Confocal Microscopy Opens the Door to 3-Dimensional Analysis of Cells

R.E. Rowland and E.M. Nickless
Institute of Molecular Bio Sciences
Massey University
P. Bag 11-222
Palmerston North
New Zealand
e-mail: R.E.Rowland@massey.ac.nz

Abstract: Biologists can now observe cells in 3D using confocal microscopy. Using laser light, a confocal microscope optically sections cells non-invasively and compiles a three-dimensional image of microscopic structures on a computer screen analogous to that of a CAT scan of humans.

Keywords: Confocal microscopy, fluorescence, 3D-imaging

Introduction

The invention of the confocal, laser-scanning microscope, known simply as a confocal microscope, heralded for biologists one of the biggest breakthroughs in microscopy for decades. The main advantage of this instrument is that it allows researchers to observe an object in three-dimensions and to gather quantitative data such as thickness, area, and volumes of cellular structures.

How does a confocal microscope work?

The first point to realize is that confocal microscopy uses a conventional compound light microscope with conventional optics. The main difference with confocal microscopy lies in the way the image is captured and conveyed to a computer screen. A conventional light microscope captures light from below that passes through the specimen and on up through the objective and eyepieces to the eye. With confocal microscopy, a laser light beam enters the head of the microscope, passes down and out of the objective onto the microscope slide, and excites a fluorescent dye in a stained specimen. The fluorescent light is then captured by the objective. The way this fluorescent light is manipulated makes confocal microscopy distinctive.

A module that sits on top of the head of the microscope receives the fluorescent image of the specimen and transfers it to a computer screen. This is much like a camera capturing an image under the microscope. The module is designed to reject light above and below the plane of focus and thus gives an optical section. Fig.1 shows how the laser light enters the back of the module, passes through a small “in pinhole” <100µm in diameter, is reflected by a beamsplitter lens (a dichroic lens that reflects the low wavelength laser light but allows longer fluorescent light to pass through) and out the objective where it is focused to a fine point onto the specimen. Different planes of the specimen are put in focus by moving the stage of the microscope.

The fluorescent dye in the specimen is excited by the laser light and emits light of longer wavelengths that scatters in all directions. Some passes back up into the objective through the beam splitter lens to the “out pinhole” and on to the detector. All fluorescent light originating from the fine point of focus (black line in Fig. 1) is refracted by the objective such that it passes through the “out pinhole;” whereas, scattered fluorescent light above and below the plane of focus (red dotted lines) is refracted at a different angle and does not hit the “out pinhole.” In summary, only light from the plane of focus is captured by the computer. This gives a sharp crisp image undistorted by cellular structures above or below the plane of focus (Fig.2). The image is considerably sharper than that observed by conventional fluorescence microscopy which collects all the fluorescence emitted.

Why the name “confocal microscope”?

A further question often asked is where the word “confocal” comes from and why scanning? The Latin prefix “con” is used here to mean together, thus confocal means together in focus. In optical terms, a common point of focus produced by two light rays is termed “confocality”. In designing a confocal microscope it is essential that the distance from the “in pinhole” to the point of focus is the same distance as the “out pinhole” to the point of focus. This can be checked by beaming light rays through both pinholes.
Figure 1. Diagram illustrating the operation of a confocal microscope. Laser light, dark shaded; fluorescent light, light shaded. The laser light enters the module on top of the microscope via an “in pinhole”, is reflected by the beamsplitter lens, and exits the objective where it can be focused to a fine point at the plane of focus. Laser light excites fluorescent stain in the specimen. Fluorescence emitted from the plane of focus (solid line) enters the objective and passes through the beamsplitter and out the “out pinhole” to the detector. Fluorescent light above and below the plane of focus (dashed lines) does not enter the “out pinhole” and thus is rejected.
so that both reached a common point of focus. The term “scanning” comes from the fact that a galvanometer in the module head is used to scan the pin-point of laser light across the plane of focus in order to obtain a square picture, much like looking face-on to a thin playing card.

Figure 2. A single 0.6µm optical section through a 10µm-thick cross-section of a maize stem showing a vascular bundle and surrounding parenchyma cells. Bar = 50µm.

Achieving serial sections
Most confocal microscopes are computer driven with a precision-made electronic step motor attached to the microscope stage. Computer commands move the stage up and down under the control of the step motor in increments as small as <1µm. One can focus precisely up and down through a specimen, whilst looking at the computer monitor, by using computer commands rather than focusing controls on the microscope. A clear fully-focused fluorescent image is achieved every step of the way. This allows one to collect any number of optical sections through the specimen.

The ability to section non-invasively allows one to collect a uniform series of optical sections through the material at steps of 0.6µm, which is the limit of resolution of a light microscope in Z-depth. The sections from each plane are stored and then assembled into a three-dimensional construct on a high-resolution computer monitor. The image can also be rotated and observed from different angles or even from the other side. One can perform all sorts of measurements, such as volume, area, and width of the structure of interest, be it a chromosome, pollen grain or fungal hypha infecting plant tissue. Some instruments can perform dual or triple scanning of double or triple-stained specimens and provide overlapping images. For hard copies, there are a number of image-capturing systems available that can be attached to the monitor.

