

What is the Confocal Volume?

David E. Wolf

Sensor Technologies LLC, 910 Boston Turnpike, Park Nine West, Shrewsbury, MA, 01545

The confocal volume is the “in-focus” volume within a sample that is efficiently detected using a system designed with confocal optics. The confocal volume is typically on the order of femtoliters and is often used to create the small detection volume required in fluorescence correlation spectroscopy (FCS) measurements.

Confocal Optics

Confocal optics are designed to reduce out-of-focus light and to limit image detection to the desired plane of “in-focus” light (the object plane). These limits increase both contrast and effective resolution.

Confocal Illumination

For confocal fluorescence measurements, a laser beam is focused in a fluorescent sample with a high numerical aperture (NA) objective. Fluorescent particles illuminated by the laser emit fluorescence. In **Figure 1**, below, the laser beam is represented by the green beam.

A dichroic mirror is used to selectively reflect the laser wavelengths through the object (represented by the lens) and onto the sample.

Confocal Signal Collection

Figure 1 also illustrates the path of light emitted from the fluorescent sample (right) to the detector (left, not shown). Emitted light passes first

through the objective, then through a dichroic mirror, then through a field pinhole or aperture in the conjugate image plane.

The aperture at the image plane serves to eliminate out-of-focus fluorescence by excluding signals focused to planes to the left and to the right of the conjugate image plane. This means that only fluorescence originating from the small confocal volume at the object plane (represented by the

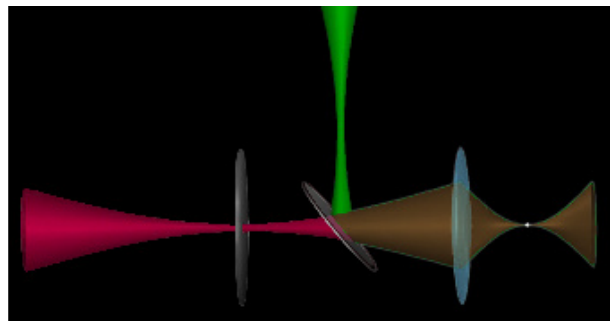


Figure 1: Confocal Illumination & Signal Collection.

The white dot indicates the object plane. The lens represents the high NA objective. The center disk represents the dichroic mirror. The left-most disk represents the pinhole or aperture at the conjugate image plane. The detector would be to the left of the figure.

white dot) makes it past the pinhole, even though much more of the sample is illuminated.

The Confocal Principle

The confocal principle describes the property of confocal systems that light emitted in the object plane is efficiently collected, but light emitted above or below the object plane is not.

Efficient Signal Collection from the Object Plane

The center diagram of **Figure 2** (pink) represents emitted light from the object plane of the objective. Because this light is focused precisely on the aperture located at the conjugate image plane, the collected signal strength is high.

Inefficient Signal Collection out of the Object Plane

The bottom diagram of **Figure 2** (blue) depicts

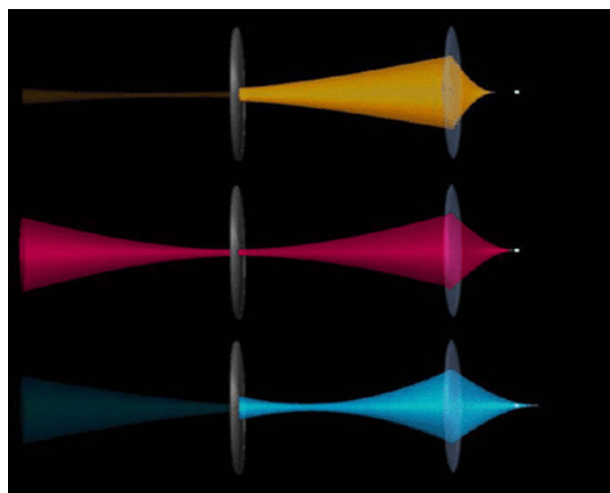


Figure 2 : The Confocal Principle. The white dot indicates the object plane. Light emitted from this plane (center diagram) is efficiently collected by the detector. Light from below (left diagram) or above (right diagram) is poorly collected.

emitted light from fluorescent particles to the right of the object plane. This light is focused to the right of the conjugate image plane, producing a weak signal to the detector. Likewise, the diagram at the top of **Figure 2** (yellow) illustrates the inefficiently collected light focused to the left of the conjugate image plane.

Confocal Mathematics

Shape of the Confocal Volume

The intensity of a focused laser beam operating in the so called TEM_{00} mode and directed along the z axis can be represented by the formula

$$I = I_0 e^{-2(x^2 + y^2 + \frac{z^2}{\kappa^2}) / w^2},$$

where I_0 is the intensity at the center, w is the e^{-2} beam radius in the xy plane, and κ is a geometric factor usually equal to the optical resolution in the z direction divided by the optical resolution in the xy plane. These properties define the confocal volume to be an oblate ellipsoid, which is an ellipse rotated around its longer, or major, axis, where κ is the ratio of the length of the major axis to that of the minor axis.

Definition of κ

If you have perfect diffraction-limited optics, then κ is the resolution of the objective in the vertical, or z , direction divided by the resolution in the xy plane. Resolution in the xy plane is defined by the Rayleigh limit to be

$$r_{xy} = \frac{0.61\lambda}{NA},$$

where λ is the wavelength of light and NA is the numerical aperture of the objective.

The resolution in the z direction is given by

$$r_z = \frac{2n\lambda}{NA^2},$$

where n is the index of refraction of the mounting media.

This gives us a lower limit for κ of

$$\kappa = \frac{2.33n}{NA}.$$

So, for instance, if your sample was in water with $n = 1.3$, and you used an objective with an NA of 0.6 , the lower limit for κ would be 5.0 .

Factors Contributing to Deviations in κ

Several factors can contribute to κ being larger than this limit. The most significant causes are

failure to expand the laser beam so that it fills the back focal plane of the objective or spherical aberrations caused by index of refraction mismatches. Microscope objectives are designed to use a particular standard-thickness coverslip, a particular mounting medium (typically: air, water, glycerin, or oil), and to focus on the surface of the sample immediately proximal to the coverslip. Failure to match any of these specifications may result in an increase in κ .

Fortunately, κ enters into the FCS correlation functions through κ^2 . Even with κ 's as low as 3.0 obtained with high NA , water-immersion objectives, κ^2 is sufficiently high enough to minimize the effect of motion along the z direction in FCS measurements.

Calculation of the Confocal Volume

The mathematical formula for the confocal volume is

$$V_C = \pi^{3/2} \kappa W^3.$$