Practical Limits of Resolution in Confocal and Non-Linear Microscopy

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ABSTRACT Calculated and measured resolution figures are presented for confocal microscopes with different pinhole sizes and for nonlinear (2-photon and second harmonic) microscopes. A modest degree of super-resolution is predicted for a confocal microscope but in practice this is not achievable and confocal fluorescence gives little resolution improvement over widefield. However, practical non-linear microscopes do approach their theoretical resolution and therefore show no resolution disadvantage relative to confocal microscopes in spite of the longer excitation wavelength. *Microsc. Res. Tech. 63:18–22, 2004.*

INTRODUCTION

From the beginnings of confocal microscopy, it was clear that the technique offered the potential for improved resolution (McCutchen, 1967), and, in fact some early reports regarded this as potentially more significant than optical sectioning (Brakenhoff et al., 1979; Sheppard and Choudhury, 1977). Simplistically, this offers an improvement of a factor of $\sqrt{2}$ so that on a Rayleigh basis, the resolution (minimum resolved distance, r) would be given approximately by $r = 0.61\lambda/\sqrt{2}NA$. Actually, the improvement for Rayleigh two-point resolution is not so large, a factor of 1.08 for confocal reflection (Sheppard and Choudhury, 1977) and 1.31 for confocal fluorescence (Cox et al., 1982). Full width half maximum (FWHM), which is often easier to measure in confocal microscopy, would be given in the incoherent (fluorescence) case by $FWHM = 0.5\lambda/\sqrt{2}NA$. Many biologists assume that the confocal microscope is automatically giving them higher resolution than they would obtain in a wide-field microscope. However, they fail to realize that these figures are based on the ideal concept of an infinitely small pinhole, and will obviously not be realized in practical imaging, particularly in fluorescence where light intensity is a limiting factor.

Non-linear microscopy—multiphoton fluorescence and second harmonic generation—would appear prima facie to offer much worse resolution at a given image wavelength since they excite the signal at twice the wavelength used in the comparable confocal mode. Three factors modify this pessimistic premise. First, for multiphoton fluorescence, since only the excitation wavelength is important, the Stokes shift inherent in all fluorescence does not reduce resolution. Second, the excitation wavelength is often rather shorter than twice the wavelength that would be used in confocal microscopy. Most microscopes are able to detect signals from 400 nm onwards, so that the excitation can be as short as 800 nm in SHG and 700 nm in TPF. Since two-photon excitation spectra are often blue shifted (Xu and Webb, 1996), and ultraviolet excitation in confocal suffers from optical aberrations limiting the resolution, the excitation wavelengths used are almost always shorter than twice those used in confocal. Third, resolution in x, y, and z is determined solely by the excitation process, so no pinhole is involved.

The consequence of these factors is that practically achievable resolution in non-linear microscopy is very little, if at all, worse than in confocal microscopy, and this report sets out to investigate this in both theory and practice.

MATERIALS AND METHODS

Samples used were 210-nm fluorescent ("Fluoresbrite") beads mounted beneath coverslips (Bio-Rad Microscience Ltd, Hemel Hempstead, UK), for fluorescence, and collagen fibers in histological paraffin sections of skin and kangaroo-tail tendon for second harmonic imaging (Cox et al., 2003; 2003).

Second harmonic microscopy was carried out using a Leica DMIRBE inverted microscope, fitted with a Leica TCS-SP2 spectrometric confocal head. The laser is a Coherent Mira titanium sapphire system, tunable between 700 and 1,000 nm, operating in the femtosecond regime, and pumped by a 5W Verdi solid-state laser. Wide-field non-descanned detectors are fitted in both epi and dia positions (Cox et al., 2002) and for second harmonic detection a 415/10 narrow band filter was used with 830-nm excitation.

