20

Measurement in the Confocal Microscope

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1. Prerequisites

1.1. Alignment

Accurate alignment of the microscope is vital for most measurements. Details of the adjustments will vary from system to system but the principles of alignment are universal. The methods given here should be appropriate for any spot-scanning confocal microscope but will not be directly applicable to Nipkow-disk, slit scanning, or spot/slit systems.

If the pinhole is not precisely in position the image will not be at its brightest when it is in true focus. This is guaranteed to destroy the accuracy of any measurement, particularly measurements of depth and intensity. If the pinhole size is adjustable, carry out the alignment with it fully closed then open it a fraction since no alignment can be perfect. As a check, if any doubt exists, take a vertical (X–Z) section of a test specimen with the pinhole fully closed—misalignment will show as double images of very thin structures (**Fig. 1**).

The axis of the scan must pivot around the back focal plane of the objective lens. Otherwise, vignetting will occur—the edges of the image will be dimmer than the center. This will affect some measurements more than others, but it will have most importance where structures are being segmented out by gray values. Some manufacturers provide a prism that fits in the objective position to check this. If not, provided that the microscope is "infinite tube length" (current designs from Zeiss, Leica, Nikon and Olympus all are, as are some older microscopes) just remove an objective and place a piece of card on the stage. *Do not use any reflective object such as a mirror or even a glass slide*. The circular patch of laser light should appear stationary. If it is not, the appropriate adjustment must be made. On a Bio-Rad system, e.g., the height of the transfer lens between microscope and confocal head is adjusted by a screw

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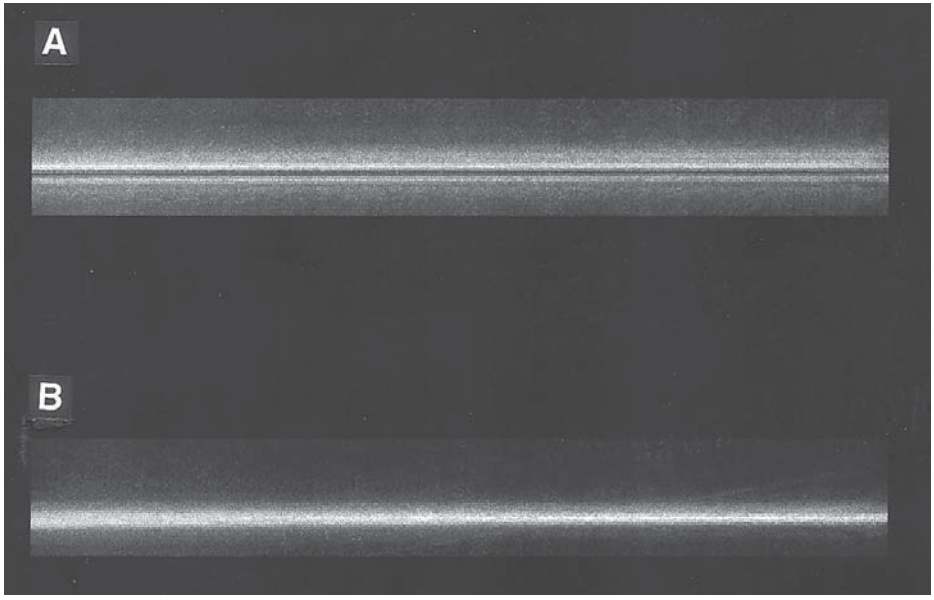

Fig. 1. Confocal reflection images of a mirrored coverslip. **(A)** XZ image with the pinhole misaligned. Note how the reflective surface appears artefactually as a double line. **(B)** With the pinhole aligned correctly the surface appears as a single line.

collar. After this adjustment, the confocal and conventional optical systems may need readjustment to make them parfocal, so that both the confocal image and that seen through the eyepiece are in focus. (This is worth doing purely as a matter of convenience, but it could also affect the magnification if it was severely out). Again taking the Bio-Rad system as an example, the whole confocal head is raised or lowered to make both sets of optics parfocal.

The field of view of the confocal optics should be centered on the optical view. It is inconvenient if it is not, and although measurements will not be affected, resolution might be, as all lenses perform at their best in the center of the field of view. The illumination should be even across the confocal image. Some microscopes are notably uneven at their lowest zoom settings—in this case it is better to use a higher zoom.

