Special section on techniques:

Taking the Confusion out of Confocal Microscopy

Nana Rezai

Pathology, University of British Columbia
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Introduction

Confocal microscopy is a major advance upon normal light microscopy since it allows one to see not only deep into cells and tissues, but to also create a three dimensional image. There are many aspects to a confocal microscope that make it a much more versatile instrument than a conventional fluorescence microscope. Although the confocal microscope is often thought of only as an instrument that can create 3D images of live cells, the great versatility of the machines means that many creative ways of examining not just the structural details, but also the dynamics of cellular processes are being developed (Hibbs, 2000).

The term "confocal" refers to the condition where two lenses are arranged to focus on the same point, therefore, sharing the same foci (Hibbs, 2000). In conventional light microscopy, two-dimensional images of the object (usually a section) are formed in the X and Y planes, generally parallel to the plane of sectioning. This is also true of confocal microscopy, but each image represents only part of the thickness of the sample. This is because the microscope optics exclude features outside of the plane of focus. The major optical difference between a conventional microscope and a confocal microscope is the confocal pinholes, which allow only light from the plane of focus to reach the detector. The principle of confocal microscopy is that the "out of focus" light is removed from the image by the use of a suitably positioned "pinhole". This not only produces images of exceptional resolution, but also allows the user to collect optical slices of the object and use them to create a 3D representation of the sample. If the plane of focus is changed, or the object moved, a series of images at different positions can be produced through the thickness of the object, i.e. a series of X-Y images at different Z positions. Such a series of images (a stack) is a three dimensional representation of the object produced by optical (as opposed to physical) sectioning. This is the main feature and a major advantage of confocal microscopy over conventional microscopy. As in conventional microscopy, it is the objective lens of the microscope that forms the image in a confocal microscope, and the quality of the image, the magnification, and resolution are dependent on the quality of the objective lens used (Hibbs, 2000).

Fluorescence imaging, using epi-illumination, is probably the most commonly used imaging technique and has advantages for confocal microscopy. Fluorescence is the emission of light from a molecule in a high-energy state as it falls to a lower energy state (Humphrey et al., 2001). The wavelength of the light produced is determined by the difference in energy. Since electrons have a limited number of possible energy states, the light emitted for a given molecule is at one or more constant wavelength(s). The excited state of the molecule is usually generated by absorbing the energy from a single photon from a light source. Because the energy carried by a photon is inversely proportional to its wavelength, and the molecule must absorb at least as much energy as it emits, the excitation wavelength <u>must</u> be lower than the emission wavelength. The quantum efficiency of a fluorochrome is a measure of how efficiently the excitation energy is converted into emission energy. Most confocal microscopes use the epi-fluorescence design for fluorescence imaging. In the epi-fluorescence method of illumination, the lens functions as both objective and condenser and so the problem of careful alignment of the two lens systems is eliminated.

In general, the confocal principle is most effectively implemented when combined with a point scanning system using a laser light source. This builds

up an image by scanning a point of laser light across the sample in X and Y directions. In the case of a laser scanning system, the detector is generally a photomultiplier tube. The signal from the photomultiplier tube is converted to a digital form that contains both information on the position of the laser in the image and the amount of light coming from the sample. A computer is used to store the intensity value of each point from the detector, and presents these in the correct order on a high-resolution video monitor to display the image. The image is displayed on the computer screen as a shaded gray image with 256 levels of gray which can be suitably coloured later for presentation or publication. To collect a series of images, the computer then shifts the focus by a fixed amount and the object is scanned again to produce the next image at the different Z position. This image is stored and the process repeated to build the 3D data set.

