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Practical Considerations for Collecting Confocal Images

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1. Introduction

Conventional microscopy delivers two-dimensional images in real time and real color to the eye of the user. Confocal microscopy adds a third dimension by imaging only one plane within the sample at a time so that variations in depth can be quantified *(1)*. This has both positive and negative aspects. The advantage is that a series of such slices can be reconstructed to give 3D views and enable volume analysis of the sample, and that any one slice is crisper and clearer than a full-field fluorescence image. The disadvantage is that the portion of the sample visible at any one time is so small that finding the most interesting parts of the specimen may no longer be possible (**Fig. 1**).

Computer control makes it easy to explore the temporal dimension, scanning time series instead of Z-series to measure the way a specimen varies over time. A wide choice of laser lines and detection filters delivers up portions of yet another dimension: wavelength. A further benefit of confocal systems is that they produce digital images, which can be manipulated easily and condensed into statistical data.

There are three main objectives to pursue during a series of experiments:

1. Identify new and interesting phenomena.
2. Collect a volume of data that proves that the first impression is real and valuable.
3. Collect a few high-quality images that explain the hypothesis and add sparkle to a publication.

1.1. Identifying New and Interesting Phenomena

It may take the novice user some time to develop skill in collecting and interpreting confocal images, and randomly perusing a sample to obtain a sense

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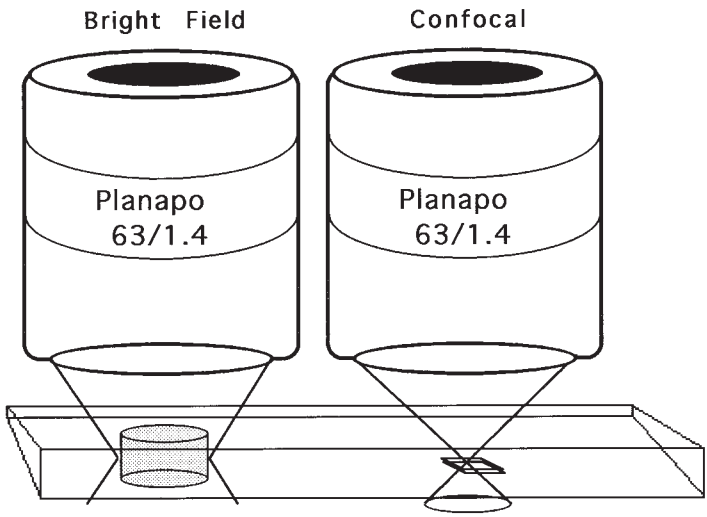

Fig. 1. Specimen space vs sampled volume in a confocal and a bright field microscope.

for how it all fits together is a necessary precursor to delving in with more detailed analyses. Most laboratories have stocks of well known animal models or cell lines, and a supply of reliable staining protocols for fluorescent imaging (**Subheading 2.0.**). Browsing an interesting sample with a well-designed confocal system is useful, but keep your notebook at hand to jot down chance discoveries.

1.2. Producing the Supporting Data

When you have seen enough evidence that something interesting is happening, you may need to design rigorous tests to prove or disprove these observations, and to uncover more subtle relationships within the test sample. If a statistical analysis is called for, care should be given to designing the most efficient sampling procedure—one that proves the point without generating unnecessary reams of data.

When the intention is to see the most detail in a sample, pixel spacing should be set at half the separation of the optical resolution. This is the so-called Nyquist criterion, and holds true for all imaging systems at any magnification. Where structures of interest are significantly larger than the maximum resolution of the microscope, a lower magnification can be used so that more structures can be counted in each image. For example, in a study of spatial separation of cell nuclei, a pixel separation of 2 μm may be acceptable; whereas if chromatin spots within each nucleus must be mapped, pixels may have to be 10 times smaller with a consequent reduction in sampled area in each image.

1.3. Producing Convincing Images

The final requirement for a confocal study is the need for really pretty images. Unlike **Subheading 1.2.**, the emphasis here is purely qualitative—pulling out all the stops to get the most attractive, most informative images possible. Exceeding the Nyquist criterion and packing in as many pixels as possible into the region of interest may not gain any information, but the smoother transition across the image from one structure to the next may make it worth the extra exposure time. If available, a good interpolation routine will achieve a similar result, but this may be less satisfying than extracting excellent images directly from the specimen.

Cleaner images come from brighter illumination, slower scan speeds, a smaller confocal aperture, and lower photomultiplier tube (PMT) gains or CCD integration times. Under these circumstances, bleach rates are much higher, so there is a limit to how often a region can be scanned. Practice on unattractive regions of the sample to optimize scan parameters, then go to draft or fast scan mode, frame a good region, then scan it once with the highest resolution parameters.

Most laboratories will have a favorite sequence of postprocessing steps, which may have to be varied considerably between samples, but should be posted on a cue card for the benefit of occasional users. The following is an example:

1. Directional smooth to eliminate random pixel noise
2. Histogram Equalize to improve contrast in the image

3. Threshold to eliminate background in each channel
4. Auto background subtraction to lower the step between threshold and zero
5. Rescale to spread the surviving pixel values across the whole dynamic range
6. Palette change, to exaggerate the most interesting channel

The most attractive confocal images are generated from 3D data sets, where the volume is rendered with simulated shadowing from an apparent light source, and where each channel is given its own opacity value to make it more or less opaque, the so-called SFP reconstruction.

2. Materials

2.1. Stains

Use these stains as test standards for practice and to be sure that everything is working properly before moving up to more challenging material. Make a set of images that describe these samples, and compare new findings with this stock of initial images to hunt for interesting differences.

1. Rhodamine B hexyl ester, 1 to 10 µg/mL live or fixed for membranes
2. Hoechst 33347, 10 µg on fixed material, or 100 µg on live cells for DNA
3. Bodipy phalloidin, 10 µg on fixed material for F-actin
4. Rhodamine 123, 1 µg/mL on live cells for mitochondria
5. Carboxyfluorescein diacetate (CFDA), 7 µg/mL, for live cells

2.2. Lens Choice

The most interesting regions for confocal study are often too small or too subtle to be spotted with low-power, low numerical aperture (NA) objectives, so confocal imaging is nearly entirely done through top-quality oil or water immersion objective lenses. By reducing the sweep angle of the scanning mirrors, pan and zoom controls can be used for homing in on details within a field of view, so the main challenge is finding fields of view that contain promising regions (**Fig. 2**).

The resolution limit of a confocal microscope is set by the NA of the lens and the excitation/collection wavelengths. This means that the ideal lens for routine confocal imaging is the lowest magnification lens with the highest available NA. A 63× oil objective is easier to use than an equivalent 100× lens simply because it offers a larger working area to the user without sacrificing performance. Working distance is also important, but for oil objective lenses performance deteriorates so quickly after 50 µm penetration that provided the sample is mounted flat, a longer working distance gives little benefit.

