My name is Ethan Costello, and I am a senior Electrical and Computer Engineering student at the Herff College of Engineering. After four years of college, I graduated in May 2023 with Magna Cum Laude distinction. I have spent my time in college making friends, studying, and finding new interests. One of those new interests is the study and design of optical systems, which I have expressed through working with my teachers and writing research papers. The time I have spent in college has been the best of my life and I am looking forward to what comes after.

Ethan's paper received a *QuaesitUM* outstanding paper award.

Ethan Costello

Design & evaluation of a confocal scanning microscope using off-of-shelf optical components by means of Zemax OpticStudio optical design software

Faculty Sponsor

Dr. Ana Doblas

Abstract

Confocal microscopes are known for optically removing the out-of-focus information (i.e., blur) in each transverse section of the sample's volume, providing a more accurate three-dimensional image of thick microscopic samples than widefield microscopes. In this work, we have designed and evaluated a confocal microscope using off-of-shelf optical components from Thorlabs' catalog, one of the major optical manufacturers. The design and evaluation have been implemented using Zemax OpticStudio, the standard optical system design software for realistic optical simulation. Here, we have also reported a practical protocol for building a confocal microscope using sequential mode.

Introduction

Biological research relies heavily on cell imaging to provide valuable morphological and functional information within cells and tissues. Among the different microscopic imaging modalities, confocal microscopes have helped biological researchers to understand cell motility, behavior, and regulation in three-dimensional environments that mimic the specific organization of organs (Singh & Gopinatthan, 1998; Matsumoto, 2003; Croix et al., 2018; Elliot, 2020). The major difference between conventional widefield microscopes and confocal microscopes is the point-based illumination and detection configuration, providing optically sectioned images (Martinez-Corral & Saavedra, 2009).

These optically sectioned images are two-dimensional (2D) images from transverse sections of a three-dimensional (3D) sample in which the out-of-focus information (e.g., blurred areas) has been optically rejected, providing clear imaging of the in-focus sections of the sample at each axial plane. Although this is the key feature of confocal microscopy compared to conventional fluorescence microscopy, another advantage of confocal microscopes is their ability to produce multicolor imaging in which different sections of the biological specimen are stained using multiple dyes, enabling colocalization studies in biological systems (Dunn et al., 2011; French et al., 2008; Jessen, 2013; Zinchuk et al, 2007). Thanks to these features, the applications of confocal microscopy are quite broad from live-cell imaging and analysis of cells and tissues to cancer pathologies and drug discovery.

Background of Confucal Microscopy

A confocal microscope is composed of two subsystems: the illumination and detection systems (Martinez-Corral & Saavedra, 2009). Figure 1 shows the optical configuration of a confocal system. The monochromatic light emitting from a laser source is collimated and focused onto the three-dimensional sample by a microscope objective lens. Although the focused beam illuminates all parts of the sample within the illumination cone, the small region surrounding the beam's focus receives the highest illumination energy. Assuming that the illumination wavelength is within the excitation band of the fluorescent dye, the sample’s region stained by those fluorophores are activated, emitting fluorescent light (e.g., light with a longer wavelength than the illumination wavelength).

In traditional confocal microscopes, the detection system is composed of the microscope objective lens, the tube lens, a pinhole (e.g., an optical aperture), and a photomultiplier as a detector. The fluorescent light scattered by the sample is collected by the microscope objective lens and focused on

the pinhole thanks to the tube lens. The photomultiplier is the ideal photometric detector for this imaging modality based on its speed and sensitivity. Experimentally, the photomultiplier is placed right after the pinhole to collect the maximum amount of light.

As previously mentioned, the feature of confocal microscopy is its optical sectioning capability (i.e., the optical removal of the out-of-focus information). This capability is a consequence of the fact that the object focal plane of the objective lens is conjugated with a pinhole plane. Therefore, parts of the sample outside the in-focus plane (e.g., object focal plane of the objective lens) are not conjugated with the pinhole (e.g., image plane), reducing the probability of fluorescent light detection (Martinez-Corral & Saavedra, 2009). In other words, point-based detection using the pinhole is responsible for providing optical sectioning capability. On the other hand, parts of the sample outside the in-focus plane receive a smaller illumination density, reducing the probability of fluorescent emission and photobleaching of the samples.

