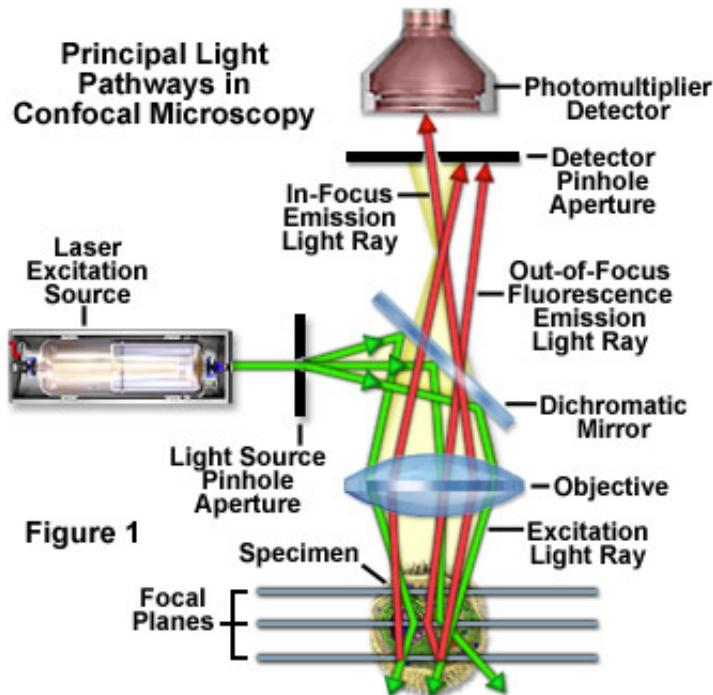


## Confocal Microscopy: Basic Concepts

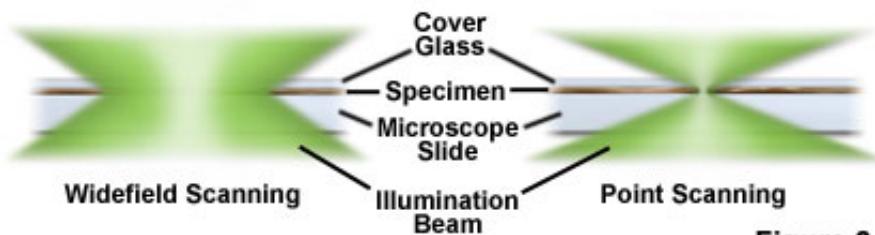
Confocal microscopy offers several advantages over conventional optical microscopy, including shallow depth of field, elimination of out-of-focus glare, and the ability to collect serial optical sections from thick specimens. In the biomedical sciences, a major application of confocal microscopy involves imaging either fixed or living cells and tissues that have usually been labeled with one or more fluorescent probes.



When fluorescent specimens are imaged using a conventional widefield optical microscope, secondary fluorescence emitted by the specimen that appears away from the region of interest often interferes with the resolution of those features that are in focus. This situation is especially problematic for specimens having a thickness greater than about 2 micrometers. The confocal imaging approach provides a marginal improvement in both axial and lateral resolution, but it is the ability of the instrument to exclude from the image the "out-of focus" flare that occurs in thick fluorescently labeled specimens, which has caused the recent explosion in popularity of the technique. Most current confocal microscopes are relatively easy to operate and have become part of the basic instrumentation of many multi-user imaging facilities. Because the resolution possible in the laser scanning confocal microscope (**LSCM**) is somewhat better than in the conventional widefield optical microscope, but still considerably less than that of the transmission electron microscope, it has in some ways bridged the gap between the two more commonly used techniques. Figure 1 illustrates the principal light pathways in a basic confocal microscope configuration.

In a conventional widefield microscope, the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye or projected directly onto an image capture device or photographic film. In contrast, the method of image formation in a confocal microscope is fundamentally different. The illumination is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen (Figure 2). The images produced by scanning the specimen in this way are called optical sections. This terminology refers to the noninvasive method by which the instrument collects images, using focused light rather than physical means to section the specimen.