1.2. Contrast

The requirements for obtaining accurate measurements in the confocal microscope are not necessarily compatible with the best image quality as judged by eye. First, it is essential, when making any measurements of intensity or resolution, that the mean background intensity nowhere goes to zero. Although this is not so essential for measurements of position or depth, it is

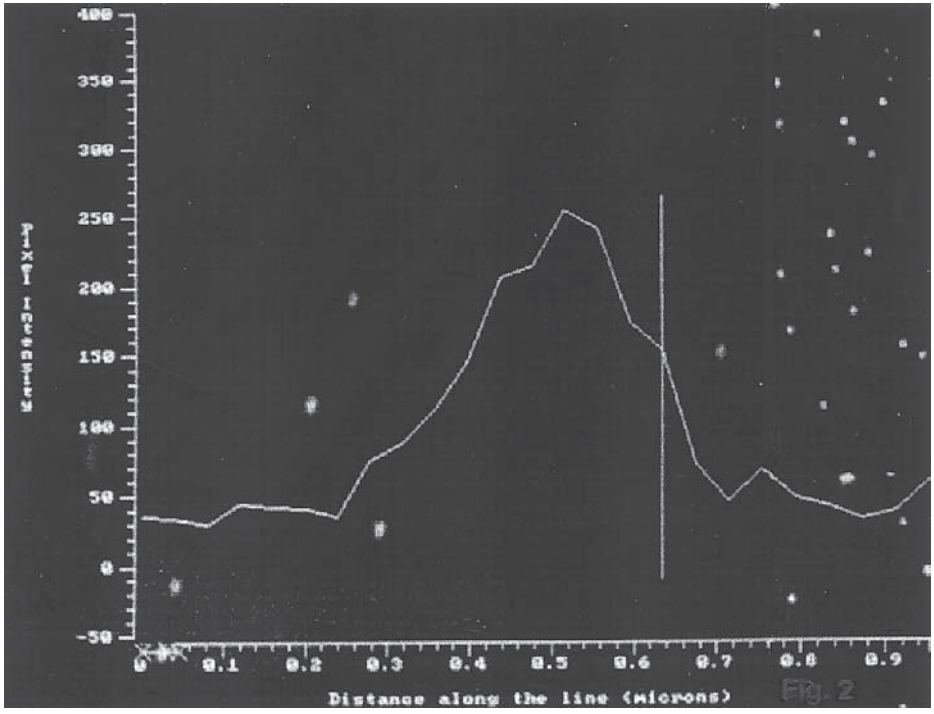

Fig. 2. Line profile through a 100-nm fluorescent bead, taken using a x63, NA 1.4 oil immersion lens. The width of the curve, taken halfway between the maximum and minimum values, gives the resolution of the system. To obtain a meaningful value the peak must not reach saturation intensity (255) and the background has to be above zero.

still advisable because otherwise the resolution, and hence the possible error, cannot be estimated (**Fig. 2**). In most cases the background level needs to be the dark current from the photomultiplier (PMT), as background fluorescence in the tissue may vary from sample to sample. Therefore the first step should be to remove the sample from the microscope and adjust the black level until a (just) nonzero value is present across the entire field of view. The resulting background will probably be higher than would be chosen for visual impact. On some systems automatic gain and/or black level controls are on by default—it is absolutely essential to turn these off.

It is even more important, for any sort of measurement, that the intensity at any point in the image never exceeds the maximum that the system can record. Some microscopes (e.g., Bio-Rad MRC 500 and 600, OptiscanF 900e) have the option of storing images in 16-bit form. In this case the maximum possible intensity is approximately 65,000 counts—a value that is unlikely to be exceeded with

any real-life specimen. But in many cases microscopes only store 8-bit images, in which case the maximum recordable intensity is 255. This is all too easy to exceed, and it is very important to set the gain of the amplifier or the PMT voltage to a value at which even your brightest sample will not saturate the image. If the intensity values saturate at any point in an object then its true intensity cannot be measured, and neither can its position laterally or in depth.

1.3. Summary

Before you start, the microscope must be aligned as accurately aspossible, and the gain (or PMT voltage) and black level must be set to avoid any overflow or underflow.

2. Depth and Thickness Measurement

Confocal microscopes are commonly used for making measurements in depth—something that conventional microscopes, whether light or electron, cannot easily do. Their major rival in this field is the scanning probe (atomic force) microscope, but this can only measure a single surface. In the confocal microscope various types of measurement are possible, with varying degrees of accuracy. The relative heights of various parts of a single surface can be measured with extreme precision, whereas measurements of vertical depths and spacings within a three-dimensional solid or liquid are constrained to some extent by the resolution of the system, which is unlikely to exceed 0.5 μm in the axial direction. Because axial resolution is related to the *square* of the numerical aperture (NA), it is absolutely essential to use the highest NA lens that is capable of covering the desired area.

