

Lab Report 3 - Optical Microscopy

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Abstract

In this work, we aim to explore the use of conventional optical microscopy and confocal microscopy. Using a standard optical microscope, image magnification and resolution were explored with a number of different tests and sampling regions. Following this, three different labs using a confocal microscope were conducted. These various experiments demonstrated key components of the microscopy technique. These measurements highlight both the advantages, as well as the limitations, of diffraction-limited optical microscopy for studying nanoscale emitters. Further, through these experiments, we are able to demonstrate the importance of diffraction effects, detector sensitivity, calibration techniques, and more.

1 Background

1.1 Stereo Microscope

A conventional microscope forms magnified images using a combination of objective lenses along with an eyepiece lens. Light transmitted through or reflected from a sample are collected by the objective, which thereby produced a real high resolution image. This image is magnified through the eyepiece and produced an image observable to the human eye. For successful imaging with a stereo microscope, one must position the sample slightly beyond the focal plane of the objective lens so that the real image can be properly formed (and magnified) when it "enters" the eyepiece.

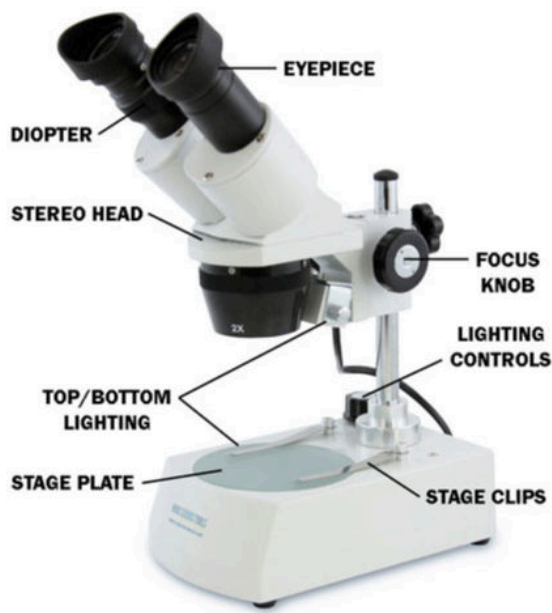


Figure 1: Stereo Microscope.

The magnification of the sample depends not only on the eyepiece, but also the objective. While magnification is a key parameter for successful imaging, it alone doesn't not determine clear image quality; the resolving power is highly dependent on both the diffraction and numerical aperture of the objective lens, and therefore are key to successful imaging.

Since conventional microscope are found to be a very simple, fast, and useful method to imaging micrometer structures, stereo microscopes are widely used in numerous field for imaging structures such as biological cells, electronic components, and geological samples.

1.2 Standard Optical Microscope

A standard wide-field optical microscope illuminates an entire sample simultaneously and collects both in focus and out of focus light at the detector/eyepiece. This is very useful for simplicity and speed, but comes at a cost when one seeks to image a thicker sample, different objective, or fluorescent sample.



Figure 2: Olympus BX51 Light Microscope.

The spatial resolution of a wide-field microscope is limited by diffraction and determined specifically by the numerical aperture of the objective lens. Numerical aperture, NA, measures the “light-gathering ability” of the objective. It measures the light-gathering ability of the objective and strongly affects resolution. Higher NA improves both resolution and image brightness. The spatial resolution is determined by NA, such that,

$$\Delta x = \frac{0.61\lambda}{NA}$$

where λ is the wavelength. This value defines the smallest lateral feature that can be resolved. Increasing numerical aperture improves both resolution and brightness because the objective collects light over a large range. The NA can also be defined as,

$$NA = n \sin \alpha$$

where n is the refractive index of the medium, and α is the half angle of the collected light cone. Oil immersion objectives increase the numerical aperture and improve resolution, brightness, and clarity. This is done by reducing the light refraction of the imaging medium. Although wide-field microscopes are limited to resolutions on the order of a few hundred

nanometers, they remain very effective for imaging samples at the micron-scale.