## Widefield Versus Point Scanning of Specimens



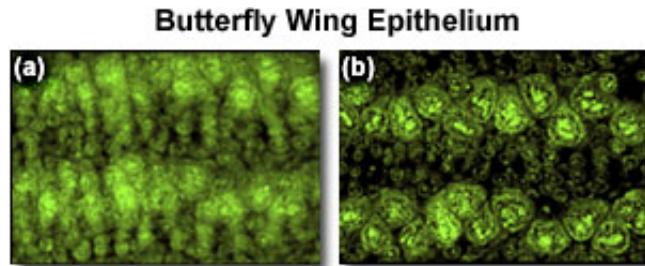
**Figure 2**

The confocal approach has facilitated much more useful imaging of living specimens, enabled the automated collection of three-dimensional (z-series) data, and improved the images obtained of specimens using multiple labeling. Figure 3 presents a comparison of a conventional epifluorescence image with a confocal image of similar regions of a whole mount of a butterfly pupal wing epithelium stained with propidium iodide. There is a striking improvement of resolution of nuclei in the LSCM image due to elimination of out-of-focus fluorescence flare.

The laser scanning confocal microscope (LSCM) is currently the most widely used confocal variation for biomedical research applications. Emphasis is placed on the LSCM in this introduction, since it is the design most likely to be encountered by the novice user. Other alternative designs of the instruments are favored in specific niches within the field of biological imaging. Most of the protocols for specimen preparation can be used, with minor modification, for any of the confocal instrument variants, as well as for other methodologies for producing optical sections such as deconvolution techniques and multiple-photon imaging.

### Evolution of Confocal Microscopy

The invention of the confocal microscope is usually attributed to Marvin Minsky, who produced a working microscope in 1955. The development of the confocal approach was largely driven by the desire to image biological events as they occur in living tissue (*in vivo*), and Minsky had the goal of imaging neural networks in unstained preparations of living brains. The principle of confocal imaging advanced by Minsky, and patented in 1957, is employed in all modern confocal microscopes. Figure 1 illustrates the confocal principle, as applied in epifluorescence microscopy, which has become the basic configuration of most modern confocal systems used for fluorescence imaging. Minsky's original configuration used a pinhole placed in front of a zirconium arc source as the point source of light.



**Figure 3**

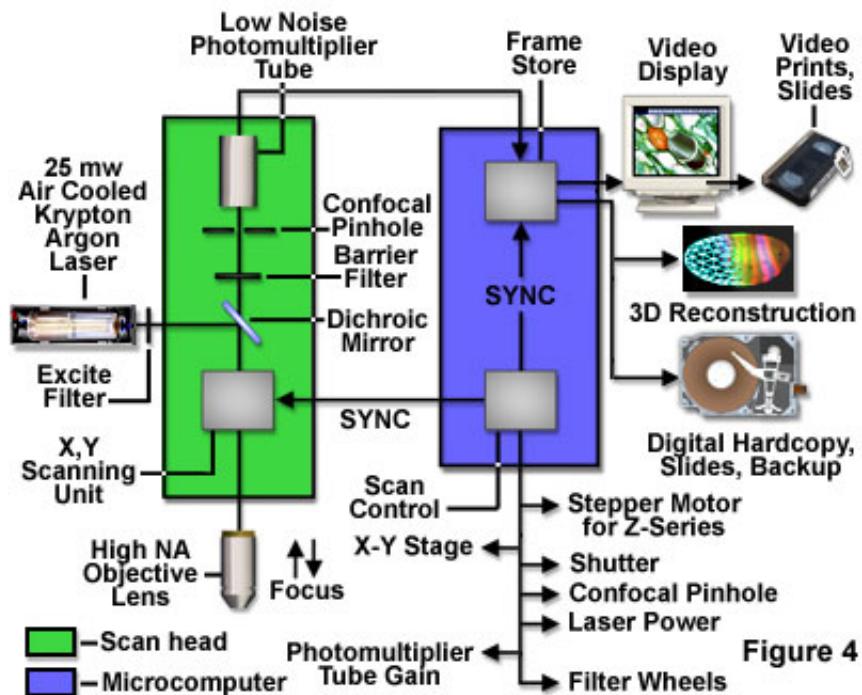
The point of light was focused by an objective lens at the desired focal plane in the specimen, and light that passed through it was focused by a second objective lens at a second pinhole having the same focus as the first pinhole (the two were confocal). Any light that passed the second pinhole struck a low-noise photomultiplier, which generated a signal that was related to the brightness of the light from the specimen. The second pinhole prevented light originating from above or below the plane of focus in the specimen from reaching the photomultiplier. The use of spatial filtering to eliminate out-of-focus light or flare, in specimens that are thicker than the plane of focus, is the key to the confocal approach. In Minsky's writings he also described a reflected light version of the microscope that used a single objective lens and a dichromatic mirror arrangement that became the basis for the systems currently in use.

