Optical modifications enabling simultaneous confocal imaging with dyes excited by ultraviolet- and visible-wavelength light

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Summary

Optical modifications to a confocal scanning laser microscope are described which allow simultaneous fluorescence imaging of living specimens excited by ultraviolet (UV)- and visible-wavelength light. Modifications to a Bio-Rad MRC 600 Lasersharp confocal microscope include the introduction of UV-path-specific lenses and a specially designed UV transmitting eyepiece and tube lens. Upon UV excitation these modifications provide similar resolution and field flatness when compared with visible confocal microscopy. The UV-path-specific optics could be adjusted to correct for varying amounts of longitudinal chromatic aberration in commercially available objectives. Eyepiece and tube lenses were chromatically corrected for UV through visible wavelengths to minimize lateral chromatic error. With these modifications, UV-wavelength light may be used to excite ratioing dyes to quantify intracellular ion concentrations, or as an energy source to release caged compounds in a spatially restricted volume, while simultaneously imaging with dyes excited by visible-wavelength light.

Introduction

Confocal fluorescence microscopy is a powerful method for obtaining thin optical sections from living specimens at high resolution (Wilson & Sheppard, 1984, pp. 47–52; White & Amos, 1987: 1989; Inoué, 1990). It is an especially important technique given the growing number of fluorescent detection molecules sensitive to ion concentrations, intracellular second messengers and proteins (Tsiens, 1989; Lechleiter et al., 1991; Adams et al., 1991; Lechleiter & Clapham, 1992). Quantitative ratioing dyes used for the measurement of many ions (Ca²⁺, Mg²⁺, Na⁺, K⁺) are all excited in the ultraviolet (UV) and emit in the visible spectrum. Photoactivated second messengers (e.g. caged Inosphosphatidylcholine, cAMP, GTP) are uncaged by UV-wavelength excitation (Kaplan, 1990), and many common dyes for the identification of specific intracellular targets (e.g. DNA) are UV-sensitive.

Optical limitations have restricted confocal microscopy to visible-wavelength dyes and prevented simultaneous visible and UV excitation in most commercially available confocal microscopes. The majority of confocal microscopes are sold as attachments to conventional microscopes which were not designed to be achromatic from UV through visible wavelengths, and in fact, often have severe chromatic aberration at UV wavelengths (Ulthake et al., 1991). In order to achieve theoretical confocal resolution and optical sectioning, the confocal microscope must have diffraction-limited chromatic and monochromatic corrections (Wells et al., 1990). Therefore, any chromatic errors introduced by the conventional microscope or the confocal optics must be corrected elsewhere in the optical system. Descriptions of several confocal microscopes with UV light sources have appeared in the literature (Arndt-Jovin et al., 1990; Montag et al., 1991; Schubert, 1991; Ulthake et al., 1991), although the degree of lateral chromatic correction has not been described in detail (see Denk et al., 1990, for a two-photon visible system that can be used to replace UV excitation). This report describes a set of chromatic corrections applied to the Bio-Rad MRC Lasersharp confocal microscope that allow confocal imaging with excitation wavelengths from 350 to 700 nm. The design has been implemented and simultaneous UV- and visible-excited fluorescence has been achieved. Quantitative estimates of chromatic aberration are compared before and after the implementation of the optical modifications.

Effects of chromatic error on confocal imaging

A confocal scanning laser microscope achieves increased
radial and axial resolution by illuminating a specimen with a single diffraction-limited spot and collecting fluorescence from the same spot (Inoué, 1990). The image is built up by scanning the excitation spot across the specimen in a raster pattern. Optical sectioning is achieved by placing a pinhole filter at an image plane of the microscope which rejects out-of-focus light, but transmits the focused image. In the Bio-Rad Lasersharp, excitation light is scanned by mirrors at images of the objective's pupil plane, and the scan is relayed through the pupil of the objective by an arrangement of lenses. Fluorescence is collected along the same path and descanned through the scanning mechanism to allow pinhole filtering of a stationary beam (Fig. 1).

Longitudinal chromatic aberration of the excitation wavelength causes the excitation spot to be displaced axially from the fluorescence collection spot. Consequently, the image of the excitation spot is displaced from the pinhole, and fluorescence radiating from the excitation spot is rejected as out of focus. This chromatic error can be corrected with additional optical elements by refocusing the excitation spot to match the fluorescence collection spot (Wells et al., 1990). This method ensures that fluorescence images from simultaneous visible and UV excitation are collected from the same plane in the specimen.

UV epifluorescence objectives for confocal imaging must not only transmit UV light and be chromatically corrected for all visible fluorescence wavelengths, but must also be chromatically corrected for UV excitation wavelengths (351 nm, in this case). The available choice of UV-passing optical materials suitable for visible achromats is limited (Kingslake, 1978), making achromat design difficult at UV wavelengths. Most UV/fluorescence objectives are chromatically corrected only for visible wavelengths (Ulfhake et al., 1991). Large longitudinal chromatic aberration at 351 nm necessitated the correction of longitudinal chromatic errors for all the commercially available objectives tested.

