MULTI-SPECTRAL CONFOCAL MICROENDOSCOPE
FOR IN-VIVO IMAGING

by

Andrew Robert Rouse

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Andrew Rouse entitled Multi-Spectral Confocal Microendoscope For In-Vivo Imaging and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Art Gmitro
Date 7/30/04

Jose Sasian
Date 7/30/04

Ronald Lynch
Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Art Gmitro
Date 7/30/04
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ABSTRACT

The concept of in-vivo multi-spectral confocal microscopy is introduced. A slit-scanning multi-spectral confocal microendoscope (MCME) was built to demonstrate the technique. The MCME employs a flexible fiber-optic catheter coupled to a custom built slit-scan confocal microscope fitted with a custom built imaging spectrometer. The catheter consists of a fiber-optic imaging bundle linked to a miniature objective and focus assembly. The design and performance of the miniature objective and focus assembly are discussed. The 3mm diameter catheter may be used on its own or routed though the instrument channel of a commercial endoscope. The confocal nature of the system provides optical sectioning with 3μm lateral resolution and 30μm axial resolution. The prism based multi-spectral detection assembly is typically configured to collect 30 spectral samples over the visible chromatic range. The spectral sampling rate varies from 4nm/pixel at 490nm to 8nm/pixel at 660nm and the minimum resolvable wavelength difference varies from 7nm to 18nm over the same spectral range. Each of these characteristics are primarily dictated by the dispersive power of the prism. The MCME is designed to examine cellular structures during optical biopsy and to exploit the diagnostic information contained within the spectral domain. The primary applications for the system include diagnosis of disease in the gastro-intestinal tract and female reproductive system. Recent data from the grayscale imaging mode are presented. Preliminary multi-spectral results from phantoms, cell cultures, and excised human tissue are presented to demonstrate the potential of in-vivo multi-spectral imaging.
Cancer is the second leading cause of death in the United States. With current technology, the time between onset of disease and detection of cancer is often ten or more years. Since cancer becomes more difficult to treat as it develops, it is crucial to detect and diagnose the disease as early as possible.

Traditional biopsy methods for diagnosis of cancer and other diseases are surgically invasive and often require a significant turnaround time for results. Often the patient will have to return for additional biopsies. There is also the risk that the specific location of the disease may be overlooked due to the inherent sampling nature of the procedure. Regular screening of easily accessible cancers such as those of the gastrointestinal track is rarely performed due in part to the invasiveness of traditional biopsy procedures.

Truly non-invasive imaging modalities such as MRI and CT have the ability to image the entire body but do not have the resolution required to visualize the morphological and cellular changes of early stage cancer and pre-cancer. Optical biopsy is a relatively new field that strives to provide the physician with real-time cellular imaging from a minimally invasive endoscopic procedure.
Conventional microscopy has limited utility when viewing thick biological tissues. Light returned from out-of-focus regions in the sample overlaps the in-plane information causing a significant reduction in image quality. Confocal microscopes\textsuperscript{2-4} are commonly used to investigate thick excised biological tissue samples because these instruments reject light from out-of-focus planes and provide high-quality images from within a localized region of the tissue. They are typically able to capture high-resolution images from a specific plane in the tissue within a few hundred microns of the surface. Since the majority of cancers are epithelial in nature\textsuperscript{1}, the confocal microscope is an excellent choice for optical biopsy. Confocal microscopes have been adapted for in-vivo imaging of skin\textsuperscript{5,6}, cornea\textsuperscript{7,8}, teeth\textsuperscript{9}, and cervix\textsuperscript{10}. Specialized endoscopic systems have been developed for imaging deeper within the body based on VCSEL arrays\textsuperscript{11}, micromachined scan mirrors\textsuperscript{12}, single optical fibers\textsuperscript{13,14}, and fiber-optic bundles\textsuperscript{15,16}.