Advantages of Confocal Microscopy

There are a great number of advantages to using confocal microscopy compared to conventional epifluorescence microscopy, the two most important being the ability to eliminate out of focus noise (obtaining better resolution) and the greatly increased sensitivity of the machine. Resolution is defined as the ability to distinguish two closely adjacent objects (Hibbs, 2000). The limit of resolution of light microscopy is determined by the numerical aperture of the objective used and the wavelength of light. For visible light, with the best objective available, the limit of resolution in the "X-Y" direction is approximately 0.1 to 0.2 microns (100 to 200 nm). This limit is approached using a very high quality conventional microscope that has been carefully and correctly set up for transmission light imaging. However, conventional epi-fluorescence microscopy, even when set up correctly, does not have the same level of resolution. A confocal microscope pushes the level of resolution of the light microscope very close to the theoretical limit of 0.1 microns. The "Z" resolution of the confocal microscope, at approximately 0.5 microns, is somewhat lower than the "X-Y" resolution. In ideal conditions, the resolution from a confocal microscope image may be up to 1.4X that achievable in conventional microscopy.

However, increased resolution without increased sensitivity would be of little use as there would not be any visible object to "resolve". Therefore, its sensitivity is the most important advantage of confocal microscopy. A laser scanning confocal microscope has greatly increased sensitivity compared to a conventional epi-fluorescence microscope. In addition to having highly sensitive light detectors, the confocal microscope has the advantage of being able to accumulate (or average) images over time. This method of collection greatly increases the sensitivity of the machine, although with the disadvantage that cellular movement will distort the image. This distortion is less marked if line averaging/accumulation is used rather than whole imaging accumulation. The confocal microscope also has the advantage of being able to readily attenuate the amount of laser light irradiating the sample. This means that a low laser level can be used to orient the image, and then a high, but relatively short exposure can be used to create an excellent image. All of these features of a confocal microscope result in images with a high level of sensitivity.

Confocal microscopy is also useful for the 3D examination of cells and tissues. At the heart of confocal microscopy is the ability to take "optical slices" through the cell or tissue of interest. This ability not only greatly improves the quality of the image, but it also allows one to obtain 3D information on the object, whereby stacks of optical slices are rendered into a 3D representation of the sample after collection. A series of optical slices contains a great deal of information on the shape of structures visible by studying adjacent optical slices. In this manner discrete structures can be readily distinguished from continuous filaments. Simple 3D reconstruction of a series of optical slices can be readily carried out using software provided by the microscope companies, however full manipulation in 3D requires separate software. A very simple way to make a 3D representation of the data is to make an extended focus image by simply adding the optical slices together. A more complex way is to use the computer to render a 3D rotation of the object, which gives the illusion of 3D as the image is rotated (or rocked back and forth). Alternatively, red and green images can be created that have a display offset that gives a distinct impression of 3D when visualized with the appropriate coloured lenses (that is, 3D glasses). 3D information is of great value in establishing not only the subcellular location of fluorescence, but also to establish the relationship between different compartments (Hibbs, 2000). 3D reconstruction does suffer from the notable drawback that it is difficult to present in conventional publications.