In order to build an image using the confocal principle, the focused spot of light must be scanned across the specimen in some way. In the original instrument built by Minsky the beam was kept stationary and the specimen itself was moved on a vibrating stage. This arrangement has the advantage that the scanning beam is held stationary on the optical axis of the microscope, which can eliminate most lens defects that would affect the image. For biological specimens, however, movement of the specimen can cause wobble and distortion, resulting in a loss of resolution in the image. Furthermore, it is impossible to perform various manipulations on the specimen such as microinjection of fluorescently labeled probes when the stage and specimen are moving.

Regardless of the means by which the illuminating beam is scanned across the specimen, an image of the specimen must be

produced. A real image was not formed in Minsky's original design, but instead the output from the photomultiplier was translated into an image on the screen of a military surplus long persistence oscilloscope that had no provision for recording. Following the debut of his invention, Minsky later wrote that the image quality in his microscope was not very impressive because of the quality of the oscilloscope display and not because of poor resolution achieved by the microscope itself. It is now clear that the technology was not available to Minsky in 1955 to fully demonstrate the potential of the confocal approach, especially for imaging biological structures. He stated that this is possibly a reason that confocal microscopy was not immediately embraced by the biological community, who were, and still are, a highly demanding group concerning the quality of their images. At the time, they had available light microscopes with excellent optics, and could easily view and photograph their brightly stained and colorful histological tissue sections onto high-resolution color film. In today's confocal microscopes, the image is serially built up from the output of a photomultiplier tube or captured using a digital camera incorporating a charge-coupled device, directly processed in a computer imaging system, displayed on a high-resolution video monitor, and output on a hard copy device, with outstanding results. The information flow in a modern laser scanning confocal microscope is diagrammed in Figure 4.

### LSCM Information Flow Schematic Diagram



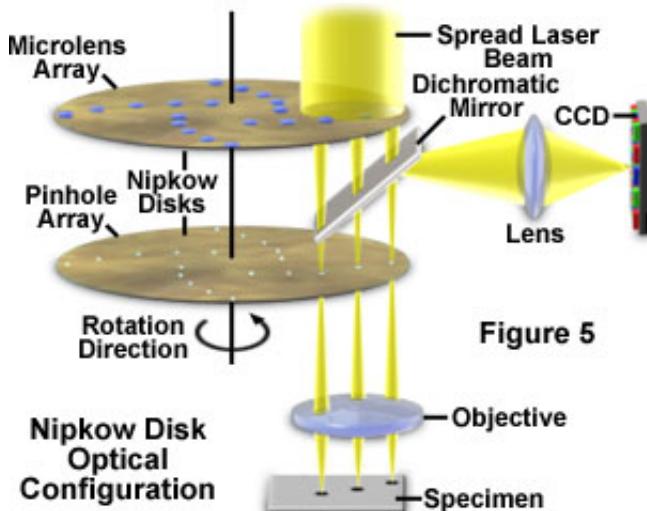
The basic optics of the optical microscope have remained fundamentally unchanged for decades because the final resolution achieved by the instrument is governed by the wavelength of light, the objective lens, and the properties of the specimen itself. The dyes used to add contrast to specimens, and other technology associated with the methods of optical microscopy, have improved significantly over the past 20 years. The growth and refinement of the confocal approach is a direct result of a renaissance in optical microscopy that has been fueled largely by advancements in modern technology. A number of major technological advances that would have been a benefit to Minsky's confocal design have gradually become available (or more affordable) to biologists and other microscopists. Among these are stable multiwavelength lasers for improved point light sources, improved dichromatic mirrors, sensitive low-noise photodetectors, fast microcomputers with image processing capabilities enhanced by availability of affordable large-capacity memory chips, sophisticated image analysis software packages, and high-resolution video displays and digital image printers.