1. *Water objectives:* There has been a recent trend in confocal microscopy toward the use of water immersion objective lenses, which are significantly more expensive than the oily types and have slightly lower NAs. These disadvantages are

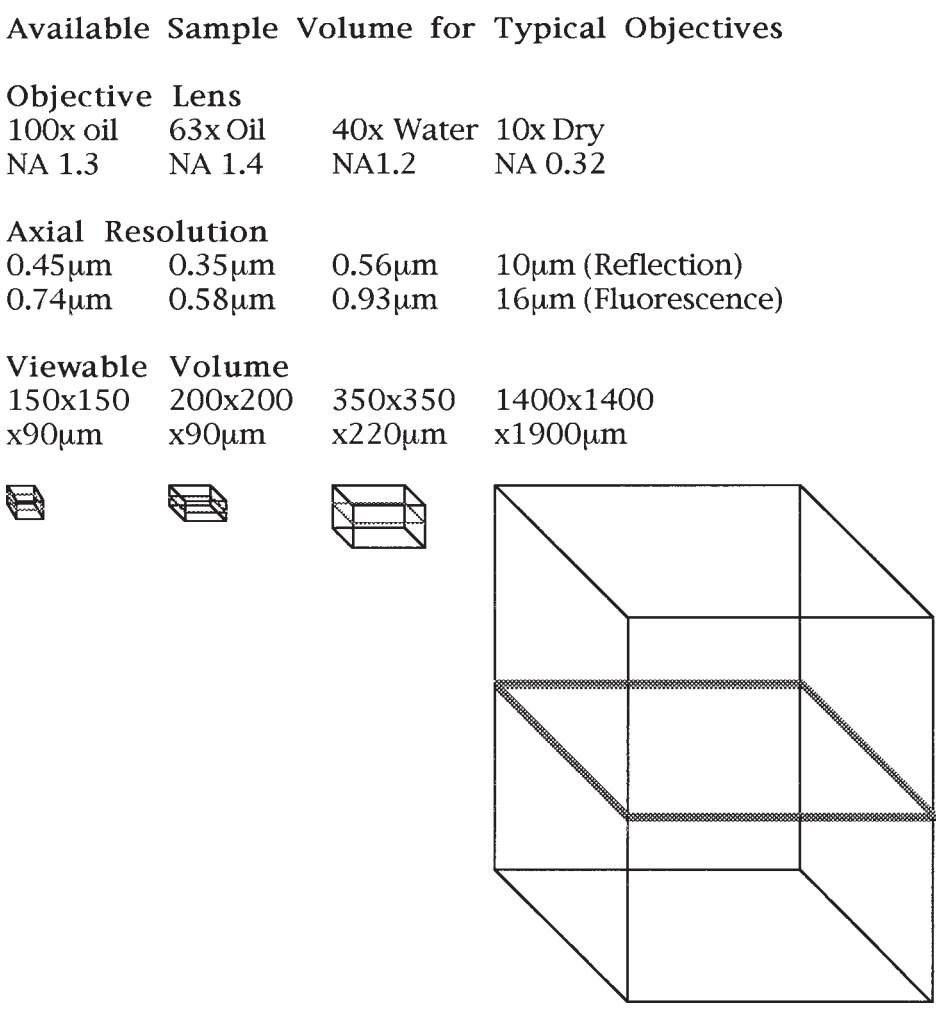

Fig. 2. Accessible sample volume: The key properties of four commonly used objective lenses are shown. Note that there is no benefit in using a higher magnification lens if the NA is not also greater, as the resolution does not increase, but the available sample volume is much reduced.

outweighed by their good compatibility with watery specimens which more than doubles the effective working distance, and the greater convenience of using water as the immersion fluid. They are also UV compatible. A good water objective lens is so versatile that it can replace a whole cluster of dry and oil objective lenses and actually saves money on the cost of a fully equipped system.

2. *Dry objectives:* There are instances when a specimen cannot be exposed to immersion fluid but must be left dry. Dry objective lenses typically have much

lower NAs and hence lower resolution than an equivalent immersion objective. But this is balanced by a longer working distance and cleaner work environment.

3. *Dipping objectives:* Dipping objectives are used where watery specimens need to be viewed directly without a coverslip between lens and specimen. These lenses have a narrow profile for insertion into dishes and are typically encased in either ceramic or Teflon for corrosion resistance in physiological solutions. This is a good type of lens for use with cultured cells on an upright system, as it has a long working distance to allow for addition of reagents, and eliminates the need for perfusion chambers.

2.3. Test Samples

1. Multiline submicron beads: The most universal test samples for confocal microscopes are made from beads that are smaller than the resolution limit of the microscope and have been doped to fluoresce at many wavelengths. Tetraspeck beads (Molecular Probes, Eugene, OR) are excited at all popular laser wavelengths from 354 nm UV to 647nm dark red, and come in a range of sizes from 0.1 to 4 μm.
2. FocalCheck™ beads: Molecular Probes sells a series of 15 μm FocalCheck beads with a visually striking staining pattern that readily shows the accuracy of instrument alignment and are easier to use than submicron multiline beads. Test slides can be made with a 5-μL aliquot of each bead suspension, spread with the pipet tip on a glass slide, and allowed to dry. Apply a drop of mounting medium and cover with a 22-mm No. 1.5 cover glass. Permafluor™ (Lipshaw Inc., Detroit, MI) works well as a mountant, as it sets completely after a few days, but is an aqueous solution that does not dissolve out the fluorophores.

   Both bead types have proved useful for factory alignment and final testing of the Meridian/TR series of instruments, which require no alignment in routine operation. The UV/Blue FocalCheck beads are excellent targets for checking the coalignment of the independent UV and visible lightpaths of the Meridian Ultima, and the Blue/Orange/Red FocalCheck beads are ideal for testing visible-only confocal systems, such as the Meridian InSight.
3. *Inclined mirror:* An easy method for testing axial resolution in reflection is to take a series of images of an inclined surface. If the *Z*-separation between images is equal to or greater than the resolution, a maximum intensity projection of the images will appear as a series of parallel stripes. If the *Z*-resolution is less, all the lines will merge together as a single, solid bar. Axial resolution is the step size at which the transition between one bar and the next can just be discriminated as a slight dip in brightness. A front silvered mirror is ideal, but if the lightpath is efficient enough, an ordinary glass slide will give enough reflection. Glue a broken sliver of slide to one edge of another such that it can be held securely on the stage and present an inclined plane to the objective.
4. *Silicon chip:* Good lateral resolution samples are more difficult to devise. The tracks on silicon chips make good test samples for reflection imaging, but can only be used for resolution tests with low NA objective lenses. Chrome on glass

test targets with exact spacing between tracks are commercially available, but are expensive and do not have features with submicron separations.