Other advantages of confocal microscopy include the ability to use optical sectioning for the analysis and imaging of thick samples (up to a few hundred microns in thickness) and the removal most out of focus information. In a conventional microscope, a single cell under the microscope will be seen in its entirety, even at a very high magnification. Therefore, the image will consist of a highly focused image that is seriously compromised by out of focus information both in front and behind the plane of focus. In a confocal microscope the image created consists of only the focal plane. This very thin optical slice contains a wealth of information
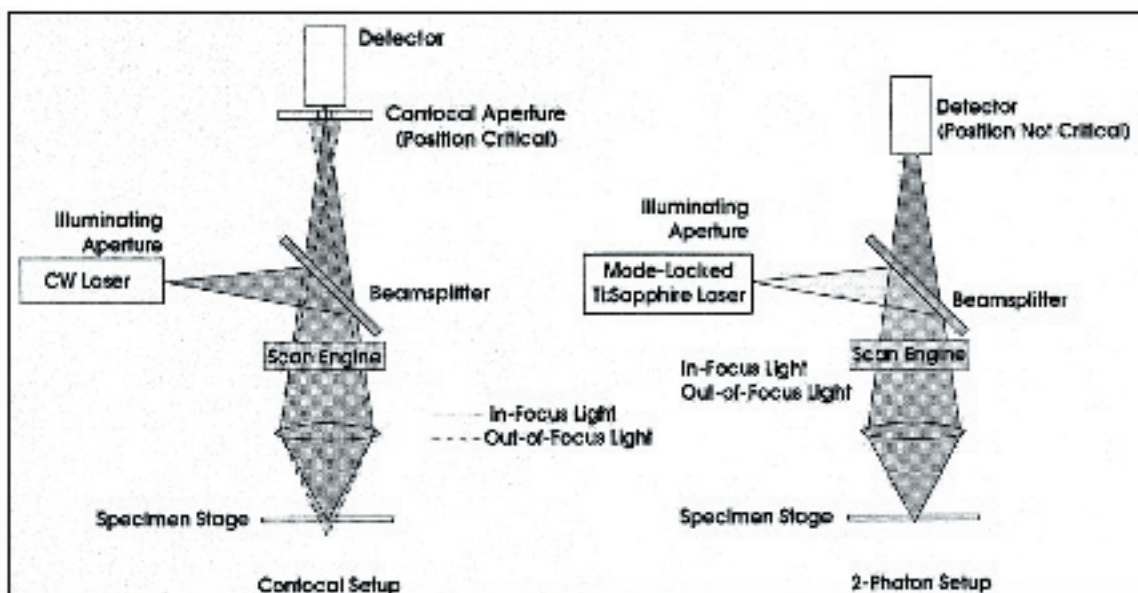
Figure 1 Laser Scanning Confocal Microscope setup versus Two-Photon Confocal Microscope setup. In a confocal microscope, the detector must be the same distance as the light source from the illuminated portion of the sample to effectively block out of focus light. The position of a detector in a two-photon system is variable. Reprinted from the May 2002 issue of Biophotonics International (Rawlings et al.)

on the 3D location of structures within the cell. The ability to take thin optical slices of the object allows one to readily distinguish between surface and internally fluorescence, information which would be difficult to determine using conventional microscopy.

Another important advantage of confocal microscopy is that there is a reduced risk of specimen or sample bleaching. The scanning system of the confocal microscope reduces the exposure of each point to the strong light used for exciting fluorochromes, thus decreasing the risk of bleaching the sample.

Limitations of Confocal Microscopy

The confocal also has serious limitations that one should be aware of if one wishes to make full use of the machine's capabilities. The first limitation of confocal microscopy is that the resolution is limited by the wavelength of light. Although a confocal microscope pushes the limit of resolution to the theoretical limit of light microscopy, it does not resolve better than about 0.1 microns (under ideal conditions). This limit in resolution is very important in biology as many subcellular structures are at or beyond this limit. Objects smaller than this resolution limit can be visualized if a suitable dye is used (such as phalloidin to stain microtubules), but if they are not resolved then two closely associated structures will appear as one. The limit to the resolution of a light microscope may cause misleading interpretations of the data when one is using two dyes or antibodies to establish whether the structures of interest are

separated within the cell. The limit of resolution may lead to the conclusion that they are associated with the same structure (for example transport vesicles), when in fact they are associated with distinct structures that are not being resolved by the microscope.

Photodamage is also a limitation in the use of confocal microscopes. Although the damage is less than that of a conventional microscope, the high intensity of the laser, when focused onto a fine spot within the sample, can still result in photodamage to both the dye being used and to other cellular components. It is possible to reduce this damage through the use of suitable antifade reagents which can control photobleaching of the dye. However, antifade reagents work well in fixed cell preparations, but are not as effective or may be toxic to live cells (Hibbs, 2000). The new 2-photon microscopes, which will be discussed below, may be the solution to the important problem of photodamage to cellular constituents.

Another limitation of confocal microscopes is that they are very expensive instruments and consequently, are often used by a diverse group of people within a department or institute. This can create a problem with the correct care and maintenance of the machine. Considerable training of personnel is required to gain maximum benefit from a confocal microscope.