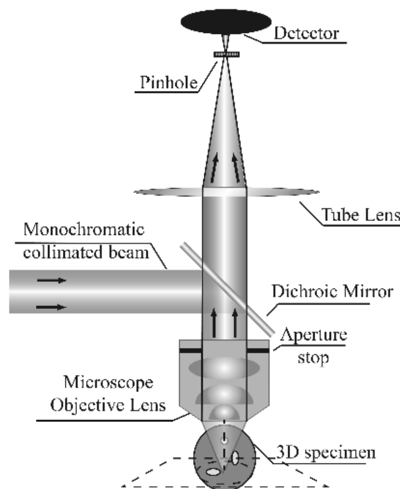

Figure 1. Optical configuration of a Confocal Microscope. The key feature of the point-based illumination and detection.

Design of a confocal microscope using Zemax OpticStudio optical design software

This section provides a practical protocol for building a confocal microscope using off-of-shelf optical components from Thorlabs' catalog, one of the major optical manufacturers. The design and evaluation have been implemented using Zemax OpticStudio, the standard optical system design software. Zemax optical design software is a readily available commercial optical design software that allows us to conceptualize, design, optimize, and analyze virtually any optical system (Geary, 2002; Siew, 2017; Sun, 2017). It is widely used in the Optics industry as a standard design tool since one can evaluate the performance of realistic optical elements from the already implemented numerical methods, including a study of the aberrations and the impulse response. Zemax has allowed engineers, scientists, and researchers bring their optical designs into real commercial systems.

In our design, we have two main changes to Figure 1. The first one is the replacement of the dichroic mirror by a beam splitter. Dichoric mirrors reflect and transmit light at two different wavelengths (e.g., color). For example, a longpass dichroic mirror with a cutoff wavelength of 490 nm reflect light with wavelength shorter than 480 nm towards the microscope objective lens in the illumination system and transmit wavelength larger than 500 nm towards the tube lens in the detection system. Conversely, beam splitters are optical elements that split incident light into two beams regardless of the wavelength. Although a beam splitter is not the desired optical element to build an experimental confocal microscope due to the loss of energy, it allows us to analyze the performance of the confocal microscope at different wavelengths without replacing the dichroic mirror. The second change is replacing the microscope objective lens with a condenser lens for design simplicity.

Let us start building a confocal microscope. The Zemax design of the microscope objective lens and tube lens are found on Thorlabs' website. The first element of the confocal microscope to insert is the beam splitter (Surface 2 in Fig. 2), which reflects the incident beam down at a 90o angle. Remember that the beam splitter separates the incident beam in two directions: one beam is transmitted through the beam splitter, and the second is reflected as a mirror does. The beam splitter was created by inserting a foldable mirror surface after the first element (a dumb surface, Surface 1 in Fig. 2) in our system with an angle of 45º. It is important to mention that a dumb surface in Zemax modeling refers to a surface that does not modify the trajectory of the optical rays in the optical design.

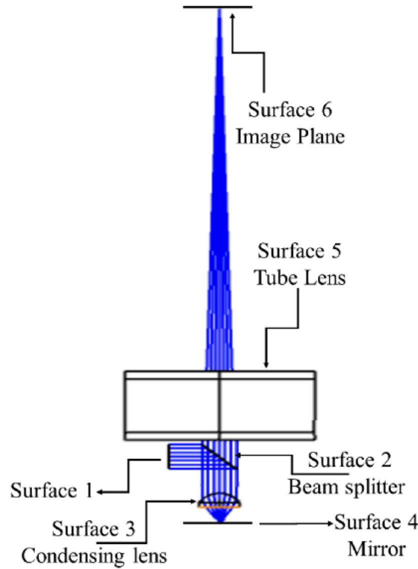

Fig. 2. Two-dimensional optical layout of the confocal microscope in Zemax OpticStudio

The second element in our system is an aspheric condenser lens (Surface 3 in Fig. 2), which replaces the microscope objective lens. Without any loss of generality, we select the AC1210U-A Thorlab's aspheric condenser lens with a diameter of 12 mm, a focal length of 10.5 mm, and a numerical aperture (NA) of 0.54. The AC1210U-A lens has a focal spot size equal to 3.953 μm at a wavelength of 633 nm.

