more than personal interest. One third of the royalties from our efforts are divided between the four inventors and the remaining two-thirds are ploughed back into the Medical Research Council's research programme. For several early years, the confocal microscope contributed the largest component of the Council's royalty income. However, this is small in comparison with that from current biotechnology developments, and, recently, a proportion has even been forfeit as a result of litigation between companies. Our acquaintance with the microscope industry has taught us the explanation why neither we nor the Council have become wealthy from this development. The reason is that microscope manufacture is a mature industry cursed with overproduction: some of the well-known companies have little or no profit margin and confocal manufacture barely breaks even.

We have come to realize that the development described here was its own reward. We certainly appreciate honours bestowed by the Royal Society and the Rank Foundation and other bodies, but our real reward was to live through those heady months, working together with Durbin and Fordham, and then see our innovations spread and stimulate research all over the world. We remain confident that the laser scanning confocal microscope will eventually prove its value in diagnostic medicine.

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