

Chapter 13

Confocal Laser Scanning Microscopy

© C. Robert Bagnell, Jr., Ph.D., 2012

You are sitting at your microscope working at high magnification trying to sort out the three-dimensional compartmentalization of your fluorescent probe. You constantly focus up and down bringing different levels of the specimen into sharp focus while letting other levels above and below go out of focus. As this frustrating job continues, you say to yourself “It would be wonderful if I could see the whole specimen in clear focus all at once”. Confocal laser scanning microscopy is the answer.

This remarkable tool provides a means for producing microscopy images of unparalleled depth and clarity. In this sense it can be compared to computer-assisted tomography but at the microscopic level of resolution. It is also a means of surpassing the resolution limits of ordinary wide field microscopy in both the horizontal and vertical plane. It is an optical microtome with a very sharp edge.

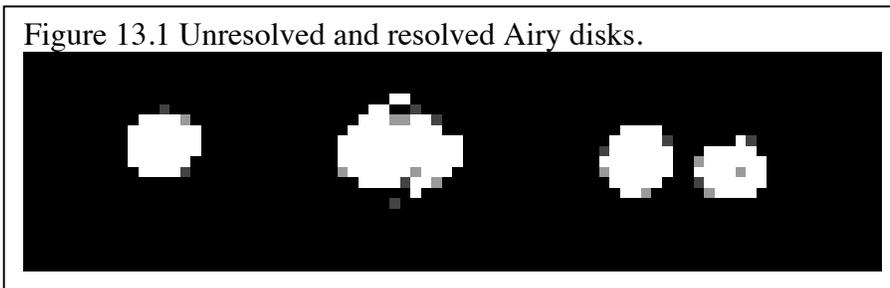
Background for Understanding CLSM

Many special and new technologies are involved in this method, but the idea is an old one. Understanding it will require that you no longer think of the microscopic image as the one you see in the eyepieces. Rather, we will build the image up out of thousands of tiny points of light and we will treat each point in very special ways. The illumination system of CLSM is not Köhler illumination. It is not a "wide field" technique, but rather a point scanning and point detecting technique.

A Point of Light makes an Airy Disk

Our first step in understanding CLSM begins with observations made by the British Royal Astronomer Sir George Airy in the 1840's. As Sir George examined the images of stars through his telescope, he discovered that the image of a star was not a solid point of light but a central bright disk surrounded by dark and light rings. Sir George described how this image resulted from diffraction of the point source of light at the circular edges of the telescope's optics. The phenomenon is known as an Airy Disk and is inherent in all optical systems. If you examine minute holes in an aluminized cover glass or sub-resolution fluorescent beads with your microscope, you will see Airy Disks.

Figure 13.1 Unresolved and resolved Airy disks.



Resolution Depends on the Separation of Airy Disks

The Airy Disk is a clear indication that there is a limit to the size of objects that an optical system can resolve. Lord Rayleigh in 1896 used the Airy Disk in his explanation of resolution in microscope systems. He found that if two point sources of light are imaged, the distance between the centers of their Airy Disks had to be at least as great as the radius of the first dark ring before the human eye could tell the two points apart. This is known as the Rayleigh criterion of resolution and is illustrated in figure 13.1.

Resolution Beyond the Wide Field Limit

In 1955, Marvin Minsky, while a Junior Fellow at Harvard University, realized that spatial resolution higher than that achieved in wide field imaging could be achieved if only the central portion of an Airy Disk is used to form an image. Minsky's apparatus is diagrammed in figure 13.2. To do this, he had to illuminate the specimen with a diffraction-limited point of light formed by an objective lens and view that same point of light (with the associated changes caused by the specimen) using an equally good second objective lens. Using a small pinhole, only the central part of the Airy Disk formed by the second lens was allowed to reach a photomultiplier type detector. The output from this detector was displayed as a point of light on a fluorescent screen. The effect of the specimen was to change the brightness of this point of light. In order to create an image of the entire specimen, Minsky had to scan the specimen in a raster fashion between the two objective lenses and he had to synchronously scan the position of the spot on the fluorescent screen. The term confocal comes from having two lenses focused on the same point.