Common to all depth measurements is the need to take account of the refractive indexes of the specimen and of the medium in which it is mounted. Wherever possible these should be the same! If specimen, mountant, and the medium between sample and lens are all equivalent in refractive index no correction is needed—the depth measured by displacement of the slide or objective will be correct. This applies whatever the actual refractive index is. Examples are:

1. Sample mounted in permanent mountant, under a coverslip, viewed with an oil immersion lens [refractive index (n) = ~1.5 throughout]
2. Surface sample such as the ornamentation on a dry pollen grain viewed under reflected light in air (n = 1 throughout)
3. Living sample, viewed in water, using a water-immersion lens, with or without a coverslip (n = ~ 1.3).

In each of these cases, although the measurement will be correct, it is necessary that the lens is appropriate to the media used. In **(1)** it is obvious that an oil immersion lens is needed, whereas in **(2)** the objective lens must be corrected

for use without a coverslip (the prefix epi- is often used). In **(3)** the lens must not only be designed for water immersion, but it will also specify whether or not a coverslip is required. The coverslip will not affect measurements because it will introduce an equal apparent displacement to all parts of the sample. But if the lens is not corrected for the medium the image will be poor, and resolution both laterally and axially will be compromised. This is a consequence of spherical aberration, which can only be corrected for a single working distance and optical medium.

Conventional "dry" (nonimmersion) objective lenses for biological use are intended for use with a coverslip, and with the specimen beneath mounted in a medium of refractive index ~1.5. The distance from the top of the coverslip to the sample should be 0.17 mm, and this distance will be found engraved on the lens. Provided that this distance is, overall, approximately correct, reasonably well-corrected images can be obtained over a distance of several microns, but relative depth measurements must be corrected for the difference between the refractive index of the medium around the lens and the medium containing the sample. **Figure 3** shows why this is so. Rays of light from the object are refracted away from the normal as they leave the coverslip, according to Snell's law of refraction. They therefore appear to emanate from the point indicated by the dotted lines. The correct measurement will be given by multiplying the apparent measurement by the refractive index of the mountant (1.5) over that of air (1)—in other words the correct figure will be 1.5 times the measured value.

Samples mounted in water should not, in theory, be measured with a dry lens but in the real world we often want to do this. We will get a reasonable image approx 0.2mm below the upper surface of the coverslip, and the measured depth should be corrected by multiplying by 1.3, the refractive index of water. Nevertheless a water immersion lens is strongly preferable—as well as not needing any correction, the image will be aberration free. Measuring depth or thickness in aqueous media under oil immersion should not be attempted—although a passable image can be obtained immediately below the coverslip the image quality deteriorates so rapidly with depth that measurements could not be trusted ***(1)***.

2.1. Measuring Heights On A Surface

Variations of height in a single plane surface can be measured with considerable accuracy in the confocal microscope; this is very commonly done in the engineering sciences but also has applications in biology. A line profile can be taken by making a vertical (X–Z) section of the surface, or an overall view can be had by collecting a series of optical sections. The accuracy to which a sur-