Bench-top and in-vivo confocal microscopes typically operate as epi-illumination systems in fluorescence or reflectance modes. However, fluorescence has emerged as the primary imaging technique due to the sensitivity and targeting specificity of fluorescent probes. The spectral emission characteristics of many fluorescent probes used in microscopy are affected by the local environment in the sample. Therefore, multi-spectral fluorescence imaging provides the ability to determine properties of the local environment in a spatially resolved manner. Confocal microspectrofluorometers have been developed to measure spatially resolved fluorescence spectra, and have been used to study drug target interactions\textsuperscript{17}, ion concentrations\textsuperscript{18}, and pH levels\textsuperscript{19} in living cells. However, these devices are essentially bench-top systems with slow mechanical scanning.
and offer no solution for evaluating remote in-vivo locations. This dissertation presents and demonstrates the concept of a multi-spectral confocal microendoscope (MCME) for remote in-vivo spectroscopic imaging.

The MCME combines the features of a catheter-based confocal microscope with those of a microspectrofluorometer. The system employs a flexible fiber-optic catheter with a maximum outside diameter of 3mm. The small size of the catheter makes remote locations in the body accessible to the MCME. In addition to working as a stand alone system, the catheter is small enough to be used as a daughter scope to many conventional endoscopes. Commercial endoscopes often incorporate empty instrument channels that enable the clinician to use the endoscope to guide additional instrumentation to the site of the disease. The small size of the catheter enables the MCME to be used in conjunction with the highly developed technology that is currently used routinely in medical endoscopy procedures.

Central to the MCME is its ability to collect multi-spectral data in addition to conventional grayscale images. In multi-spectral mode, a custom built spectrometer is used to collect a spectral signature from each spatially resolved location in the object. For a specific focal depth in the tissue under observation, a three-dimensional data set containing two spatial dimensions and one spectral dimension is obtained. A four-dimensional data set (3D spatial and 1D spectral) may be acquired by collecting information from successive depths in the tissue.

The MCME was developed from a first generation confocal microendoscope that was designed and built by Gmitro and students\textsuperscript{20-24}. The original system was capable of
high-resolution grayscale imaging and employed a 7mm diameter catheter. To the first
generation design, the MCME adds multi-spectral imaging and a new 3mm diameter
catheter that greatly broadens the scope of potential applications for the system.

Chapters 2 and 3 of this dissertation provide a general theoretical foundation of
confocal microscopy and multi-spectral imaging with a focus on those areas pertinent to
the MCME. Chapter 4 gives a detailed description of the optical and mechanical sub­
assemblies of the system with an emphasis on the new components. Chapter 5 provides a
detailed characterization of the optical performance of the MCME. Chapter 6 is a
compilation of results from various imaging experiments as well as a general discussion
of the potential application for the system.
CHAPTER 2: CONFOCAL MICROSCOPY AND FIBER BUNDLE THEORY

Before describing the details of the MCME it is important to review the basic concepts of a fiber-based confocal fluorescence imaging system. For a theoretical discussion of the multi-spectral aspects of the system refer to Chapter 3. This chapter will discuss the basic principles of fluorescence imaging, review the theoretical foundation for the spatial imaging performance of a confocal microscope, and introduce the key features of imaging fiber bundles.

Since a detailed mathematical description of fluorescence and confocal microscopy would be repetitive, this chapter is intended as a compilation of the important results obtained by key investigators in the field\textsuperscript{3,4}. For a comprehensive theoretical description of the slit-scan confocal microendoscope, from which the MCME is based, please refer to PhD dissertations written by David Aziz\textsuperscript{21} and Yashvinder Sabharwal\textsuperscript{22}.

2.1 Fluorescence

Before discussing the theoretical performance of conventional and confocal microscopes, it is first important to review the basic phenomena of fluorescence\textsuperscript{25}. During the process of fluorescence, a molecule is excited to a higher energy state by a
photon of appropriate wavelength. As the molecule returns to its ground state, the energy absorbed from the excitation photon is released in the form of a fluorescent photon. In most cases, the emitted photon is of lower energy, and therefore higher wavelength, than the absorbed photon because some of the energy associated with high vibrational levels of the excited state is dissipated in the form of heat. The entire process of excitation and emission happens on the picosecond to nanosecond time scale.