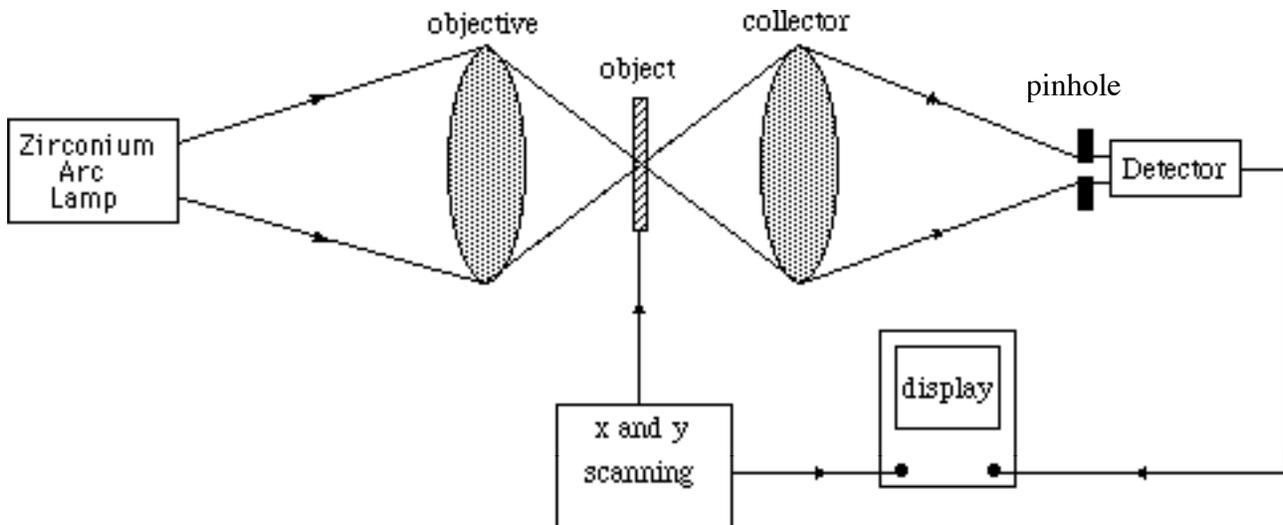


Figure 13.2 - Schematic of the First Confocal Scanning Microscope.

As far as I know, Marvin Minsky was the first person to construct a confocal scanning microscope. His discovery went unappreciated for many years. The invention of the laser in 1960 finally provided a light source of sufficient brightness and stability for practical confocal scanning microscopy. The first reports in the scientific literature describing a confocal laser scanning microscope were from Egger and Davidovits at Yale in 1971, Shepherd and Wilson at Oxford in 1978, and Brakenhoff in Amsterdam in 1979.

Although Minsky built his microscope with two objective lenses he realized that the system would work using a single objective lens operated in a reflected light mode. This mode is, in fact, how all, current commercial CLSM systems work (Fig. 13.3).

The Importance of Laser Light

Lasers had not been invented when Minsky built his confocal scanning microscope. At first he used a carbon arc lamp but later used a zirconium arc for its stability. Laser light provides a number of advantages over all other lights for confocal microscopy. Each type of laser produces light of only one or a very few well defined wavelengths. This minimizes the need for excitation filters in fluorescence microscopy. The light is both spatially and temporarily coherent. Spatially coherent light is light in which all the waves have the same frequency, direction, and polarization angle. Temporally coherent light is light in which the waves have exactly the same phase and speed. This combination produces a beam of light that is very bright and tightly focused. The high intensity of laser light is useful in getting a sufficiently bright spot of light onto the specimen. The fact that the laser light is polarized is useful when doing reflected light microscopy (not all lasers produce polarized light). In this technique, materials in the specimen reflect the laser's light. In doing so they change the polarization angle of the light. This provides a means of separating the specimen derived reflected light from any background primary laser light. All of the reflected light is simply passed through a polarizer that is crossed to the laser's direction of polarization.

How a CLSM Works

The basic systems described here will always be present in one form or another on any modern CLSM.

A variation of the CLSM is the spinning disk CLSM. In these systems the illumination is scanned across the specimen very rapidly by the action of a disk that contains a series of holes or micro-lenses distributed in a pattern similar to an Archimedes spiral. This type of system is especially useful for live cell imaging. I do not cover spinning disk systems in my notes.