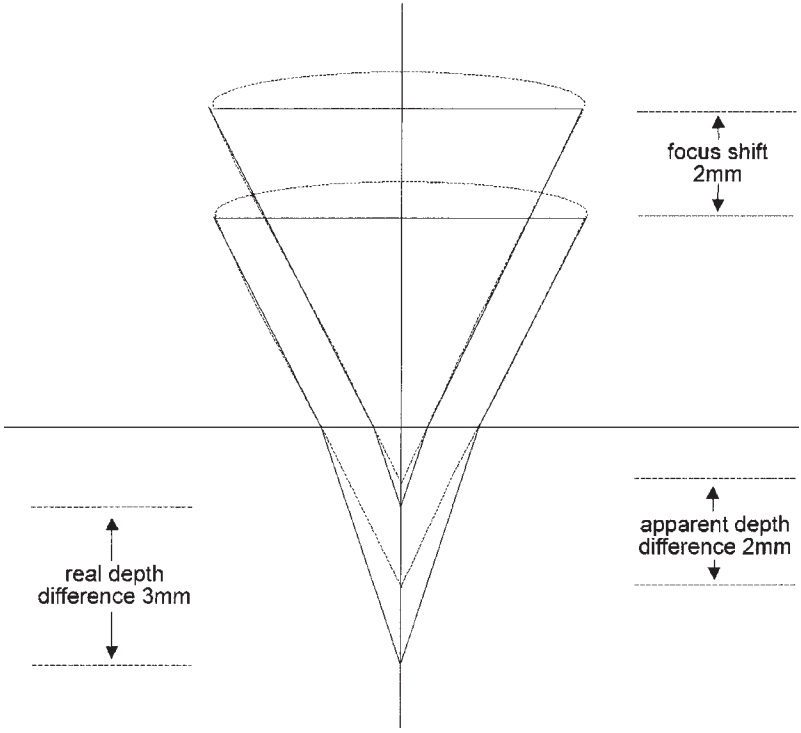

Fig. 3. The relationship between real and measured depth when using a dry lens with a permanently mounted sample. (In real life the actual value could not be as large as 2 mm without causing other errors).

face can be located considerably exceeds the vertical resolution of the microscope (resolution being the vertical distance between two objects that can be distinguished from each other). The spacing between planes (or lines in an X–Z section) should therefore be as fine as is compatible with a reasonable size for the resultant file.

The resulting dataset, viewed as an X-Z section or a side view in a 3D reconstruction, will generally look very disappointing at first glance. The plane surface will be stretched or smeared in depth—possibly to the extent that measurement seems impossible (**Fig. 4A**). However a line intensity profile through the surface will show that there is in fact a strong maximum at the actual location of the surface. This can be used directly as the basis for measurement, or various image processing techniques can be used. Thinning or erosion algorithms ***(5)*** can produce a single-pixel wide line indicating the profile of the surface to the nearest depth step (**Fig. 4B**). Some systems (e.g., Zeiss LSM 410) include this feature as part of their standard software, but in other

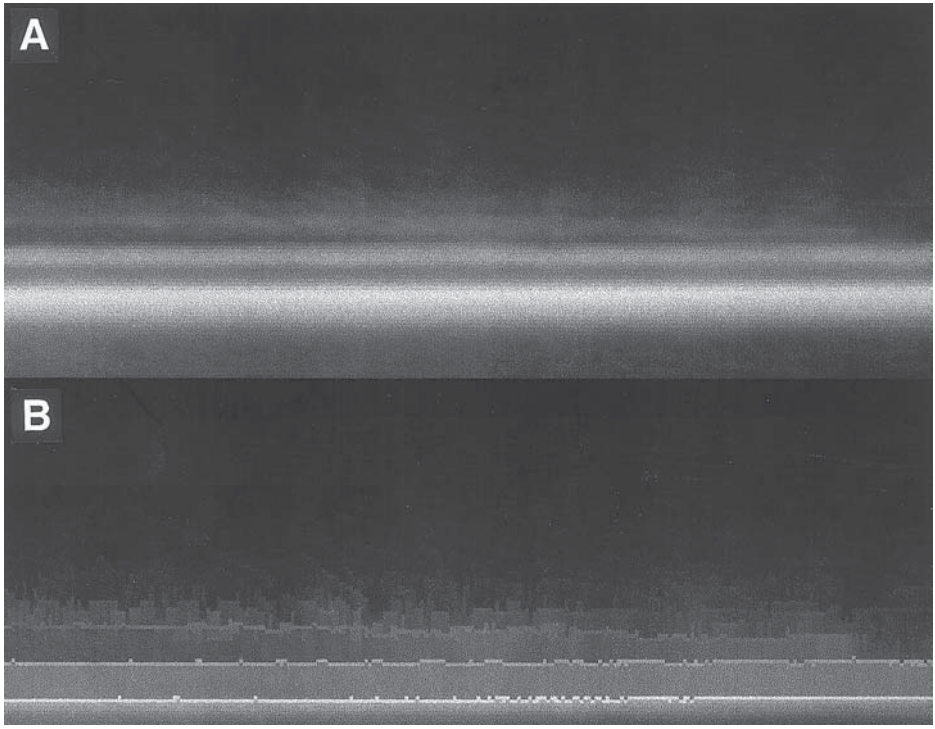

Fig. 4. X–Z section through a sample consisting of a 3 μm layer of SiO_2 on a silicon substrate. Over the oxide is a thin layer of oil (not normally a recommended practice but deliberate in this case!). In the original image **(A)** although the actual position of the surface of each layer is actually the brightest part this is not apparent to the unaided eye, and accurate measurements of layer thickness would seem impossible. After only five iterations of an erosion (thinning) algorithm, which preserves the peak intensity ***(5)***, the actual position of each surface (to the nearest pixel) is clearly visible.

cases third-party software will be needed. Free software is available that will not only extract a surface in this way but also carry out hyperbolic interpolation to give subpixel accuracy (**Note 1**). Another alternative is to create a height-coded image, which is effectively a contour-colored map of the surface, plotting the depth of the brightest pixel as the intensity in the output image.