Figure 2.1 shows a simplified energy diagram for a fluorescent molecule. An incoming photon causes the molecule to move into one of the sub-energy levels of an excited singlet state. From there, the molecule will drop back down to the ground state in either a direct transition or via a triplet excited state. A photon may be released when the molecule relaxes directly from the excited singlet state to the ground state or when it relaxes from the excited triplet state to the ground state. The two processes have an important practical difference in that molecules that have electrons in a triplet state are often more susceptible to photobleaching and the creation of potentially damaging free radicals. Photons emitted as the molecule relaxes from an excited singlet or triplet state are called fluorescence and phosphorescence respectively.
Figure 2.1 Jablonski energy level diagram. Absorbed photons excite electrons, which then emit fluorescence as they relax to their ground state.

When properly excited, most biological tissue will produce a natural fluorescent signal; a process known as autofluorescence. While certain microscopy systems are sensitive enough to image autofluorescence, the majority of experiments use man-made exogenous dyes to increase sensitivity and targeting specificity. The use of manufactured dyes in fluorescence microscopy became popular in the early 1940's when Albert Coons\textsuperscript{25} developed a technique to conjugate exogenous fluorescent molecules to specific antibodies. Since then, advancements in microscope objectives and optical filters have made fluorescent imaging systems commonplace in the scientific community.

Figure 2.2 shows the excitation and emission spectra for Alexa Fluor 488 dye\textsuperscript{26}. The general features in this plot are similar to those found in most exogenous fluorophors. The separation between the peaks of the excitation and emission spectra is called the Stokes shift. In general, the smaller the Stokes shift for a given fluorophor, the
larger the overlap of the excitation and emission spectra and hence the harder it is to
distinguish the fluorescence from the excitation source.

**Figure 2.2** Excitation and emission spectra for Alexa Fluor 488 goat anti-
mouse IgG antibody at a pH of 8. Alexa Fluor dyes are fluorescence
derivatives manufactured by Molecular Probes.

It is often desirable to label a specific tissue with multiple fluorophors in order to
increase the contrast and highlight specific features of the individual cells and/or cell
morphology. The dye selections for such an experiment will be limited by the
requirement that the emission profiles of the dyes maintain sufficient separation to be
individually detectible by traditional microscopy filter sets. The spectral capabilities of
the MCME provide for a broader selection of fluorophors by enabling the user to more accurately distinguish the individual emission profiles.

In certain cases, the spectral characteristics of the excitation and emission profiles are affected by various properties of the tissue such as pH, ion concentration, and oxygen levels. The MCME is able to record the spectral signature of a fluorophor and characterize these changes in-vivo.

2.2 Conventional Fluorescence Microscopy

2.2.1 Optical system

Figure 2.3 shows a simplified layout of a conventional fluorescence microscope. A fluorescent probe is applied to the tissue sample and excited by a broadband high radiance source, such as a mercury or xenon arc-lamp. The source in Figure 2.3 represents a complex optical system designed to provide uniform illumination over the entire field of the sample. The source is spectrally limited by a band-pass filter such that the energy reaching the sample is within the excitation wavelength band for the fluorophor. The probe’s lower energy, higher wavelength emission is collected by the objective and separated from the illumination wavelength by a dichroic beamsplitter commonly located in a filter cube. An emission filter is usually placed between the beamsplitter and the eyepiece to limit the spectral range of detection to a specific region of interest.
2.2.2 Lateral Resolution