The CLSM Optical System

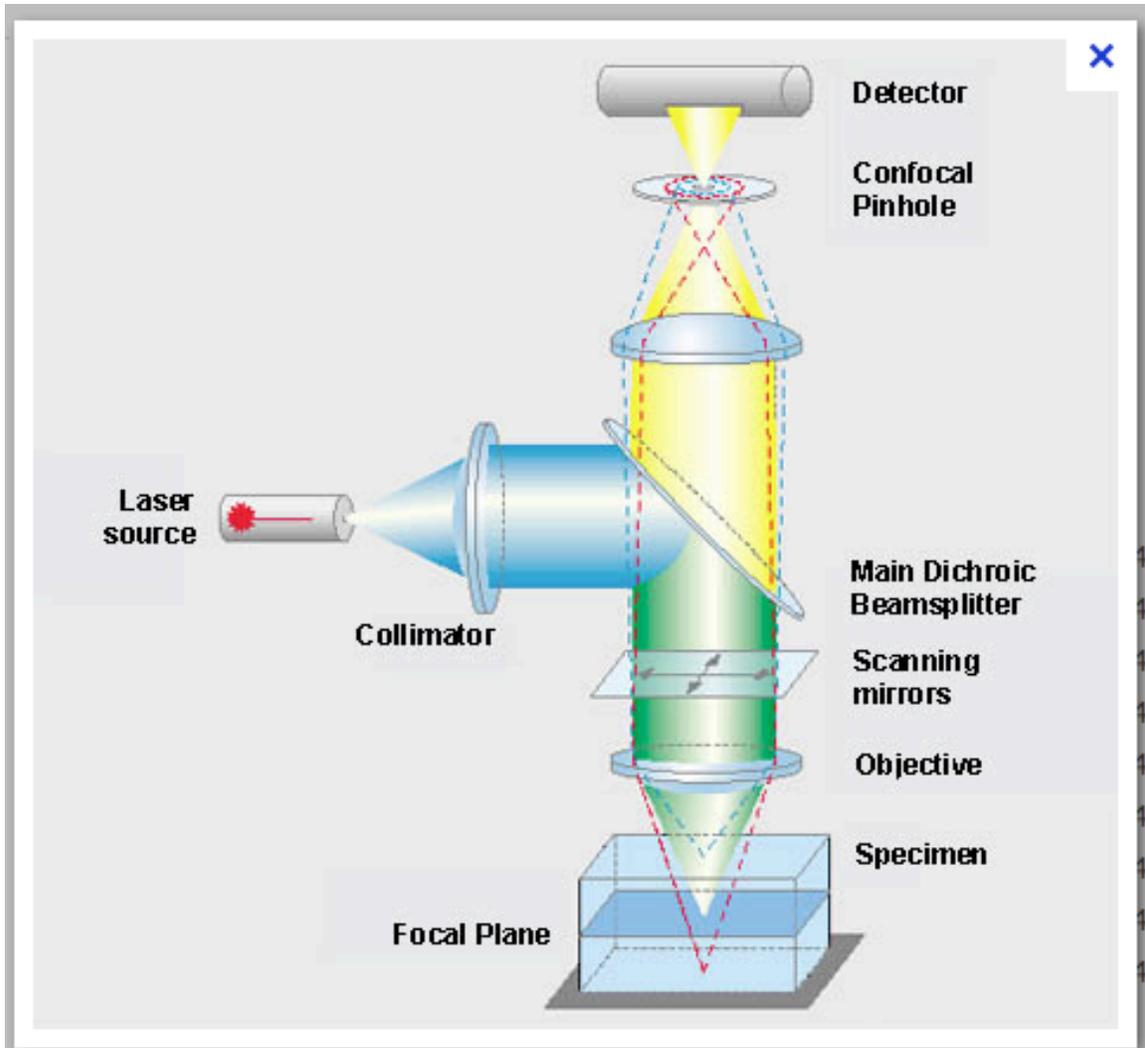
Figure 13.3 illustrates the optical elements and light path of a CLSM. The following discussion is in reference to this figure. Note that CLSMs can be on upright or inverted stands.

One or more lasers are present. A shutter or some type of optical coupling mechanism is used to allow the laser beam to enter the microscope. The laser beam is quite small in diameter (usually less than 1 mm) and must first be expanded to a diameter of about 1 cm. The intensity of the laser light is next adjusted by a set of neutral density filters. The beam is next brought to a set of scanning mirrors. These mirrors are mounted on a mechanism that can move them very precisely and very rapidly. One mirror tilts the

beam in the X direction, the other in the Y direction. Together, these mirrors tilt the beam in a raster fashion. The beam is then brought to the back focal plane of the objective lens. The objective lens focuses the beam onto the specimen. The result of all this is that a diffraction-limited spot of light is scanned in a raster fashion over a small area of the specimen. The size of this area is equal to or smaller than the objective lens's field of view.