5. *Diatoms:* Repetitive structures in various species of diatom can be used as resolution tests, and these can be purchased from most biological supply houses, e.g., Carolina Biological Supply, as assortments, or as more expensive individually mounted target sets. Once the system is properly aligned for reflection, take a representative image and keep it for future reference. Diatoms can also be used as fluorescence standards by mounting them in immersion oil doped with fluorophore. This produces negative images, where the specimen appears dark against a bright background.
6. *Pollen:* Pollen grains make notoriously effective demonstration specimens for confocal imaging. The lignified outer skeleton of the grain can be stained with acridine orange to enhance its natural fluorescence and make it stable against bleaching. Lily pollen grains have triangular features along the main ribs that blend together when the system is poorly aligned, and become crisper and more distinct as alignment is optimized.
7. *Fluorescent paper:* Many grades of paper are naturally fluorescent, e.g., linen bond thesis paper, which fluoresces moderately under blue excitation. Special papers, such as bright yellow sticky notes, are considerably brighter. Alternatively soak filter paper in any of the commonly available fluorescent dyes. To reduce the amount of light scatter, the paper can be soaked in immersion oil before it is mounted for observation. This renders it translucent and allows for imaging at great depth.
8. *Fluorescent plastic:* The brightly colored plastic used to make children's toys is uniformly fluorescent, and can be used either as whole sheets or as thin sections for aligning an instrument.
9. *Thin fluorescent dyes:* Extremely thin fluorescent targets can be made by mounting a small quantity of fluorescent ink between slide and coverslip. A suitable mixture of dyes will fluoresce with similar intensity for three or four laser lines simultaneously.
10. *Mixed bead slides:* Populations of fluorescent beads can be mixed and mounted together to make test standards for many experimental procedures. For example, a slide containing a tiny minority of fluorescent beads can be used to test automated search routines, while known proportions of beads with differing size or brightness are useful for confirming population characterizing procedures.
11. *Live cells:* A dish of cells can be stained in just a few minutes with a membrane dye such as Rhodamine B hexyl-ester (hexyl-rhodamine, 10 μg/mL), which labels all membranes throughout the cell, but has very little fluorescence in free solution. Fixed cells can be stained as quickly with a DNA stain such as Hoechst 33342 (10 μg/mL), which will also penetrate live cells at higher concentrations (100 μg/mL, 1 h).
12. *Live plant tissue:* Instant samples can be made from plants or algae, which have chlorophyll that is brightly fluorescent blue to red without any pre-treatment. Slices of spring onion leaf are easy to handle because they are circular and can be

mounted between slide and coverslip without falling over. The outer cuticle of the leaf fluoresces green, and lignified xylem vessels have broad excitation and emission ranges. The epidermis between layers at the root end can be stained with hexyl-rhodamine, and the DNA with ethidium bromide. Provided that the staining solution has low osmolarity, vesicles will be seen parading around the cell due to cytoplasmic streaming.

3. Methods

3.1. Test of Instrument Performance Using Multiline Submicron Beads

1. *Field flatness:* In a perfectly aligned system, the whole field of view will come into focus together as the Z-drive of the confocal system is adjusted. If there is an error in field flatness, it can be characterized by collecting a Z-series of the whole field of view, using a very small step size, then making a depth shaded reconstruciton of the data volume.
2. *Axial resolution:* A monolayer of beads can be used to test axial resolution in fluorescence for any combination of excitation and detection wavelengths. Take an *X–Z* image through a patch of Tetraspeck™ beads, then take a vertical line query through them. Axial responses are recorded as full width half max (FWHM) values, which is the distance between the two points at which the *X–Z* image records half its maximum brightness.
3. *Chromatic aberration:* Most objective lenses have excellent color correction at the center of the field, where a line query across a bead image will show each channel rising to a peak at the same location. To test the whole field of view, take a line query across beads at each corner of the field of view and at the middle. Radial errors are more likely than errors tangential to the optical axis, so line queries in a direction that passes through the center of the field of view are more likely to register an error. If an aberration is detected, a second line query perpendicular to the first will fully characterize its magnitude. Alternatively, a "number 4" shaped line query can be used to summarize chromatic separation in a single, folded line query.
4. *Z-drive reproducibility:* Reproducibility of the Z-drive mechanism can be tested by collecting two Z-series through a plane of Tetraspeck beads. The beads should be in sharpest focus and hence brightest at corresponding levels in each series.
5. *Particle analysis:* A mixed population of beads is useful for testing the ability of the whole system to discriminate differences in size brightness. Flow cytometry test kits are available for calibrating a system against a known number of fluorophore molecules per bead. However, they may be too precise and hence too expensive for everyday use.

3.2. Test of Instrument Performance Using FocalCheck Beads

1. *Chromatic aberration:* It is often easier to use FocalCheck beads for characterizing chromatic aberration, as they are more visually striking, and present a larger

target than submicron beads for testing with a line query. The large beads are easier to locate, and it requires less dexterity with the mouse to accurately bisect the bead image with a line query. Colocalization scattergram plots can be used on a single FocalCheck bead to present the accuracy of color registration, and chart its improvement as an alignment procedure is carried out.

2. *Image analysis:* Images of FocalCheck beads should always be circular, and their maximum size should be constant regardless of their position on the field of view. This can be tested by compiling a maximum intensity projection of a field of beads, then querying the size and shape of each bead in the field.
3. *Calibration of Z-drive:* A simple *X–Z* image of a FocalCheck bead is sufficient to show that the Z-drive (and image rendering software) is indeed performing as expected, as these beads are spherical and should always appear circular in a confocal image. When preparing samples of large beads, be generous with the mounting resin so that it will not compress the beads between slice and coverslip as it shrinks and hardens.
4. *Refractive index correction:* When there is a mismatch between the refractive index of a specimen and that of the immersion fluid, a distortion in the *Z*-direction is introduced ***(2)***. If the two refractive indices are known, and the NA of the objective lens is entered, this error can be compensated for so that the final data set is accurate in all dimensions.
5. *Coalignment of excitation beams:* By taking a multichannel *X–Z* image of a FocalCheck bead, the coalignment of laser lines can be confirmed. Slight errors in coalignment will appear as a slight vertical shift between channels, producing single-color fringes above and below the circular image of each bead. Standard *X–Y* images will show any lateral alignment errors between excitation lines, and a Z-series will show up both types of error in a single data set.

3.3. Sampling Strategies: Finding Regions of Interest Under Bright Field

The main benefit of confocal imaging is also its greatest limitation—that only a tiny portion of the specimen is visible at any given time (**Fig. 1**). For example, a standard microscope slide with a 22-mm coverslip viewed through a 100 µm working distance objective lens has a specimen volume of up to $20{,}000 \times 20{,}000 \times 100$ or 4×10^{10} µm^3. A single confocal image may be only 200 µm^2 and half a micron thick, or 2×10^4 µm^3 in volume. If each image takes just a second to acquire, it would take 2 million s (46 h) of imaging time to thoroughly scan the whole sample!

Clearly, it is impractical to scan all of a sample at high resolution with a confocal microscope, so strategies must be developed to help navigate through specimen space and find regions of interest quickly.