In a confocal scanning laser microscope, lateral chromatic aberration at the excitation wavelength in both the relay lenses (in this case the eyepiece and tube lenses) and/or objective causes magnification error and the excitation spot is laterally displaced from the fluorescence collection spot at any field angle which is not zero (Wells et al., 1990). As the field angle increases, the displacement of excitation and collection spots also increases. Fluorescence from the displaced excitation spots may be descanned through the scanning mechanism, but the resulting beam will no longer be stationary and will be deflected across the pinhole during the course of a scan. As a result, the visible field of view is reduced since the fluorescence beam will only be focused on the pinhole at sufficiently small scan angles. Lateral chromatic aberration must be corrected optically after the beam is scanned. The Lasersharp's scanning mechanism consists entirely of mirrors (White, 1991) and thus does not add lateral error to the system. However, the relay optics and microscope objectives do add lateral error. An additional problem is that UV light is blocked by the Lasersharp's confocal attachment eyepiece, made of conventional glass elements.

Our goal was to build a confocal microscope that could adjust for varying amounts of chromatic aberration in commercially available objectives, achieve confocal resolution and visualize large fields. This was accomplished in
three steps: (1) all optics were replaced with UV-transmitting materials; (2) an adjustable telescope arrangement was added to the UV excitation path to correct for UV longitudinal aberration with different objective lenses; and (3) lateral chromatic aberration introduced by the relay lenses was minimized with a special lens design. No attempt was made to correct for lateral chromatic errors in the objectives, as the magnitudes of the errors were unknown and varied between objectives.

Methods

A Bio-Rad MRC 600 confocal microscope was adapted for use with an inverted Zeiss microscope (IM), using a telon lens (a negative lens that refocuses the image formed by the objective from 160 mm to infinity) supplied by Bio-Rad. The visible light source was an Ion Laser Technology 5000 argon laser rated at 25 mW in the 457–514.9-nm wavelength range. The UV source was a Coherent Innova 90 argon laser with UV optics supplying 300 mW total power at approximately 351 nm. Figure 1 shows the layout of the confocal system. Images were stored on a Nimbus VX (Intel 80386 processor) PC, and processed on a Silicon Graphics Personal IRIS system using ANALYZE (copyright Mayo Foundation Software).

All custom lenses were made with fused silica and calcium fluoride (CaF₂), transmitting light above 200 nm. Olympus D-APO 100×, Dapo 40× UV and PlanApo 10× UV objectives were chosen for these studies due to their ability to transmit UV light at 351 nm. Dichroic mirrors were ordered to specifications from Omega Optical (Brattleboro, Vt.). Calcium fluoride lenses were purchased from Janos Technology, Inc. (Townshend, Vt.). Fused silica lenses were custom ground and the eyepieces constructed by E & W Optical (Minneapolis, Minn.). The optics were designed for both inverted and upright microscopes with the aid of a ray tracing program (OPTEC, Scoipt Enterprises, San Jose, Calif.).

Confocal images were collected from a dish containing Fluo-3 (Molecular Probes, Eugene, Ore.), a Ca-sensitive dye in a Ca-containing saline solution, in order to compare field sizes using different optics. To demonstrate resolution across the field, 0.1-μm fluorescent beads were glued to a cover slip with polylysine and covered with saline solution. The size of the beads was smaller than the resolution limit of the optics; images of the beads were considered to represent the point spread functions (PSFs) of the optical system at the beads' positions (Agard et al., 1989). Confocal z-section images of the beads were collected at 0-2-μm steps using a 40× objective. Projected images were calculated through the resulting image volumes in ANALYZE software and displayed to show PSFs across the field of view with UV- and visible-wavelength excitation.

Results

Replacement of UV-attenuating optics

Eyepieces from Nikon, Zeiss and Olympus were all found to block substantial amounts of UV-wavelength light and could not be tested for UV-visible imaging. An achromatic eyepiece (Fig. 1f, inset) and relay lens (Fig. 1g) were designed and fabricated. Lenses 1, 3, 5, and 8, shown in the inset to Fig. 1, were made from fused silica, and lenses 2, 4, 6 and 7 were made from CaF₂. In addition, the telon lens of the Zeiss microscope was replaced with a quartz lens. Objectives were chosen from a variety of sources with the aim of passing as much UV light as possible.

The coating thickness across the MRC 600 concave mirrors (Fig. 1d) was too variable for use with UV light. These mirrors were replaced with Al-coated mirrors from the MRC 500, resulting in some attenuation of visible light (c. 15%), but no detectable variation in UV across the field. New coatings will be required to recover more light.

Longitudinal chromatic error correction

To correct for UV longitudinal chromatic aberration encountered in the common ray path, an additional lens was added to the UV excitation beam (Fig. 1, lens b). The UV excitation beam was thus refocused to match the visible-wavelength focal plane of the objective. For maximum resolution, a focusing beam expander (Fig. 1, optic a) was used in conjunction with lens b to ensure that the back aperture of the objective was filled. The beam expander focus was adjusted to correct for the various magnitudes of chromatic error from different objectives. UV and visible beams were combined at the dichroic mirror (Fig. 1c). This approach left the visible excitation focus unaffected, and allowed simultaneous UV and visible excitation. Details of alignment and experimental UV focus determination are given in the Appendix.