After the beam reaches the specimen, the light takes two different paths. Part of the light may be transmitted through the specimen and subsequently through the condenser and field lens, just backwards from the normal illumination path. In this case, a detector placed in about the position of the normal tungsten lamp can be used to create a transmitted image of the specimen. This image cannot, however, be confocal.

If the specimen is fluorescent or reflective, part of the light will pass back into the objective lens, and it is this part in which we are most interested. This light travels backwards through the same path that the laser travels. The effect of the scanning mirrors on this light is to produce a spot of light that is not scanning - that is it is standing still.



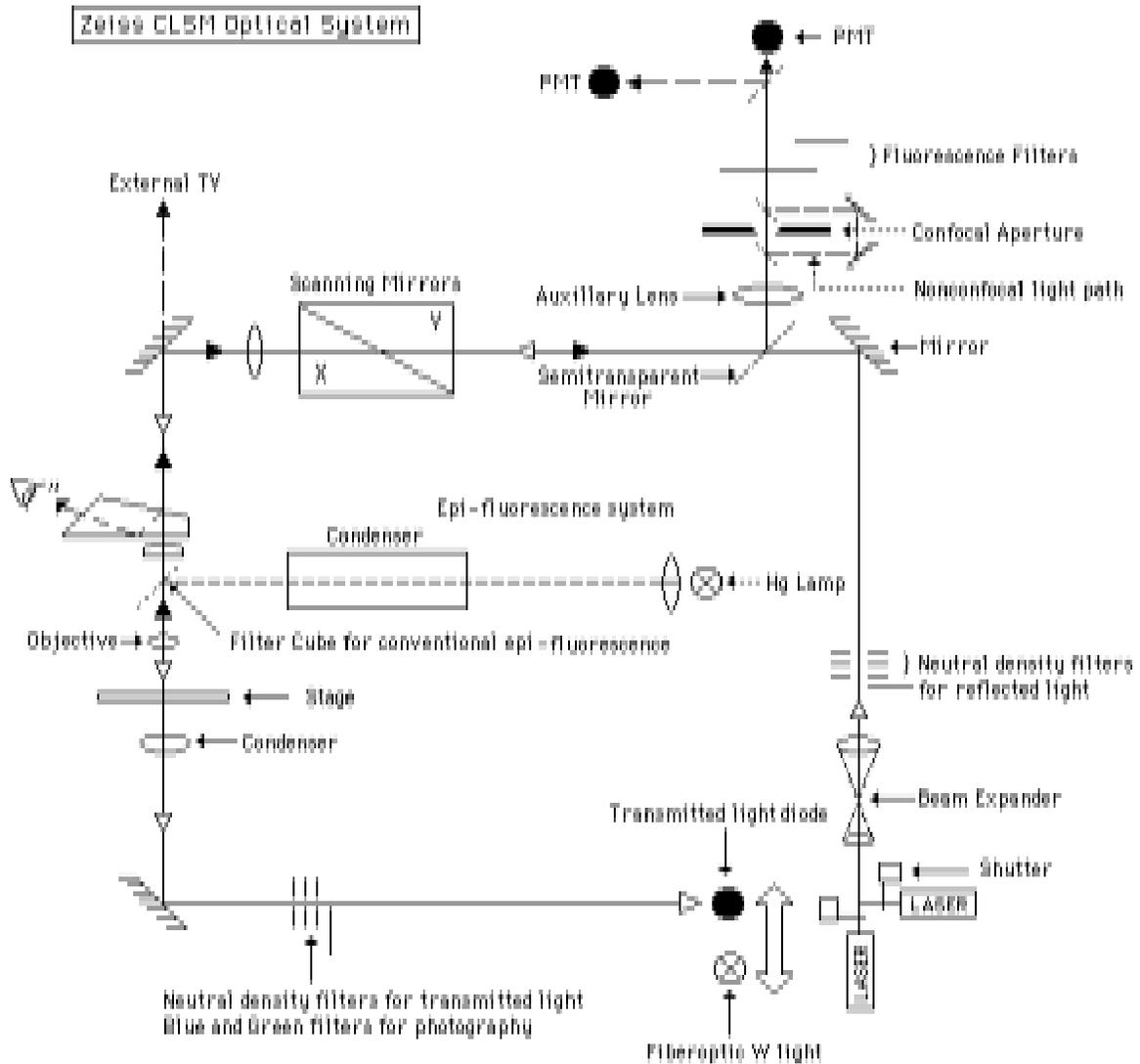


Figure 13.3 LSM Light Path

This light then must pass through a semitransparent mirror or a dichroic mirror that reflects it away from the laser and toward the detection system. The first object in the detection system is the pinhole aperture. This aperture is in the intermediate image plane of the microscope. It is at this position that the objective forms a diffraction limited Airy Disk of light. The aperture serves to allow only a small central portion of this light through to the light detectors. If this is fluorescent light, it will be a different color from the laser light, and emission filters are next used to separate it from laser light that has been reflected from the specimen. If reflected light is being examined, it will be passed through a polarizer that will allow only laser light with a different polarization angle from the initial laser light to pass. Finally, the light enters a photomultiplier tube type detector.

The CLSM Detector and Display System

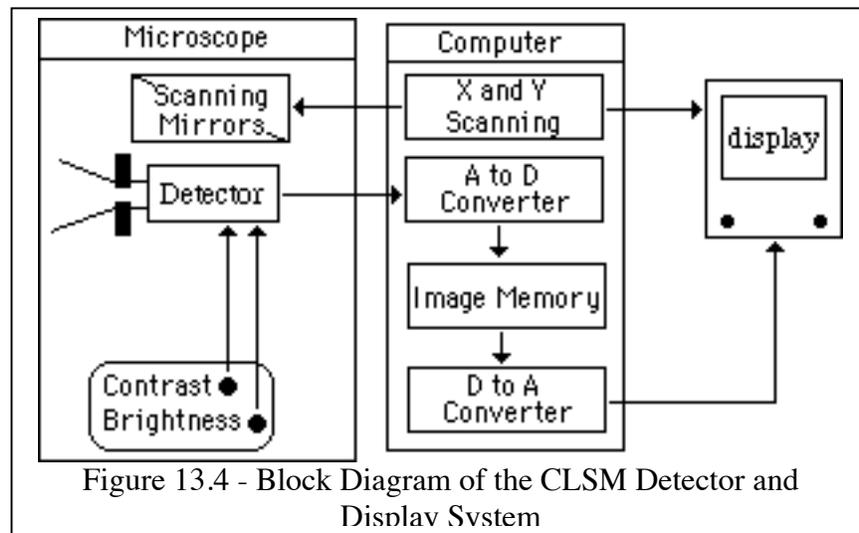


Figure 13.4 - Block Diagram of the CLSM Detector and Display System