Confocal microscopy was carried out on the same microscope using an argon-ion laser at 488 nm, and on a Bio-Rad Radiance 2000 fitted to a Nikon E800 upright microscope, also using 488 nm. Multiphoton microscopy was carried out on a Bio-Rad MRC 1024 with a Coherent Mira titanium sapphire laser, using both confocal and non-descanned (epi) detectors.

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RESULTS AND DISCUSSSION

Confocal Microscopy

Figure 1 shows a plot of expected resolution (as FWHM) against pinhole size for confocal fluorescence, assuming a Stokes shift of 1.2 (e.g., excitation at 500 nm and detection at 600 nm) and a 0.5NA objective. The FWHM becomes approximately equal to the excitation wavelength (FWHM/λ = 1) once the pinhole reaches one Airy unit and remains the same at 2 Airy units; that is, for pinhole sizes greater than 1 Airy unit, the resolution is essentially equivalent to wide-field microscopy (except that in wide-field microscopy it is the emission wavelength that determines resolution, whereas in non-confocal scanned microscopy it is the excitation wavelength, and in confocal microscopy it is both).

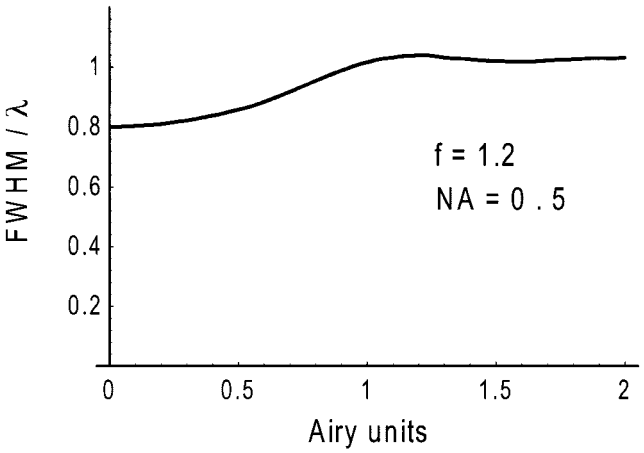

Fig. 1. FWHM values for different pinhole sizes assuming a Stokes shift (f) of 1.2 and N 0.5, based on formulae of Gu and Sheppard (1991, 1992).

Figure 2 shows intensity plots of sub-resolution (210 nm) beads using a lens of NA 0.5 on a BioRad Radiance confocal microscope. The same pair of beads is plotted at each pinhole setting. FWHM measurements for these curves are given in Table 1.

These values are substantially worse than expected from Figure 1, which predicts ~400 nm at a pinhole size of 0.5 Airy (this corresponds to a diameter of 0.7 mm and is the smallest size selectable on this microscope). Assuming a mean detection wavelength of 580 nm (Stokes shift 1.2), the wide-field FWHM should also be ~580 nm, so even with the smallest pinhole size the confocal microscope barely exceeds wide-field resolution. Furthermore, opening the pinhole wider than 1 Airy unit, which should have no further effect, actually worsens the resolution quite substantially.

These effects are almost certainly a consequence of the incoming laser beam not being expanded to provide uniform illumination at the back focal plane (BFP) of the objective lens, so the full NA is not being used in the excitation pathway. On the Radiance, the beam expansion is fixed and not under user control, but the

