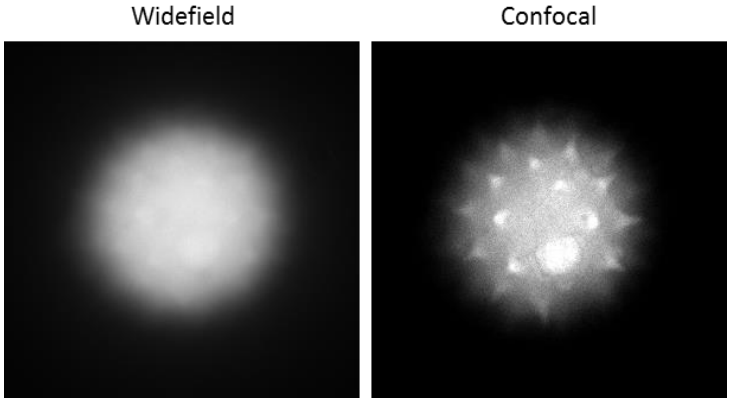
Laser Scanning Confocal Microscopy

Why confocal microscopy?

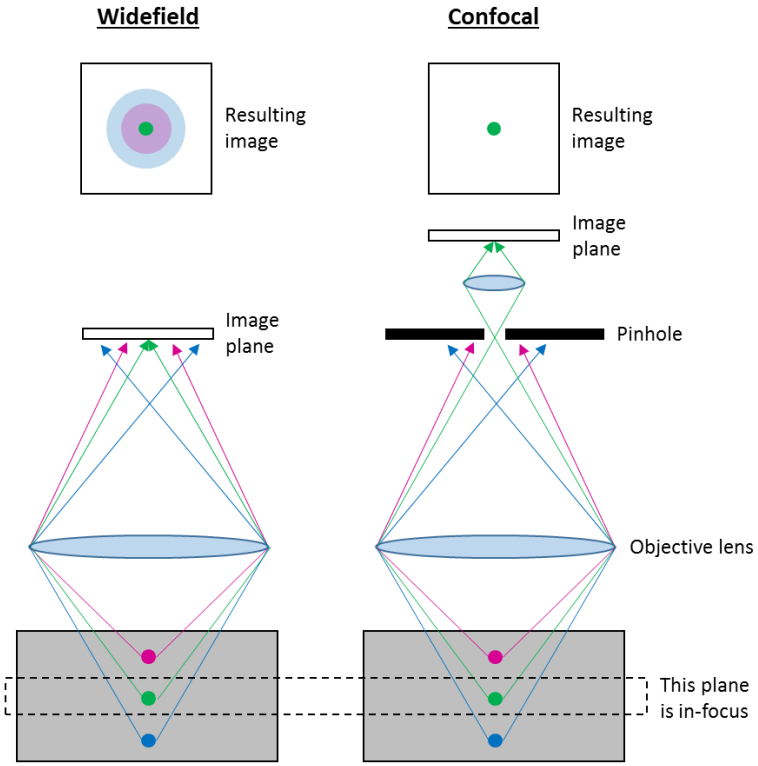
One problem with fluorescence microscopy is that thick, three-dimensional objects often appear blurred due to out-of-focus light. Consider this scenario: you have three lit candles in a dark room at varying distances from you. If you focus on the candle in the middle and take a photo, your picture will also include blurred images of the light coming from the candles that are closer and farther away. This could be a nice effect for a photo, but if there were hundreds of candles it would be difficult to see the candle in the middle clearly. The same thing happens in fluorescence microscopy except instead of candles, we have fluorophores.



Confocal microscopy eliminates out-of-focus light so that you can see the in-focus objects clearly. The two images on the right are of the same pollen grain, imaged using widefield ("normal") and confocal fluorescence microscopy. By reducing out-of-focus light, confocal reveals the texture of the pollen grain surface.

The basic confocal principle

In widefield fluorescence microscopy, light from all planes in a 3D specimen is collected by the objective and formed into an image at the "image plane" (the camera). Notice how the light rays from the green dot converge to a point at the image plane while the blue and magenta dots converge too early and too late, respectively. The resulting image contains in-focus light from the plane of the specimen that is in-focus (green dot), but also blurred light from the planes that are out-of-focus (magenta and blue dots). Confocal microscopy removes the out-of-focus light from your image by sending all the light coming from your specimen through a tiny **pinhole** through which only in-focus light can pass. Light from the in-focus plane (green dot) converges just in time to go through the pinhole, while the light from the out-of-focus areas converges too early or too late to pass through the pinhole. In a certain sense, light from in-focus

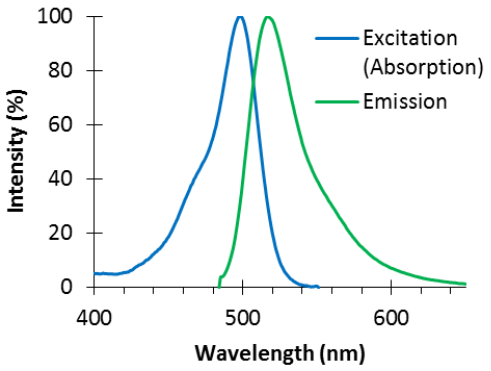


3D specimen with fluorescent objects at three different depths

areas is “sharp” and can pass through the small pinhole while light from out-of-focus areas is blurry and too “diffuse” to pass through the pinhole.

Differences between confocal and widefield fluorescence

Besides excluding out-of-focus light, confocal microscopy has several major differences with widefield fluorescence microscopy:



- Excitation light is provided by a laser tuned to specific wavelengths instead of white light sent through an excitation filter. Consider the fluorophore spectra shown on the right. A widefield microscope would use a filter to excite the dye with a range of light between 450 and 500 nm. A confocal microscope would use a single wavelength located at the peak excitation wavelength (490 nm).
- Images are acquired pixel-by-pixel instead of all at once. The laser scans across the specimen very fast, imaging the pixels from left to right, row by row, from top to bottom. The computer builds an image from each of the pixels to display on the screen. Our eyes cannot do this. To view your specimen through the oculars, you need to use normal widefield fluorescence excitation. You can only view a confocal image using the computer.
- Because the tiny pinhole excludes almost all the light coming from the specimen (it only lets the in-focus light through), confocal images tend to be dimmer and noisier than normal fluorescence images. You will learn how to compensate for these problems during your training.
- Confocal microscopes can image multiple different color fluorophores simultaneously. Simultaneous detection increases the speed of imaging, but can cause problems if the excitation and emission spectra of the fluorophores are too close together. This can result in the fluorescence of one channel “**bleeding-through**” to the next channel, causing you to see a fluorescent object in the incorrect channel.

We will discuss these issues in greater depth and cover additional considerations during your training.

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