Figure 13.4 is a schematic representation of the CLSM's detector and display system. Unlike Minsky's confocal microscope, today's CLSM's are operated by computer. The computer controls scanning of the laser beam, collection, processing, display and storage of the image, and even movement of the microscope's stage and focus mechanisms. Marvin Minsky (founder of the Artificial Intelligence Laboratory at MIT) is undoubtedly amused to see how far his invention has come.

Light that emerges from the CLSM's complex optical system may have a very low intensity, that is, there are very few photons. A photomultiplier tube (PMT) is used to detect and amplify this light signal. Photomultipliers are capable of amplifying a faint signal approximately one million times while introducing almost no noise. The sensitivity

of a PMT can be adjusted by electrical controls that represent brightness and contrast in the final image. The output from the PMT is an electrical signal with an amplitude that is proportional to the initial light signal. This analog electrical signal is next converted to a series of digital numbers by an analog to digital converter in a computer. The range of digital numbers that represent the analog signal is limited. Older systems use the range of 0 to 255 (eight bits; 2 to the 8th). In this scheme, 0 represents black in the image and 255 represents white (or visa versa). Newer systems use 12 bit A to D converters. (0 to 4095 gray level). As the laser beam sweeps along the specimen, the detection system constantly samples and converts the PMT output. There is a limit to the number of samples that can be taken on each raster line. Older systems take 512 samples on each line and there are 512 lines in the raster. This amounts to more than 256,000 samples for each complete scan of the raster. Each sample is called a picture element or pixel. Newer systems can take 1024 X 1024 or 2048 X 2048 pixels. As each sample is taken, its value is saved in the computer's memory. The computer converts each of these values back into an analog signal through a digital to analog converter and displays them on the computer monitor in the proper order. All of this occurs so fast that the display appears to be showing a real time image of the specimen.