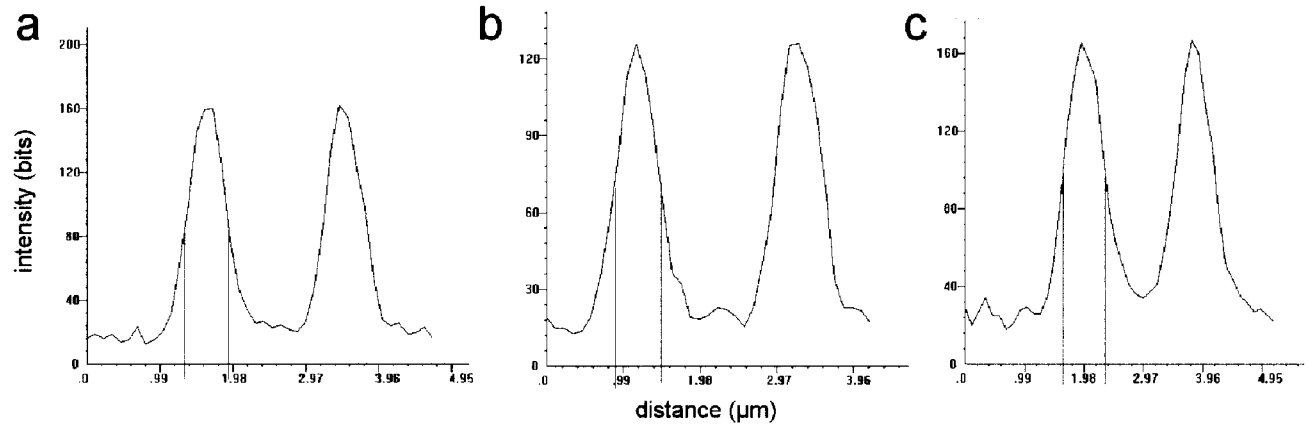

Fig. 2. Intensity profiles of 210-nm subresolution beads, taken with a ×20 NA 0.5 lens (**a**) confocal pinhole set at half the Airy disk diameter (0.7 mm), (**b**) confocal pinhole set at the Airy disk diameter (1.4 mm), and (**c**) confocal pinhole set at twice the Airy disk diameter (2.8 mm). The vertical lines indicate the FWHM (values are in Table 1).

TABLE 1. FWHM measurements from the Radiance 2000/Nikon confocal microscope at three different pinhole openings, and predicted values from Figure 1 (488 nm excitation, 500 LP detection)

	Pinhole (Airy)		
	0.5	1	2
FWHM (nm)	560	620	715
Expected FWHM	400	488	488

TABLE 2. Measured FWHM at 4 pinhole sizes on the Leica TCS SP2 using the beam expander[a]

	Pinhole (Airy)			
	0.25	0.5	1	2
FWHM (nm)				
Expander 6	417	439	620	786
Expander 3				1000

[a]The numbers 6 and 3 refer to the actual magnification of the laser beam at the objective compared to the size with no expansion lens in the beam path.

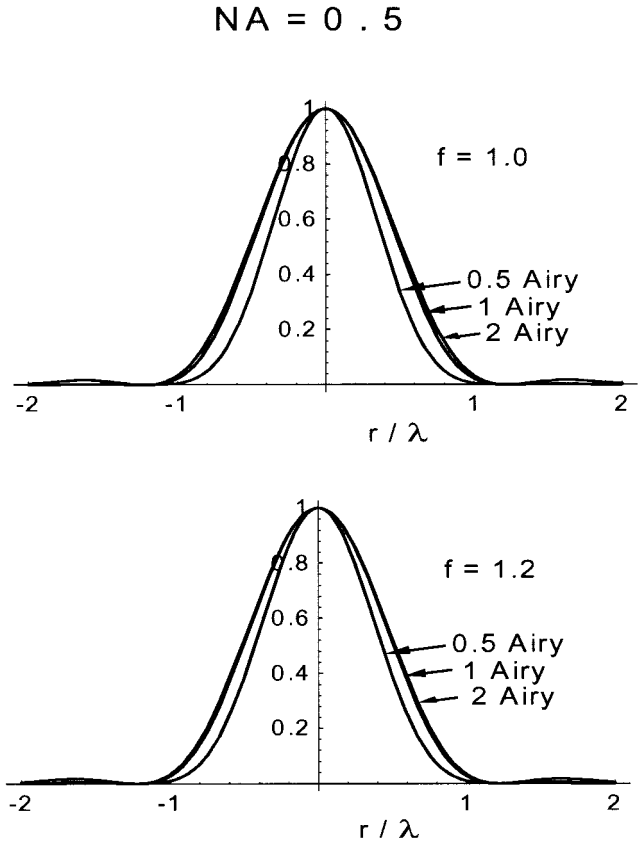

Fig. 3. Response curves, expressed as minimum resolved distance/λ, for confocal microscopes (NA 0.5) with different pinhole sizes at two different values of Stokes' shift (f). It is clear that in principle a moderate resolution improvement should be obtained with a pinhole size of 0.5 Airy in both cases. This is much more significant than the Stokes' shift.

