

# Optimum Optical Design Characteristics for Confocal and Multi-Photon Imaging Systems

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## Introduction

The principle of a confocal microscope is simple: it must direct a laser beam to a point focus, scan the focus over the specimen and collect the light emitted only from the immediate vicinity of the focus. Putting this principle into practice and optimising the design is, however, very difficult and requires a thorough understanding of both optics and the applications of the technology. This was done in an innovative way by White, Amos and Fordham in the mid-1980s. Key steps were the use of a fully variable confocal aperture and a novel form of all-reflective scanning system. Other designs have been proposed and implemented for confocal systems but these have typically resulted in reduced sensitivity and compromises in image quality and/or flexibility.

The Radiance series, introduced in 1997, incorporates the best features of the original designs together with new features which improve the sensitivity, stability of operation, ease of use, and, polarization performance. A unique optical design and optimised manufacturing procedures eliminate the need for any user adjustment of the scan head. All adjustments necessary for perfect operation are accessible and controlled via an intuitive computer interface while all unnecessary or confusing adjustments have been eliminated.

## Characteristics of the ideal scanning system

The innovative nature of the basic optical design of the MRC/Bio-Rad confocal systems received international recognition in the form of a Rank Prize for Optoelectronics awarded to J. White and B. Amos in 1995, along with M. Minsky and G. Brakenhoff for earlier work on confocal systems. Bio-Rad and the MRC have also received Queen's Awards in the UK for both Technology and Export Achievement.

It is also recognised that the entire field of multiphoton microscopy has its origins in the work of Denk, Stickler and Webb at Cornell University in the USA. This innovation is embodied in patents held by the Cornell Research Foundation. Bio-Rad quickly recognised the importance of this work and was granted an exclusive licence to this technology in 1996.

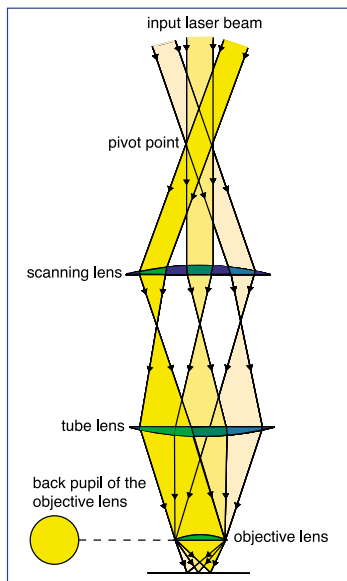
**Good Duty Cycle** – The proportion of time the scanning system spends actually scanning the sample should be maximised. This means that all non-useful scanning of the beam (dead time) should be minimised and is a prerequisite to optimum scan speed.

**Stationary Pivot Point** – The ideal scanning system directs the laser beam into a light microscope in such a way that the light is brought to a point focus in the specimen. The size of the focus should be as small as possible and this requires that the objective lens should be filled with light at all stages of the scan. This can only be achieved by rotating the beam about a so-called pivot point which is imaged in the back pupil of the objective

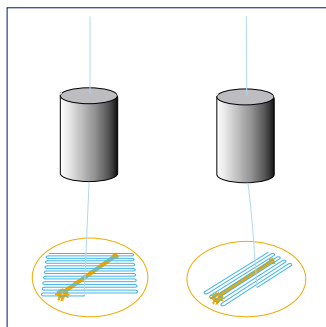
lens (see Fig 1). As the beam scans over the specimen, the part of the beam on the back pupil of the lens should remain constant in position and should undergo a 'pure' rotation as shown in the diagram. In practice this ideal of a truly stationary point during the scan is very difficult to achieve.

**Speed of Scan** – The point focus in the specimen is scanned raster-fashion like the spot on a TV screen. There is fast horizontal scan (line scan) and a slower vertical scan (frame scan). It is advantageous to increase the framing rate as much as possible since this allows a wide degree of flexibility in setting up scanning modes to suit the requirements of the application. This requirement indicates that the duty cycle of the scanning system should be as high as possible. That is the 'dead time' between scans should be minimised.

**Scan Rotation** – A third desirable feature of a scanning system is that the entire raster should be capable of rotation as



**Fig 1** An idealised scanning system: the input laser beam rotates around a pivot point and the beam remains stationary within the back pupil of the objective lens. The scanning mechanism is not shown.