**Magnification and resolution**

Because confocal microscopy utilizes a conventional compound light microscope, the magnifying power and resolution are the same. However, it is possible to increase the magnification of the image obtained with the confocal microscope by using the zoom facility. The limit of resolution of a confocal microscope is also governed by the same optical laws as bright field microscopy, which is 0.2µm in X and Y and 0.6µm in Z depth. However, the image obtained by confocal microscopy is usually much clearer than conventional fluorescence microscopy. In practical terms, this means that a confocal microscope can resolve objects that range in size from bacteria or organelles to something as big as a small insect, providing the laser light can penetrate the structure and provide an undistorted fluorescent signal.

**Achieving a 3-dimensional image**

Most commercial confocal microscopes are equipped with the software necessary to achieve 3-dimensional images by assembling a stack of serial sections. Commands allow one to present a direct side-on view of a structure and color it green, then overlay with an image taken at an angle of =10 degrees and colored red. This color combination is called an anaglyph and when viewed with red/green cellophane glasses, gives a stereoscopic view of the structure. This is illustrated in Figs 3 - 8 (color image on cover of this issue).

**Exploiting the technology**

Biologists quickly realized that confocal microscopy offers one big advantage: the ability to perform optical sectioning at the cellular level in a non-invasive way. It eliminates the need to embed material and cut serial sections. The confocal microscope offers biologists the chance to investigate the microscopic world in 3 dimensions. A second advantage is the ability to quantify the visual data that greatly enhanced the use of confocal microscopy. The scientific literature contains many confocal microscope studies ranging from the cellular to the macroscopic. Molecular biology in particular is exploiting the technology. It is being used to unravel the 3-dimensional functioning of genes in nuclei, for example to show when they become active (Rowland and Nickless 1999). In fact, confocal microscopy is now applied worldwide not only in all avenues of the biological sciences but also the physical sciences (see Further Reading for range of applications). At one conference I attended, a geologist showed wonderful 35mm colored slides of vertical channels through
Figures 3 - 5. 3-D anaglyphs of pollen grains stained with acridine orange. Bar = 10µm. Fig.3 shows a single Hoheria sp. pollen grain; Fig.4 shows Myosotis monroi pollen grains on the surface of a stigma; Fig.5 shows Agathis australis (kauri) pollen grain obtained from a swamp profile, estimated age 10,000 years. Figures 6 and 7. 3-D anaglyphs of dividing somatic cells of Vicia faba, broad bean, root-tips stained with Feulgen. Fig.6 shows mostly interphase cells, note the bright heterochromatic knobs. Also note the early prophase at left, late prophase in middle, and metaphase top right. Bar = 5µm; Fig.7 shows a prominent anaphase in the center and a prophase cell at top left. Bar =5µm. Figure 8. 3-D anaglyph of germinating yeast spores stained with acridine orange. Bar = 5µm. Color images of these anaglyphs are found on the cover of this issue. Anaglyph 3-D glasses are included with this issue. View the color image using the glasses: Left eye = blue, right eye = red.
granite into which the fluorescent dye had penetrated to a depth of 100µm!

**Biological application of confocal microscopy**

In this article, we elucidate the advantages of confocal microscopy for biologists by including illustrations of a range of research that has been conducted in our laboratory. The Confocal Microscope Facility at Massey University is a multi-user facility used by plant biologists, veterinary researchers, food technologists, dairy product researchers, microbiologists, cyto geneticists, molecular biologists and even technologists examining bitumen (asphalt-like products). The potential of the machine is limited only by the type of questions one wishes to ask. Unquestionably, it has created a sense of inquiry where features relating to growth and development, injury and disease, mode of infection of pathogens, chromosomal behavior, milk processing and product development all require some quantitative understanding of events that unfold. The accompanying illustrations, Figs. 3 to 8, are colored anaglyphs of various cellular structures that can be viewed with red/blue cellophane glasses to obtain the 3D effect; these usually can be obtained from the local theater.

**Dr R E (Al) Rowland** (email: R.E.Rowland@massey.ac.nz), is a Senior Lecturer in Plant Biology and Cyto genetics in the Institute of Molecular Bio Sciences, Massey University, P. Bag 11-222, Palmerston North, New Zealand. **E M (Liz) Nickless** is Manager of the Confocal Microscope Facility in IMBS, Massey University.

**Literature Cited**


---

**Call for Nominations**

**President-Elect & Steering Committee Members**

ACUBE members are requested to nominate individuals for the office of President-Elect and two at large positions on the ACUBE Steering Committee.

If you wish to nominate a member of ACUBE for a position, send a Letter of Nomination to the chair of the Nominations Committee: Dr. Nancy Sanders, Division of Science, Truman State University, Kirksville, MO 63501-0828, Voice -- (816) 785-4619 FAX (816) 785-4045, E-mail -- sc26@nemo.mus.edu