2.2. Thickness Measurements

When measuring the thickness of a cell or similar structure the simplest approach is again to take an XZ section, or multiple XZ sections. Where the boundary of the object is stained (e.g., where a cell is bounded by a labeled

membrane or wall) the brightest pixel can again be taken as an indicator of the true position of the surface. The depth (with any necessary correction for refractive index) can then be measured from a line profile.

If the object to be measured is stained or labeled throughout its thickness the problem of determining the true edge of the structure is more complex. The best general approximation is to take the halfway point between the background level and the peak intensity. This will be reasonably valid provided that there is no refractive index difference between the object and the surrounding medium.

2.3. Summary

Refractive index effects must always be borne in mind when making this class of measurement. Measuring surface profiles and relative depths is straightforward and can be carried out to a higher accuracy than the depth resolution of the microscopes, even though the actual images may look poor. Measuring the thickness of objects that are labeled throughout is less accurate.

3. Length, Area, and Volume Measurement

3.1. Two-Dimensional Measurements

There is little here that is specific to the confocal microscope. Because the image is already in digital form these measurements are simple to do, and most microscopes include software permitting segmentation of single or multiple grayscale ranges for simple area measurement, and line cursors for measurements of straight-line lengths. More sophisticated measurements will require the files to be exported to specialist image analysis software. Because most confocal microscopes offer export to standard file formats, and many image analysis packages will directly read confocal microscope formats, this is no longer the problem that it was as recently as 3 or 4 years ago.

3.2. Surface Area and Volume

Volume measurement from a confocal dataset is straightforward, as a count of the voxels in the volume of interest (with appropriate corrections for scaling in the vertical dimension) is all that is required. Many packages, including the Lasersharp software provided with Bio-Rad confocal microscopes and 3D analysis packages such as Voxblast™ (**Note 2**) and VoxelView® (**Note 3**), provide a facility for seeding (identifying a voxel within the desired volume) and then setting intensity thresholds to segment out the required volume. This will give a numerical result very simply but in the case of complex structures it may still be preferable to segment out the structure of interest in the individual layers and measure each one. The considerations outlined above for determining the vertical extent of the object will still apply.

Surface area is much less simple—a count of voxels making up the surface is *not* a measure of the actual area. Some specialist 3D packages will fit a surface to a 3D dataset and measure it but at the time of writing commercial systems that can do this remain rare. Alternatives are (1) approximating the shape to a regular solid (cuboid, ellipsoid) and estimating the area from that or (2) adopting a stereological aproach (below).

3.2.1. Stereological Approaches for Cases in Which the Volume of Interest is Not Fully Contained Within the Confocal Dataset

Measurements made on a random sample of sections can provide very accurate estimates of surface area and volume and this is often a more appropriate approach to determining these parameters in many biological systems. Textbooks on stereology will offer a bewildering array of solutions that may well be necessary in difficult cases but two very simple formulae will be usable in a wide ranges of circumstances ***(2)***.

These are:

$$V_V = A_A$$

In other words, the volume of a structure or compartment, per unit volume (V_V), is the same as the measured area of that component in a section, per unit area (A_A). Many confocal microscopes will enable the area of interest to be segmented out, either by gray levels or by drawing, and its area (and that of the full image) calculated automatically. This will (after measuring a suitable number of images) give an accurate estimate of the volume of the structure of interest.

$$S_V = (4/\pi)L_A$$

Thus by measuring the length of intercept of a membrane (or other surface) per unit area of section (L_A), and multiplying it by $4/\pi$, or ~1.27, we can estimate the surface area of our structure in a unit volume (S_V).

Both these measurements depend on sections being taken at random. If your structure has a particular orientation in the specimen you must cut (real or optical) sections in a range of directions, and you must sample the section so as to include all parts equally. Picking views that contain the structure you are measuring will **not** give the correct result! It is probably best, to avoid any unconscious bias, to sample your specimen in a grid pattern, taking images at a series of predetermined coordinate patterns.