**Fig 2** Raster rotation. If the scan system is able to change the orientation of the scan from that shown on the left to that on the right the image on the screen rotates and the elongated object shown can be scanned with better time resolution.

shown in Fig 2. The value of this is in getting the best possible image quality and time resolution when imaging elongated objects such as neurones or muscle fibres. With such specimens, rotation of the microscope stage is often impossible. They can be accommodated however by orienting the scan pattern such that the sample is along the fast direction of scan.

Scan rotation is also very convenient in purely morphological work, for fitting cells optimally into the image frame.

## Implementations of scanning systems

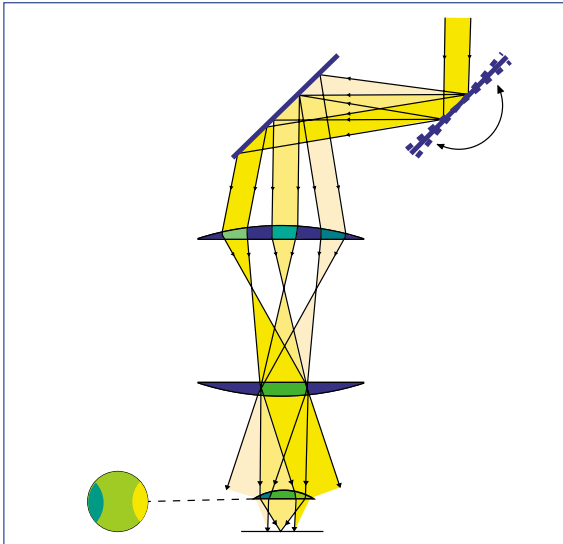
Having considered the ideal characteristics of a confocal scanning system, we can now look at how the engineering can, or could, work in practice. There are a number of ways in which some kind of scanning mechanism can be implemented. Each of these has specific characteristics and in most cases, they fall short of the ideals described above.

**Polygon mirror** – A rotating polygon is used in bar code scanners and many other optical devices. It can scan very fast, but has three serious drawbacks when used in microscope imaging. Firstly the angle between successive facets cannot be made accurately enough to guarantee registration of successive scans. Secondly the duty cycle is very poor. Finally, the reflected beam changes position as well as angle. This makes it very difficult to achieve the 'stationary point' described above as essential for ideal scanning. For this reason in particular, polygon mirror scanning is not currently implemented in any commercial confocal system. **Conclusion** – Very far from ideal characteristics in a number of important ways.

**Close-coupled galvos** – A simple form of raster scanner can be constructed by reflecting the laser beam off first one and then another mirror; each mirror being oscillated about an axis lying in the surface of the mirror. This kind of oscillation is usually brought about electromechanically by a moving-coil mechanism similar to that in a galvanometer, hence the term 'galvo' mirrors and this arrangement is termed 'close-coupled galvos'. One galvo oscillates quickly and produces the line scan and the other, slower mirror produces the frame scan perpendicular to it.

Close-coupled galvos are used in some confocal systems but have a serious defect; if the beam is stationary on the first mirror then it cannot be stationary on the surface of the next. The subsequent scanning in the microscope is incorrect, as shown in Fig 3. The beam moves out of the pupil of the objective at the extreme ends of the scan. The practical consequences are that the intensity of the image changes across the field and the resolution may even be reduced at the extremes of the scan. This seriously compromises the quality of the imaging performance of such a system.

In practical implementations of this scanning mechanism, an attempt is made to reduce these negative effects by



**Fig 3** Scanning system with two close-coupled galvo mirrors. The oscillation of the first mirror (blue dotted) produces beam movement on the second. Of the three successive beams shown, only the central one enters the back pupil of the objective lens correctly.

increasing the beam size until it overfills the lens pupil and by bringing the two galvo mirrors as close as possible on either side of the correct pivot point. These modifications though, have further drawbacks. Overfilling the lens pupil wastes laser light and for critical samples may make it impossible to achieve good imaging conditions. Furthermore, putting the mirrors as close together as possible still leaves them in a non-ideal configuration. The emitted beam coming back from the specimen therefore oscillates in position, necessitating the use of complex highly-corrected lenses to try to achieve a uniform intensity at the detector aperture. This invariably results in a poor compromise in performance. **Conclusion** – A non-ideal configuration which compromises performance and wastes laser excitation light.