These technologies were developed independently, and since 1955, have been gradually incorporated into confocal imaging systems. As one example, digital image processing techniques were first applied effectively in the early 1980s by researchers at the Woods Hole Oceanographic Institute. Using what they termed "video-enhanced microscopes" they were able to image cellular structures such as microtubules, which are just beyond the theoretical resolution of the optical microscope. The apparent increase in resolution was enabled by digital enhancement of images that were captured using a low light level silicon intensified target (**SIT**) video camera connected to a digital image processor. The cellular structures were imaged using differential interference contrast (**DIC**) optics, and the images were further enhanced using digital processing methods.

The classification of confocal microscope designs is usually done on the basis of the method by which the specimens are scanned. The two fundamental means of scanning are to scan either the stage or the illumination beam, and there are at least two fundamentally different methods of beam scanning. The original design by Minsky was a stage scanning system that was driven by a primitive tuning fork device, and it was rather slow in building an image. Current stage scanning confocal designs that have evolved from the original concept are primarily used in materials science applications such as the microchip industry. Systems based upon this principle have recently become popular in biomedical applications involving screening of

## DNA on microchips.

A more practical alternative for most imaging of biological systems is to scan the illumination beam across a stationary specimen. This approach is the basis of many of the systems that have evolved into the research microscopes that are in vogue today. The technical details involved in confocal microscopy are not dealt with in this introduction, but basically two fundamentally different methods of beam scanning are used; multiple-beam scanning and single-beam scanning. Single-beam scanning is currently the most popular, and is the method used in the LSCM. Here the scanning of the beam is most commonly achieved by use of computer-controlled mirrors driven by galvanometers at a rate of one frame per second. To achieve faster scanning, at near video frame rates, some systems use an acousto-optical device or oscillating mirrors. The alternative method uses two beams to scan at near real time, and usually relies on using some form of spinning Nipkow disk. Such systems have been derived from the tandem scanning microscope (**TSM**), with improvements to make them more efficient for collecting images from fluorescently labeled specimens. Figure 5 illustrates one such improved system that employs dual Nipkow disks and microlenses to enhance the detection of the low fluorescence levels of real time image collection.



**Figure 5**

There are currently two alternative methods to confocal microscopy that are in use for producing optical sections: deconvolution and multiphoton imaging. They differ technically, but like confocal methods, are based on the conventional optical microscope. Deconvolution uses computer-based algorithms to calculate and remove out-of-focus information from fluorescence images. Due to more efficient algorithms and much faster mini computers, this technique has become a practical option for imaging. Multiphoton microscopy uses the same scanning system as the LSCM, but does not require the pinhole aperture at the detector. The pinhole is unnecessary because the laser excites the fluorochrome label only at the point of focus, eliminating the out-of-focus emission. An additional benefit in the imaging of living tissues is that photobleaching is reduced in the specimen due to the reduced energy absorbed from the laser beam.

The conventional optical microscope forms the basis around which the LSCM is built. Instead of a tungsten or mercury lamp, a laser is used as a light source, and is combined with a sensitive photomultiplier tube (**PMT**) detector, and a computer to control the scanning mirrors or other scanning devices and to facilitate the collection and display of images. Following acquisition the images are stored on digital media and may be analyzed by any of the numerous image processing software packages available using either the microscope system computer or a second computer.

By design of the LSCM, illumination and detection are confined to a single diffraction-limited point in the specimen. This point of illumination is brought to focus in the specimen by the objective lens, and scanned across it using some sort of scanning device under computer control. The sequences of points of light from the specimen are detected by the photomultiplier through a pinhole (or in some cases, a slit), and the output from the PMT is built into an image and displayed by the computer. Although unstained specimens can be viewed using light reflected back from the specimen, they usually are labeled with one or more fluorescent probes.