3.2.2. Summary

Length and 2D area measurements are common image analysis problems and easily carried out with image analysis software. Volume measurements are conceptually equally simple but require manual techniques or 3D analysis soft-

ware. 3D surface area measurements require specialist software but are probably better carried out with stereological techniques.

4. Fluorescence Intensity Measurements

Making relative intensity measurements is relatively simple, while making absolute measurements is absolutely impossible. Within its limitations the confocal microscope is probably the most accurate tool available for measuring intensity within a defined voxel space, although fluorescence photometry may be preferable for measuring the integrated intensity from a specific organelle, at least if that organelle can be isolated from the cell. In either case bleaching of the fluorochrome is likely to be at least as significant a factor as instrumental parameters ***(3)***.

4.1. Linearity Calibration

Before making any actual measurements the linearity of the response should be checked using neutral density filters. If the transmitted light detector uses the same PMT as the fluorescence detector, an initial check can be made using the neutral density filters which control the incoming laser power. On a Bio-Rad MRC 500/600, for example, these provide 1%, 3%, and 10% of full power, which should enable a calibration graph of adequate accuracy to be constructed. Ideally this should be done with each available laser line although one could hope that the detector would be equally linear at all wavelengths even if it is not equally sensitive.

If the transmission detector is a separate system this calibration must be done using a fluorescent sample. In this case great care must be taken, if using the neutral density filters in the beam path from the laser, to ensure that the fluorescence does not saturate (all available molecules in the excited state) at the higher intensities. Otherwise saturation of the fluorochrome could be mistaken for nonlinearity of the detector. To avoid bleaching a solution of dye is best (e.g., DiI in immersion oil) and by using several dilutions one can check whether the response is linear in relation to concentration. Alternatively, fluorescent plastic sheet (available from any sign-maker) is reasonably resistant to fading.

It is preferable, however, to calibrate the system by placing a series of neutral density filters in front of the detector. On a Bio-Rad MRC500/600 this can be done simply enough, provided a single-channel filter block is used, by placing neutral density filters (which do not need to be any particular size) inside the "tunnel" for the second filter block so that they cover the hole leading down to the detector. (The cover plate must be put back in position each time, of course.) Many other microscopes will offer a similar spot at which a neutral density filter can be placed in the path of the fluorescent light. Kodak sells gelatine neutral density filters that can easily be cut to any desired shape or size.

One way or the other, this exercise will lead to a calibration curve relating detected light intensity to PMT output. Ideally this should be linear, and in practice this will probably be true provided that one keeps the brightest pixels at least 10% below the maximum of the system.

4.2. Measurement

The black level should still be at the "no sample" setting established in **Subheading 4.1.** Place the brightest sample you have in the microscope and (quickly, so that bleaching is avoided) set the gain to keep the maximum intensity comfortably below saturation. From now on the gain, PMT voltage, and black-level controls must not be touched. If they are calibrated, write down the values, and if they are lockable lock them at these values. (It is a good idea to unlock them once the experiment is over because the next user may well do some damage by forcibly turning a locked knob).

There are three measurements you will normally need to make. First, measure an unlabeled sample, to get a value for background fluorescence with no label present. Second, measure your labeled control sample. Then measure your various experimental treatments. The relative differences in the amounts by which the fluorescence in the control and experimental samples exceed the background fluorescence of the unlabeled sample, corrected if necessary for nonlinearity in the detection, will be a reasonable measure of the differences in fluorescence resulting from the experimental treatments.

4.3. Ratio Imaging

Many fluorochromes can be used to measure pH or other ion concentrations, as the wavelength of their emission or excitation peak (or both) will change with the concentration of the ion in question. Examples of curves for both emission-ratioing and excitation-ratioing dyes are shown in **Fig. 5**. It will be clear from these that because all curves are distinct, *in principle* measurements of intensity at any two wavelengths could be used to determine which particular curve we are on, and therefore the ionic concentration present. In practice this is less simple because the signal-to-noise ratio of the image will not be good enough for any reasonable accuracy unless we choose points where large changes can be expected. In general, for a given integration time, a confocal microscope will always give a worse signal-to-noise ratio than a wide-field CCD camera. During a 1-s exposure acquiring a 768×512 pixel image, each point in the sample will be sampled for 1 s in the wide-field camera, but only for 2.5 µs in the confocal microscope! This disadvantage is offset by the ability to sample in a particular focal plane, so that the measurement is not degraded by interference from cells or free dye in higher or lower planes.