1. *Ink and scratch:* Simple steps such as mounting the specimen in the center of the coverslip, and marking the slide with a sharpie or scratch mark can save time in

locating your specimen on the confocal instrument. On an inverted microscope, the underside is readily accessible, so regions of interest can be dabbed or circled while the specimen is previewed under low power. These markings can be found easily with the condenser light, and will not be exposed to immersion oil or cleaning fluid that may wash them off.

For thin samples, it may be helpful to mark the specimen side of the slide with indelible ink or a diamond scratch before the sample is mounted, to make it easier to home in on the plane of focus. None of these marks will interfere with confocal imaging, as both the light source and the detection path are on the coverslip side of the specimen.

2. *Full-field flash:* When the sample is moved into the light path, scattered bright field light or fluorescence color changes show up through direct observation, even if the focal plane is not close enough to show any structural details through the ocular lenses. This is easily seen on an inverted microscope which offers a clear view of the underside of a slide-mounted sample, but even on an upright system, enough light is scattered through the slide itself to make its edges brighten when the sample is encountered. This is not the case when using the confocal microscope for locating the specimen because here the image of the specimen is either in focus or out of focus, and it can be extremely hard to find the specimen, especially for the novice user. It is therefore necessary to search for the specimen using the conventional light microscope.
3. *Combing a slide for regions of interest:* Uniform samples, such as cells grown on coverslips, will not flash when a region of interest is swept through full-field illumination, so more careful observation through the ocular lenses may be needed to find interesting locations.

   To survey the entire area under a coverslip, use the lowest power objective that will show sufficient detail, and start looking in one corner of the coverslip. While looking through the oculars, scan the stage in one direction until the opposite edge of the coverslip is encountered, then move down one field of view and sweep back over the coverslip. Continue this across then down the pattern until the whole slide is scanned, or until enough locations of interest have been found. Where possible, it is easier to adjust the density of targets such that a random walk starting anywhere on the slide will quickly uncover enough sites for closer examination.
4. *Recording and revisiting regions of interest:* Marks on the specimen itself are easy to find again, and can be related to notes in a lab book. All microscope stages also have a numbered scale to indicate approximate *X* and *Y* positions, although reading and reproducing them accurately is often difficult.

   If it is necessary to correlate coordinates from one microscope stage to another, a correction table can be made with graph paper glued to a slide. Take stage readings on opposite corners of each stage, then use the coordinates to calculate intermediate values for every location.

   Universal grids are available that have a numbered/lettered grid etched on a standard slide. These can be used to correlate positions to a standard scale. Such

grids can be constructed using an EM finder grid placed onto a coverslip, and depositing a thin layer of carbon over it onto the coverslip below. Alternatively, when a region of interest has been found and imaged, simply remove the slide without moving the stage, then insert the calibration scale and read off the grid location through the oculars.

5. *Epi-illumination of opaque specimens:* When the specimen is too large or opaque for conventional bright field range finding, locating the right plane of focus within the specimen can be frustrating. If there is any fluorescence, a mercury arc system will suffice, but the beam splitter needed for reflection imaging is not a common peripheral.

   The meter routine used for automatic alignment of the pinhole can also be used as a range finder. Select a very large pinhole and alternately adjust the gain and focus until the brightest plane is found.

   When neither is available, try obliquely illuminating the specimen with a flashlight. The new Meridian/TR system has a narrow gooseneck task light permanently mounted on the benchtop. This can be held very close to the objective lens below the specimen, or shone through an empty arc lamp port and reflected onto the specimen with a plain microscope slide.
6. *Full-field fluorescence from mercury arc or laser:* Most samples can be located and previewed under conventional bright field illumination. Many fluorochromes have enough color under white light to show where a specimen is on the slide, and in water-based samples there is often enough refractive difference between specimen and medium to resolve the sample with bright field or phase contrast. However, it is much easier to home in on promising areas using full field fluorescence from a mercury arc lamp. The color balance will be widely different from that experienced when scanning the sample because the excitation wavelengths are different and the human eye has a natural bias toward detecting greener colors.

   The Meridian ACAS and Ultima systems have beam diffusers that allow the laser frequencies to be used as a full field fluorescence source. This gives a more accurate perception of what the confocal optics will detect, as it uses exactly the same excitation and detection wavelengths.

   Camera-based confocal systems such as the Meridian InSight offer the user direct ocular viewing of the confocal fluorescence image. The human eye has a dynamic range many times greater than that of a CCD or PMT at a given setting, so a slight increase in brightness will be seen as the plane of focus is moved towards the specimen. Alternatively, the aperture can be opened wide until the specimen is found, then stopped back down to return to confocal imaging mode.

3.4. Homing in: Finding Regions of Interest in Confocal Viewing Mode or Matching the Experiment to the Instrument

Every confocal instrument has advantages and disadvantages, and until all confocal facilities are equipped with several different systems, the user will be aided or constrained by whatever instrument is available. To make the best use

of available equipment, its benefits and limitations should be understood. Confocal manufacturers have developed a range of user friendly functions to help the user find regions of interest as quickly and easily as possible. When used properly, these capabilities can save a great deal of time and effort, and make the use of the confocal system a more enjoyable than frustrating experience.

1. *Confocal ocular viewing:* InSight and spinning disk systems: Slit scanning laser confocal microscopes, Nipkow and tandem spinning disk systems all have the advantage of delivering a real-time fully confocal image directly to the eye of the user. Focus and stage controls can be used as easily as for a bright field microscope to rapidly survey the specimen and find regions of interest. Moving the prism on the trinocular head of the microscope then sends the selected image to a CCD camera for collection and storage.

   The light throughput of spinning disk systems is so low that they are mostly used for reflection applications, whereas the slit scanner is actually more efficient than a point scanning laser confocal, and can be used to collect video rate images of dim fluorescent samples.

   The human eye boasts a much greater dynamic range and pixel density than any camera or PMT system with the greatest density of 150,000 cells/mm^2 ***(3,4)***, and is exquisitely sensitive to detection of movement within the specimen. An image delivered straight to the eye has far more impact than a rendered image on a computer screen, and on good samples can be quite mesmerizing.

   Confocal facilities that have an InSight and a point scanner typically use the InSight on all samples to quickly check the quality of staining, before moving on to the slower, more difficult to use point scanner ***(5)***. The newest infinity corrected InSight has a resolution comparable to point scanners in *X* and *Y*, and the latest version, the InSight Point, is capable of point scanning as well for optimal *Z* resolution.
2. *Turbo preview scanning:* When navigating around a specimen, hunting for regions of interest, image quality can be sacrificed for higher scan speeds. A simple way of switching between slow scanning for data collection and fast scanning for navigation is to toggle averaging on and off. For faster scan rates, the beam steering system usually has a speed optimized mode that will allow several images to be scanned in a second, often at the cost of pixel density and field flatness. This feature is worth trying, but generally image quality suffers so much that full-field methods are more productive.
3. *Fast X–Z imaging:* The Leica confocal has an optional fast Z drive whereby the sample is mounted on an arm which is rotated by a galvanometer. This arrangement is so fast that *X–Z* images can be acquired as quickly as *X–Y* image. Finding the plane of focus of the specimen is then as simple as scanning a single *X–Y* image, then moving the stage up or down until the plane of focus enters the specimen.
4. *Kalman averaging:* Averaging of data reduces PMT noise and produces much better images. It can be done on a point-by-point basis, taking many data samples from one pixel before moving to the next; line-by-line, taking a complete line of

data many times, then reading out the average; or frame-by-frame, taking a complete image many times and averaging together whole frames. These approaches are used by the Meridian Ultima, TR, and InSight, respectively.

