# Building a Confocal Microscope: Principles and Design

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The confocal microscope has become a powerful tool in biological research, able to image thick (typically 50 $\mu$m) tissues and living cells in 3D. In light microscopy, thick samples pose a major challenge as light out of the focal plane blurs the image. The confocal microscope gets around this problem by focusing the illumination light into a small, diffraction limited spot (typically diameter around 400 nm) on the sample and then using a pinhole to block unfocused light before it hits the detector. In this report, we will discuss how to build a simple confocal microscope in order to image stamen and compare it to a brightfield microscope. With the confocal, we were able to produce a one dimensional image of the sample at 3X magnification with a higher signal to noise ratio than brightfield.

## I. INTRODUCTION

The confocal microscope was patented in 1957 by Marvin Minsky, a postdoctoral fellow at Harvard University. [1] He was studying biology and neuroscience, but little was known about the brain and the wiring of nerve cells. Minsky wanted to create a 3D map of the neural network, but the density of the web of cells made this impossible due to the excessive scattering of light. [2] Minsky designed a scanning microscope that only illuminated one spot on the sample at a time while blocking out the unfocussed light with a pinhole. His new invention—the confocal microscope—allowed him to image such thick samples with high resolution. [3]

However, Minsky's work gained little attention. In the 1950s, interest had moved on from light microscopy to the developing technology of electron microscopes for biological research. The confocal microscope's design was also costly. It required lasers strong enough to illuminate the sample with high intensity and powerful computers to put the scans together into an image, neither of which were readily available. It was not until 1987 that the technology for small reliable lasers and inexpensive computers improved, allowing the confocal microscope to appear commercially. [3] The confocal microscope quickly gained popularity for imaging biological specimens. It is able to image thick samples such as living cells, it reduces the background noise from other parts of the cell when using fluorescence, and it is able to produce 3D images by stacking scans. [4]

To acquire an image from a confocal microscope, the sample needs to be scanned. There are multiple approaches to doing so, such as stage scanning, laser scanning, Nipkow (spinning) disk, or slit scanning. [3] In this report, we will use the stage scanning method. This approach keeps the light source and the rest of the microscope still while moving the stage back and forth. The advantage of this technique is that all points will have the same optical properties as nothing else in the microscope changes, edge artifacts are minimized as only the center of the objective lens is used, and the completed image size is only limited by how far the stage can move. However, this approach is slow, requires the stage to be moved with high mechanical precision, can leave motion artifacts, and can rearrange the sample as the stage movement puts force on it. [4]

The high resolution of confocal microscopes in thick samples is due to the pinhole. A microscope is diffraction limited when it achieves the maximum theoretical resolution, given the numerical aperture (NA) of the objective and wavelength of light used. Resolution describes the minimum distance at which two points can be distinguished as separate. For a confocal microscope, the spatial resolution doesn't just depend on NA and the wavelength of light, but also the size of the pinhole that filters out unfocused light. [1] The image is diffraction limited when the pinhole is one Airy unit across. (An Airy disk is the size of the central diffraction spot of a point of light.) [3] Decreasing the pinhole size reduces the thickness of the focal plane, allowing for higher resolution along the optical axis. However, with dim samples, the pinhole often needs to be open wider at the cost of resolution. [4]

We want to image the stamen of a flower. In sexually reproductive flowering plants, genetic material is exchanged through pollination. [5] The male part of the flower is called the stamen, which consists of a filament with two lobes at the end called anthers that produce pollen. [6] Pollen grains are transported to the female part of the flower called the stigma, where the ovules are fertilized in order for seeds to develop. The structure of the flower's reproductive system can tell us about its function and how the plant is pollinated whether it be by wind, insects, birds, or animals. [5] Understanding the stamen through microscopy can give us information about this complex system.