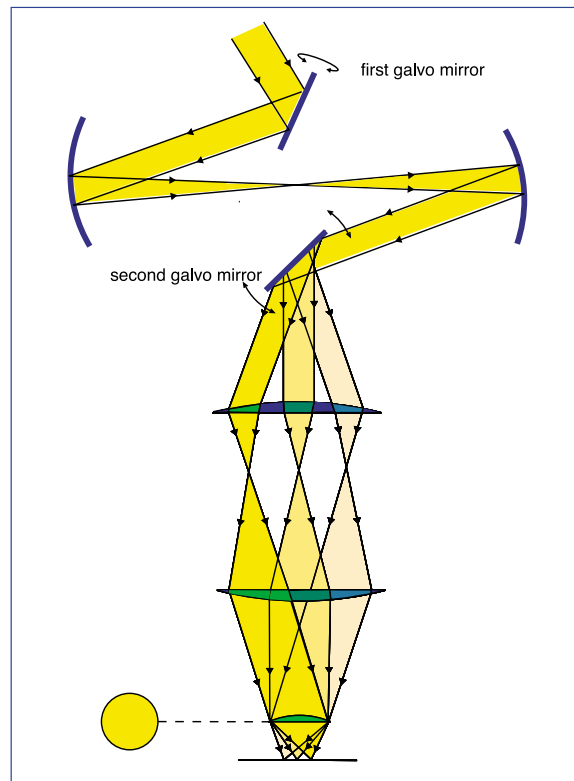
**Framing mirror on an arm** – This design has been produced in response to the problems created by the close-coupled design. The first mirror to be hit by the laser beam is mounted on an arm and oscillates slowly around an axis remote from the point of reflection. By this means, the position of the beam can be made almost constant on the second mirror, which is of the normal galvo type and produces the fast line scan.

Although this design almost cures the problem of the stationary point, it introduces another problem: the high inertia of the first (slow) mirror. This makes it impossible to rotate the raster to produce scan rotation as described above since the first mirror cannot be made to scan fast enough for a raster rotation. **Conclusion** – Almost overcomes one of the shortcomings of the close-coupled design but introduces another serious compromise.

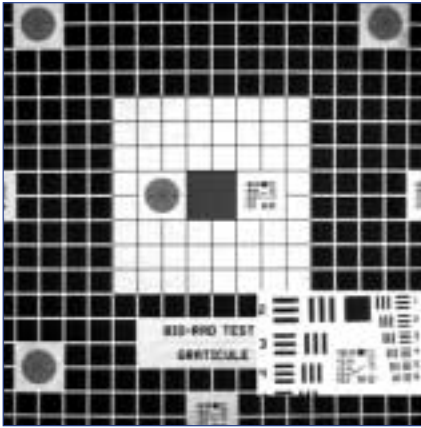
**Single mirror on a gimbal** – If a single galvo mirror is mounted on a gimbal mechanism it can be tilted simultaneously in two

directions and can produce the ideal rotational scan without any shift in beam position. The major disadvantage of this system, as with the previous one, is that there is a huge inertia in one of the axes of rotation since the entire galvo drive mechanism together with its mirror must be tilted. This means that flexibility in scanning modes is severely compromised; scan rotation for neurological or other similar studies is impossible. **Conclusion** – A good design from the point of view of optical configuration, but again compromises the flexibility of scanning modes.

**Bio-Rad confocal scanning system** – This solution is an original one in which the first galvo mirror is imaged on the second by means of two concave reflecting surfaces (Fig 4). The beam scans over the concave reflectors but is always directed to the same point on the second mirror; the optically ideal solution. The problem of shift of beam position in the close-coupled galvo arrangement is avoided, yet it is still possible to achieve fast framing, scan rotation and other modes of operation which depend on the fact that either mirror, or both, can be oscillated very rapidly. This design is patented (W.B.Amos, US Patent 4,997,242) and has been licensed exclusively to Bio-Rad for use in its confocal and multi-photon systems. **Conclusion** – A novel, patented design which corresponds to the ideal scanning system in all respects.



**Fig 4** The Bio-Rad scanning system. Ideal scanning geometry is produced by imaging one galvo mirror on another by means of concave reflectors. Since both scanning mirrors have low inertia, full flexibility of scanning is achieved, including scan rotation and fast framing.



**Fig 5** In order to perform this test, a fluorescent test specimen was inserted into the scanning eyepiece of the Radiance scan head. The confocal aperture was opened maximally to eliminate the improvement in resolution resulting from the confocal optics, since the objective was to test the intrinsic resolution of the scanner as a flying-spot device.