The lateral resolution of any optical microscope is closely related to the numerical aperture (NA) of the microscope objective used in the experiment. Given an objective that collects light over a half-angle of $\theta$ in an index of refraction of $n$, the NA is defined as,

$$NA = n\sin \theta.$$  \hspace{1cm} (2.1)
The relationship between the NA of the microscope objective and the overall lateral resolution of the optical system may be calculated using Fourier scalar diffraction theory and has been presented in numerous publications\(^{3A,27}\). The result of this calculation is that the point spread function (PSF) of a conventional fluorescence microscope is simply proportional to the incoherent PSF of the detection optics. For a traditional radially symmetric optical system using incoherent illumination the PSF is given as

\[
I_{PSF}(r) \propto \text{somb}^2 \left( \frac{2NA_d r}{\lambda_d} \right),
\]

where \(\text{somb}(r)\) is defined as

\[
\text{somb} \left( \frac{r}{d} \right) = \frac{2J_1 \left( \frac{\pi r}{d} \right)}{\left( \frac{\pi r}{d} \right)},
\]

and \(J_1\) is the first order Bessel function of the first kind. It is important to note that equation (2.2) is a function of the numerical aperture of the microscope objective \(NA_d\), and the fluorescent emission wavelength \(\lambda_d\). The PSF is independent of the illumination optics and excitation wavelength. The distribution in equation (2.2) is often referred to as the Airy disk pattern and is plotted in Figure 2.4.

By the Rayleigh\(^{27}\) criteria, two points are said to be resolvable if they are separated by a distance such that the peak of one Airy disk pattern coincides with the first zero in the second Airy disk pattern. Therefore, the lateral resolution of a conventional fluorescence microscope may be represented as
Another common measure of lateral resolution, which will be used later to describe the resolution of the confocal microscope, is the full width half maximum (FWHM) of the PSF given by

\[ FWHM = \frac{0.5\lambda_d}{NA_d}. \]  \hspace{1cm} (2.5)

Functional Form of the Lateral PSF for Conventional Microscopy

Figure 2.4  Airy disk pattern common to diffraction-limited circularly-symmetric optical systems. This is a plot of equation (2.2) with \( \lambda_d = 0.5\mu m \) and \( NA_d = 0.5 \).
2.2.3 Axial Resolution

A conventional fluorescent microscope is excellent at imaging thin samples. However, its performance is significantly limited when viewing thick biological tissue and samples with varying surface topography. The portion of the object that is beyond the in-focus region of the microscope contributes a bright defocused component to the image. In many circumstances the signal returned from the out-of-focus regions is significant enough to severely degrade the quality of the image. For this reason, imaging beyond the surface of biological tissue is virtually impossible with a conventional fluorescence microscope. Even imaging the tissue surface can be problematic for samples that are highly fluorescent throughout the tissue.

A mathematical description for the axial response of a conventional fluorescent microscope does not exist because such a microscope essentially has no axial resolution. The axial irradiance distribution of a point source imaged through a conventional optical system is derived in Born and Wolf\textsuperscript{28} and given by,

\[
I(z)_{\text{conv.pt.}} \propto \text{sinc}^2 \left( \frac{NA^2}{2\lambda} z \right).
\]

(2.6)

However, this on-axis point response is rarely applicable in traditional tissue imaging situations were thick fluorescent objects are imaged. If one were to scan a thin fluorescent planar target through the focus of a conventional fluorescence microscope one would find no appreciable fall off in irradiance for reasonable amounts of defocus (\textit{i.e. no axial resolution}).
2.3 Confocal Microscopy

2.3.1 Optical System

As discussed in the previous section, imaging thick biological samples in a conventional fluorescence microscope is difficult due to the virtually non-existent axial resolution. By contrast, a confocal microscope rejects light from out-of-focus planes and provides a clear in-focus image from a thin section within the sample. Figure 2.5 shows a simplified layout of a point-scan confocal microscope. Illumination optics focus a point source onto a specific location in the sample. Detection optics collect the fluorescent emission signal and image it onto a point detector. Signal originating from areas outside the focal plane in the tissue is largely rejected by the pinhole aperture in front of the detector. This out of plane rejection is the key to optical sectioning in the confocal microscope. In this simplified example, a 2D image is formed by scanning the sample over a plane perpendicular to the optical axis. A 3D data set may be obtained by acquiring several 2D images for different depths in the sample. The MCME adds an additional spectral dimension to this data set, the details of which will be discussed in subsequent chapters.
Figure 2.5 Optical sectioning in a confocal microscope. Fluorescence from a specific point in the sample is collected by the detector. Out-of-focus fluorescence (dotted lines) is blocked by the pinhole aperture.