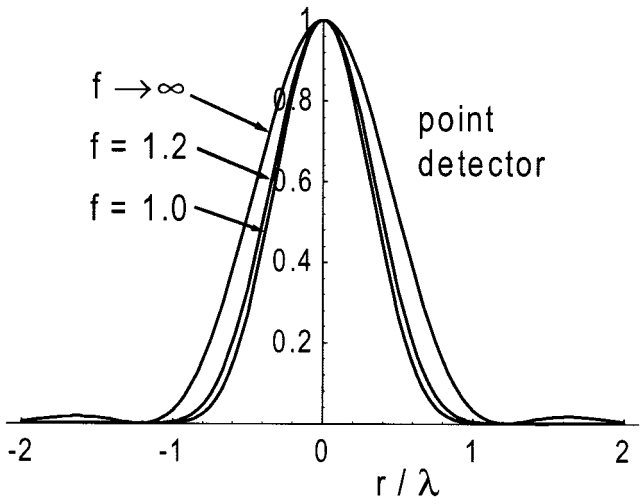

Fig. 4. Response curves, expressed as minimum resolved distance/λ, for a perfect confocal microscope (point detector) at three different values of Stokes' shift (parameter *f*). Eventually, with an infinite Stokes' shift, resolution reverts to the wide-field value, and the smaller the shift the better the resolution will be. NA = 0.5.

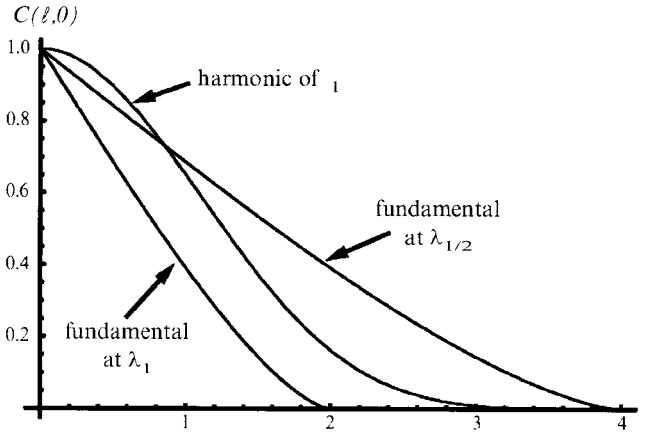

Fig. 5. Cross-section through the two-dimensional weak object transfer function *C (l; 0)* of the Type 1 scanning harmonic microscope.

TABLE 3. Full width half maximum expressed in units of wavelength for a multiphoton microscope and for confocal microscopes of different pinhole sizes and Stokes' shift

	FWHM/λ
Conventional	1.03
Confocal, $f = 1.0$	0.74
Confocal, $f = 1.2$	0.8
1 Airy, $f = 1.0$	1.0
1 Airy, $f = 1.2$	1.02
Multiphoton (at 1P λ)	1.48

TABLE 4. Two different resolution criteria where η is the refractive index and θ the half-angle of acceptance (η · sin θ is the NA), calculated for NA 1.4 and wavelengths λ of 800 nm and 830 nm

		800 nm	830 nm
2-point	$r = \frac{0.61\lambda}{1.31\,\eta \cdot \sin\theta} =$	266 nm	276 nm
FWHM	$f = \frac{0.5\lambda}{\sqrt{2} \cdot \eta \cdot \sin\theta} =$	202 nm	210 nm

Leica has a variable beam expander, and these tests were therefore repeated on it with results shown in Table 2.