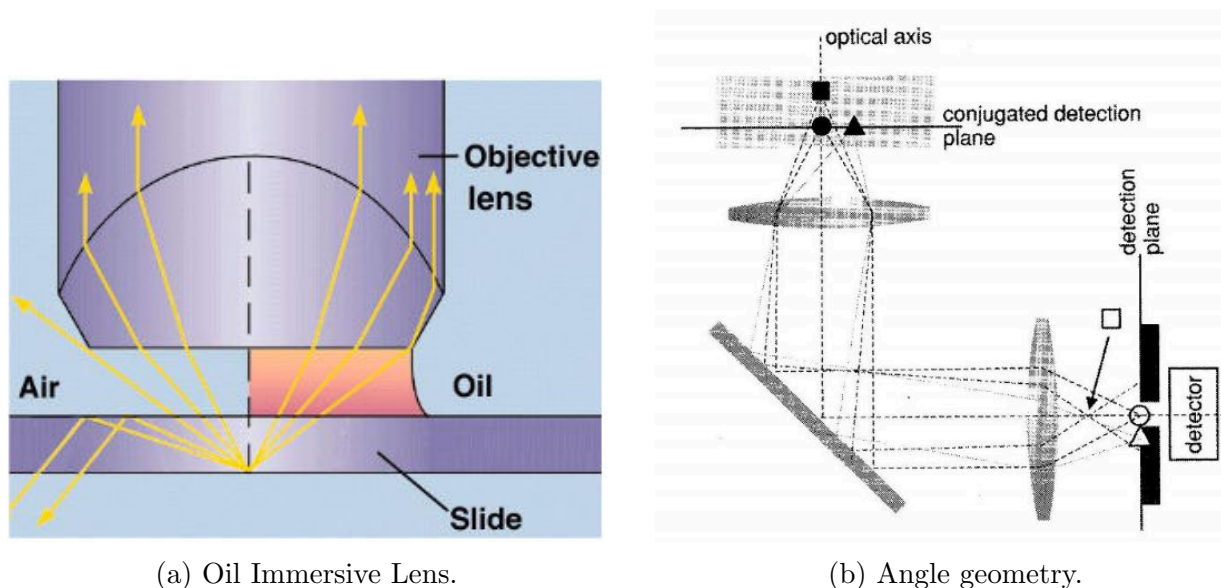


Figure 3: Numerical Aperture.

1.3 Confocal Microscope

Confocal microscopy is an optical imaging technique that improves image contrast by selectively detecting light from a small focal volume within a given sample. Rather than illuminating the entire sample at once like a wide-field microscope, a confocal microscope uses focused light to acquire images, allowing signals to be collected from specific depths inside the sample. This technique enables one to image structures within thicker samples that would otherwise appear blurred in conventional microscopy.

The main components of a confocal microscope include the laser (used as the light source), objective, sample table, scanning system, pinhole, various filters, and the detectors. The laser excites fluorescence within the sample, and the emitted signal is separated from the excitation light using a dichroic mirror. A key feature of the system is the pinhole aperture, which rejects light that is out of focus, allowing only light from the focal plane to contribute to image formation.

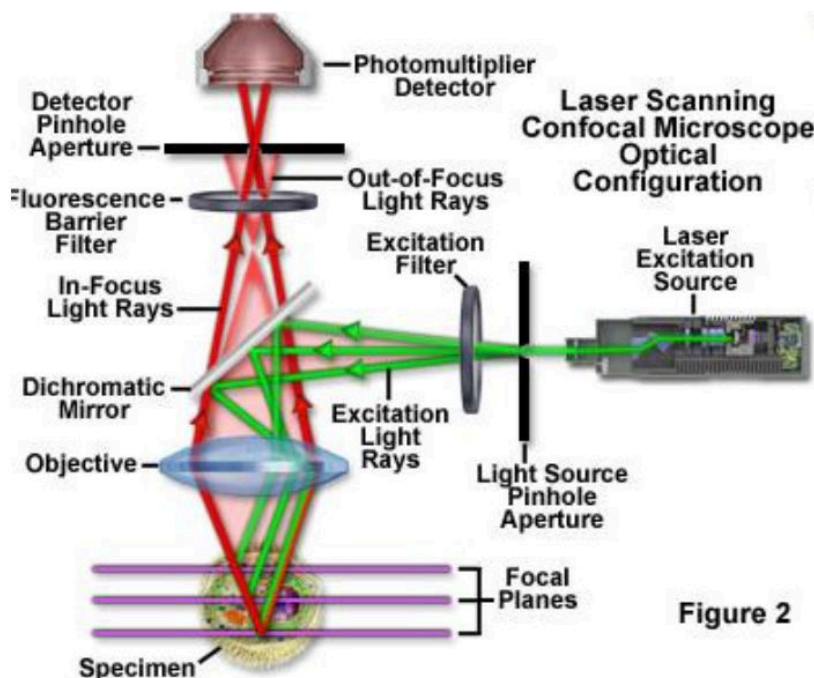


Figure 4: Confocal Microscopy Setup.

Because out of focus light is removed, confocal microscopy greatly reduces background information away from the focal plane and provides improved contrast compared to wide-field microscopy. Another advantage of this technique is that it collects serial optical sections from the sample (at different depths), which makes it possible to reconstruct 3-D images of the sample.

Compared to conventional microscopes, confocal microscopes allow one to carefully control the depth of the field and thereby obtain more precise information from specific regions of interest. However, wide-field microscopes still have advantages in that they image much faster and produce higher signal intensity. Further, they require less intense illumination for imaging, making them useful when rapid data acquisition is needed. Both techniques remain very widely used, and it is important to consider the sample being imaged, and what data one hopes to acquire, when determining which technique to use.

1.3.1 Photon Statistics

In the confocal fluorescence setup, single photon detection was performed using avalanche photodiodes (APDs), which act as sensitive photon counting detectors capable of recording individual photon arrival events. These detectors were arranged in a Hanbury Brown–Twiss setup, which allows measurements of photons over time and provides information about whether a light source behaves as a classical emitter or a true single photon source.