No real color from the specimen is recorded using this type of detection system. The photomultiplier is sensitive to a wide range of colors, but its output is a single analog electrical signal. Normally each of the possible numbers that represent intensity values is displayed as a shade of gray. False color can be added to the displayed image by having the computer assign a color to each of these numbers. Pseudocoloring the image does have practical value in some instances, for example to highlight areas of the image that fall within a certain range of gray levels or easily telling one spectral channel from the other in multi channel imaging.

With the digital image stored in the computer's image memory, certain operations can be performed on the image before it is displayed. This is called digital image processing. For example, an image may be given more contrast by applying a high pass filter to it. Many digital images may be stored as files on the computers disk system. They can subsequently be recalled and added to one another. If the images represent a through focus series, the result of adding them together is to produce one image that has vastly greater depth of focus than can be achieved in any other way. The computer can also produce a stereo pair from the image series. These are only a few of the many operations that can be applied to the digital image.

Magnification, Resolution and Sampling Frequency

When should you use a 512 X 512 setting on the CLSM and when a 1024 X 1024? First it is important to realize that in each of these settings, for a given objective lens, the same area is being scanned on the sample. So why use twice as many pixels to sample the same line? It has been known for a long time from sampling theory that to accurately sample a signal of a given frequency, the sampling frequency must be twice as fast as the frequency to be sampled. This is known as the Nyquist sampling theorem. The

same holds true in the special domain of microscopy. In microscopy, the highest “frequency” that must be sampled is the resolution of the objective lens i.e. the area of the sample represented by the Airy disk after the confocal aperture. For example, a 63X / 1.4 oil lens using blue light has a resolution of about 0.14 micrometers in confocal x/y. If it is used to scan its largest optimal square raster on a specimen, the line length on the specimen will be about 144 micrometers. This line length divided by the number of samples should give a value less than half of the lens resolution in order to satisfy Nyquist sampling.

The calculations are:

$$144 / 512 = 0.28 \text{ micrometers per pixel}$$

$$144 / 1024 = 0.14 \text{ micrometers per pixel}$$

$$144 / 2048 = 0.07 \text{ micrometers per pixel}$$

Here the 2048 choice is best. It is also valid to change the microscope zoom setting so that the line length is cut by half, that is, the magnification is doubled. In that case:

$$72 / 512 = 0.14 \text{ micrometers}$$

$$72 / 1024 = 0.07 \text{ micrometers}$$

$$72 / 2048 = 0.035 \text{ micrometers}$$

Here the 1024 choice is best. There is nothing to be gained by using 2048.

Magnification in a CLSM is the magnification of the objective lens times any additional magnification up to the pinhole.

Advantages of CLSM

Confocal Advantages

- Improved horizontal resolution

The best horizontal resolution produced by a wide field light microscope is approximately $0.25\mu\text{m}$ using a high NA objective and condenser lenses and blue light. The CLSM can produce about $0.16\mu\text{m}$ resolution using the same objective lens. For a Wide Field (non confocal) system the X Y resolution equation is:

$$R_{xy \text{ wide field}} = 1.22 \lambda / 2 \text{ NA}$$

For a confocal system, the pinhole radius is set somewhat smaller than r_{airy} and thus the X Y resolution equation is:

$$R_{xy \text{ confocal}} = 0.8 \lambda / 2 \text{ NA}$$

The mechanism by which this is achieved has been indicated above. Perhaps a simple way of summarizing this is to state that for each spot imaged in the specimen, the photomultiplier detector measures brightness from a specimen volume much smaller than the lenses diffraction spot. This is because of the pinhole aperture that is placed between the diffraction spot and the detector.