The third element of the confocal microscope is a flat mirror. This mirror mimics a perfect thin fluorescent sample, reflecting the light back into the condenser lens. Since the confocal microscope is a point-based illumination, the mirror should be located at the focal plane of the condensing lens. Based on the manufacturer's datasheet, the focal plane is placed at a distance equal to 6.725 mm distance from the last surface of the lens.

The diverging light reflected by the mirror is collimated by the condenser lens, generating a collimated plane wave after the condenser lens. A collimated plane wave is such a wave whose size does not change, generating a point source at infinity. The collimated plane wave meets the tube lens (TTL200-A, Thorlabs), which is Surface 5 in Fig. 2. The separation between the condenser lens and the tube lens is irrelevant since the wave is collimated. Nonetheless, we ensure that the distance was enough so that the tube lens is after the beam splitter in Fig. 2. Since the tube lens is illuminated

by a collimated plane wave, focusing the light into a point at its image plane. Based on the manufacturer's datasheet, the image plane is located at 152.008 mm to the last surface of the tube lens. In an actual experiment, the image plane is the plane to set the pinhole. It is important to mention that the tube lens is shown as a black box in Fig. 2. We have chosen this lens to optimize the performance of the confocal microscope. This type of tube lens is known for its careful design, which contains multiple optical surfaces to compensate the optical aberrations further.

Evaluation of the confocal microscope using Zemax-Optic Studio

Zemax OpticStudio software allows the evaluation of the performance of any optical system using different metrics, including the analysis of the spot diagram, the aberrations present at each surface of the system, and the impulse response and its reciprocal Fourier transform. All these analyses are available on the Analyze tab of the software. Without any loss of generality, we have evaluated the performance of the proposed confocal microscope by analyzing the aberrations through the Seidel, the system's frequency response through the Modulation Transfer Function (MTF), and the variation of the spot size.

First, let us start with the Seidel diagram, which provides information about the aberrations up to the third order. In other words, Zemax OpticStudio informs us about the following aberrations: spherical aberration, coma, astigmatism, field curvature, distortion, axial color, and lateral color. A more detailed information of these optical aberrations can be found in Dereniak, (2008). Based on the Seidel diagram, we found that spherical and axial color aberrations were the only ones present in the system. Spherical aberration is present when the outer parts of an optical element do not focus the light rays at the same axial plane, generating a blurred image. On the other hand, axial color aberration is a type of chromatic aberration caused by the fact that light rays focus on different axial planes based on wavelength/color. In other words, if the material of the optical element presents normal dispersion, blue light always focuses on planes before red light. The aspheric condenser lens introduced these aberrations. However, since the light passes through the condenser lens twice (e.g., forward in the illumination system and reverse in the detection system), these aberrations cancel each other, not affecting the final performance of the confocal microscope (e.g., at the image plane there are not aberrations distorting the confocal system).

The second metric used to analyze the performance of the confocal microscope was the MTF plot. This curve informs us how the information is

transferred through the imaging system. In particular, we can find which and how much the spatial frequencies are distorted by the system. In other words, the MTF plot provides a quantitative measurement of the image contrast versus the spatial frequency. A key feature of Zemax OpticStudio software is that we can plot the system's MTF and compare it with the theoretical limit (known as the diffraction limit), providing us with another way to visualize if our system is distorted by aberrations. Although we cannot identify the type of aberrations by observing the MTF plot, as a general rule, the lower the area under the curve of the MTF plot, the higher the number of aberrations. Figure 3 shows the MTF plot of the confocal microscope for different wavelength (blue curve in Fig. 3) and its comparison with the diffraction limit (black curve in Fig. 3).