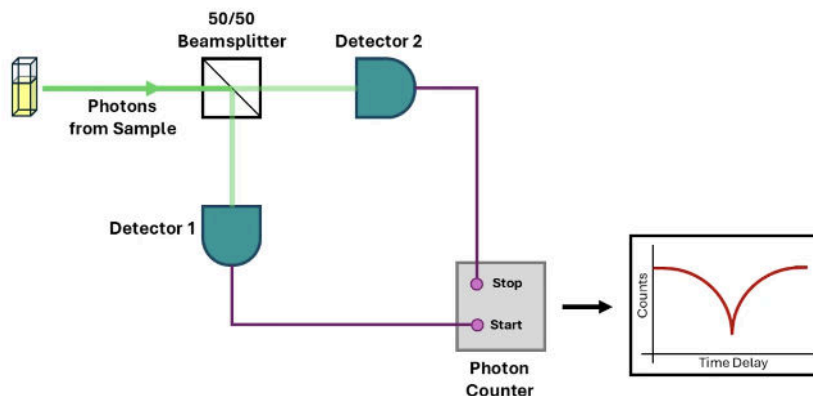


Figure 5: Hanbury Brown-Twiss Setup.

Using two detectors instead of one is necessary since when an APD detects a photon it goes through a dead time where it cannot record another photon, which limits detection efficiency. By introducing a second detector, the dead time for each cancels each other out resulting in there always being a time where a photon can be recorded. This improves the reliability of photon counting measurements and allows coincidence detection between the two detector channels.

In this configuration, coincidence counts are measured as a function of time delay between detection events on APD1 and APD2. These measurements are recorded using a timing electronics system that produces a histogram of photon arrival delays. The most important region of this histogram corresponds to zero time delay, which represents simultaneous photon detection events at both detectors. If the detected signal originates from a true single-emitter, then only APD1 or APD2 can make the detection event, meaning that coincidence counts at zero delay should be nonexistent.

This effect is known as antibunching, which is a defining signature of single photon emission. For a single photon source emitter, one should only detect a single photon at a time since photons cannot be detected simultaneously when only one emitter is present. In contrast, attenuated classical light sources (such as lasers) can still produce photon pairs, and therefore do not exhibit true antibunching behavior.

By analyzing coincidence counts near zero delay, it is possible to determine whether the fluorescence signal originates from a single photon emitter or from a classical light source. These measurements demonstrate how photon correlation techniques can be used to characterize nanoscale emitters beyond the diffraction limit of a conventional optical microscope.

2 Procedure

All imaging was conducted in Wilmot Building. The stereo microscope used in this lab was property of Professor Svetlana Lukishova, and the standard optical microscope was an Olympus BX51 Light Microscope used in Wilmot second floor. All confocal microscopy measurements were conducted in Professor Svetlana Lukishova's lab in Wilmot 323.

3 Stereo Microscope Procedure

1. The stereoscopic microscope was powered on and the illumination source was adjusted to provide sufficient lighting for the sample.
2. The sample was placed on the microscope stage and centered within the field of view.
3. The distance between the eyepieces was adjusted for comfortable viewing.
4. The focus knob was used to bring the sample surface into clear focus.
5. The magnification was adjusted using the zoom control to observe different surface features of the sample.
6. The illumination angle and intensity were modified as needed to improve contrast and visibility of the sample features.
7. Representative regions of interest were visually inspected and recorded for comparison with higher magnification optical microscope observations.

4 Standard Optical Microscope Procedure

1. The optical microscope illumination source was turned on and adjusted using the condenser and diaphragm to provide uniform lighting across the sample.
2. The sample was placed on the microscope stage and secured in position.
3. A low magnification objective lens was first selected in order to locate regions of interest on the sample before switching to higher magnifications.
4. After locating a feature at low magnification, the objective magnification was increased step by step to observe smaller configurations in greater detail.
5. The coarse adjustment knob was used to bring the sample into approximate focus, followed by the fine adjustment knob to obtain a sharp image.

6. The imaging software QCapture Pro was used to record images and perform measurements of selected sample features.
7. Before performing measurements, the microscope scale was calibrated in QCapture Pro by selecting the calibration settings, entering the appropriate magnification, and verifying the measurement scale.
8. After calibration, the special measurement scale option was used to check feature dimensions at each magnification level.
9. The magnification setting was verified each time the objective lens was changed, and calibration was repeated as necessary to maintain measurement accuracy.
10. Representative features of interest were recorded at multiple magnifications for comparison and analysis.

Note: Sometimes the scale for the NA is not accurate (one must enter data for the scale and everyone sharing the microscope used different values). Therefore there is a special scale one can use to check for special measurements.

5 Confocal Microscope Procedure

1. The confocal microscope system was prepared and aligned prior to the start of the experiment by the instructor.
2. Two avalanche photodiodes (APDs) were connected to the detection outputs of the confocal microscope for single photon counting measurements.
3. The APD signals were routed to the PicoHarp 330 timing electronics module for coincidence counting and photon correlation measurements.
4. The PicoHarp 330 was connected to the control computer carts used for data acquisition and histogram visualization.
5. Coincidence counts between APD1 and APD2 were recorded as a function of delay time using the PicoHarp 330 software interface.
6. Add filtration to the microscope if this will be your first scan with a sample, or the first scan using a different laser power.
7. Remove beam block.

8. Turn off lights. Plug in APDs and power on.
9. Open the application for the SCOM.
10. Press the start button.

Note: The laboratory lights were turned off in order to minimize background photon noise during measurements (these detectors are very sensitive, and keeping the lights on during use can be very damaging) and the fluorescence signal from the sample was monitored using both APDs simultaneously.