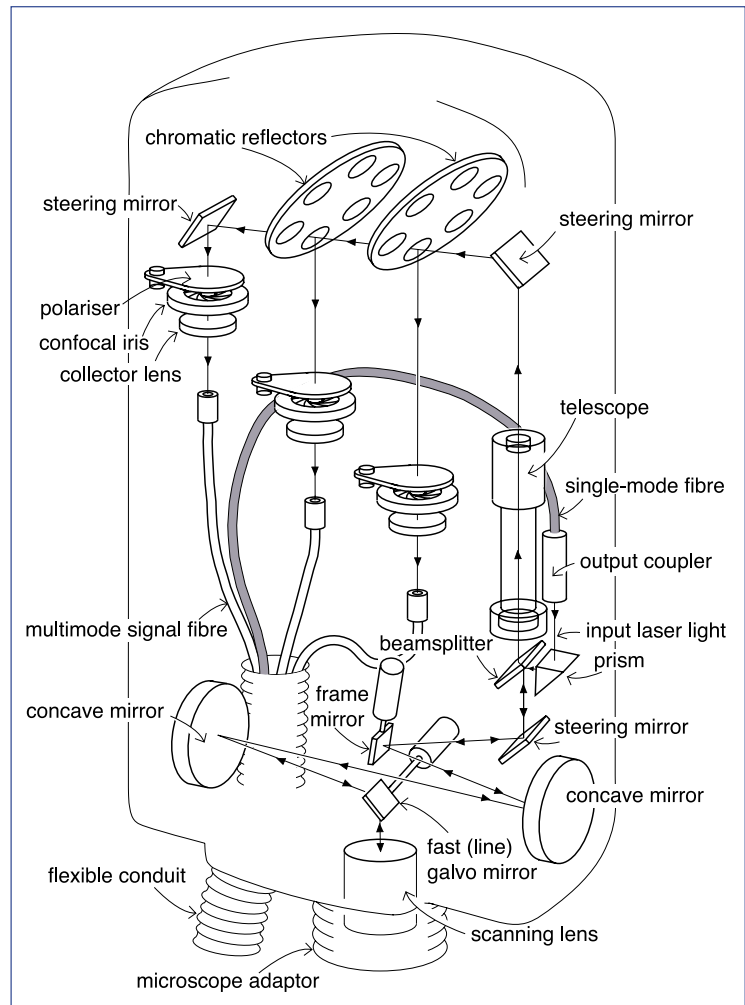
## Putting the Design into Practice – Image Quality

The overall layout of the Radiance series scanheads is shown in *Fig 5*. The design conforms closely to the ideal characteristics, is extraordinarily compact and is manufactured by a specially developed process which guarantees permanent alignment of all the key optical components.

The high quality of the Bio-Rad scanning system can be confirmed by the use of standard test patterns. The inset in *Fig 5* shows a small area of the entire scanned field. The area is chosen from the bottom part of the scanned raster where aberrations would be expected to be maximum, yet the scanner resolution is 2,400 x 2,400 pixels. This is greater than the resolution in the microscope image generated by any high-magnification objective.

### COMPARISON OF SCANNING SYSTEMS

	Polygon mirror	Close-coupled Galvos	Mirror on an arm	Gimbal	Bio-Rad Patented scanning system
Good Duty Cycle	x	✓	✓	✓	✓
Stationary Point Focus	x	x	✓	✓	✓
Fast Frame Scan	✓	✓	x	✓	✓
Scan Rotation	x	✓	x	x	✓



**Fig 6** Layout of Bio-Rad Radiance scan head.

## The Detector Aperture

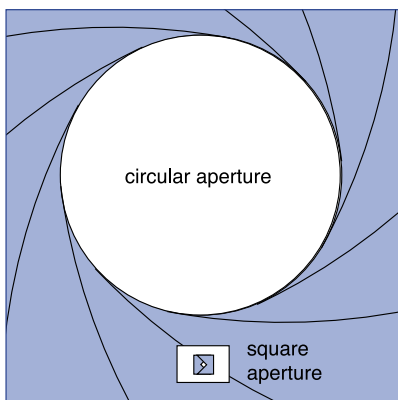
A formidable obstacle which blocked the early development of confocal laser scanning microscopes was the problem of making and using a sufficiently small detector aperture. For example, if the detector aperture were placed in the intermediate image plane of the microscope, which was how very early confocal systems were designed, the diameter of the aperture would have to be 12 micrometres to give acceptable confocal performance. This assumes very common operating conditions with a 60X, NA 1.4 objective lens. An aperture of this size would be impossibly difficult to engineer, align and keep clean. The White design solved this problem in a striking way by enlarging the microscope image a further 70 times before casting it on the detector aperture. The aperture could consequently be 0.8mm in diameter with equivalent optical effect. This novel approach opened up many possibilities which were exploited in the MRC series Bio-Rad systems and subsequently in the Radiance designs.