In order to image the stamen, we will use both a brightfield and a confocal microscope. Due to the sample's relative thickness, using a confocal microscope will reduce the scattered light from other focal planes and increase resolution. This will result in a higher signal to noise ratio (SNR) than the brightfield microscope. The sample doesn't need to be suspended in media, allowing us

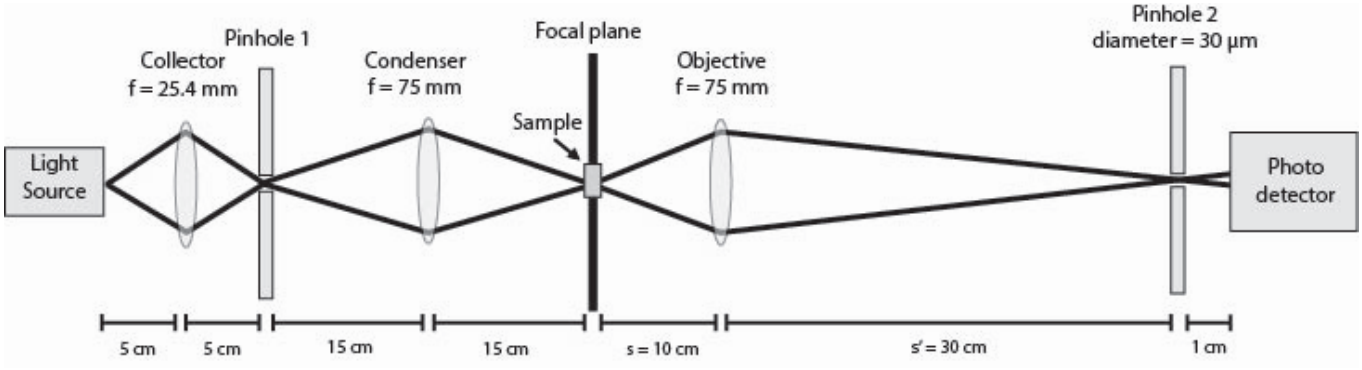


FIG. 1: Diagram of a simple confocal microscope where the sample is placed in the focal plane. The distance between the sample and single lens objective (s) and objective to the pinhole in the conjugate image plane (s'). Pinhole 1 is positioned at the point where the rays from the collector converge, blocking stray light from entering into the system. Pinhole 2 is positioned in the conjugate image plane, blocking unfocused light from planes above and below the focal plane before they hit the photo detector.

to use a stage scanning method for the confocal without having to worry about motion artifacts or rearrangement of the sample. We will build a simple confocal microscope as well as a brightfield microscope to image the stamen, calculate their magnification, and compare the signal to noise ratios.

## II. METHODS

### A. Brightfield Microscope

Brightfield microscopes illuminate the sample, collect the scattered light, and then magnify the object with lenses to create an image. To build a compound brightfield microscope we used an optical mounting board, objective lens (10X, 0.25 NA, tube length = 160 mm, working distance (WD) = 6 mm), condenser lens (f = 75 mm), a viewing screen (a flat, white piece of plastic), and a light box (Pasco, OS-8470, in bright point source mode). The sample was illuminated by the light box 15 cm away. A condenser lens was placed in between the light source and the sample 12 cm away from the sample to produce a beam of parallel rays. Light rays pass through the sample, are diffracted, and are collected by the objective to converge at the image plane. The objective was clamped in place by a stand and base and placed 0.6 cm away from the sample at the WD. The viewing screen was placed behind the objective at the tube length.

### B. Confocal Microscope

To build a confocal microscope, the sample needs to be illuminated by a single spot. (Figure 1) Light travels from the light box through two lenses, the first collecting it and the second condensing it onto the sample. The condenser lens (f = 75 mm) was placed behind the sample at 15 cm, the collector lens (f = 25.4 mm) was placed at 30 cm, and the light box at 35 cm. Between the two lenses, a pinhole removes stray light from the system. It was placed 15 cm from the condenser (5 cm from the collector) where the rays from the collector lens converged.