6 Results and Discussions

6.1 Stereo Microscope Results

Initial imaging of the fabricated alignment array were performed using the stereo microscope. The array features were clearly visible across a large field of view. The stereo microscope provided a useful baseline of the sample layout along with the spacing between structures. While one could clearly see the array in full view, this method did not allow for precise quantitative measurements due to its lower magnification.

The wide field of view made it possible to quickly identify multiple alignment markers simultaneously and confirm the overall uniformity of the patterned array prior to higher magnification imaging. However, as mentioned, fine feature edges and small separations between structures were not resolved with enough clarity for detailed measurement.

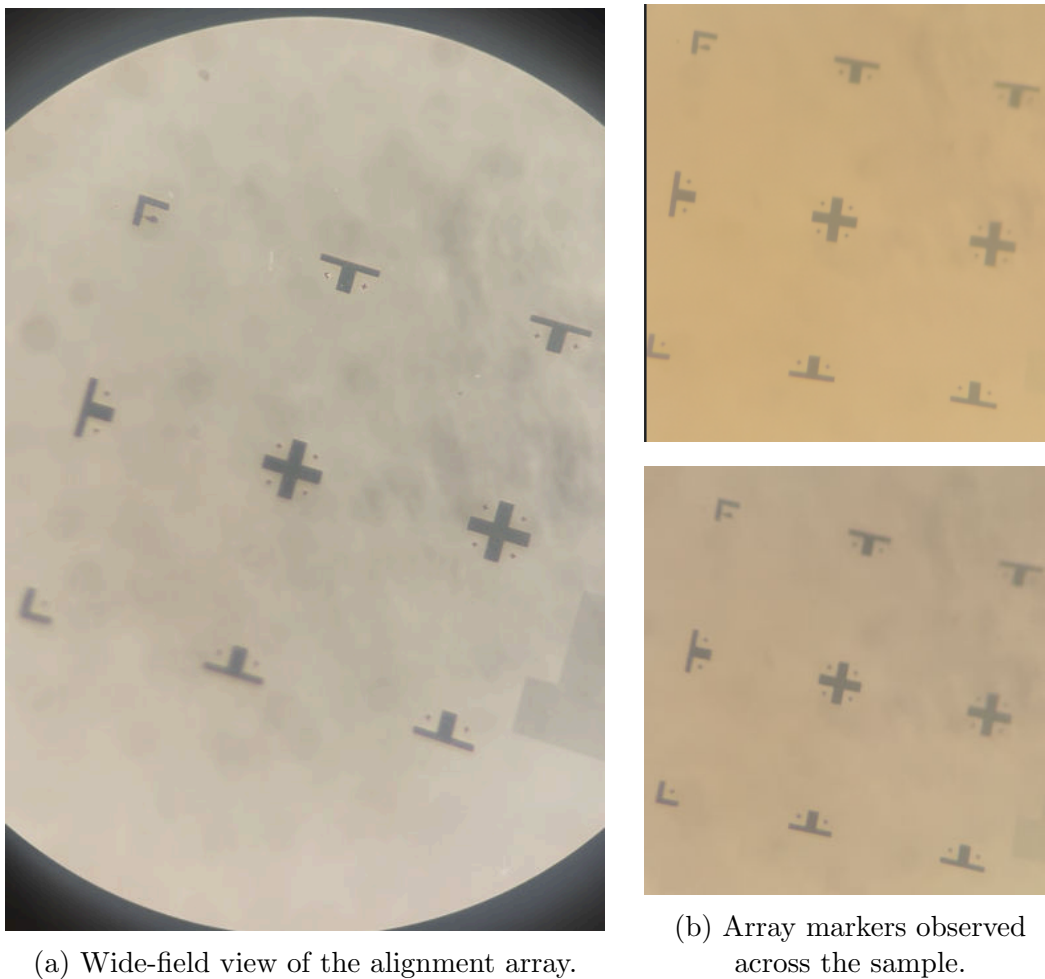


Figure 6: Stereo microscope images of the fabricated alignment array.

6.2 Standard Optical Microscope Results

6.2.1 Array Measurements at 10x

Initial inspection of the array was performed at 10x magnification. The spacing between alignment features was measured to be $68.44\mu\text{m}$, while the width of a single arm was approximately $9.801\mu\text{m}$. Additional measurements taken from a neighboring array region included horizontal and vertical separations of $39.29\mu\text{m}$ and $49.71\mu\text{m}$, respectively, along with an additional spacing of $29.48\mu\text{m}$ and an arm width of approximately $19\mu\text{m}$. These measurements confirmed that the overall geometry of the array could be resolved at low magnification and provided a baseline estimate of the array's dimensions prior to higher resolution imaging.