Firstly, the detector apertures consist of photographic-type irises which are engineered to be almost perfectly circular and can easily be opened and closed across a continuous range of diameters from 0.7 to 12.0 mm. In this way, the best compromise between confocal performance (small aperture) and high signal strength (large aperture) can be found without interruption of imaging.

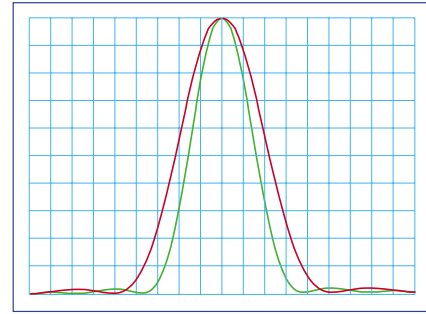
Secondly, the signal emitted beam can be divided by chromatic reflectors and each of the coloured beams is made to fall accurately on one of several detectors, each with its own independently variable aperture. In other designs this approach is either impossible, because of the unachievable engineering tolerances which would be needed, or is implemented in a way which both compromises the ideal aperture geometry and is highly susceptible to misalignment.

In all the Radiance series systems the 'White' design principle continues to be used (see Fig 6). The emission beam is split into fractions according to wavelength and the positioning of the beams on to three separate motorised irises is laser-jig-aligned and fixed during manufacture. In the Radiance designs the extra magnification of the beams is achieved using a five-element achromatic telescope. This has allowed the whole scan head to be miniaturised without compromise of optical resolution.

Competing designs have attempted to follow the principle applied by White in using some degree of extra magnification, but have been constrained to using very tiny confocal apertures (Fig 7). As a result of the very difficult engineering involved in manufacturing a very small aperture, a compromise is usually adopted by fabricating the aperture in the form of two partially-overlapping metal plates. The plates have V-shaped notches facing each other which are moved back and forth to give a variable square-shaped aperture. This introduces performance problems because the use of a square aperture is optically very poor. For example, bright punctate specimens generate square images when slightly out of focus with such an aperture.



**Fig 7** Continuously-variable circular aperture, which can be opened to a diameter of twelve millimetres, compared with a small, square piezo-driven aperture (shown approximately to a scale).



**Fig 8** Calculated intensity profile of the Airy disk for light of wavelength 500 nm (green) and 670 nm (red). The convention for setting the confocal iris size is that it should just include the central peak: the diameter should be 34% greater for red light than for green.

Furthermore, it is very difficult to align separate beams accurately onto different apertures if the apertures are very small and because of this, some designs resort to elaborate alignment and re-alignment mechanisms to try and overcome this day-to-day problem.

In some systems, the engineering is simplified by dispensing with continuously adjustable confocal apertures altogether and using a series of fixed apertures. Typically the number of choices is limited to 5 or 6. While this does ease the engineering design problems, it also imposes major limitations on the ability of the user to optimise performance.

In an alternative approach, one design side-steps this issue completely by acknowledging the impossibility of successfully engineering multiple tiny apertures and accepting the major compromise of having only one confocal aperture for all detector channels. In this case, the chromatic separation of the signal is postponed until after the single confocal aperture. This creates two problems. First, there is no possibility of opening the aperture wide for the dim fluorescence of one colour while retaining a confocally ideal size for another colour which is emitted more strongly by the specimen. Achieving the best image quality is therefore impossible. Secondly, since the ideal aperture size changes with wavelength (see Fig 8) there is no single ideal size for a single aperture. What is ideal for green light will be 34% too narrow for red and again this forces the user to accept poor imaging performance.

### Conclusions

- Large apertures give greater stability of performance and eliminate the need for constant re-alignment.
- A circular iris-type aperture is optically superior to square-shaped designs.
- Continuously-variable apertures allow true optimisation for the characteristics of the sample.
- One aperture per detector gives far more flexibility when imaging multi-labelled samples.
- One aperture per detector allows an optimum optical section to be collected for each colour.

## Enhanced Photomultiplier Tube Optics

It is a fact that the most serious losses of light emitted from the specimen in any confocal system occur in the photomultiplier tube (PMT). Thus, addressing and improving this issue is invariably the most rewarding as far as increasing the sensitivity of the system is concerned.