Effects of eyepiece lateral chromatic aberration on field size

Initially, a UV-transmitting fused silica eyepiece (eyepiece 1) was designed without consideration of chromatic correction. Although total longitudinal chromatic error in the optical system was corrected with the UV refocusing lenses, large lateral chromatic errors in the fused silica eyepiece reduced the field size dramatically. We explored the effect of eyepiece lateral chromatic errors on the confocal image by modelling the microscope and relay lens with equations for thin lenses. The model gave estimates for the required degree of chromatic correction in the eyepiece to achieve a full field of view at confocal apertures and demonstrated the effect of eyepiece and objective magnification on lateral error introduced by the eyepiece.
Fig. 2. Thin lens estimation of lateral scan errors. (a) Eyepiece chromatic error produces a lateral scan error that limits field size. Solid line, visible scan line; dashed line, aberrated UV scan line; L_e, eyepiece; L_1, 160-mm tube lens; L_{obj/obj}, 160-mm telon lens and objective combination; O_o, visible focal plane; R_o, maximum scan height; δP, chromatic pupil error; δY, lateral chromatic displacement error. Aberrated scan lines proceed from point A, and converge again at image point L_1. The point L_1 always precedes the focal plane O_o in the absence of objective chromatic error because the distance from L_1 to L_o is necessarily greater than R_o. If the eyepiece focal length is shortened, the distance from L_1 to L_{obj/obj} is reduced. Point L_1 moves closer to the focal plane O_o, and the effects of eyepiece lateral chromatic error are decreased. If the UV scan lines fall short of the visible pupil, the UV field will be smaller than the visible field. Tube lens and objective chromatic aberration can increase, decrease, cancel or reverse the sign of the lateral error. (b) Upright configuration. L_e eyepiece; L_{obj/obj}, objective.

Figure 2(a) demonstrates the effect of eyepiece lateral chromatic aberration (inverted microscope configuration), with an achromatic objective, telon lens and 160-mm tube lens. It was assumed that the confocal aperture was small enough for the resolution limit to be determined by the size of the confocal diffraction-limited PSF and not by the size of the pinhole (Brakenhoff et al., 1990). Longitudinal chromatic error in the eyepiece was assumed to be corrected with lens b of Fig. 1, focusing the UV beam to the visible focal plane (point O_o, Fig. 2a). A ray (scan line) was traced through the centre of a visible beam, deflected by the scan angle θ (solid line, Fig. 2a). A scan line traced through the centre of the UV beam was deflected by the same scan angle, but was distorted by the lateral chromatic error in the eyepiece (dashed lines). The lateral aberration caused the excitation spot to be laterally displaced from the fluorescence collection spot (δY, Fig. 2a). As θ increased from zero, the lateral displacement also increased, predicting a decrease in field intensity with increasing scan angle, and limiting field size as the lateral displacement surpassed the size of the fluorescence collection PSF.

This model was used to estimate the degree of chromatic correction necessary in the eyepiece to image the entire field of view when lateral chromatic errors in the tube, telon and objective lenses is negligible. The height of the scan across the eyepiece was set to a maximum value (R_o) to examine lateral chromatic errors at the edge of the field (Fig. 2a). The distance between the points where the visible and distorted UV scan lines cross the optical axis (δP, or chromatic pupil error; Fig. 2a) is related to δY by thin lens equations (Eq. 2, Table 1), and gives a convenient measure of eyepiece lateral chromatic error provided the design of the tube lens is sufficiently achromatic. Estimates of the required chromatic aberration correction in the eyepiece were calculated by assuming that the lateral error, δY, may not be larger than the full-width at half-maximum (FWHM) intensity (Born & Wolf, 1987) of the radial fluorescence PSF (δr), and calculating the corresponding maximum pupil error (δP_{max}, Table 1) permitted between excitation and fluorescence wavelengths.

δP_{max} increased with increasing objective or eyepiece power when the numerical aperture (NA) of the objective was kept constant (Table 1). When the objective power is increased, field size is decreased due to the shorter focal length of the lens. The absolute size of the lateral chromatic error is also reduced. With constant NA, the PSF remains the same absolute size and larger eyepiece chromatic errors can be tolerated. For a given eyepiece, this model predicts that the visible fraction of the UV-excited field will increase with increasing objective power when eyepiece chromatic errors are significantly larger than objective chromatic errors.

When eyepiece power is increased, the distance between the eyepiece and tube lens is reduced due to the shorter focal length of the eyepiece. Although δP may be the same for a given 6.5× and 10× eyepiece, the shorter distance between the eyepiece and tube lens for a 10× eyepiece results in better performance from the 10× eyepiece (Fig. 2a).