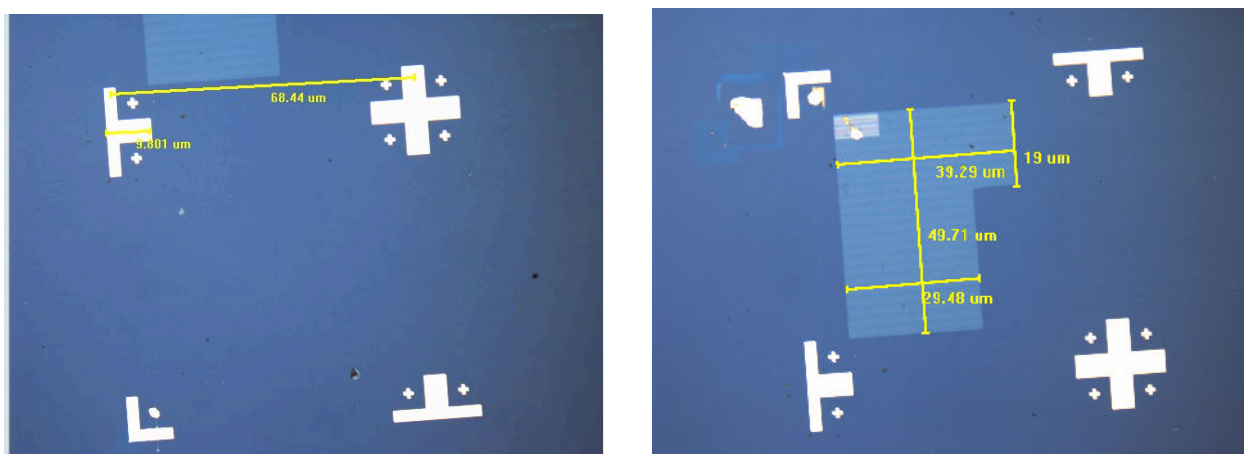


Figure 7: Array structures observed at 10x magnification.

6.2.2 Array Measurements at 20x

The microscope was then set to 20x magnification using both the patterned array and a calibrated stage graticule. The graticule verified a spacing of $100\mu\text{m}$ between major divisions and a total measured span of approximately $501.5\mu\text{m}$, confirming correct calibration of the measurement scale. Measurements of a rectangular array region at this magnification yielded horizontal and vertical dimensions of $397.2\mu\text{m}$ and $186.1\mu\text{m}$, respectively.

A cluster of silver nanocubes was also observed at 20x magnification. While individual nanocubes could not be resolved due to diffraction limited resolution, the cluster structure and contrast relative to the substrate were clearly visible.

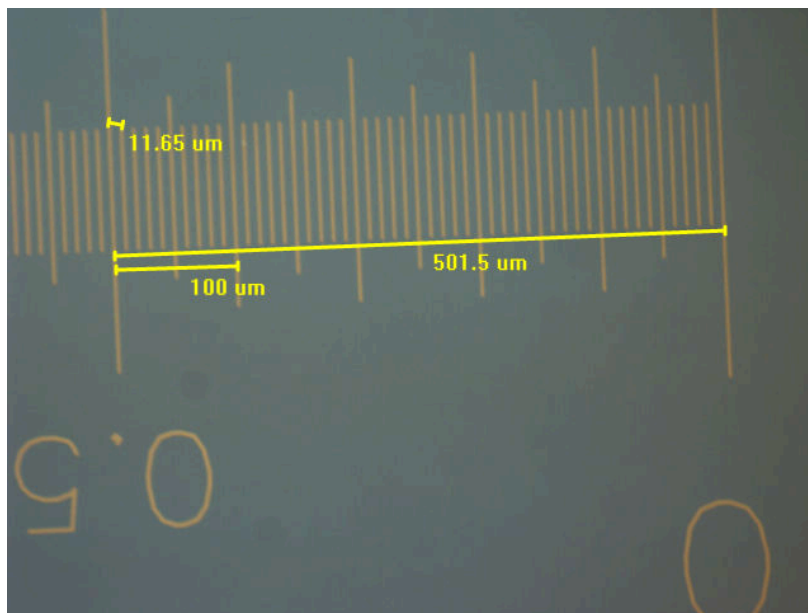


Figure 8: Stage graticule calibration image acquired at 20x magnification.

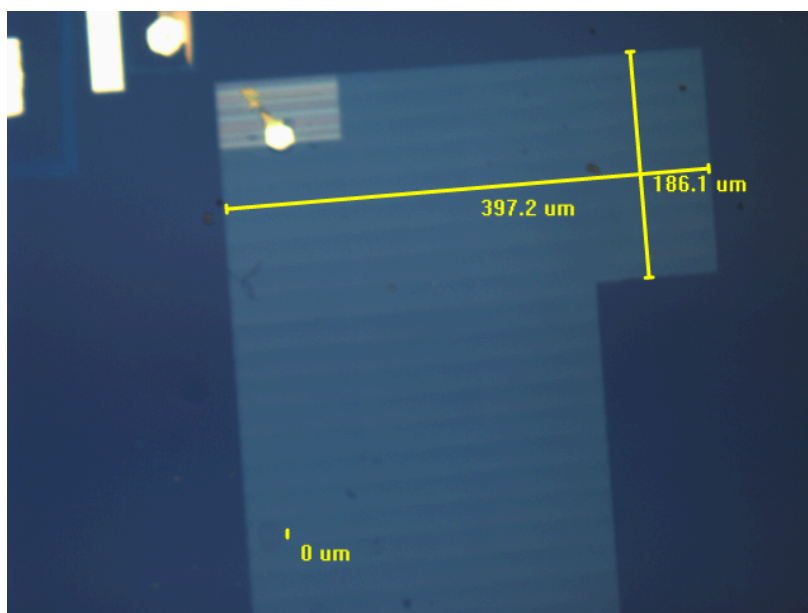


Figure 9: Array feature measurements at 20x magnification.