- Improved vertical resolution or short depth of field

A plan apochromatic objective of NA 1.4 operating in green light has an in focus depth of field of approximately $1\mu\text{m}$ with considerably more depth out of focus. When this same objective is used on a CLSM with an optimum pinhole diameter, the depth of field is approximately $0.5\mu\text{m}$, all of which is in focus. For a wide field system the Z resolution equation is:

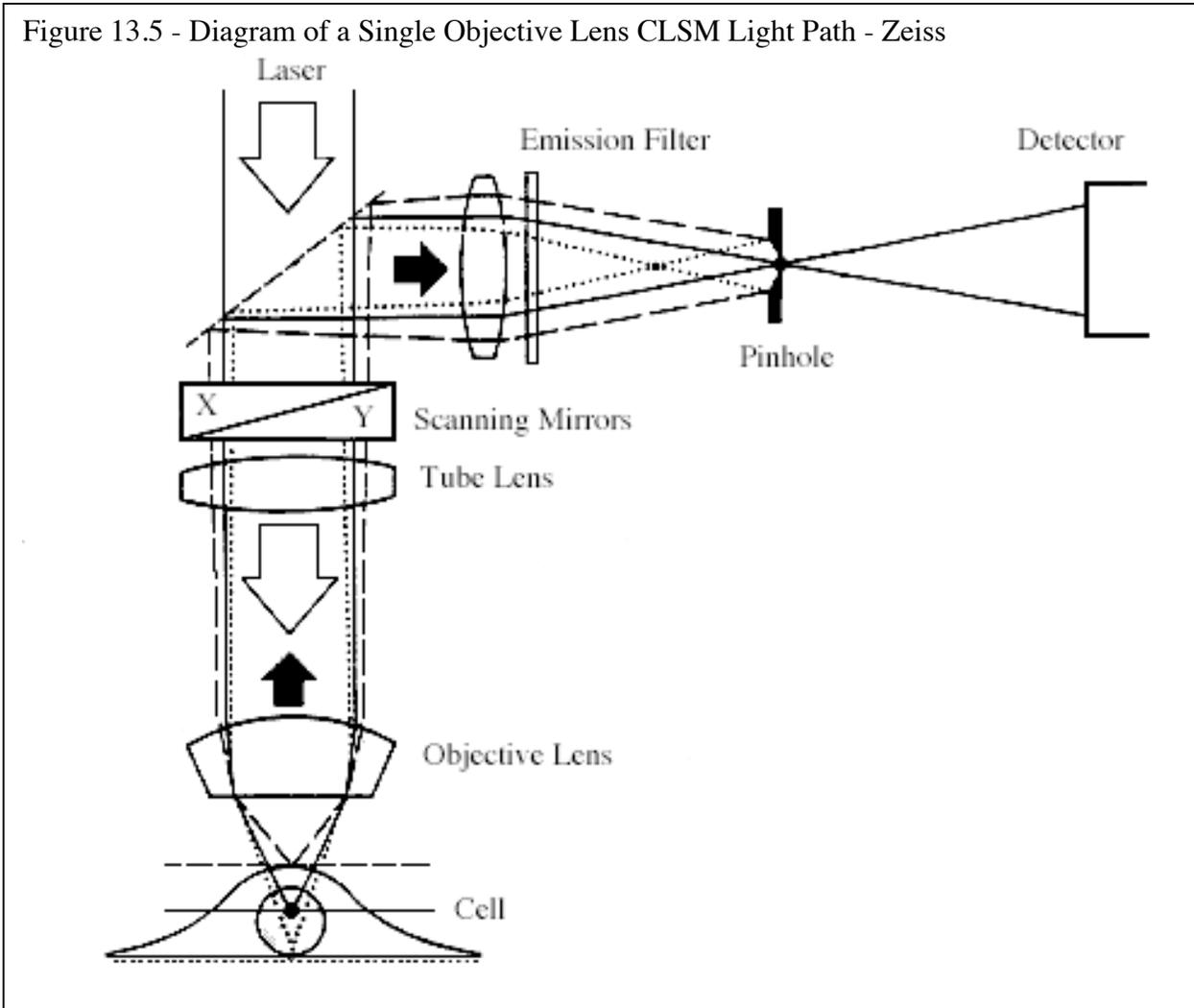
$$R_{z \text{ wide field}} = 2\lambda\eta / \text{NA}^2$$

For a confocal system, the pinhole only allows light from a much shorter vertical distance to pass through to the detector so the Z resolution equation is:

$$R_{z \text{ confocal}} = 1.4 \lambda\eta / \text{NA}^2$$

Figure 13.5 offers a geometrical explanation of reduced depth of field in confocal microscopy: The laser is focused to a point within a specimen by the objective lens. Light from that same point is imaged onto the confocal aperture. Light originating above and below the focal point is stopped by the aperture before reaching the detector. The pinhole aperture effectively eliminates all light except that from the plane of focus, thus there is very little out of focus information in the image. This short depth of field allows direct optical sectioning.