Effects of objective chromatic aberration on field size

Lateral chromatic aberration in an objective is dependent on the effective focal length and the position of the principal planes for each wavelength. The relationship of effective focal length and principal planes is determined by many factors, including type of glass, lens curvature and lens thickness, and is not predictable related to objective back focus errors, which could be easily measured. Objective designs are proprietary, thus making it difficult to use them to correct for lateral chromatic errors in other elements in the optical system. Objective lateral error can increase, decrease or cancel lateral error introduced by the rest of the
optical system. As a practical alternative to redesigning objectives, commercial objectives may be tested and chosen for minimal lateral error after the longitudinal corrections are made.

**Minimizing eyepiece chromatic error**

The scanning eyepiece and tube lens (Fig. 1f and 1g, respectively) were chromatically corrected for rays passing through conjugate points at the last scanning mirror (Fig. 1e) and the centre of the objective/tube lens combination (Fig. 1h) for 300-, 464-, 560- and 656-nm design wavelengths. Fused silica and CaF₂ were chosen as lens materials because they transmit UV light, are fairly hardy under normal laboratory conditions, do not cloud with prolonged UV light exposure (under 200 mW of power), and have sufficiently different dispersion characteristics to allow chromatic corrections with reasonably attainable lens surface curvatures. Other UV-transparent optical glasses may be substituted for the fused silica lenses but the dispersion characteristics of CaF₂ glass are unique. Calcium fluoride lenses are particularly useful for reducing secondary spectra at visible wavelengths (Smith, 1990).

The eyepiece and tube lens were corrected chromatically for visible wavelengths and were corrected monochromatically for visible and UV wavelengths. Monochromatic errors for UV wavelengths were checked at different image points at the small beam diameters typical of actual use of the eyepiece (< 2 mm). Since most of the chromatic error was introduced by the eyepiece, the effects of chromatic error in the tube and telon lenses will not be discussed in detail. To date, prototypes of the eyepiece lens have been made and tested only for the inverted microscope. A design for the upright microscope has not yet been tested.

Trade-offs between monochromatic aberrations, chromatic aberrations and other practical requirements are apparent in the eyepiece design process. Two-lens and four-lens fused silica eyepieces, and 6.5×, 6.25× and 8×, six-lens fused silica and calcium fluoride eyepieces were made. Tables 2 and 3 list the chromatic pupil error, ΔP (Fig. 2a), for four of the eyepieces in combination with a 160-mm lens design. Thin lens equations predict that, for maximum resolution, an 8× eyepiece will require c.20% less pupil error correction than that required by the 6.5× eyepiece (ΔP<sub>max</sub>, Table 1). However, chromatic error was more than halved by designing a longer focal length 6.5× eyepiece (compare eyepieces 2 and 3, Table 2). The 8× design required stronger surface curvatures to achieve the same chromatic correction as the 6.5× lens, increasing monochromatic corrections to unacceptably large values. Therefore, the chromatic correction was necessarily reduced for the 8× eyepiece. Eyepiece 3 had the smallest amount of chromatic aberration but was flawed by significant distortion and was redesigned (eyepiece 4). Again, with eyepiece 4, the chromatic correction was compromised to optimize monochromatic corrections. In both the 8× and 6.25× (eyepiece 4) designs, lenses 5 and 6 (Fig. 1) were reversed in order to enclose the humidity-sensitive CaF₂.

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**Table 1. Maximum pupil errors, ΔP<sub>max</sub> (mm), with various objective and eyepiece magnifications (Born & Wolf, 1987).**

<table>
<thead>
<tr>
<th>Objective magnification</th>
<th>NA</th>
<th>n</th>
<th>Δr at 488 mm (μm)</th>
<th>Field size (μm)</th>
<th>ΔP&lt;sub&gt;max&lt;/sub&gt; at eyepiece magnifications (250 mm/F₅) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10×</td>
</tr>
<tr>
<td>100×</td>
<td>1.3</td>
<td>1.516</td>
<td>0.225</td>
<td>200</td>
<td>2.30</td>
</tr>
<tr>
<td>60×</td>
<td>1.3</td>
<td>1.516</td>
<td>0.225</td>
<td>333</td>
<td>1.38</td>
</tr>
<tr>
<td>40×</td>
<td>1.3</td>
<td>1.516</td>
<td>0.225</td>
<td>500</td>
<td>0.92</td>
</tr>
<tr>
<td>20×</td>
<td>0.6</td>
<td>1</td>
<td>0.488</td>
<td>1000</td>
<td>1.00</td>
</tr>
<tr>
<td>20×</td>
<td>0.4</td>
<td>1</td>
<td>0.732</td>
<td>1000</td>
<td>1.50</td>
</tr>
<tr>
<td>10×</td>
<td>0.4</td>
<td>1</td>
<td>0.732</td>
<td>2000</td>
<td>0.75</td>
</tr>
</tbody>
</table>