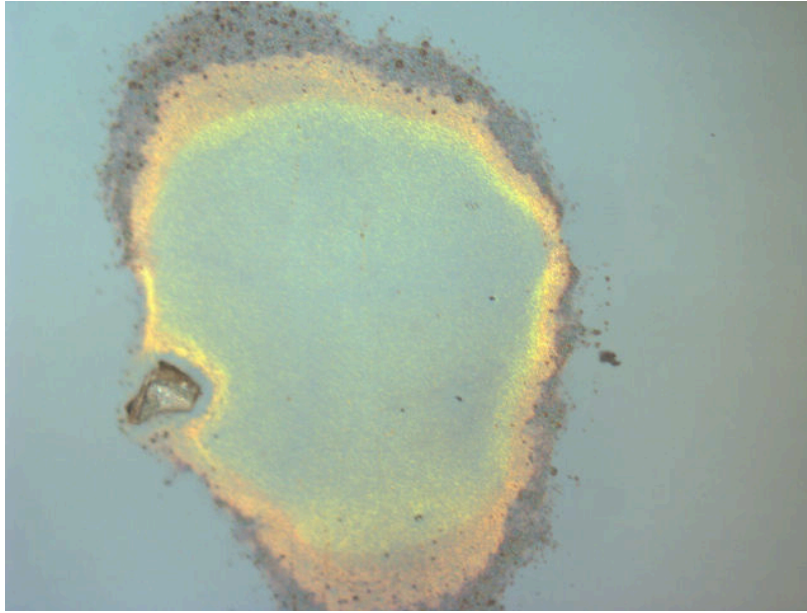


Figure 10: Silver nanocube cluster observed at 20x magnification.

6.2.3 Array Measurements at 50x

The array was then observed at 50x magnification. At this higher magnification, the field of view was reduced to a smaller region of the patterned structure, allowing improved visualization of feature edges and local surface contrast relative to lower magnifications. No additional quantitative measurements were recorded at this magnification, and the image was used primarily for qualitative comparison with the 10x and 20x observations.



Figure 11: Array region observed at 50x magnification.

6.3 Confocal Microscope Results

6.3.1 Lab 1: Beam Spot Alignment

Initial alignment of the confocal microscope was performed by imaging the focused excitation beam on the sample plane. A localized bright spot was observed near the center of the scan window, confirming that the excitation path was properly aligned through the objective before fluorescence measurements were performed. Establishing a well defined focal spot ensured that later scans probed a confined region of the sample.

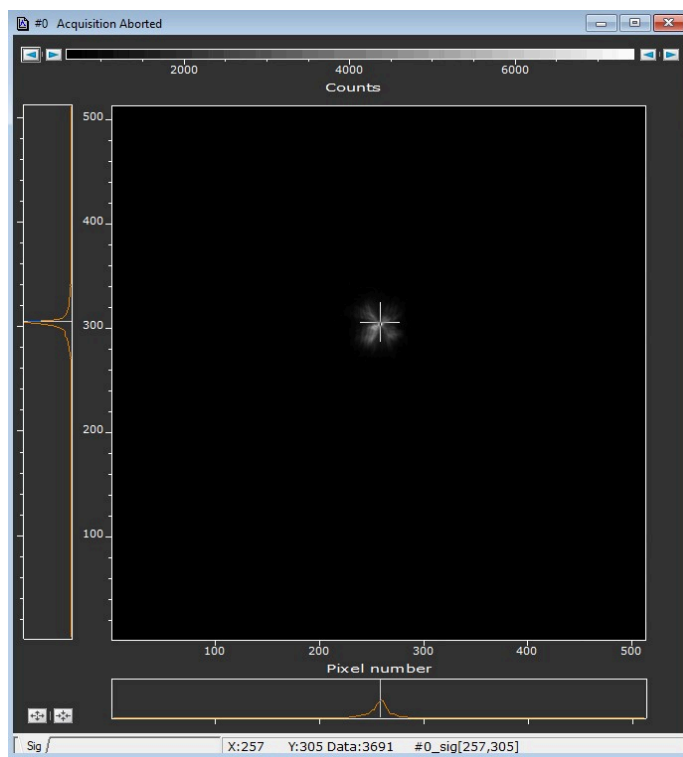


Figure 12: Focused excitation beam spot observed during confocal alignment.

This experiment confirmed that the excitation beam was properly focused onto the sample, ensuring that the following confocal scans were localized to the region we intended for imaging rather than background fluorescence from out of focus regions.

6.3.2 Lab 2: Localization of Fluorescence Sites

Raster scans of the sample surface were performed to identify localized fluorescence spots. Several bright regions were observed that were stronger than the surrounding background signal. A smaller scan region was then selected to isolate one localized fluorescence site for further spectral and photon correlation measurements.