The illumination is scattered by the sample to pass through the objective lens. (Figure 1) To magnify the image, we used a single lens objective (f = 75 cm) placed on the opposite side of the sample from the light box at 10 cm. The lens converged the light to a point 30 cm away where a pinhole (diameter = 30 $\mu$m) was placed to block unfocused light. This point is in the conjugate image plane meaning that it is in the same focus as the sample. Behind the pinhole, a photodiode (Thorlabs, DET100A2-Si Detector) reads a signal output in volts.

### C. Sample Preparation

The sample was constructed by sandwiching dandelion stamen between two glass slides and mounting them to a post and base with tape. The base was able to be moved with precision along the x-axis (perpendicular to the optical axis) such that the sample could be scanned in one dimension.

### D. Data collection

To acquire an image from the brightfield microscope, we took a picture of the viewing screen in a darkened room.

For the confocal microscope, a 1D image was obtained by moving the sample over by 0.01 mm, recording the voltage output from the photodiode, and then repeating the process 100 times for a total movement of 1 mm.

### E. Magnification Measurements

For the compound brightfield microscope, the magnification (M) can be calculated using:

$$M = \frac{h'}{h} \tag{1}$$

Where h' is the height of the image, and h is the height of the specimen.

For the single lens confocal microscope, the magnification can be calculated using:

$$M = -\frac{s'}{s} \tag{2}$$

Where s' is the distance between the single lens objective and pinhole in front of the photodiode, and s is the distance between the objective and specimen.

The SNR is calculated using:

$$SNR = \frac{P_{signal}}{P_{noise}} \tag{3}$$

Where $P_{signal}$ is the power of the signal and $P_{noise}$ is the power of the noise. To find the SNR for the brightfield microscope, we used the 2D image to get a 1D line in the same location as where the confocal imaged. We graphed the gray values along this line. We found $P_{signal}$ by measuring the change in gray values for a peak in signal due to the edge of the sample. $P_{noise}$ was the variation in the gray values for a flat readout section. To find the SNR for the confocal microscope, we used the recorded voltage values from the 1D image line. We found $P_{signal}$ and $P_{noise}$ in the same way as for the brightfield, except instead of using gray values, we used the voltage. A higher SNR value means the image is more clear.

## III. RESULTS

We were able to image the dandelion with both the brightfield and confocal microscope. (Figure 2) For the brightfield microscope, h and h' were 4 ± 0.5 mm and 40 ± 1 mm respectively. $P_{signal}$ and $P_{noise}$ were 69 ± 2 and 16 ± 2 gray values respectively. The magnification for this microscope was 10 ± 1.5X and the SNR was 4.3 ± 0.7. For the confocal microscope, s and s' were measured to be 9.5 ± 0.2 cm and 28.5 ± 0.2 cm respectively. $P_{signal}$ and $P_{noise}$ were 0.022 ± 0.001 and 0.001 volts respectively. This gave a magnification of -3 ± 0.1X and a SNR of 22 ± 1. The negative sign on the confocal's magnification means that the image is inverted. The brightfield microscope had a higher magnification, but the confocal had a higher signal to noise ratio.