While the simplified view of Figure 2.5 is useful for describing the optical sectioning capability of a confocal microscope, most confocal systems operate using epi-illumination with high speed scanning optics to provide fast in-plane scanning without moving the sample. Figure 2.6 shows the major components of a typical commercial confocal microscope. The fluorophor is usually excited by a laser source and the same optics that provide the focusing and scanning of the illumination profile collect and de-scan the fluorescent emission. A dichroic beamsplitter separates the emission spectra from the source. The detection arm usually includes an emission filter, a low-noise detector, and a computer to aid in data acquisition and image display.
Figure 2.6 Layout of a confocal microscope. A monochromatic point source is scanned in two dimensions to build up a full 2D image.

2.3.2 Infinitely Small Point Scan Performance

The theoretical performance of a confocal microscope can be modeled using common Fourier theory\textsuperscript{27,29}. The process used to derive theoretical values for the lateral and axial performance of a confocal system is rather involved and is well documented by other authors\textsuperscript{3,4}. This dissertation will review the important aspects of their calculations and present a concise review of their findings.
2.3.2.1 Infinitely Small Point Scan: Lateral Resolution

In its most basic form, the confocal microscope can be thought of as a point-scanning system employing an infinitely small point source and a corresponding infinitely small detection pinhole. In this ideal situation, one finds that the PSF for a confocal microscope is simply the multiplication of the PSF's of the illumination and detection optical systems or

\[
I_{PSF}(r) \propto \text{somb}^2 \left( 2 \frac{NA_i}{\lambda_i} r \right) \text{somb}^2 \left( 2 \frac{NA_d}{\lambda_d} r \right),
\]  
(2.7)

where \( NA_i \), and \( \lambda_i \) are the numerical aperture and wavelength of the illumination system and \( NA_d \), and \( \lambda_d \) are the numerical aperture and wavelength of the detection system.

For typical epi-illumination experiments \( NA_i = NA_d = NA \) and the excitation and emission wavelengths are often within a few hundred nanometers of each other. Under these circumstances equation (2.7) can be approximated by

\[
I_{PSF}(r) \propto \text{somb}^4 \left( 2 \frac{NA}{\bar{\lambda}} r \right),
\]  
(2.8)

where \( \bar{\lambda} \) is the average wavelength. By the Rayleigh criteria, the ideal confocal microscope has the same lateral resolution as that of a conventional fluorescence microscope since functions (2.2) and (2.8) have their first zero at the same location.

However, the FWHM of the confocal PSF is

\[
\text{FWHM} = \frac{0.37 \bar{\lambda}}{NA_d},
\]  
(2.9)

which is significantly smaller than that of the conventional system.
Figure 2.7 shows the profile of equation (2.2), the lateral PSF of an ideal conventional fluorescence microscope, and equation (2.8), the lateral PSF of an ideal confocal microscope. For this plot, $NA_i = NA_d = 0.5$ and the excitation and emission wavelengths were chosen as 488nm and 520nm respectively. The figure reveals a significant reduction in the width of the profile for the confocal system. The reason for this is the presence of the illumination PSF in the confocal PSF equation, which effectively leads to a somb$^4$ shape rather than the somb$^2$ shape. Since the illumination PSF is dependent on $\lambda_i$ and not $\lambda_d$, the larger the difference between the illumination and detection wavelengths, the greater the difference between the conventional and confocal profiles.
2.3.2.2 Infinitely Small Point Scan: Axial Resolution

While the enhanced lateral resolution is significant, the real benefit to a confocal system is its axial resolution. Unfortunately, the method by which to experimentally test the axial resolution of a confocal system is not well defined. Many publications use a flat mirror scanned through focus. Often the emission filter is removed to increase the signal returned from the mirror. While this is the simplest way to test the axial resolution, it does not accurately reproduce the actual working environment of the microscope since
with the mirror, energy collected by the detection system is specularly reflected excitation light rather than diffuse fluorescence. Another, more accurate method of measuring axial resolution is to scan a thin fluorescent target through focus. However, the problems associated with constructing such a target often make this method impractical. A third, and perhaps best, method is to scan a thick fluorescent planar target through focus and thereby measure the axial response to a fluorescent edge. As with the knife edge test commonly used to measure lateral resolution in a traditional optical system, a simple derivative of the axial edge response yields the axial PSF for the confocal microscope.