Different Types of Confocal Microscopes
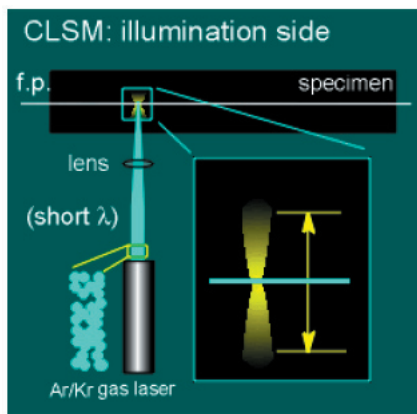
In standard light microscopy, a relatively large

Figure 2 Gas lasers in laser scanning confocal microscopes focusing on the focal plane (f.p.) within a specimen.

Figure 3 Solid state lasers in two-photon confocal microscopy focusing on the focal plane (f.p.) within a specimen.

volume of the sample is illuminated, and the light gathered by the objective lens comes not only from the point of focus, but also from below and above the focal plane. The resulting image contains the in-focus light as well as the haze or blur caused by the light from the out of focus planes. The basic principle of confocal microscopy is to eliminate the out of focus light, thus producing an accurate, sharp and high-resolution image. As mentioned previously, this is achieved by the use of pinhole apertures.

In a typical setup for a laser scanning confocal microscope, a pinhole is placed in front of the light source to produce a distinct and spatially constrained illumination point. The light passing through this aperture is focused on the sample, while a second pinhole is placed in front of the detector (Figure 1). If the optical distance from the detector aperture to the focal point is exactly the same as that between the focal point and the illuminating aperture, only the light generated at the focal point will reach the detector since the pinhole will block out the out of focus light. The signal from the detector is then digitized and passed to a computer, which handles the data collection. Given that the confocal microscope illuminates and collects only from a point source, the image has to be digitally built up by scanning the sample in the X and Y directions. Therefore, the computer receives data from each illuminated point in the sample as it is scanned and uses special software to reconstruct a digital image.

Two-photon (also referred to as multi-photon) microscopy setups are virtually the same as laser scanning confocal microscopy setups, except that the continuous-wave laser is replaced with a mode-locked Ti: sapphire laser which operates in the near-infrared range (Figure 1). The laser produces tens of kilowatts of peak power in a series of low-energy pulses which are approximately 10nJ per pulse. It is tuned to a wavelength
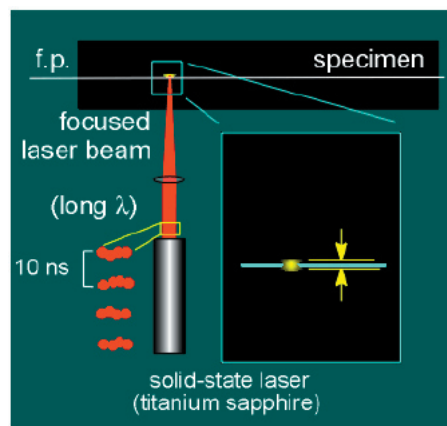
about twice that of the intended absorption wavelength of the sample, which requires a nonlinear two-photon (or more) process to excite the chromophores. Since the cross section for the two-photon process varies as the inverse fourth power of the distance from the focus, only at the focal point will there be enough energy to induce fluorescence (Rawlings et al., 2002). Thus, the need for pinholes is eliminated because there is virtually no fluorescence outside of the focal plane.

There are several major advantages in the use of the rather expensive and technically sophisticated two-photon confocal microscopy versus laser scanning confocal microscopy. First, out of focus bleaching is reduced, since chromophore fading is decreased at the focal point and eliminated in the surrounding areas (Rawlings et al., 2002). Two-photon microscopy also increases sample penetration because of the reduced absorption of near-infrared radiation. This allows thick, live tissues to be imaged with little damage to the environment (Michalet et al., 2003). In addition, two-photon microscopy is believed to result in an increased sensitivity since the elimination of the pinhole allows all of the signal to reach the detector (Rawlings et al., 2002).