A variation pioneered by Bio-Rad applies a weighted frame average, where the most recently acquired frame has the most significant effect on the final image. The stage and focus can be moved around while Kalman filtering is in effect, and as soon as the location is left unchanged for a significant number of frames, a high-quality image will appear. This is an interesting solution to the problem of being able to move around, and needing to see a good quality image of promising locations.

5. Locate: Where very large preview scans are possible, as in stage/mirror scanning systems, regions of interest can be homed in on by scanning with a large pixel size, then using a Locate function to specify the center point of the next scan. This can be used to acquire higher resolution *X–Y* images, or to specify the exact cross-section position for an *X–Z* image. The Zeiss 410 allows the user to specify a vertical optical section along any line by drawing an oblique Locate line on a preview image.
6. *Pan/Zoom:* Both the zoom factor and the position on a preview image may be specified in one stroke with the Meridian/TR by clicking on the Pan/Zoom tool button, then drawing a rectangle around the previewed image. This may be used repeatedly to home in in several steps until the maximum available zoom value has been reached. A Pan/Zoom page on the scan bar can also be used to alter the position and zoom settings (**Fig. 3**).
7. *Fast Z-series:* A camera-based confocal system such as the Meridian InSight has the advantage that it acquires data from all points in the image simultaneously ***(6)***. Acquisition rate is limited only by camera speed, so video rate data acquisition is readily attainable. Alternatively, the camera can be left acquiring data while the focal plane is cycled up and down. In this way, an average 3D projection can be collected through a 50-µm thickness of sample in under a second (**Fig. 4**).

   Some confocal systems can acquire maximum intensity in a similar way, by acquiring a sequence of whole images at different depths, and saving the brightest pixels from each location in the image. This takes much longer, but still succeeds in condensing a 3D volume into a single image that can be saved as a summary. It is a common way of saving *four-dimensional* data sets, where a single Z series reconstruction is saved for each time point.
8. *Auto focus, gain, and black level:* In theory, it is possible to have an instrument automatically adjust focal plane, gain and black level to instantly produce a high-quality image of a specimen, in much the same way as a scanning electron microscope optimizes imaging parameters. In practice, there are many difficulties with trying to do this reliably with a confocal system, so these automations are less likely to find universal favor in the confocal field.

   An SEM has an extremely thick focal plane, whereas confocal imaging is extremely thin. Automatically adjusting to give the highest contrast works with

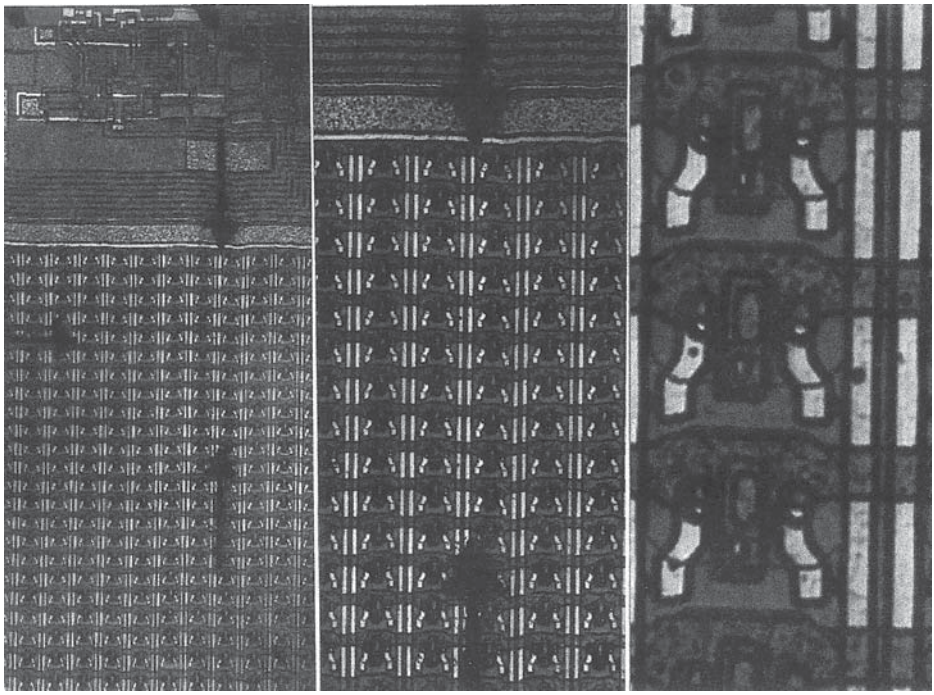

Fig. 3. Automatic zoom. These images were all taken through the same objective lens, but with different areas within the sample being scanned by the laser beam. By taking a wide-field image, then zooming in, locations of interest are identified much more rapidly than could be done at a constant, high magnification. The bright tracks on this silicon chip are 5 μm thick.

EM, but the optical plane with the highest contrast is not necessarily the most interesting to the investigator. Because confocal filtering is so very effective, a system presented with an unfocused sample is lost in blackness, and searching in the wrong direction could crash the objective into the sample.

Automatically setting gain and black level is somewhat easier, and can be done by randomly sampling the image while varying settings until the dimmest pixels fall to zero and the brightest to saturation, and this capability has been available on the Zeiss 410 for many years.

3.5. Systematic Screening Procedures

Examining a sample on a microscope is prone to subjective biases that may not be apparent to the casual user. The more time spent looking at a particular slide, the more likely it is that features of interest or positive results will be discovered *(7)*. Also, when a phenomenon has been noticed and described, it