Figure 13.5 - Diagram of a Single Objective Lens CLSM Light Path - Zeiss



It is the improved vertical resolution that gives confocal imaging its main advantage. Even though vertical resolution is not as high as horizontal resolution it represents a huge improvement over wide field imaging and makes possible the optical sectioning nature of confocal microscopy.

- Extended depth of field and 3-D reconstruction

Since the confocal image is in focus throughout its depth, several images taken at different depths can be added together to give an extended depth of field or used directly in digital imaging to create a 3-D reconstruction without the need of first removing out of focus information.

Laser Advantages

- Small spot size

Low divergence and monochromaticity of the laser allow the objective to form an accurate point focus.

- High-energy light

Using laser light the objective lens can form an extremely bright point source allowing excitation and subsequent detection of weakly fluorescing structures and greater penetration depth into the specimen.

- Precise excitation wavelength

The wavelength for illumination can be selected precisely by choosing the appropriate laser to match the application.

Scanning Advantages

- Improved contrast

Optical interference of light from nearby objects (halation or glare) is greatly reduced by scanning a point light source to create an image.

- Various magnifications with one objective lens

Changing the raster size scanned on the specimen gives variable magnification. Typically a 1:8 ratio is available.

- No restriction on excitation or emission wavelengths

Wavelengths suited to the study can be used with an electronic detector. Thus wavelengths not suitable for observation, such as IR can be utilized on the emission side. The image is recreated on a video monitor for observation.

- Z axis imaging

Scanning along the X-Z or Y-Z axis is possible with a motorized focus control and motorized stage. This creates a cross-sectional view of the specimen. In fact, scanning can also be carried out at any angle creating any desired section.

- Electronic filtering

Improved contrast and lowered background can be achieved by electronically filtering the detector signal. A simple example of this is averaging the detector signal over many samples of the same image point. The detector signal contains some noise produced by its electronic components. Since the amount of noise is random over time, averaging many samples tends to level out the noise while that part of the detector signal resulting from the specimen remains constant. Thus the signal to noise ratio is improved and the image looks better.

- Digital image processing

By digitizing the detector's signal an image is produced that can be processed by a computer. Digital image processing methods provide powerful tools for improving image quality, for measuring structures in the image, and for looking at images in entirely new ways.

Disadvantages of CLSM

Currently Restricted to Fluorescent or Reflected Light

Today, commercial versions of the confocal laser scanning microscope all use the single objective lens, epi-illumination method. Therefore, the specimen must produce either fluorescent or reflected light. Some means must be available to correlate the confocal image to normal specimen morphology. The transmitted light methods of bright field, phase, and DIC all work properly even though the image is created by a scanning beam technique. A method of observing one or more of these types of images correctly superimposed on the confocal image is necessary.

Cost and Upkeep of the Microscope

Confocal laser scanning microscopes require very good optics, one or more lasers, and an integrated beam scanning - image acquisition, image processing system. All of these items carry a high cost. Additionally, lasers, computers, and beam scanning equipment require expert maintenance, usually by the manufacturer or by a trained representative of the microscope seller. It is not unusual to insure the operability of a CLSM system by purchasing an annual service contract much like those for electron microscopes.

Exercises

If you have access to a CLSM, here are a few things you can do to get better acquainted with it. A standard H&E stained histology slide is a good test specimen since the eosin will fluoresces in all channels.

- 1) Align the microscope for Kohler in its bright field visible mode.

- 2) Use the dye selection software to choose dyes that are excitable by each of your major laser lines: on my system with three PMTs I choose FITC, TRITC, Cy5 for green, red and far red emission respectively.
- 3) Use the visible mode of the CLSM to focus on the H&E slide in bright field using a 20 X lens. This is a good time to test the setting of any DIC prisms that might be in the system.
- 4) Switch to the LCM mode without scanning and set the PMT voltages to a low setting and set the amplifier gain and offset to their minimum values.
- 5) Start the scope scanning and adjust the PMT settings and the focus to give a good quality image in all channels.
- 6) Acquire an averaged image.

If you can do these things, you are well on your way to becoming a good confocal microscopist. There are many more things you can do with this instrument, but that is for another course and another time.