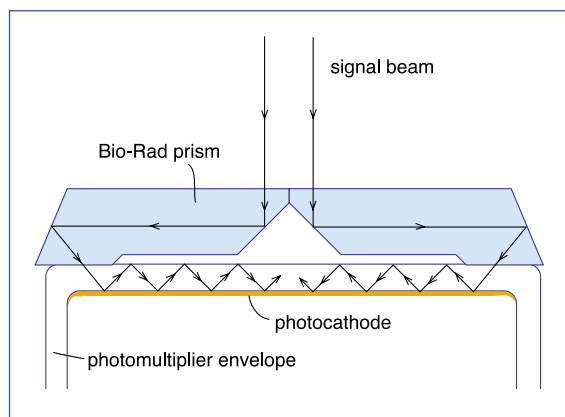
Bio-Rad has co-operated for a number of years with one of the world's leading PMT manufacturers to develop unique enhancement optics for the PMTs used in Bio-Rad confocal and multi-photon systems. The result is that particularly for dimly-fluorescing samples, the Bio-Rad systems exhibit unequalled sensitivity.

The Bio-Rad PMTs are equipped with specially-designed prisms to trap the light by total internal reflection within the PMT envelope, so the quantum efficiency increases by a factor of approximately two in the green, four in the red and even more in the infra-red. This prism system (*Fig 9*), coupled with the high efficiency large size PMT tubes used in all Bio-Rad systems means that excellent image quality is obtainable on the Radiance systems even with difficult, dimly-fluorescing samples.

## Multiphoton Optics

Two-photon fluorescence microscopy was invented by Denk, Strickler and Webb in Cornell University, using a slightly modified Bio-Rad MRC-500 confocal scan head for the original demonstration. Bio-Rad has an exclusive world-wide licence to develop and market multi-photon instrumentation and there has been an extensive collaboration with the group at Cornell to optimise the optical system. The Cornell researchers discovered that the signal strength in a multi-photon system could be increased by increasing the diameter of the detector aperture far wider than needed for confocal function, without significant loss of resolution. In fact, in practice in multi-photon imaging, the resolution is fixed by the illumination spot size and there is no need for an aperture in the detection optical path at all. Indeed, there is a great increase in signal strength if methods can be developed to bypass the confocal detector optics.

As a result of extensive experience in multi-photon, Bio-Rad has developed detector systems for multi-photon such that light can be efficiently detected even if it has undergone significant scattering in the specimen. There is abundant evidence that this increases the depth within the specimen at which imaging is possible and increases the available signal up to five-fold, which in turn allows lower laser powers to be used. This improvement in sensitivity and hence imaging depth is valuable in many applications areas including neuroscience, physiology and developmental biology.



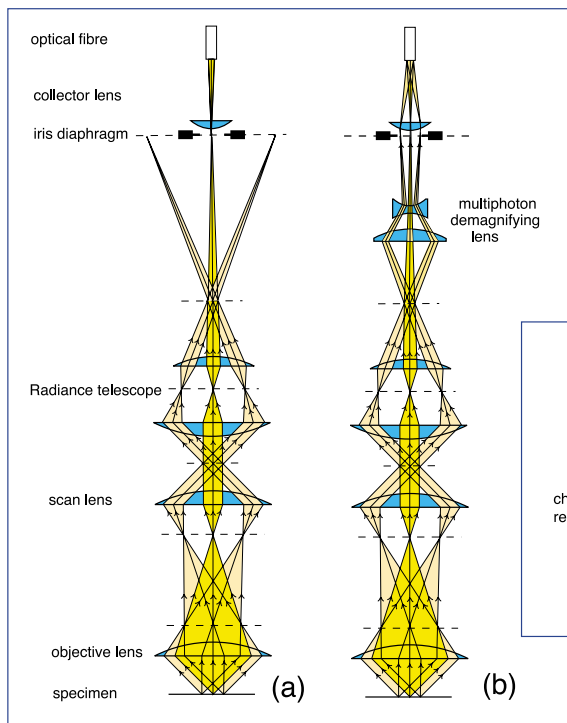
**Fig 9** Proprietary optical arrangement for enhancing the quantum efficiency of an end-window photomultiplier tube by repeated reflections at the photocathode.

To explain the optical arrangement of the detectors, *Figs 10a, b and c* show ray paths from a central point in the specimen, corresponding to the illuminated spot and from two points on either side which are illuminated by scattering and serve as secondary sources of signal.