At the edge of the field, the maximum tolerable lateral error was set to the size of the fluorescence collection PSF FWHM (Δr, Eq. 1). The corresponding maximum pupil error (ΔP<sub>max</sub>, Eq. 2) was used to compare eyepiece chromatic corrections:

\[
\Delta r = 0.6λ/n \sin \alpha_0,
\]

\[
\Delta P_{\text{max}} = \frac{M_0 F_l^2 \Delta r}{F_5 (R_5 + \Delta r)}
\]

where \(M_0\) = objective magnification, \(F_5\) = eyepiece focal length, \(F_l\) = tube lens focal length of 160 mm, \(\Delta P_{\text{max}}\) = maximum allowed pupil error, NA = numerical aperture of the objective, \(n\) = refractive index of the immersion medium, and \(R_5\), or maximum scan height above the axis at eyepiece = 10 mm (see Fig. 2). The field sizes listed are larger than many objectives achieve without vignetting. Eyepieces corrected to within \(\Delta P_{\text{max}}\) will have even smaller lateral errors at smaller field sizes.
lenses inside the eyepiece, making monochromatic and chromatic corrections slightly more difficult to achieve. Anti-reflection coatings can be used to reduce power loss.

Remaining chromatic errors in eyepiece 4 are, in general, larger than those stipulated in Table 1 ($\Delta P_{\text{max}}$), limiting theoretical high-resolution field size to varying degrees, depending on the objective used. However, with the

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Eyepiece 1 (10× fused silica)</th>
<th>Eyepiece 2 (8×CaF$_2$/silica)</th>
<th>Eyepiece 3 (6.5×CaF$_2$/silica)</th>
<th>Eyepiece 4 (6.25×CaF$_2$/silica)</th>
</tr>
</thead>
<tbody>
<tr>
<td>330</td>
<td>-29.77</td>
<td>-9.09</td>
<td>-3.28</td>
<td>-4.22</td>
</tr>
<tr>
<td>494</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>656</td>
<td>9.53</td>
<td>1.38</td>
<td>-0.76</td>
<td>-0.21</td>
</tr>
</tbody>
</table>

40× objective, the entire field was visible at partially confocal apertures (variable pinhole size 0.7 mm) using UV excitation. When eyepiece 4 was replaced with the less-corrected 8× silica/CaF$_2$ eyepiece, or with an uncorrected fused silica eyepiece, a large portion of the field was not visible.

**Table 2. Chromatic pupil errors, $\Delta P$ (mm), for inverted configuration. Each eyepiece design was combined with a 160-mm tube lens design in OPTEC and rays of different wavelengths were traced along scan lines (Fig. 2) through the optics. The difference in scan line focus (pupil error $\Delta P$; Fig. 2) was measured against the reference wavelength, 494 nm. $\Delta P$ was 29 mm at UV wavelengths with the uncorrected fused silica eyepiece, and was reduced to a few millimeters with the 6.5× aplanatic.**

**Table 3. Chromatic pupil errors, $\Delta P$ (mm), with eyepiece 4 and a 160-mm fused/silica/CaF$_2$ triplet.**

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>$\Delta P$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>656</td>
<td>-0.21</td>
</tr>
<tr>
<td>540</td>
<td>0.13</td>
</tr>
<tr>
<td>494</td>
<td>0</td>
</tr>
<tr>
<td>488</td>
<td>-0.05</td>
</tr>
<tr>
<td>450</td>
<td>-1.01</td>
</tr>
<tr>
<td>351</td>
<td>-3.19</td>
</tr>
<tr>
<td>330</td>
<td>-4.22</td>
</tr>
</tbody>
</table>

The effects of eyepiece and objective chromatic error on field size were reproduced by thick lens models (using a ray tracing program). The simulations support the conclusion from thin lens calculations that increased eyepiece chromatic error increases UV magnification errors. However, field curvature errors were also increased, predicting a more pronounced decrease in field size than expected from thin lens calculations (Fig. 3). Significantly, the 6.5× eyepiece reduced the magnification and field curvature errors almost to the magnitude of the objective error alone.

**Fig. 3. Field curvature and chromatic aberration for corrected and uncorrected eyepieces. Field curvature at the specimen plane was calculated by tracing rays of light at incremented scan angles through theoretical thick-lens models (OPTEC program). As the scan angle was incremented, the root-mean-square position of the objective focus was recorded in the axial and radial directions, and plotted above. To model the full optical train, a theoretical infinity-corrected, 10× objective (achromat in visible but with some residual chromatic aberration in UV) was designed from calcium fluoride and fused silica. A 160-mm lens with similar chromatic properties was designed. Rays were traced through the optimal system at three wavelengths: red (r), 656 nm; blue (b), 488 nm; and UV, 330 nm. Chromatic corrections in the calcium fluoride/fused silica eyepiece dramatically reduced lateral magnification errors, $\Delta y$, as well as field curvature errors, $\Delta z$, compared to errors introduced by the uncorrected fused silica eyepiece.
Observed lateral chromatic aberration effects

The predicted effects of minimizing eyepiece chromatic aberration are demonstrated with measurements from two prototype eyepieces (Fig. 4). With a fused silica eyepiece, the field intensity dropped below 50% of maximum at half the width of the field of view for UV or visible excitation (eyepiece 1, 40× Olympus objective; Fig. 4a). However, when the CaF₂/fused silica optics were used (eyepiece 3), more than 50% of the field intensity remained over the entire field of view (Fig. 4a, 0.7-mm pinhole). When using a 10× Zeiss eyepiece with visible excitation, the field intensity drop-off was less than 90% at this pinhole aperture (not shown).