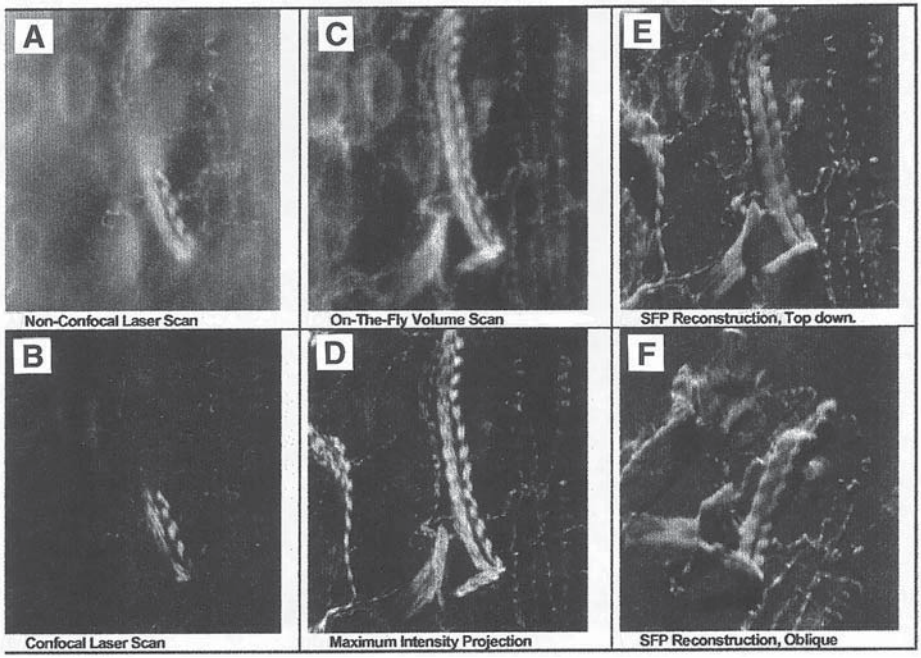

Fig. 4. Rapid 3D reconstructions. **(A)** Image of insect midgut taken on a Meridian InSight Plus using laser scanning at a single level, with the confocal aperture wide open. This indicates the level of optical sectioning achieved by concentrating the light source at a single plane within the specimen, rather than bathing the whole specimen in Kohler or full-field illuminaiton. **(B)** The same location in the specimen scanned with the confocal aperture in place. Note the absence of out-of-focus blur. **(C)** Single-second exposure taken at the same location, with the Z-drive cycling the plane of focus through a 10-µm volume. This image has the sharpness of a confocal image, but samples a larger volume of tissue than the nonconfocal laser scan shown in (A). **(D)** Maximum intensity projection representing 30 video frames of data taken at different levels such that the brightest pixel in each location is retained in the final image. Collecting an image at each level results in a sharper reconstruction than that shown in (C) but takes slightly longer to collect. **(E)** and **(F)** If individual slices are saved, the volume data set can be reconstructed from any vantage point, including top-down and oblique; and can be rendered with a variety of visual enhancements, in this case SFP shadowing.

becomes much more apparent in later studies because the human brain is phenomenally good at pattern recognition. Fortunately, most confocal applications do not require the stringent screening criteria used by clinical pathologists, but there are cases in which strictly objective sampling procedures are needed. Adherent cell cytometry systems have built-in routines that will automatically

identify, list, visit, and image all regions of a sample that obey given criteria of fluorescence intensity.

1. *Quick Look:* The Quick Look program on the ACAS and Ultima instruments sweeps a diffuse laser cursor in a raster pattern over the entire specimen and displays a fluorescence map on the screen. Selecting a range of pixel intensities reduces the scanned area to a list of locations, any of which can be visited to test the accuracy of the chosen criteria. Once satisfied with the stringency of the threshold criteria, a complete cell list is compiled, from which the system can be asked to automatically scan and analyze all locations.

   This procedure is used to find rare events, but can also be used to make subcultures of positive cells. Cells are grown on a heat absorbing film in Cookie Cutter dishes. The cell population is queried and each positive cell is visited in turn. Healthy positive cells are then cut around with a high-intensity laser beam that welds a disk of membrane onto the bottom of the dish. Peeling away the backing removes all the negative cells and leaves the positive cells to repopulate the exposed floor of the dish.
2. *Pattern list:* Rather than querying the sample itself to collect a list of locations of interest, a random or uniformly spaced pattern of locations can be generated for objectively and repeatably sampling the specimen. First, the edges of the sample are entered, then a predetermined number of scan locations is generated, which will fit inside this area without any overlap (**Fig. 5**).
3. *Sampling the whole specimen:* If sampling choice locations or spaced locations within a sample are insufficient for a particular study, a grid pattern of locations where all images butt against each other will scan the entire sample at whatever resolution is necessary. This is useful for collecting and displaying the morphology of histological sections where the sample varies enormously from location to location (**Fig. 6**).

   Stage scanning the whole section is a more economical way of summarizing a large specimen, with additional high resolution images being taken at choice locations. The ACAS and Ultima can scan any area up to 10×8 cm with a pixel density of anything up to 1536 square.

3.6. Troubleshooting

The most common difficulty encountered with a confocal microscope is what to do when the image screen remains completely blank during scanning. This may be due to a lack of data at the current position, something blocking the lightpath, incorrect instrument settings, or a problem with the hardware or software. When this happens, check that there is laser light reaching the specimen; change to a false color palette and increase detector gain to see that some PMT signal is being displayed on the screen; and go back to bright field or full-field fluorescence to see that something is visible under conventional optics that should be detected with the confocal scanner. If these quick checks fail to

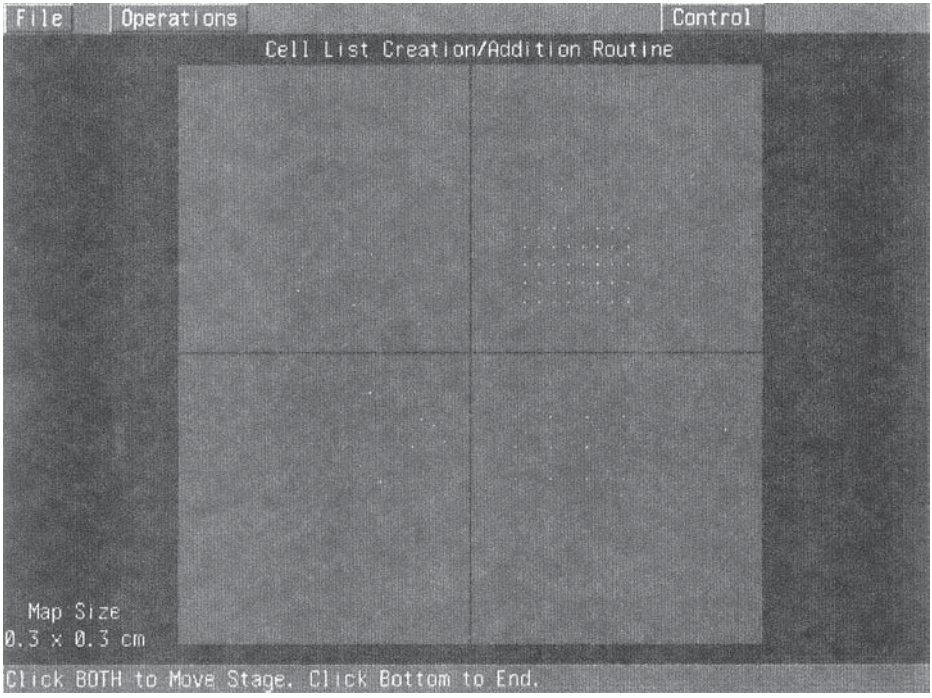

Fig. 5. Automatic imaging. **(Top left)** Manual identification of locations of interest using the ocular lenses of the microscope and clicking on each found location for future automatic confocal imaging. **(Bottom left)** Random Cell List of 10 randomly assigned locations within an area of interest designated by the user. **(Bottom right)** Grid Cell List of 12 regularly assigned locations, laid out in a grid pattern within an area of interest defined by the user. **(Top right)** Montage Cell List of 40 fields of view automatically calculated to butt against each other and completely sample the area of interest defined by the user.

reveal the problem, change to using a well-characterized, reliable sample and go through every step in the imaging system until the problem area is identified.