**Multi-photon Demagnification Lens (MDL)** – The first Bio-Rad enhanced detection system (W.B.Amos, patent pending) consists of an optical assembly on a motorised mount, which can be placed between the telescope and the detector apertures in the Radiance 2000 (*Figs 10a and b*). The lens reduces the magnification of the image at the level of the detector aperture, while preserving the correct beam parameters for efficient focussing into the multi-mode optical collection fibre. The MDL can be inserted and removed under computer control without disturbing the alignment of the Radiance telescope, and hence rapid switching is possible between confocal imaging and multi-photon imaging with or without an aperture. The Radiance MDL is already being used successfully as a means of obtaining deep multi-photon sectioning, while retaining confocal operation for comparative purposes (see Higdon, Toruk and Wilson, 1999).

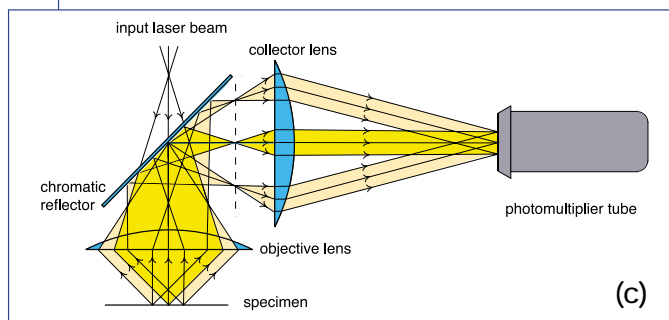
The second, more profound modification, giving the ultimate signal enhancement, is shown in *Fig 10c*. Here a chromatic reflector is inserted close to the objective of the microscope to pick off the emitted light and pass it all directly, via a collector lens, to the detector, without any additional optics. The detection is referred to in this case as 'external' or 'non-descanned'. The Bio-Rad external detectors are linked to the microscope by a carefully-designed optical arrangement which results in the direct focussing of the light emerging from the objective back aperture into the entrance pupil of the enhanced PMT. Additional relay stages and chromatic reflectors are used to provide detection at multiple wavelengths.

External detection is without question the best optical arrangement for multi-photon imaging. It gives approximately



**Fig 10 (a) & (b)** Normal Radiance confocal optics (a) compared with the use of the multiphoton demagnifying lens (b) which gives improved collection efficiency. Beams from a central (directly illuminated) point in the specimen are shown, together with beams from two points on either side of the centre. For simplicity, the tube lens is omitted.

**Fig 10 (c)** Optimised detection efficiency achieved by the use of an external detector. A chromatic reflector diverts all of the light emitted by the specimen which enters the objective lens into a collector lens, which images the back pupil of the objective on to the photomultiplier tube. Note that the light from all three points in the specimen reaches the detector. In reality the rays falling on the chromatic reflector are more nearly parallel than shown. Also, multiple PMTs can be incorporated by the addition of further chromatic reflectors and relay lenses.



30% greater depth of sectioning than the MDL approach and can be used even with short emission wavelengths which do not pass efficiently through the MDL pathway.

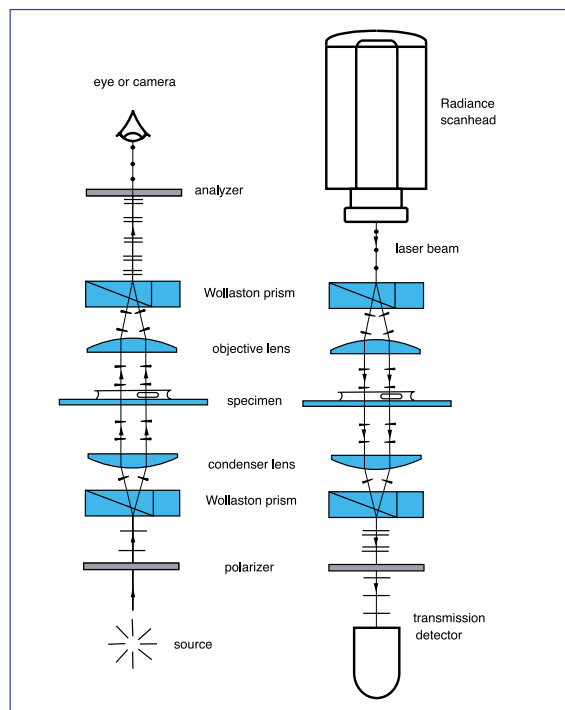
Both methods have the perhaps unexpected bonus that they make the imaging immune to chromatic aberrations introduced by the objective lens or the specimen. They are set to become the methods of choice for co-localisation studies where complete elimination of chromatic aberrations is vital.