When eyepiece chromatic aberrations were large (i.e. with the fused silica eyepiece, eyepiece 1), an increase in the fraction of visible field of view was observed with increasing objective power as was predicted by the thin lens model (Fig. 4b). However, this trend was not observed when the chromatically corrected eyepiece was used, indicating that objective lateral chromatic aberrations caused magnification errors that were similar or larger in size than the errors introduced by this eyepiece.

To demonstrate attainable field size with a 6.5× eyepiece (eyepiece 3) and 40× objective, a field of 100-nm fluorescent beads was imaged with UV and visible excitation (Fig. 5) using a 0.7-mm pinhole aperture. The beads act as point sources of fluorescence and help identify optical aberrations. Single beads are visible across the full field with UV or visible excitation. Some aberration is visible at the edge of the field for both cases with this objective/eyepiece combination.

Collection efficiency improvement with increased path length

Many of the test images were collected with the 40×

![Collection efficiency improvement with increased path length](image)

Fig. 4. Eyepiece aberration. Confocal sections (pinhole size 0.7 mm) were collected through the centre of a thick slab of fluorescein dye, excited with blue or UV light (40× Olympus objective). Curves were fitted to a profile of the fluorescent image, drawn through the centre of the field. The curves were shifted slightly to match their peaks (peak-shifting was caused by small mirror alignment differences). A full field of view was 768 pixels. (a) Fluorescent field brightness was compared with UV or visible excitation for two eyepieces, a chromatically corrected calcium fluoride/fused silica design (curves a.v. and a.u.v., eyepiece 3), and a chromatically aberrated fused silica design (curves b.v. and b.u.v., eyepiece 1). Image intensity across the field was dramatically improved with the chromatically corrected eyepiece. (b) Field intensity improved with increasing objective power when eyepiece lateral chromatic errors were much larger than objective lateral errors. The data in this plot were collected for the chromatically uncorrected fused silica eyepiece and the 100×, 40× and 10× Olympus objectives.

Fig. 5. (A) Full-field (274 μm) confocal images of 100-nm fluorescein-coated beads (0.7-mm pinhole). Visible excitation (488 nm) using a 40× Olympus, 1.3 NA, UV-D-Apo objective lens. (B) Full-field (274 μm) confocal image of 100-nm Hoechst beads excited with UV (350–360 nm) light (0.7-mm pinhole).
Olympus objective because of its high NA, large field of view and excellent UV transmission. Of those tested, (see Methods) this objective imaged the largest fraction of the field of view. At the smallest pinhole setting (0.5 mm), some reduction in collection efficiency was observed with the 40× Olympus objective using visible excitation and the 6.5× eyepiece. Improved resolution and increased collection efficiency was observed with UV excitation if the UV beam was focused a few tenths of a micrometre above the visible plane of focus. To explain this behaviour, the system was analysed for collection efficiency using techniques outlined by Shao et al. (1991).

In the Bio-Rad confocal microscope, the pinhole is placed sufficiently far from the confocal optics to appear to be at infinity, eliminating the need to position a pinhole at the image plane (White, 1991). Shao et al. (1991) demonstrated and characterized a decrease in light collection efficiency with this design if the path length from the eyepiece to the variable pinhole was too short. Estimates of the minimum path length to maintain a collection efficiency of 75% for different objectives were calculated (Table 4). For the 40× objective/6.5× eyepiece combination, the current path length (c. 1600 mm) was found to be too short for optimal light collection efficiency with a point detector. The decrease in collection efficiency is caused by an axial shift between the detection (fluorescence collection) PSF and the excitation PSF. The calculated shift, δ, was 0.878 μm (Eq. 6 in Shao et al., 1991). A normalized measure of the shift, η, was 5.29μ (Eq. 8 in Shao et al., 1991). From comparison with Fig. 2 (Shao et al., 1991), the minimum collection efficiency was near zero for a point detector. At large pinhole sizes, the collection function would be much larger than the excitation function (Brakenhoff et al., 1990), and provided δ is small compared to the size of the collection function, the collection efficiency will be near 100%. As the pinhole size is reduced, diffraction effects govern the response and the collection efficiency decreases. At a pinhole size of 0.7 mm, the radial (Δr) and axial (Δz) geometrical collection spot FWHMs are 0.421 and 0.357 μm, respectively (Eq. 2 in Brakenhoff et al., 1990), near the diffraction limits for the collection FWHM (Δr = 0.225 μm and Δz = 0.876 μm at a wavelength of 488 nm). At this intermediate pinhole size, both diffraction effects and pinhole diameter will determine resolution, optical sectioning and collection efficiency (Brakenhoff et al., 1990). For high-resolution, visible-excitation visible fluorescence (VEVF) imaging with maximal collection efficiency, a 100× objective would be recommended with this system (Table 4). Unfortunately, the 100× Olympus objective had a small high-resolution fraction of the field of view at UV wavelengths, presumably due to lateral chromatic aberration in the objective. For a 60× objective, the calculated collection efficiency was c. 50% for a point detector. With objectives of any magnification, the collection efficiency can be improved to 100% with UV excitation by refocusing the UV excitation spot to match the axial position of the visible collection spot. Since the Bio-Rad Lasersharp incorporates separate pinholes for filtering multiline fluorescence images, the pinhole that filters the UVEF image can be closed for highest resolution, while the pinhole that filters the VEF image can be opened for highest collection efficiency. However, a small axial registration error results between simultaneous VEF and UVEF images.