1. *Is laser light reaching the sample?*

Unless the sample is completely opaque, it should be easy to determine what laser lines are reaching the specimen by glancing at the scattered light from the specimen on the stage, or moving the turret to an open port and looking through a frosted glass surface held against the opening. Use fluorescent plastic or paper to detect UV, and take care to avoid direct exposure to the eye.

2. *Is the laser lasing?*

Large lasers typically require a cooldown period during which they are off, but their cooling system and power supply is still active. The sound of the cooling system does not therefore indicate that the laser is active.

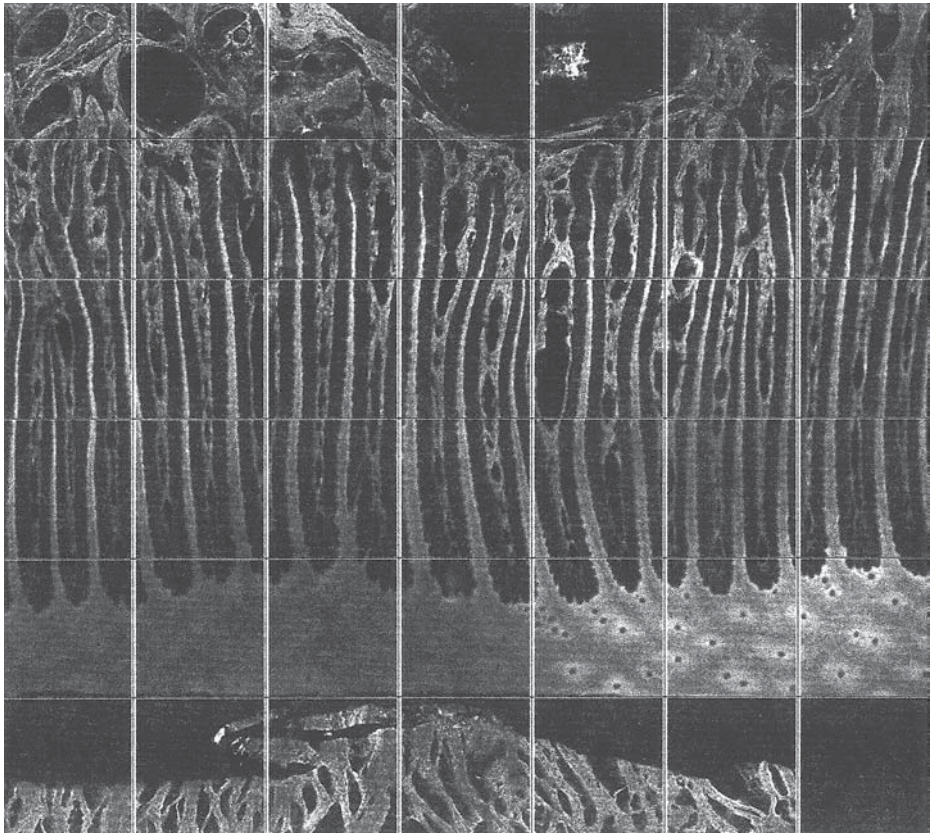

Fig. 6. Montage. Example of Montage Cell List of a histological sample of horse hoof lamellae. The size of each image was selected so that the resolution was just sufficient to determine the length of the lamellar junction between bone and hoof.

3. *Are all shutters and filters correctly set?*

All research grade microscopes have a surplus of knobs and levers, which can block access to the confocal port or the oculars. Make sure the correct positions are clearly marked and correctly set.

4. *Is the confocal aperture set too small?*

Avoid the temptation of routinely using extremely small confocal apertures for fluorescence imaging. Very small pinholes are of value only in reflection, or with extremely bright fluorescent samples, when signal intensity is not a limitation.

5. *Is the sample properly stained and positioned for viewing?*

Full field fluorescence may yield widely different illumination intensities than laser excitation, but if an image is seen under full field, the confocal

microscope should also be able to detect it. Try a well-stained slide to confirm that it gives a good image. If so, the staining protocol is suspect and fresh specimens with brighter staining may be necessary.

6. *Is the detector gain or black level set too low?*

Monitor displays are sometimes turned down or turned off to reduce glare in the room and enable a user to get dark adapted for hunting down a dim specimen under full-field fluorescence. If no image appears on the screen, try loading a saved image to ensure that monitor presentation is active and working properly.

7. *Are detection dichroics and filters excluding all signal?*

Make sure that the right cube and filters are being used for the fluorophore of interest. On automated systems, try switching to a less stringent detector filter (say 515LP instead of 530/30) to increase detection efficiency.

8. *Is the computer displaying data from the right channel?*

Set the software to display all channels on the screen, and choose a palette that clearly shows the difference between very low intensity signals and zero output. Increase gain until some signal is seen, then try to increase it by adding condenser light to the signal. If bright field illumination makes no difference on a transparent sample, signal is not getting through to the detectors.

9. *Are all components turned on and properly connected?*

When running a video signal through a video tape recorder, it is usually necessary to keep the VCR turned on, even when data are being acquired directly to computer.

Make sure that all cables are secure and connected to the right ports, that the monitor is turned on and has normal values for brightness and contrast. When a monitor is on and receiving a video signal, its indicator light is green. This either turns yellow when the signal is lost, or goes out completely.

3.7. Ergonomics: High Comfort Equals Low Stress

Time available on a confocal instrument can be both limited and expensive, but working under a perpetual sense of urgency can be distracting and stressful. To be as productive as possible, the user should be comfortable and relaxed. Pieces of technology that cost hundreds of thousands of dollars should be a pleasure to use.

1. Instrument layout: The very occasional user has little control over how the room and the instrument itself are laid out, but if you are using an instrument regularly, make a mental note of any aspect of your time on the instrument that was distracting or annoying. Spend time between confocal sessions thinking up solutions. The chances are that if it bothers you, a suggested change will go down well with other users. Are the room lights too bright or inaccessible? Does the keyboard give you cramps? Is the chair high enough/soft enough? Is there enough workspace? Is the hard drive always full? Is the user manual close at hand? Do the cooling fans broil your feet?

Take a look around a well established EM facility, and note the dedicated room for each instrument, the subdued lighting, the big comfortable armchairs, and the neat banks of drawers for specimens, plates, and useful paraphernalia. Such is the comfort level that is demanded for flagship instrumentation, and high-performance laser scanning systems should be no exception.