The type of sample often has a significant impact on the optical sectioning of a confocal microscope. For example, the optical sectioning performance differs if one is looking at individual punctate cells in an otherwise non-fluorescent extracellular matrix vs. a densely packed tissue sample with relatively uniform fluorescence throughout. There is, therefore, a need to characterize a confocal system for point and plane detection scenarios.

Since several different methods are commonly used to measure the axial PSF it is important to review the theory of each of these situations. Starting with the simplest case, Corle and Kino\(^4\) present a detailed derivation of the scalar theory for a point and planar mirror reflector scanned through focus. A summary of their results follows. Refer to Corle and Kino’s book for an in-depth derivation of these equations. For an ideal point-scan confocal microscope, the axial point and plane responses to a mirrored reflector are
For equations (2.10) and (2.11) the microscope is assumed to be operating in epi-illumination under the ideal conditions of an infinitely small point source and an infinitely small detection pinhole. These equations should be compared to the axial response of a conventional microscope to the same targets (see equation (2.6))

\[
I^R(z)_{\text{confocal point}} \propto \text{sinc}^4 \left( \frac{NA^2}{2\lambda} z \right) \quad \text{FWHM} = \frac{1.24\lambda}{NA^2} \quad (2.10)
\]

\[
I^R(z)_{\text{confocal plane}} \propto \text{sinc}^2 \left( \frac{NA^2}{\lambda} z \right) \quad \text{FWHM} = \frac{0.9\lambda}{NA^2} . \quad (2.11)
\]

Figure 2.8 shows a graphical representation of equations (2.10) through (2.13) for the case of \( NA = 0.5 \) and \( \lambda = 500\text{nm} \). One can see significant improvement in the axial response to a point reflector for the case of the confocal microscope. It is even more interesting that the confocal response to a plane is even better than the confocal response to a point.
Figure 2.8 Ideal reflectance axial PSF for conventional and confocal microscopes. The confocal microscope shows considerable improvement for both point and planar targets scanned through focus.

While the reflectance mode axial PSFs presented in equations (2.10) and (2.11) are important to understand, they do not accurately represent the fluorescent imaging conditions of most confocal microscopes. Wilson\(^3\) provides a comprehensive discussion of the theoretical axial response to point and planar fluorescent targets. For an ideal point-scan confocal microscope, the axial point response to a fluorescent target is

\[
IF(z)^{confocal}_{point} \propto \sin^2 \left( \frac{NA^2}{2\lambda_i} z \right) \sin^2 \left( \frac{NA^2}{2\lambda_d} z \right) \quad \text{FWHM} \approx \frac{1.24\lambda}{NA^2}.
\]
This equation shows that the axial response to a fluorescent point is virtually identical to the axial response to a mirrored point reflector.

The axial fluorescent planar response is significantly more complex. In radial coordinates the planar response to a fluorescent target \( f \) is

\[
I^F(z)_{\text{confocal}} = \alpha |h_i(z, r)h_d(z, r)|^2 \otimes f(z, r) = \int_{0}^{\infty} |h_i(z, r)h_d(z, r)|^2 r dr,
\]

where \( f(z, r) = \delta(z) \) for an infinitely thin planar fluorescent target and \( h_i \) and \( h_d \) are the three-dimensional impulse responses of the illumination and detection subsystems, respectively. Notation from Born and Wolf may be used to introduce the three-dimensional impulse response of a simple lens of diameter \( 2a \) and focal length \( f \) as

\[
h(r, z) \propto \int_{0}^{1} e^{\frac{i\pi \rho^2}{2}} J_0(\nu \rho) \rho d\rho
\]