The visible-wavelength collection efficiency can be improved in several ways. First, visible excitation could be refocused to match the collection spot (C. J. R. Sheppard, personal communication) or visible fluorescence could be focused to the pinhole with a single lens. With both of these options, magnification of the image spot is not increased at the pinhole and the minimum photomultiplier diaphragm diameter of 0.5 mm may not be small enough to achieve the response. Table 4.

<table>
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<tr>
<th>Objective magnification (nFp/F)</th>
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<th>Eyepiece magnification (250 mm/Fp)</th>
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theoretical limit of optical sectioning for some objectives. Alternatively, the path length after the eyepiece may be increased with additional mirrors, or a two-lens optic may be introduced into the fluorescence path. The two-lens optic could be used to magnify and focus the image of the fluorescence spot at the pinhole. Both of these options magnify the image sufficiently, but fluorescence intensity is attenuated by the extra elements. These options are currently being pursued.

Implementation
Experiments were performed on various biological specimens to test simultaneous use of dyes excited by UV- and visible-wavelength light. Human embryonic kidney (HEK) cells were stained with a membrane dye, Rh 414, and Hoechst chromatin dye. The chromatin dye is excited by UV and fluoresces in blue, while the membrane dye is excited by green and fluoresces at red wavelengths. Simultaneous, separate registered images of the cell membranes and the cell nuclei (Fig. 6A) were obtained by separating the blue and red fluorescent wavelengths and collecting them via separate photomultiplier tubes. To test the simultaneous use of a UV-excited caged compound and a visible-excited dye, caged IP₃ and Fluo-3 were injected into a *Xenopus laevis* oocyte. The UV laser shutter was opened for a fraction of a second to expose a banded region of the oocyte to UV light, releasing caged InsP₃ in the localized region (Fig. 6B). To demonstrate use of a UV-excited ratioing dye, a field of HEK cells was loaded with Indo-1 AM and the fluorescence wavelengths were separated and collected at 495 nm (Fig. 7A) and 405 nm (Fig. 7B). The ratio of the two wavelengths was calculated and normalized (Fig. 7C). A filtered ratio was also calculated (Fig. 7D), representing resting levels of calcium in the cell.

Discussion
Confocal microscopy using simultaneous UV and visible excitation has been achieved and adapted to commercially available objectives. The design corrects longitudinal chromatic aberrations in the confocal optics and the objective as well as most of the lateral chromatic aberration introduced by the confocal optics. However, improved objective designs will be necessary to eliminate lateral chromatic errors and achieve full field of view, diffraction-limited, confocal imaging at UV excitation wavelengths. Alternatively, objective lens lateral chromatic errors can be compensated by overcorrections elsewhere in the optical path (if the errors are known). We are currently

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**Fig. 6.** (A) Simultaneous confocal images of doubly stained A293 (human embryonic kidney) cells. The red and blue images (false colour) are Rh 414 (membrane-staining rhodamine) and Hoechst (chromatin; nuclei c. 10 μm) dyes. (B) Confocal caged release of InsP₃. A *Xenopus* oocyte was injected with Fluo-3 (c. 1 μm) and caged InsP₃ (c. 170 μm). Just prior to confocal imaging, the oocyte was also injected with the non-hydrolysable analogue InsP₃S₃. Initial images exhibited regenerative Ca²⁺ activity (Lechleiter & Clapham, 1992). Caged InsP₃ was then released by UV excitation in a confocal strip across the oocyte (left image, purple region). The subsequent confocal scan revealed spatially restricted Ca²⁺ release (green fluorescence). These images were collected with a 10× Olympus, 0.4 NA, UV Planapo objective. Field width = 764 μm.
Fig. 7. Resin casting Ca levels in human embryonic kidney cells loaded with Indo-1AM. This image was taken with a 40x, 1.3 NA Olympus objective. Fluorescence was separated and collected at 495 nm (a) and 405 nm (b), the ratio calculated (c) and the ratio image filtered (d). Intensity pseudo-colour is demonstrated in the 8-bit intensity bar. Field width = 7.5 mm.

Testing commercially available objectives for minimal lateral chromatic aberrations. A theoretical analysis of diffraction effects on confocal resolution and optical sectioning in the presence of longitudinal and lateral chromatic aberration would be useful for precise determination of the design criteria for an achromatic confocal system. In practice, lens designers generate modulation transfer function (MTF) plots (Kingslake, 1978) to determine if a system is diffraction limited. This technique does not take into account the superposition of the fluorescence and excitation spots, and resulting increase in resolution in confocal microscopy (Sheppard & Choudhury, 1977; Wilson & Sheppard, 1984, pp. 47–52; Brakenhoff et al., 1990).