As we can see, both blue and black curves overlap for the different wavelength, meaning that the system does not present any aberration. Observing Fig. 3, we have estimated the maximum spatial frequency passing through the confocal microscope. This maximum spatial frequency is known as the

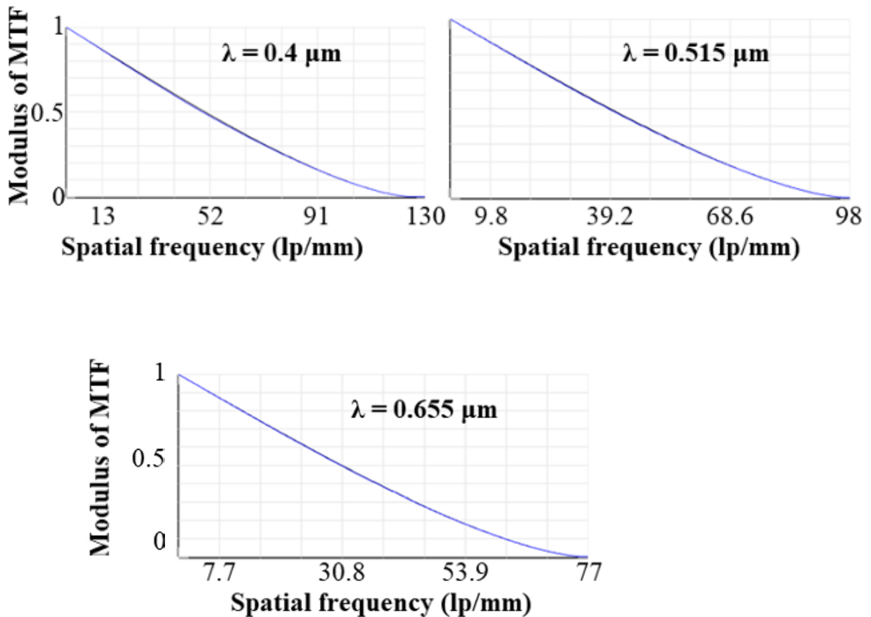

Figure 3. System's MTF for different wavelengths.

cutoff frequency, the first frequency at which the MTF value is null. The cutoff frequency of the confocal microscope is wavelength dependent, being 130 lp/mm for violet color ($\lambda = 0.4$ μm), 98 lp/mm for green color ($\lambda = 0.515$ μm), and 77 lp/mm for deep red color ($\lambda = 0.655$ μm). The higher the value of the cutoff frequency, the higher the resolution of the confocal microscope, being able to discriminate finer details. This behavior is already known in Optics; the same optical instrument has a better resolution capability using purple-blue color light than red.

Finally, we have evaluated the spot diagram of our confocal system, see Fig. 4. The spot diagram shows the image produced by our confocal system assuming that the object was a point source (e.g., the spot of light). Aberrations such as astigmatism and coma are instantly viewed on the spot diagram since they produce asymmetric images. Figure 4 shows that the spot diagrams for the different wavelengths are symmetrical, confirming that astigmatism and coma aberrations are not present. This result already agrees with the conclusion provided by the Seidel diagram. From Fig. 4, we can see that the spot size is wavelength dependent. The root-mean-square (RMS) radius is: 1.015 μm for purple color ($\lambda = 0.400$ μm), 1.138 μm for green color ($\lambda = 0.515$ μm), and 1.339 μm for deep red color ($\lambda = 0.655$ μm). Note that there is a variation of 0.329 μm within the visible spectrum.

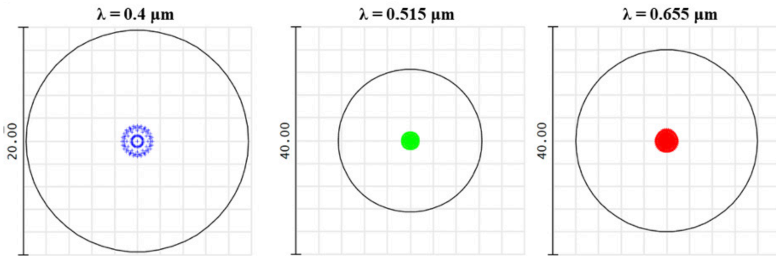

Figure 4. Performance of the confocal microscope by comparing the spot size at different wavelengths