\[
u = \frac{2\pi a}{\lambda f},
\]

\[
u = \frac{2\pi a}{\lambda f},
\]

where \( u \) and \( v \) are normalized axial and radial coordinates, respectively. Equation (2.16) is simply the Hankel transform of the lens pupil with a quadratic phase factor to account for axial defocus from the paraxial image plane. Combining equations (2.15) and (2.16) yields the mathematical description of the fluorescent planar response for an ideal point-scan confocal microscope. Unfortunately, there is no simple analytical solution to this set of equations. However, mathematical software such as Mathcad may be used to calculate a numerical solution for a given optical system. Figure 2.9 shows the
PSF for various excitation and emission wavelengths. The plot assumes $NA = 0.5$ for both the illumination and detection arms, and the data are presented in terms of the normalized Stokes shift variable

$$\beta = \frac{\lambda_d}{\lambda_i}. \quad (2.17)$$

The axial response to a planar fluorescent target depends on the Stokes shift of the dye used in the experiment. However, several of the plots in Figure 2.9 depict extreme Stokes shifts. Typically $\beta$ ranges from 1.1 to 1.3. The axial response presented in Figure 2.9 represents a remarkable improvement over the virtually nonexistent axial response to planar targets found in conventional microscopy.

It should be noted that the plots presented in Figure 2.9 do not agree exactly with data presented by Wilson. Every attempt has been made to precisely duplicate Wilson’s steps but the results do not agree with his published work. It is unclear which calculation is correct although there seems to be no errors in the calculations that led to the plots in Figure 2.9.

The plots in Figure 2.9 may be used to estimate the FWHM of the axial response to a fluorescent planar object for specific values of $NA$ and $\beta$. For $NA = 0.5$ and $\beta = 1$ the FWHM is roughly $2\mu m$. For a typical value of $\beta = 1.2$ the FWHM increases to $2.2\mu m$ and at $\beta = 2$ the FWHM is $3\mu m$. By comparison, for the same optical system Wilson estimates a FWHM of $2.5\mu m$ and $5.3\mu m$ for $\beta = 1$ and $\beta = 2$ respectively.
2.3.3 Infinitely Small Slit Scan

The primary drawbacks to the point-scan confocal microscope are slow scan rates and low light collection efficiency. Unfortunately, speed and sensitivity are crucial when visualizing tissue in-vivo. The sample is rarely perfectly stationary and medically approved fluorophors often suffer from low quantum yield. A Nipkow disk configuration offers very high speed acquisitions but at the cost of extremely low light efficiency.

The approach taken by our lab was to build a slit-scanning confocal microscope. Under this configuration, the two-dimensional raster scan of a point-scanning system is
replaced by a less complex one-dimensional scan. Slit illumination replaces the point source and an image is collected in a single sweep of the scan mirror. Powered by a slit scanner, a confocal microscope can achieve video frame rates with sufficient sensitivity to measure common exogenous fluorophors.

The faster frame rates and higher sensitivity of a slit-scanning confocal microscope are counterbalanced by a reduction in optical sectioning. As with a point-scan system, there is no simple analytical solution to the axial PSF of a slit-scan confocal microscope. However, Corle and Kino and Wilson present an estimate of the FWHM one would expect for plane mirrored reflectors and plane fluorescent targets,

\[ FWHM_{slit, plane}^R = \frac{1.08 \lambda}{NA^2} \]  

\[ FWHM_{slit, plane}^{F, \lambda_i = \lambda_d} = \frac{1.90 \lambda}{NA^2} \] 

It not surprising that these numbers are worse than the corresponding values for a point-scanning system. The response to a flat mirror is worse by 20% and the response to a flat fluorescent target is worse by 40% (compared to Wilson’s estimates). This implies that the size of the optical slice imaged by a slit-scanning confocal system is larger than that of a point-scanning system. However, depending on the application, this may be an acceptable tradeoff for the increased speed and light collection efficiency of the slit-scanning system.
2.4 Effects of Finite Apertures

The discussion up to this point has assumed the ideal case of infinitely small illumination and detection apertures. Clearly the sizes of the illumination profile and detection aperture are inherently linked. If the detection aperture is too small, the system will suffer from poor sensitivity to the fluorescence signal. However, if the detection aperture is too large the system will have excellent sensitivity but very poor optical sectioning.