Since the Leica can select a smaller pinhole size than the Bio-Rad, the ultimate resolution is better with this objective, but at 1 Airy pinhole size (the value normally used in confocal fluorescence microscopy and selected by default on most systems) both are equivalent. At small pinhole sizes, the signal/noise ratio was poor and the values given should not be taken as precise. Neither microscope attains the value of 390 nm predicted by Figure 1.

These experiments show that some current confocal microscopes do not expand the beam sufficiently to provide effectively uniform illumination of the objective BFP, and this is a limiting factor on their ultimate resolution. More significant, though, is the consequence that opening the pinhole will *not*, as commonly expected, give wide-field resolution, but will give very substantially worse resolution than a wide-field microscope. Therefore, opening the pinhole wider to give a brighter image of dim objects (Reichelt and Amos,

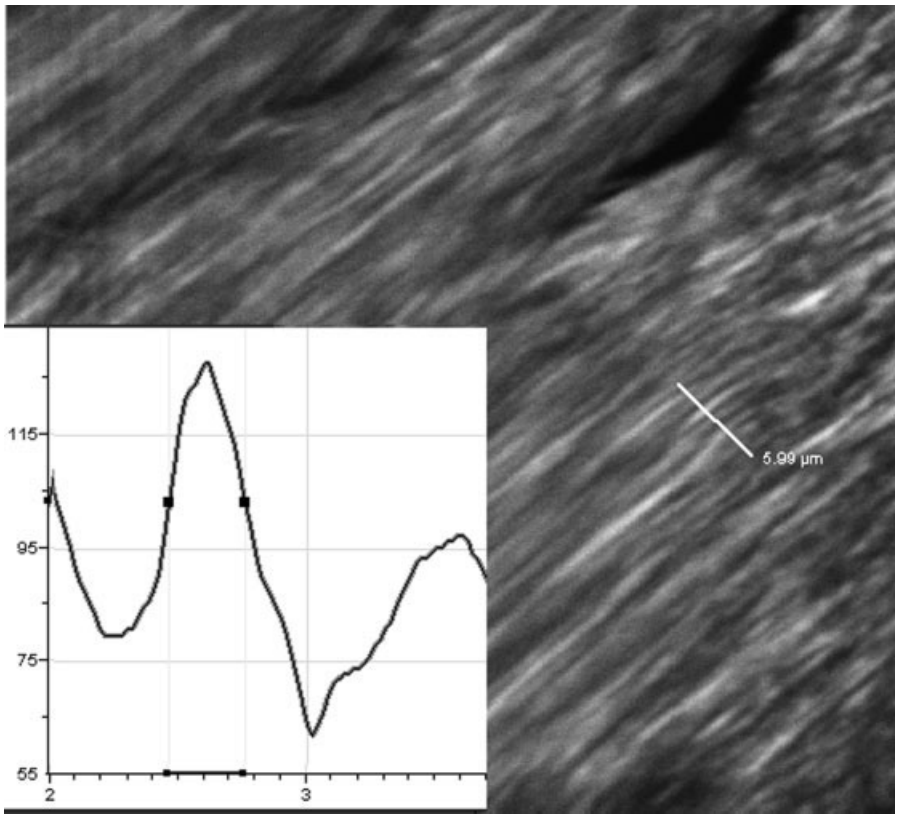

Fig. 6. Kangaroo tail tendon, excitation 800 nm, ×40 oil, NA 1.25: Second harmonic image of collagen. **Inset:** Part of the profile along the measurement line, 6 μm long, showing a FWHM of 300 nm.

2001) is in practice not at all a good idea. (It is also not clear how this is expected to work; at 1 Airy unit the entire central lobe of the Airy disk reaches the detector so opening the pinhole further would only increase the background, not the signal, except for very thick objects.)