## Optimised DIC Performance

By careful attention to the optical design, and particularly by arranging all the reflections to be in planes orthogonal to the polarization direction, the Radiance has been made to preserve the linear polarisation of the input laser beam. A key component in this is a high-performance polarisation-preserving single-mode optical fibre developed at the University of Southampton (UK) for conveying the light from the laser to the scan head. The polarisation direction is horizontal (in relation to Fig 11), resulting in a 30% s-plane reflection by the primary reflector, even though this plate transmits 80% of the (randomly-polarised) fluorescent emission. As a result of this careful attention to the design, the light emerging from the scan lens is highly plane-polarised.

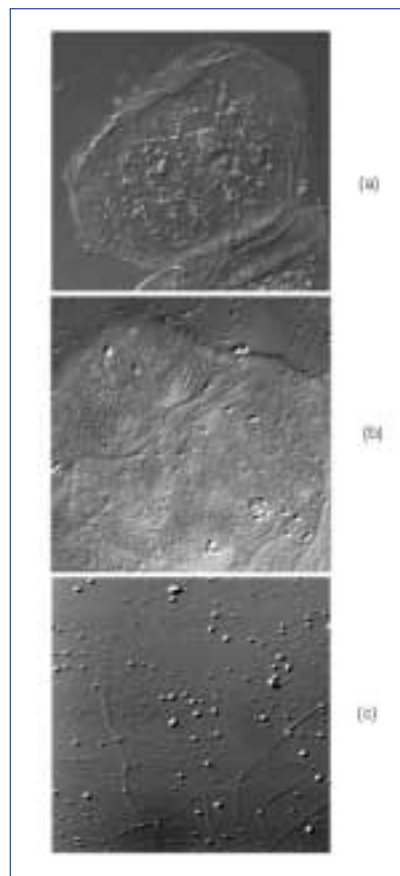
This feature of the Radiance optics permits simultaneous differential interference contrast imaging (DIC) and confocal or multi-photon fluorescence. Normal DIC, using a camera,

requires the insertion of an analyzer into the epifluorescence light path, with a consequent drastic reduction in sensitivity to fluorescence. With the Radiance there is no need for an analyzer, so fluorescence may be imaged at the highest sensitivity simultaneously with DIC.



**Fig 11** Comparison of conventional DIC imaging with an upright microscope (left) with scanned DIC with a Radiance scan head (right). Note that with the Radiance an analyzer is not necessary, so simultaneous epifluorescence and DIC transmission imaging are possible without compromising the efficiency of the epifluorescence signal.

For example, if blue excitation is being used to excite FITC, the blue laser light can be used to form the transmitted (non-confocal) image simultaneously with the fluorescence image. For prolonged imaging with minimal photodamage, red excitation at 638 nm can be used both to form the DIC image and to excite an IR-emitting fluorochrome such as Cy5 (Amersham). Figs 12a, b and c show examples of scanned DIC images. The buccal epithelium is the standard test for correct DIC performance, particularly the finger-print-like pattern on the cell surface. It is well known, however, that by careful adjustment of a conventional DIC microscope, followed by analogue and digital image enhancement techniques, objects such as single microtubules can be seen, even though they are only 25 nm in diameter (ten times smaller than the resolution of the light microscope) (Salmon, Walker & Pryor, 1989). A striking validation of the Radiance optics and associated electronic design is that a DIC image of this type, in which individual microtubules are visible can be produced quite easily (Fig 12c). In this case, no additional enhancement methods were used other than the standard zoom, gain and offset facilities present in all Radiance systems. Researchers who at present use enhanced video (e.g. for motility assays) may well prefer to use a Radiance rather than a video camera-based system, since the green HeNe laser is vastly preferable to the usual mercury arc in terms of stability, heat output, cost, longevity and safety. The simplest model of MicroRadiance would be sufficient for such work, but even with such a basic system it would be possible to combine this transmitted 'super-resolution' imaging with confocal fluorescence and so achieve interesting new experimental possibilities.



**Fig 12** This diagram illustrates the superiority of scanned DIC transmission imaging with the Radiance as compared with the use of a conventional camera. With the Radiance, no analyzer is present, with the result that the DIC image can be obtained simultaneously with the fluorescence image without the loss of more than half of the fluorescence signal by absorption in the analyzer.

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