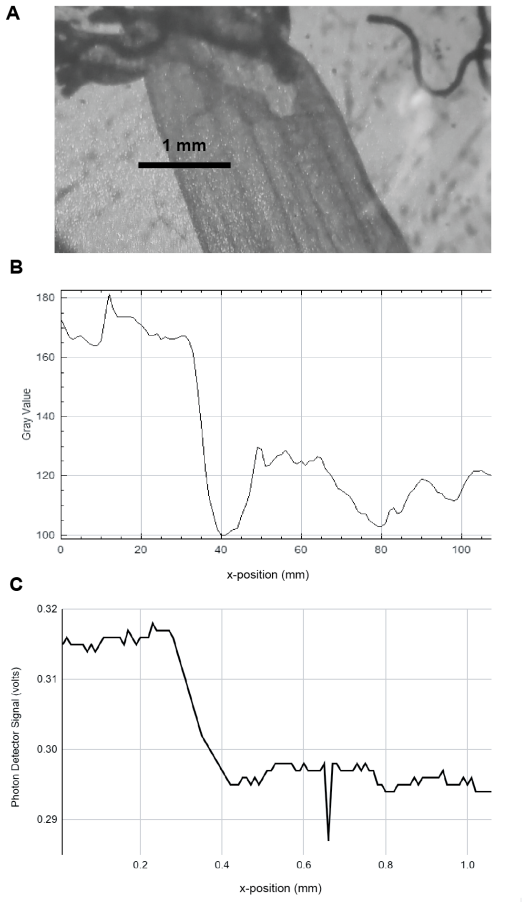


FIG. 2: (A) Image of dandelion stamen taken using a brightfield microscope with a 10X, 0.25 NA objective. The location imaged by the confocal microscope is shown as a line. (B) Plot of gray values from the 10X brightfield microscope along the line shown in A. Larger gray values correspond to lighter shades. (C) Photodiode signal (volts) collected as a sample of dandelion stamen was scanned in one dimension by a confocal microscope with 3X magnification along the line in A. A higher voltage corresponds to more collected light and lighter shades. In a darkened room, the voltage was recorded, the sample stage moved over by 0.01 mm, and the process repeated 100 times for a 1 mm line.

## IV. DISCUSSION

Our brightfield microscope imaged the sample with a magnification of 10 ± 1.5X and a signal to noise ratio of 4.3 ± 0.7. The confocal microscope was able to image a 1mm 1D section of the sample with 3 ± 0.1X magnification and a signal to noise ratio of 22 ± 1.

The images acquired from the confocal and brighfield microscopes revealed parts of the dandelion stamen's structure. (Figure 2) We were able to see wide, transparent sections cut through by darker, thick lines. The

lines were parallel to each other, about 0.4 mm apart and ran from the base to the end of the stamen. These structures may be used like veins for transportation of resources through the stamen or as supports to keep the stamen's shape.

The confocal microscope was able to achieve a higher signal to noise ratio than the brightfield. This is important when imaging biological samples as it produces a clearer image where faint details in the sample can be distinguished from the background noise. This advantage is due to the pinhole that blocks out unfocused light which would add noise to the image. However the confocal's design comes at a cost.

Our confocal microscope was limited in its magnification, 1D image dimensions and scanning capabilities, and photodiode sensitivity. The confocal microscope had a much lower magnification than the brightfield, preventing it from capturing finer details in the sample. It was only able to scan in 1D, whereas the brightfield microscope took an image in 2D. Unlike the brightfield microscope, the confocal image isn't produced instantaneously. Our method for scanning the sample was not automated, so it was tedious, imprecise, and time consuming as the sample had to be moved and voltage recorded from the photodiode at each interval. The photodiode and voltmeter readout were limited in their sensitivity and number of significant values.

To improve our design of the confocal microscope, we could increase the magnification and increase the signal to noise ratio. Higher magnification could be achieved by increasing s' and decreasing s or by using a compound objective with a higher magnification rather than a single lens. Noise in the readout could be reduced by enclosing the microscope in a dark box. Some of the noise in the brightness while scanning can be attributed to fluctuations in the brightness of the surroundings.

Confocal microscopes are also harder to build than brightfield microscopes. They require more components, they need a high precision in alignment so that light is able to correctly pass through the lenses and hit the pinholes, they use a photodiode instead of projecting the image onto a screen, and they involve moving parts for the stage to be scanned. All of these factors introduce challenges into the construction process from higher costs due to more parts to more hours spent fiddling with alignment.

So, which is better, the confocal or brightfield microscope? It depends on what is being imaged, what one wants to learn about the specimen, and how many resources are available. The confocal microscope is the better option when imaging thicker samples and if one needs a high signal to noise ratio to distinguish faint details from the background. However, it requires more time to build and image the sample. If one is looking for an easier, faster approach to get a quick 2D image, the brightfield microscope would be better.

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