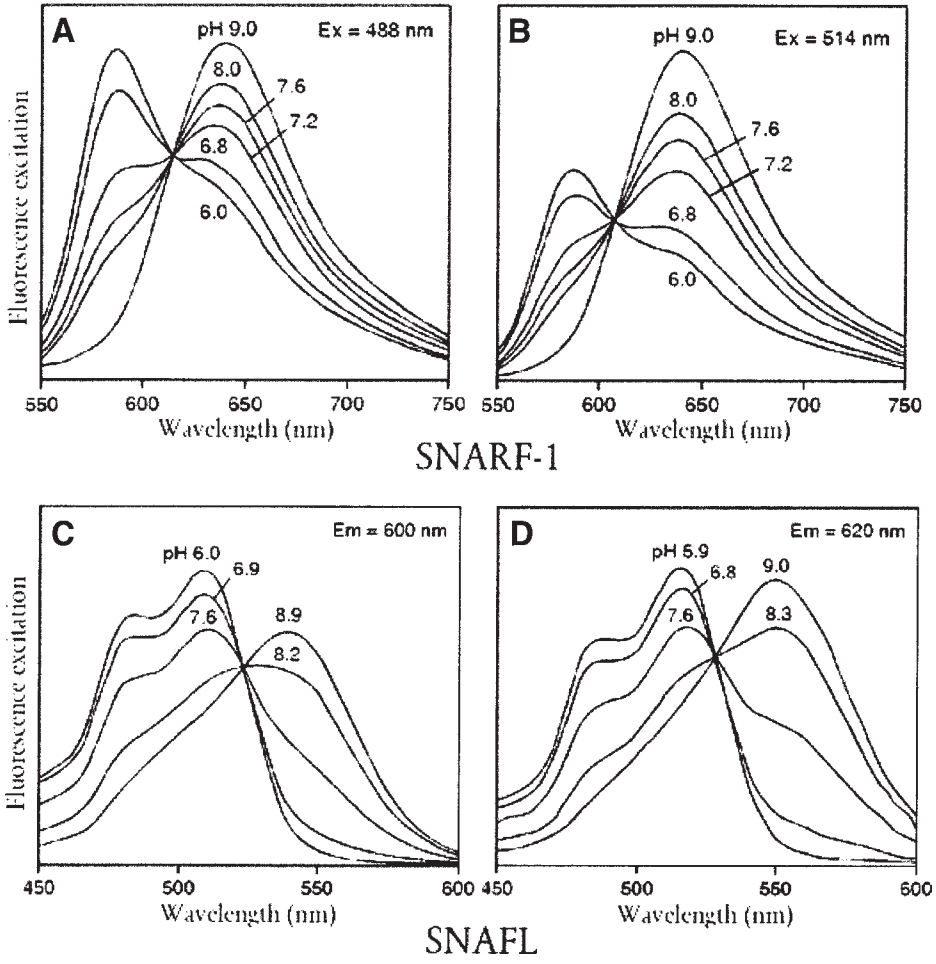

Fig. 5. pH-sensitive ratiometric dyes suitable for confocal microscopy. **(A)** Emission curves of carboxy-SNARF-1 from pH 6.0–9.0 with excitation at 488nm. **(B)** Corresponding curves using 514 nm excitation. **(C)** Excitation curves of carboxy SNAFL-1 from pH 6.0–8.9 measuring emission at 600 nm. **(D)** Excitation cuves of carboxy SNAFL-2 measuring emission at 620 nm. Both would be suitable for ratiometric measurement in a confocal system using 488 nm and 568 nm excitation. (Data reproduced from Haugland 1996, by kind permission of Molecular Probes, Inc.)

The great advantage of ratiometric measurement is that it is the *ratio* of the intensities at the two wavelengths that defines the ion concentration—the absolute intensity is unimportant. It is therefore unaffected by dye concentration, uneven loading, partitioning among cell components and bleaching. It *will*, however, be affected by background fluorescence so care needs to be taken

to minimize autofluorescence. Dynamic range can also be a problem especially with confocal microscopes that store only 8-bit images. It is important to be sure that even high dye concentrations do not take the intensity at either wavelength to saturation.

In wide-field ratiometric imaging excitation ratioing is often preferred. For calcium measurement the dye of choice is fura-2. Images are taken using excitation wavelengths of 340 and 380 nm with a 510 nm emission filter, and the ratio is taken between the two images. In the confocal microscope suitable wavelengths are not available, even from ultraviolet lasers. In fact, because confocal microscopes only have very limited excitation wavelengths the possibilities are much more restricted than with a mercury arc source, so emission ratio measurements are often more practical. They offer the added advantage that, because almost all confocal microscopes have two or more photomultipliers, both images can be captured simultaneously.