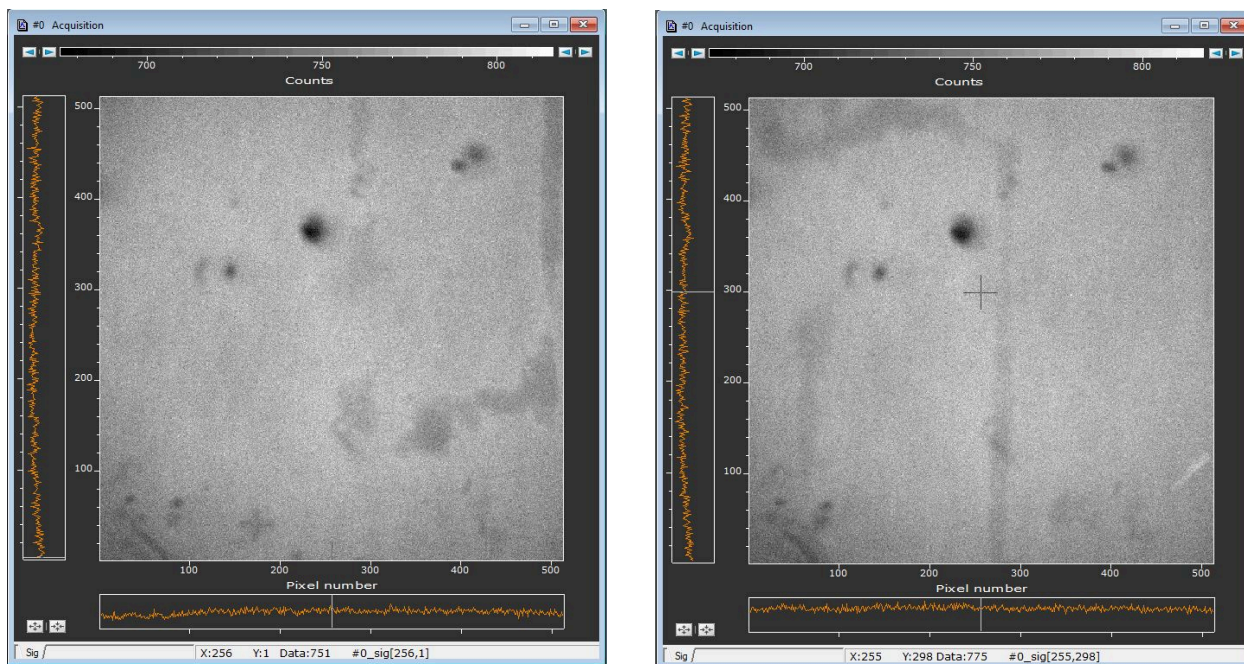


Figure 13: Scans used to identify isolated emission regions in the sample.

The presence of spatially isolated bright regions indicates localized emission sites within the sample.

6.3.3 Lab 2: Emission Spectrum Measurement

After selecting a region, the emitted signal was directed into the spectrometer for wavelength resolved analysis. The spectrometer was configured with a 600gr/mm grating and a blaze wavelength of 500nm. The center wavelength was set to 600nm, and the detector exposure time was 20000ms with high analog gain. The detector temperature was approximately -60°C during data acquisition. The measured signal extended across the visible wavelength range from approximately 480nm to 710nm.

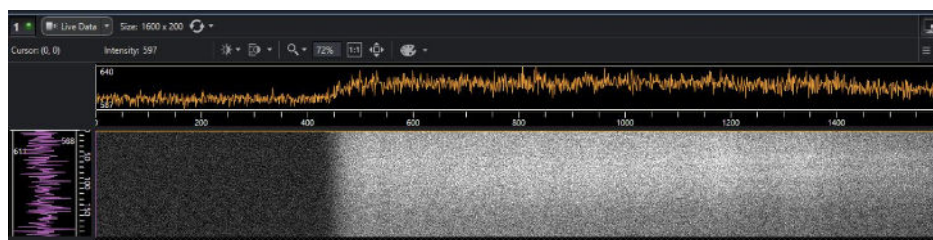


Figure 14: Emission spectrum recorded from the selected fluorescence site.



Figure 15: Spectrometer configuration used during wavelength resolved measurements.

The broad spectral distribution across the visible range confirms that the detected signal originated from the sample rather than reflected excitation light.

6.3.4 Lab 2: Photon Correlation Measurement

Photon correlation measurements were performed using the PicoHarp 330 time correlated single photon counting module. The recorded coincidence histogram contained 3438 channels with a bin width of 0.032ns, corresponding to a total measurement window of approximately 110ns. The coincidence counts were centered near 130 counts and ranged between approximately 90 and 170 counts across the histogram.