Lasers in Confocal Microscopy

Lasers are used as the light source in confocal microscopy because they provide images from samples at a single, specific wavelength, thus producing images of a very pure colour. Lasers provide sufficient intensity to compensate for the light lost after passage through the detector pinhole, thereby producing a distinct and spatially constrained light point. Laser light has very different properties from normal light. First, the light released is **monochromatic** – it is released at one specific wavelength of light, producing an image of one specific colour. The wavelength of light is determined by the amount of energy released when the electron
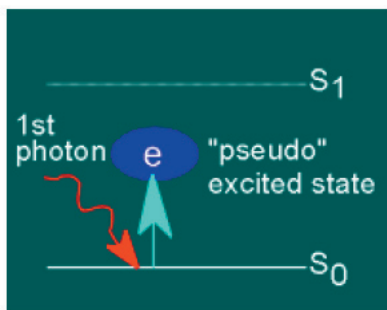
Figure 4 An electron being launched into a "pseudo" excited state.

Figure 5 An electron being elevated into the true excited state from the pseudo excited state.

drops to a lower orbit. A second important feature of lasers in confocal microscopy is that the light released is **coherent** or "organized" in that each photon moves in step with the others. Finally, laser light is also very **directional**. The light released has a very tight beam and is extremely strong and concentrated. In comparison, a flashlight releases light in many directions, therefore producing a light signal which is very weak and diffuse.

As mentioned previously, different forms of confocal microscopes use different types of lasers as light sources. Laser scanning confocal microscopes use gas lasers, such as an argon/krypton laser. Gas lasers give a steady stream of photons (Figure 2). While the laser light is focused on the focal plane considerable fluorescence is also created above and below the focal plane. To get rid of this light a pin hole is introduced between the detector and the specimen. Light outside of the focal plane is largely excluded from hitting the photon multiplier tube while light in the focal plane passes through the pin hole and is thus detected by the photon multiplier tube. This combination creates a sharp image from the specimen of interest. On the other hand, Multiphoton or 2-photon confocal microscopy uses solid state lasers, such as Ti-sapphire lasers. These types of lasers give off photons in short (approximately 10 ns) pulse intervals while giving light off at a longer wavelength (Figure 3). Therefore, the energy of the light is lower so when a probe absorbs a photon from the first pulse of light the electron is not elevated completely to an excited state. Instead, the electron is launched into a "pseudo" excited state and from this state falls back to the ground state (S0) without giving off any fluorescence (Figure 4). If the probe is to absorb a second photon, coming from a subsequent pulse of the laser, then the electron can be elevated to the true excited state (Figure 5). Thus, florescent light will be given off as it returns to the ground state.

Tissue Preparation

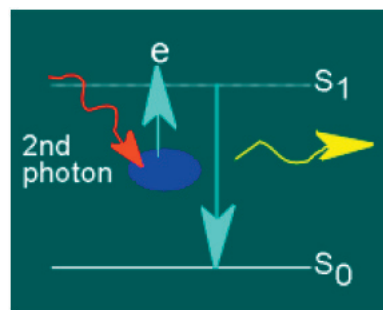Unlike samples for conventional microscopy, tissue and cell preparations for confocal microscopy do not need to be thin. In fact there is little or no advantage in viewing a thin section with a confocal microscope. There are, however, physical limits on how deep it is possible to optically section into a sample. These limits are dependent on the magnification required and are mostly determined by the working distance of the objective lens, the opacity of the tissue and the strength of the signal to be detected (Humphrey et al., 2001). It is possible to optically section several millimeters into a transparent tissue at low magnification (20X objective) while the practical limit with a normally opaque tissue with a 63X objective lens may be 20 micrometers or less.

It is advised that samples should be put onto glass slides and mounted with a glass coverslip. This is critical since confocal optical sectioning involves moving the objective lens rapidly towards the sample and back again, it is thus important to have a physically robust mounting system so that samples do not move with the objective lens. When dealing with living cells, it is advantageous to use an inverted microscope to view the living cells growing in chambers.