Figure 3 presents response curves for confocal microscopes with different pinhole sizes at two different values of Stokes' shift (values of the parameter *f*). This makes it clear that in principle a moderate resolution improvement should be obtained with a pinhole size of 0.5 Airy in both cases. At values usually used in practice, the effect of pinhole size is much more significant than that from the Stokes' shift, though, as Figure 4 shows, the Stokes shift does make a difference and in the limiting case it will reduce the resolution to close to the wide-field value.

Nonlinear Microscopy

Whereas in practical confocal microscopy the theoretical resolution limit is not attainable, in two-photon and second harmonic microscopes there is no pinhole and no constraint on the practical attainment of the resolution predicted by theory. The only limitation is whether the beam fills the BFP of the objective. Fortunately, the normally lengthy beam path from the laser to the microscope means that the beam is much larger before expansion than in the confocal case, so this is less likely to be a problem.

As Table 3 shows, a multiphoton microscope will have a minimum resolved distance twice that of a confocal microscope considered on the base of the equivalent single photon wavelength, but identical in terms of the actual wavelength used. Excitation wavelengths in typical confocal microscopes extend to as long as 647 nm, and in typical two-photon and second harmonic microscopes can be as short as 700 nm, so assuming twice the wavelength is not really accurate.

In Figure 5, we see the transfer function for a harmonic microscope, which is identical to that for an "ideal" confocal microscope. So "confocal" super-resolution, mythical in practical confocal microscopy, should be an everyday reality in practical non-linear microscopy.

Figures 6 and 7 present attempts to measure resolution of a harmonic microscope on collagen samples. Since the imaging is partially coherent, a line object such as a collagen fibril imaged in SHG mode could be expected to show a slightly worse resolution. Nevertheless, it is almost an ideal test sample since the harmonic signal is very strong and, unlike fluorescence, the signal will not fade with time.

We normally use an excitation wavelength of 830 nm for second harmonic since below 415 nm the signal is substantially attenuated by absorbance in the filter coatings. 400 nm harmoic (i.e., 800 nm excitation) represents the absolute shortest wavelength detectable in our system. Table 4 shows the values of two different resolution parameters at these wavelengths, using an NA 1.4 objective.

Figure 6 shows our first attempt at measuring SHG resolution (Cox et al., 2003). An NA 1.25 lens was used,

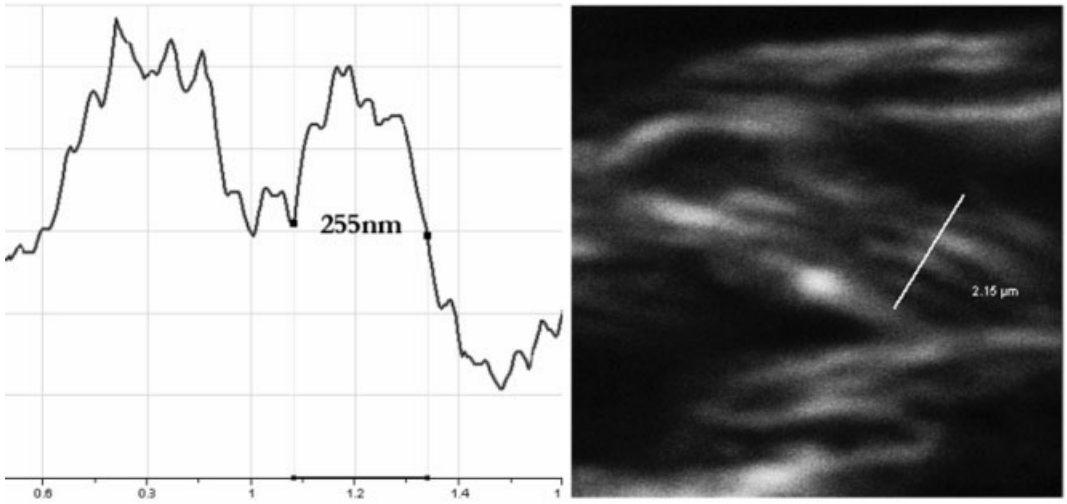

Fig. 7. Collagen fibrils in mouse dermis. Oil immersion objective, NA 1.4; 830-nm excitation, BP 415/10 detection. **Right:** Image of the fibrils. **Left:** Profile of the trace, showing FWHM approximately 255 nm.

slightly lower than in Table 4, but the excitation wavelength is 800 nm (harmonic 400 nm). This would give us a calculated FWHM of 236 nm, and our measured value is 300 nm, only 27% larger than the predicted value.