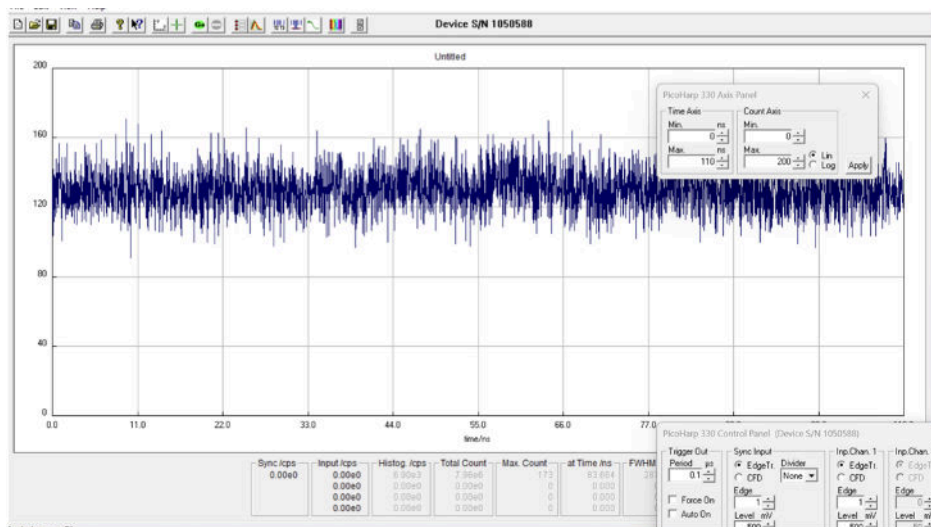


Figure 16: Coincidence histogram recorded during photon correlation measurements.

6.3.5 Lab 3: Beam Splitter Timing Calibration

A beam splitter was inserted into the detection path to divide the collected signal between the two APDs prior to data collection. The detector pulse had a width of approximately 248ns and a peak amplitude on the order of 4-5V, based on the 1.00V/div oscilloscope scale. This confirms a stable detector response suitable for coincidence timing calibration. This measurement established the zero-time delay reference required before interpreting the photon correlation histogram recorded with the PicoHarp 330.

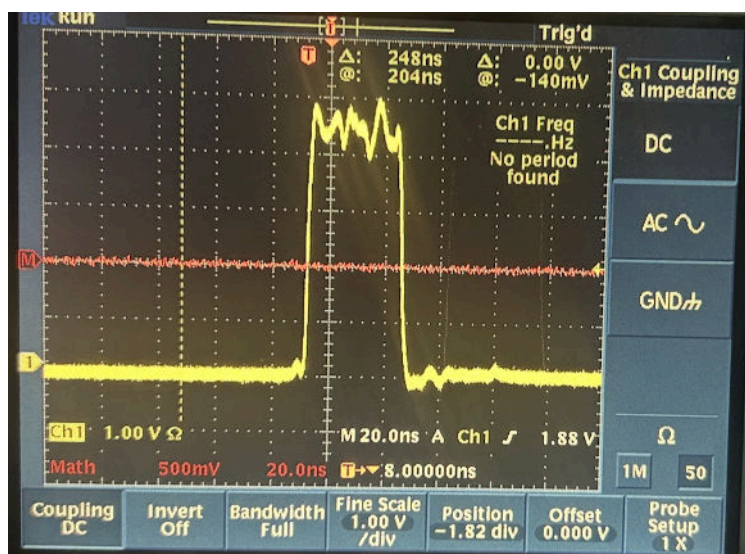


Figure 17: Oscilloscope trace of the APD output signals used to verify timing alignment between detector channels during beam splitter calibration.

Establishing this timing reference ensured that any future measurements could be interpreted relative to a calibrated zero-delay position between detector channels.

Note: Some images, including the histogram discussed earlier, are not pictured in this report. Some data was not accessible at the time of this report's completion.

7 Conclusion

The data analysis above follows our previous understanding of optical microscopy as a high imaging, successful set of techniques. Multiple optical microscopy methods were used to characterize fabricated structures and investigate localized fluorescence within the sample. Stereo microscope imaging verified the presence of alignment markers across the patterned array, while optical microscope measurements allowed feature dimensions and spacings to be estimated at several magnification levels. These observations confirmed that the fabricated structures were resolved clearly at larger scales and that their geometry was consistent across the sample surface.

Confocal microscopy measurements were then used to localize fluorescence within the sample and isolate regions of enhanced emission. Raster scans revealed spatially confined bright spots that were stronger than the surrounding background signal, indicating localized fluorescence sites suitable for further investigation. Wavelength resolved measurements of these regions showed emission extending across the visible range from approximately 480nm to 710nm. Photon correlation measurements were performed using the PicoHarp 330 used to evaluate the statistics of the detected photons. Finally, a beam splitter timing calibration was performed to verify synchronization between APD detection channels. This process confirmed stable detector response and established the zero-time reference required for co-incidence measurements.

Overall, these measurements demonstrate the use of many optical microscopy techniques to locate localized fluorescence sites, characterize their spectral properties, and evaluate their photon correlation behavior. The experiment provided practical experience with confocal alignment, wavelength resolved detection, and time correlated single photon counting methods used in the study of nanoscale optical emitters. This broad set of techniques can be used separately and together to image and characterize sample structures across multiple length scales, from millimeter-scale alignment features observed with the stereo microscope to micron-scale patterns resolved with the optical microscope and localized regions identified with confocal microscopy.

References

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- [2] Home Science Tools, *Using a Stereo Microscope Science Lesson*, Website, <https://learning-center.homesciencetools.com/article/using-a-stereo-microscope-science-lesson/>.
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