Sometimes mixtures of dyes are used for emission ratiometric imaging, e.g., fura red and fluo-3 to measure calcium concentration. Using 488 nm (argon) excitation, ratio imaging can be accomplished by detecting images at 520 and 650 nm [in practice fluorescein isothiocyanate (FITC) and rhodamine filter sets would be used]. The problem with this approach is that is difficult to ensure equal loading of the two dyes in all cells of a population. Single dyes are preferable, and more are now being developed with confocal microscopy in mind. However, the discussion of individual dyes is beyond the scope of this chapter.

The fundamental protocol for making an emission ratiometric measurement is to record images at each wavelength (which can normally be taken simultaneously), subtracting the background level (determined previously), then displaying the ratio of the two images. Confocal microscope manufacturers normally provide (at extra cost) software for doing this automatically. In the case of the Bio-Rad confocal microscopes, e.g., this software will do these operations as the scan proceeds, so that the ratio image, as well as the two original images, are displayed live. It will also display graphs of ion concentration against time for multiple points over the image. However, if speed is less of a consideration it is quite possible to make ratiometric measurements without additional software.

The detection wavelengths will need to be chosen with some care. As **Fig. 5** shows, SNARF-1 will work very effectively with the two-channel filters designed for 514 nm excitation in an argon-ion system, such as Bio-Rad's D1/D2 or A1/A2 combination. It cannot, however, be measured efficiently with filters designed for two-channel detection with an argon/krypton laser, as the fluorescence at 550 nm is very low and the low pH peak at ~580 nm will be blocked as it lies close to the 568 nm excitation wavelength. However,

the barrier and dichroic filter sets designed for argon-ion lasers (A1/A2) will work quite effectively on an argon/krypton system using 488 nm excitation only and may prove a much less costly alternative than purchasing a custom filter set.

Excitation ratioing will require sequential collection of the two images. On systems in which the emission line selection is under software control, and there is a macro language available to automate the process, the time delay can be made quite small. On older or simpler systems, however, filters will need to be changed by hand and time-course measurements will be difficult. However, if a suitable dye is available it may be worth taking the trouble. The precision of the laser lines (relative to the rather wide-band filters usually used for emission ratioing) can give a very accurate measurement if they match the characteristics of the indicator. **Figure 5** shows an example—carboxy SNAFL-1 will give very effective ratio images from the 488 nm and 568 nm lines of an argon/krypton laser.

In any ratio imaging, accurate calibration is essential if actual numeric output is required. Because both dissociation constants and fluorescent properties may be influenced by the environment inside a cell, this calibration cannot be done in vitro but must be done on the cell system being investigated. The principle is to clamp or buffer the concentration of the ion in question to a known value, or series of values. EGTA can be used to produce defined calcium ion concentrations, and nigericin is used to calibrate pH. In general the measured intensity ratios obtained from these known concentrations can be entered into the microscope's ratiomentric software so that actual concentrations are shown on the live display.

4.4. Summary

First check that your system is linear in its response—and draw up a calibration curve if it is not. Set gain and black levels to be well clear of overflow and underflow. Then measure an unlabeled sample as well as a labeled control and experimental samples. For ratiometric measurements filters and/or laser lines should be chosen to optimise the response and calibration should be done in conditions as close as possible to the experimental ones.

Notes

1. 3-D View, by Iain Huxley. A modified version of NIH Image, the freeware image analysis system developed at the National Institute of Health, USA. Available from http://www.physics.usyd.edu.au/physopt
2. Voxblast. Vaytek Inc, 305 West Lowe St., Fairfield, IA.
3. Voxel View. Vital Images Inc., PO Box 551, Fairfield IA.

References

1. Sheppard, C. J. R. and Török, P. (1997) Effects of specimen refractive index on confocal imaging. *J. Microscop.* **185,** 366–374.

2. Underwood, E. E. (1970) *Quantitative Stereology*. Addison-Wesley, Reading, MA.
3. Pawley, J. (1995) Fundamental limits in confocal microscopy, in *Handbook of Biological Confocal Microscopy*, (Pawley, J. B., ed.), Plenum Press, New York, pp. 19–38.
4. Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals.* Molecular Probes, Eugene, OR.
5. Cox, G. C. and Sheppard, C. J. R. (1998) Appropriate image processing for confocal microscopy, in *Focus on Modern Microscopy*. World Scientific Publishers, Singapore, in press.