To attempt to assess the best resolution attainable, we carried out further tests with a ×100 NA 1.4 objective, as in Table 4, using 830-nm illumination. These are the conditions used for Figure 7, which shows an FWHM of 255 nm, just 21% larger than the calculated value. By way of comparison, the widefield Rayleigh resolution for a fluorescent object imaged at 550 nm is 240 nm, and we have seen that a confocal microscope is not likely in practice to improve on this.

In reflection mode, we have measured the 200-nm pitch grating on the Cornell confocal test specimen (Centonze and Pawley, 1995) so a true resolution (not FWHM) at this level is obtainable with a closed pinhole. The 170-nm lines on the same sample are not resolvable with any microscope we have tried. As a rule of thumb, we therefore regard 200 nm as the limit of resolution of a commercial confocal microscope in reflection mode, and around 250 nm as the best achievable in fluorescence. In practical terms, therefore, nonlinear microscopy achieves effectively the same resolution as single photon confocal fluorescence microscopy.

CONCLUSIONS

Confocal fluorescence microscopy offers little if any improvement in resolution over wide-field fluorescence microscopy. The widely used habit of opening the pinhole beyond 1 Airy unit when imaging weakly-fluorescent samples will degrade this substantially, often leading to images with much worse resolution than in wide-field microscopes. Non-linear microscopy at practically used wavelengths will perform much better than popularly expected since the full "confocal" resolution improvement is always available. This is particularly easy to achieve in harmonic imaging since there is no dye to bleach, and the signal-to-noise ratio can, therefore, be quite good. In practical terms, resolution can approach the 250-nm value, which is as good as confocal fluorescence will achieve in normal use.

REFERENCES

Brakenhoff GJ, Blom P, Barends P. 1979. Confocal scanning light microscopy with high aperture immersion lenses. J Microsc 117: 219–232.

Centonze V, Pawley J. 1995. Tutorial on practical confocal microscopy and use of the confocal test specimen. In: Pawley J, editor. Handbook of biological confocal microscopy. New York: Plenum Press. p 549–570.

Cox IJ, Sheppard CJR, Wilson T. 1982. Super-resolution by confocal fluorescent microscopy. Optik 60:391–396.

Cox G, Kable E, Jones A, Fraser I, Manconi F, Gorrell MD. 2002. 3-dimensional imaging of collagen using second harmonic generation. J Struct Biol 141:53–62.

Cox G, Kable E. 2003. Second harmonic imaging of collagen. In: Cell Imaging Techniques. Taatjes D, ed. Humana Press (in press).

Cox G, Xu P, Sheppard C, Ramshaw J. 2003. Characterization of the second harmonic signal from collagen. SPIE Proc 4963:32–40.

Gu M, Sheppard CJR. 1991. Effects of a finite-sized detector on the OTF of confocal fluorescent microscopy. Optik 86:65–69.

Gu M, Sheppard CJR. 1992. Confocal fluorescent microscopy with a finite-sized circular detector. J Optic Soc Am A 9:151–153.

McCutchen CW. 1967. Superresolution in microscopy and the Abbe resolution limit. J Optic Soc Am 57:1190–1192.

Reichelt S, Amos WB. 2001. SELS: a new method for laser scanning microscopy of live cells. Microsc Anal 13–15.

Sheppard CJR, Choudhury A. 1977. Image formation in the scanning microscope. Optica Acta 24:1051–1073.

Xu C, Webb WW. 1996. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. J Optic Soc Am B 13:481–491.