In confocal microscopy, as in conventional microscopy, it is necessary for the tissue specimen of interest to demonstrate some form of optical contrast between different areas of the sample for visualization. This requires staining of the tissue specimen using a label which either absorbs or reflects light or is fluorescent. As a general guide, fluorescent labels are most versatile and represent a good starting point. Most staining protocols which work well for conventional microscopy can be easily adapted for confocal microscopy, although for thick tissue sections, the diffusion of labels into tissue is often a major limiting factor. It may be useful for some tissue sections to increase the degree of staining for confocal microscopy since thin optical sections of the samples are used (Bacallo et al., 1990; Bacallo et al., 1994). In other cases, it may be necessary to considerably increase incubation periods and to use detergents, such as 1% Triton x100 in PBS for 4 hours, or other treatments to allow the label to penetrate (Humphrey et al., 2001). Vibratome sections of solid tissues (50-100

im thick) are useful way to prepare thick tissue for confocal microscopy (a Vibratome instrument has a rapidly vibrating razor blade which cuts samples into slices).

Applications

Confocal microscopy is now being used in a very large number of fields of science. These applications include the use of confocal microscopy for live cell imaging to image within a cell or tissue, protein trafficking where green fluorescent protein (GFP) or other fluorochromes can be used to track intracellular protein movement by attaching them to proteins of interest, and for locating genes on chromosomes, in which hybridization and fluorescence PCR can be used to locate individual genes to specific locations on individual chromosomes. Confocal microscopy is also used to analyze subcellular functions, such as pH gradients and membrane potentials, using specific fluorescent dyes and to measure intracellular changes in ion concentrations of molecules such as calcium, sodium, magnesium, zinc and potassium.

Confocal microscopy is a highly-regarded imaging and analysis tool which is generally believed to have a high publication value. Although the image capture process can often be quite time-consuming, with the length of time to image approximately 50 z optical slices being close to 15 minutes, it is an extremely useful method for imaging a wide range of biological specimens.

Due to the high costs involved in purchasing and maintaining confocal microscopes, there are only a few facilities in Vancouver which own the machines. The Bioimaging Facility at the University of British Columbia, supervised by Dr. Elaine Humphrey, is home to a large supply of microscopes, including both a Bio-Rad Radiance laser scanning microscope and a Bio-Rad Multiphoton confocal microscope. These microscopes are available for use by visitors at an hourly fee. In addition, the *iCAPTUR⁴E* Centre/McDonald Research Laboratories at St. Paul's Hospital has recently purchased a Leica confocal and multiphoton microscope which will also be available for use by outside personnel at a cost of approximately $50 per hour.

Confocal microscopy, whether laser scanning or the novel two-photon system, has generated a tremendous amount of excitement in the research community. This technique has rapidly become the technique of choice for researchers, particularly in the biosciences. With the recent availability of more affordable confocal and two-photon microscopes, this technology holds a great deal of potential in the future of science.

References

1. Bacallo, R., Bomsel, M., Stelzer, E. H. K., and De Mey, J. (1990). Guiding principles of specimen preservation for confocal fluorescence microscopy. *In* "Handbook of Biological Confocal Microscopy" (J. Pawley, ed.), pp. 197-205. Plenum, New York.

2. Bacallo, R., and Garfinkel, A. (1994). *In* "Three-Dimensional Confocal Microscopy: Volume Investigation of Biological Systems." (Stevens, J. K. et al., eds.), pp. 172-4. Academic Press, London.

3. Hibbs, A. (2000). Confocal Microscopy for Biologists: An Intensive Introductory Course. (BIOCON, ed.), pp. 2-9.

4. Humphrey, E., and Norman, K. (2001). A Practical Introduction to Confocal Microscopy: for Users of the Bio-Rad Radiance 2000 Confocal Microscope. (University of British Columbia), pp. 1-15.

5. Michalet, X., Kapanidis, A.N., Laurence, T., Pinaud, F., Doose, S., Pflugghoefft, M., and Weiss, S. (2003). The Power and Prospects of Fluorescence Microscopies and Spectroscopies. *Annu. Rev. Biophys. Biomol. Struct*. 32: pp. 161-182.

6. Rawlings, S., and Byatt, J. (2002). How Microscopy Produces a Sharper Image. *In* "Scanning Microscopy" (Biophotonics International), pp. 1-4. Laurin Publishing Co. Inc.