Any refractive or diffractive elements (i.e., lenses, acousto-optic modulators, prisms) in a confocal microscope design will introduce chromatic errors that must be compensated. Off-the-shelf optics are normally only corrected in visible light for chromatic error, and many use flint glasses that transmit UV light poorly (especially at focal lengths shorter than ±50 mm). However, lens manufacturers will sometimes supply designs of their most common achromatic optics and the chromatic errors in UV can be determined by ray tracing programs.

UV/visible achromats present a difficult design problem because of the limited variety of optical materials available for transmitting UV light (Kingslake, 1978). To avoid strong lens surface curvatures and therefore large monochromatic aberrations, achromats must be made of at least two glasses with very different relative dispersion characteristics (Hecht & Zajac, 1979, pp. 175–194). Unfortunately, UV-transmitting materials have similar dispersion characteristics with the exception of a few crystals (i.e., CaF₂, LiF). Development of new materials would be helpful. Recent developments in diffractive lens production may simplify UV/visible achromat designs; chromatic aberration in diffractive elements is opposite to aberration in refractive elements and the two may be combined into superior achromat designs (Veldkamp & McHugh, 1992).

Imaging mirrors and flat mirrors are achromatic for all wavelengths, but have significant light losses on reflection. High-reflectivity mirror coatings for broad band from UV to red are still being developed and must be custom ordered. If these coatings are successfully developed, more mirror elements could be incorporated into UV/visible confocal microscopes, simplifying the design problems. However, mirror systems must: often be made much larger than lens systems to decrease monochromatic aberrations and field curvature to acceptable levels.

Once the chromatic errors are reduced sufficiently in a confocal design, unique experiments can be performed with simultaneous UV/visible microscopy. For example, we have examined the roles of Ca and InsP₃ in Ca release by releasing caged InsP₃ or caged Ca in localized areas, while monitoring Ca levels with Flu-3 (Lechleiter & Clapham, 1992). Quantitative, spatial and temporal information can be obtained about Ca and other ions in cells by using the UV-excited ratiing dyes. With an additional PMT and dichroic mirror in the collection path, structural information from visible membrane or mitochondrial-staining dyes could be collected simultaneously with ratiing dyes. UV-excited fluorescent dyes could be used with structural or ion-sensitive dyes to track DNA or RNA movement during cellular events. Higher resolution is theoretically possible using UV excitation and blue fluorescence, and would be particularly helpful in examining DNA-marked chromosomes (Arndt-Jovin et al., 1990; Montag et al., 1991).

In summary, we have designed a confocal microscope capable of simultaneous imaging of UV and visible fluorescence. Besides expanding the biological range of experiments, the shorter wavelength UV excitation should result in a further improvement in radial and axial resolution. Although the current resolution and field of
view are acceptable for most applications, further modifications are needed to improve collection efficiency and resolution. Improved eyepiece designs, including a reflecting eyepiece/scan-head combination are currently being evaluated.

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References


Appendix

Experimental alignment and objective correction procedure

The visible eyepiece was replaced with the UV/visible eyepiece. The Zeiss IM microscope turret was detached to remove the telon lens and objective from the system. A stationary 488-nm laser beam was projected through the optics using the 'park' feature on the Bio-Rad microscope. The distance from lens f to lens g (Fig. 1) was adjusted by moving the microscope until the beam emerging from lens g was collimated, ensuring correct visible focusing in the objective. The turret was replaced, and the normal visible alignment procedure was followed.

The UV beam expander mounts (Fig. 1a) were adjusted so that axial movements of the lenses did not deflect the beam. Two beam steering mirrors were adjusted after the UV laser to direct the UV beam to the centre of the UV/visible dichroic (Fig. 1c). A small visible back reflection and a small amount of UV transmission formed two spots to the right of the dichroic that were centred during this part of the alignment. Lens b was placed in the UV beam and adjusted so as not to deflect the beam. Scanning was begun
and the position of the visible scanned beam was observed in front of lens g. One of the beam steering mirrors before lens b, and the dichroic c, were adjusted until the UV scan matched the visible scan in front of lens g, and then for brightest fluorescence. The first sample used was a piece of paper immersed in saline and fluorescent dye, giving a strong fluorescent signal and some detail. While scanning the sample, the UV beam expander, a, was adjusted for the brightest, most detailed image. The second sample consisted of a field of 0.1-μm UV- and blue-excited fluorescent beads, attached to a cover slip by polylysine and immersed in saline. Visible excitation light was focused on the specimen at the smallest pinhole setting and the stage was locked at the best axial focus position. Visible excitation was replaced with UV excitation, and the pinhole was again opened. The beam expander was adjusted and the pinhole was closed until the best UV focus was determined. The micrometer reading for the beam expander stage was then noted. Dichroic c was adjusted to maximize the observed field.