Discussion and Conclusions

We have implemented a confocal microscope using off-of-shelf optical components and evaluated its optical performance in terms of the aberrations, the spot size, and the MTF plot. Because confocal microscopes are typically used for multicolor imaging, it is important to ensure that the design is invariant to different wavelengths within the visible range. To simplify the design of the microscope, we replaced the dichroic mirror with a beam splitter. Note that another alternative exists to build a confocal microscope for inexperienced users of Zemax OpticStudio. This alternative is the design of a confocal microscope following an inline configuration. In other words, no dichroic mirror and beam splitter are considered, and we insert all the elements in a sequence. If one follows this configuration, the microscope objective lens must be inserted twice and in reverse order since the light passes through it twice. In the proposed confocal microscope, we have also replaced the microscope objective lens with an aspheric condenser lens.

The latest change would reduce the cost of the experimental system since the cost of the proposed aspheric condenser lens is $32.61 versus $1,2667 for a Nikon microscope objective lens with the similar NA (N20X-PF, Thorlabs). The results shown here demonstrate the effectiveness of our design in comparison with the theoretical expectation. We have also shown that the proposed confocal microscope is quite robust to the change of the wavelength, showing a slight variation of 0.329 μm in the spot size from 400 nm to 655 nm. The small variation ensures the use of the same pinhole size for multicolor imaging without affecting the performance of the experimental confocal microscope. Future work should be focused on a more detailed comparison of the system's performance using an aspheric condenser lens, including experimental results since no reported system has used it.

This work allows undergraduate students interested in Optical Engineering to increase their knowledge in optical system design using the Global Academic Program of Zemax (e.g., free license for academia). In the event of insufficient funding to implement an optical laboratory, Zemax OpticStudio also allows undergraduate students to become familiar with different imaging systems, providing them with a virtual hands-on tool to design and analyze optical systems.

References

Dereniak, E. L., and Dereniak, T. D. (2008). Geometrical and Trigonometric Optics. Cambridge: Cambridge University Press.

Dunn K.W., Kamocka M.M., and McDonald J.H. (2011) A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol. 300(4):C723-42. doi: 10.1152/ajpcell.00462.2010.

Elliott, A. D. (2020). Confocal Microscopy: Principles and Modern Practices. Curr. Protoc. Cytom. 92(1), e69. doi: 10.1002/cpcy.68.

French, A. P., Mills, S., Swarup, R., Bennet, M. J., and Pridmore, T. P. (2008). Colocalization of fluorescent markers in confocal microscope image of plants cells. Nature Protocols 3, 619-628. doi.:10.1038/nprot.2008.31.

Geary, J. M. (2002). Introduction to lens design: with practical ZEMAX examples. Richmond, Va: Willmann-Bell.

Jessen, E. (2013) Technical review: colocalization of antibodies using confocal microscopy. American Association for Anatomy 297(2), 183-187. doi:10.1002/ar.22835.

Matsumoto, B. (2003). Cell Biological Applications of Confocal Microscopy. Elsevier.

Martinez-Corral, M., and Saavedra, G. (2009). The resolution challenge in 3D optical microscopy. Progress in Optics, 1-67. doi:10.1016/S0079-6638(08)00201-1.

Siew, R. (2017) Perspective on Modern Optics and Imaging: with practical examples using Zemax OpticStudio. Independently Published.

Singh, A., and Gopinathan, K. P. (1998). Confocal microscopy: a powerful technique for biological research. Current Science 74(10), 841-851.

St. Croix, C. M., Shand, S. H., & Watkins, S. C. (2005). Confocal microscopy: comparisons, applications, and problems. Biotechniques, 39(6), S2-S5.

Sun, H. (2017) Lens design: a practical guide. CRC Press, Taylor & Francis Group.

Zinchuk V., Zinchuk O., and Okada T. (2007) Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. Acta Histochem Cytochem. 40(4):101-11. doi: 10.1267/ahc.07002.