2. *Illumination:* Microscopists do it in the dark. But they need light for taking notes and finding their equipment. To avoid having to keep getting up and down to use the main room lights, a desk lamp near the microscope is essential. A lamp with a dimmer switch is even better, as the user can then match ambient light intensity with the brightness through the oculars. The Meridian TR has an integral gooseneck task light, which has both a dimmer switch and a removable red shade. This lets the user work in comfort and makes it easy to maintain dark adapted vision for locating dim specimens through the oculars.
3. *Bench space:* Early confocal microscopes were placed on standard size antivibration tables, which help improve the scanned image, but are most uncomfortable for performing the nonconfocal functions of finding the sample through the microscope. Most modern instruments are now configured on custom-built antivibration tables, which are less restrictive. However, immunity to bench vibrations can be built in to a microscope so that it is either used on a regular desk top or is enclosed in its own integral chassis.

   Very few systems are equipped with adequate work space, so it is often necessary to place a workbench on one side of the instrument for holding notes, immersion fluid, tissues, and specimens. Printers and storage devices also need space near the instrument, and can have surprisingly large footprints.
4. *Storage space:* The media used for storage need to be kept in an organized fashion. Many multiuser facilities provide backup storage to minimize the risk of useful data being permanently lost. Date stamped files can be accessed by delving into the booking log to see when a particular run of work was completed.

   Users should always keep their own data, usually organized by subject rather than date. Some facilities offer no long-term storage because they are too busy to administer it. There is no point having an archive copy somewhere if the archive is not readily accessed. The onus is then entirely on the user to take good care of their data, and make their own backup copies.
5. *Noise:* Laser power supplies and cooling systems can be noisy, but the sound of rushing air is rarely intrusive to the user. If the room itself suffers from external noise, have a pair of headphones handy and a good collection of CDs. The data CD of most computers is audio compatible, a creature comfort that is often overlooked.

3.8. Software Design

Most menu-driven software is laid out as a simple toolbox, which groups commands under a number of headings, and grays out those that are temporarily unavailable, but otherwise offers very little help to the user in deciding which commands to use in what order. A more productive alternative is to have software modules, each of which walks the user through a known experimental

procedure. This logical software design is much more difficult to implement because it requires intimate knowledge of how it might be used so that it can guide the user rapidly to the final result, without being too restrictive.

A promising compromise is to have toolbox type software that contains in it extensive use of wizards, macros, templates and cue cards. With all these aids, the complete palette of tools is always available to the user, but streamlined pathways are made available that guide the user through the mass of options and allow for rapid repetition of experimental procedures.

1. *Macros:* A macro is simply a recorded sequence of commands that is accessed with a programmed keystroke or keyword. They are useful to speed up tediously repetitious procedures, or to ensure that exactly the same instructions were applied to each sample.
2. *Templates:* Most confocal programs save the parameters used in acquiring a data set, either as a separate parameter file, or as a custom header within the image file itself. If an identical scanning procedure is required, the scan parameters can be reset by loading the header or parameter file as a template. This is especially useful for quantitative analyses where all data must be acquired under identical scan conditions.
3. *Wizards:* Wizards are a standard feature of Windows® programs, which guide the user through an involved procedure, asking for appropriate choices to be made at each step of the process. For example, the TR software has a Dye Wizard that uses an internal database of fluorophore spectra to calculate the most efficient optical layout for simultaneous detection of multiple dyes. It lists the dyes in its database and allows users to add their own. Then it asks which dyes are to be used in combination, then it tests whether this combination is feasible and determines the best layout for optimal detection. Finally, it reconfigures the hardware to match these predictions, and changes the labels on channels to reflect what is being imaged in each channel.
4. *Cue cards:* Cue cards offer information and short cuts to performing different commands and can be used like macros for consistently repeating user defined procedures, or like Wizards to sequentially inform the user about appropriate options that carry out the selected commands. For example, to make a good SFP reconstruction, a Z-series must be scanned, the data should be filtered to minimize noise effects, then thresholded to choose a range of interest. A preliminary reconstruction should be made to confirm image quality, then a complete series of reconstruction views assembled to make a movie loop. An SFP cue card could contain action items for each of these steps, with descriptive details of what settings would be appropriate. But unlike a Wizard, any of the steps can be undertaken at any time, and all the other menus on the main screen are still available so the power user can mix and match at will.

3.9. Economics: Time Management, Billing, and Laser Conservation

1. *Laser life:* Lasers all have a rated longevity, expressed in mean hours of lifetime. Ion gas lasers are the shortest lived but most popular lasers for confocal work,

mainly because of their high intensity and good color. Helium/neon lasers are now available with very good colors, but are barely bright enough unless the delivery system is very efficient. A typical 100 mW argon laser is rated about 2000 h at full power, whereas a 5 mW red helium/neon laser has a 25,000 life expectancy. Water-cooled UV argon lasers have an intermediate life span, about 5000 h.

Longevity of ion lasers can be extended considerably by running them at less than full power, and by switching them to standby mode when not in use. If the laser is not going to be used for an hour or more, it can be powered down to further extend operating life, but care must be taken to leave the cooling fan on for at least 10 min, as a hot shutdown can damage the tube. Omnichrome now has laser power supplies with built-in control of cooling fan shutdown, and new confocal systems such as the Meridian/TR have integrated one-button shutdown. Large water-cooled argon lasers should not be turned on and off repeatedly because startup stresses the tube and reduces life more than an additional few hours of constant operation. They should be turned on once and kept running until the day's work utilizing them is finished.

2. *Mercury arc life:* Mercury arc bulbs have much shorter lifetimes, about 200 h, although the cost of a replacement is comparatively trivial. Again, the latest versions have better power regulation which can vary light intensity and extend bulb lifetime.
3. *Booking time on an instrument:* Billing and time management are important aspects of running a multiuser facility. Every user needs adequate access to the instrument, but demand for instrument time will often outstrip availability. In a university setting, 4-h time slots may be appropriate, morning, afternoon, and evening. High-productivity systems such as the InSight may be booked in 1 h or even half-hour slots because they are so much faster and easier to use than a typical point scanner.

   A good self-correcting booking scheme is to allow each user to sign up for a maximum number of time slots. This allows some users to book the same time each week, while others who need a whole day to scan samples can also allocate appropriate time. As soon as a time slot is used up, more time can be booked so each user never has more than three slots on the sign up sheet. If the instrument is not in use, any user has access, so early morning and lunch times may be free for users to quickly preview samples without using up a time slot.

   Keeping track of printer paper, specific laser usage, and mercury arc burn time is more difficult, as the hourly charges cannot adequately reflect frugal or excessive usage. Inkjet printers are now good enough to generate draft images for lab notes, allowing the facility to keep its dye sublimation printer separate for publication quality printing. Alternatively, an occasional check on the amount of unused paper may be enough to keep all users honest. As a last resort, the ink ribbon can be unwound to reveal negative images of every print taken.

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