One of the more commercially successful LSCMs, reported in the literature about 1990, was designed in response to a perplexing fundamental problem encountered by developmental biologists. Many of the structures and specific macromolecules inside immunofluorescently labeled embryos are impossible to image after the two-cell stage using conventional epifluorescence microscopy because as the cell numbers increase, the overall volume of the embryo remains approximately the same. This means that with more and more closely packed cells, increased fluorescence from cells out of any given focal plane of interest interferes with image resolution.

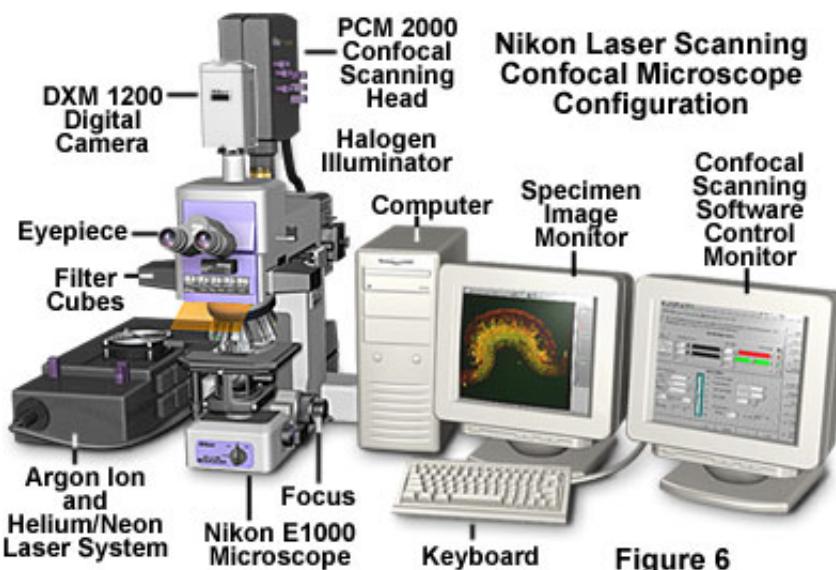


Figure 6

A group of researchers working on the problem discovered that none of the confocal systems available at the time would satisfy their needs. The technology of the time consisted of stage scanning microscopes that were too slow in producing images, taking about 10 seconds for one image, and the multi-beam scanning instruments, which were not practical for fluorescence imaging at that time in their development. A LSCM was designed that was suitable for conventional epifluorescence microscopy, and along with several others that were developed during the same time period, became a forerunner of the sophisticated instruments that are now available to the biomedical community from several commercial vendors. A typical example of a currently available system (the Nikon E1000) is illustrated in Figure 6.

In the specialized instrument that was developed, the thickness of the optical sections could be varied by adjusting the diameter of the pinhole in front of the photodetector. Compared to some other designs that use a fixed pinhole size, this optical variation is extremely flexible for imaging biological structures. The image can be zoomed with no loss of resolution by decreasing the area of the region scanned in the specimen, and placing the scanned information into the same size of digital array for storage or display (in similar fashion to changing magnification in the scanning electron microscope). The ability to do this imparts a range of magnifications to one objective lens, and can be extremely useful when imaging rare or transient events that may be missed or the location lost if lenses must be changed.

Due to the sophistication and flexibility of the LSCMs available from commercial vendors now, there has been a tremendous explosion in the popularity of confocal microscopy in recent years, with many multi-user laboratories purchasing these instruments in preference to electron microscopes. Confocal microscopy has an advantage in the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional optical microscopy, and in its great number of applications in many areas of current research interest.

The first-generation LSCMs worked well for fixed specimens, but were extremely wasteful in their use of light energy from the lasers, and tended to kill living specimens unless great care was taken to preserve their viability while they were being imaged. In spite of the limitations, the images of fixed material produced by the microscopes were so good that the confocal approach was fully embraced by the biological imaging specialists. Technological improvements have been made to every aspect of the imaging process in subsequent generations of instruments. In addition, the ergonomics and usability of the newer instruments is much improved so that alignment, changing filter combinations, and adjusting laser power, now usually controlled by software, are all much easier and less time consuming. It is now possible to image up to three fluorochromes simultaneously, and more than that sequentially. The image processing stage is also more highly developed due to improved, more reliable software, and much faster computers with more disk storage space and more, and less expensive, random access memory.

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