To calculate the ideal size for the pinhole in a point-scanning system one must first know what the lateral irradiance distribution is at the location of the pinhole. With Fourier theory it can be shown that the irradiance distribution at the pinhole is

\[ I(\rho_{\text{pinhole}}) \propto \text{somb}^2 \left( \frac{2 NA_p}{\lambda_d} \rho_{\text{pinhole}} \right), \]  

(2.20)

where \( \lambda_d \) is the wavelength of the detected signal and \( NA_p \) is the numerical aperture of the optical system at the pinhole. According to Corle and Kino \(^4\) the optimal pinhole size clips this irradiance profile at the FWHM points. Therefore,

\[ r_{\text{pinhole}} = \frac{\lambda}{4NA_p}. \]  

(2.21)

The magnification from pinhole to detection is defined as

\[ m_p = \frac{h_d}{h_p} = \frac{NA_p}{NA_d}, \]  

(2.22)
where $h_p$ and $h_d$ are the lateral extents of the image at the pinhole and detector respectively. Equation (2.21) can now be written as

$$r_{\text{pinhole}} = \frac{\lambda}{4m_d N_A_d}.$$  \hspace{1cm} (2.23)

The same argument may be applied to a slit-scanning confocal microscope, where equation (2.20) would be replaced by an equivalent one-dimensional sinc² function. The result would be the one-dimensional equivalent of equation (2.23) or

$$w_{\text{slit}} = \frac{\lambda}{2m_p N_A_d}.$$  \hspace{1cm} (2.24)

Note that the pinhole equation is describing a radius or half-width, whereas the slit equation is describing a full-width. This is the only reason for the factor of 2 difference between the two equations.

### 2.5 Imaging Fiber Bundles

The ability of the MCME to image remote in-vivo locations is due largely to the use of a fiber-optic imaging bundle in the catheter. Such products are commonly fabricated by packing individual optical fibers into a dense hexagonal pattern and drawing the fiber down to an appropriate total width in a controlled high heat environment. Throughout this process, the packing orientation of the individual fibers in the bundle must be maintained. This allows an image on one end of the finished product to be relayed to the other side of the fiber with minimal distortion.

To understand the performance and optical properties of the bundle, one must first review the properties of an individual fiber. The simplest way to understand the guiding
phenomena of an optical fiber is using geometrical optics. Assume light is incident on an interface where the refractive index, \( n_2 \), of the medium the light is entering is lower than the index, \( n_1 \), it is leaving. If the angle of incidence is greater than

\[
\theta_{TIR} = \sin^{-1}\left(\frac{n_2}{n_1}\right),
\]

(2.25)

then the light will be reflected as if the interface were a perfect mirror. To make an optical fiber, a cylindrical core of index \( n_{core} \) is surrounded by a concentric ring of cladding with index \( n_{clad} \). If the refractive indices are chosen correctly, the resultant fiber will guide light up to a certain angle of incidence with respect to the front face of the fiber. The numerical aperture of the cone of light that will be guided by the fiber is

\[
NA = n_{core} \sin\left(90 - \theta_{TIR}\right),
\]

(2.26)

where \( n_1 \) and \( n_2 \) are replaced with \( n_{core} \) and \( n_{clad} \) respectively to calculate \( \theta_{TIR} \). Any light incident on the fiber from outside this acceptance cone will be transmitted into the cladding region and will not be guided by the fiber.

In practice, the core of many optical fibers is sufficiently small that this simple geometrical model is not enough to fully characterize the properties of the fiber. In this case, a more detailed physical optics model must be used that treats the light as an electro-magnetic wave rather than a ray\(^3\). The result of such a consideration is that the fiber will only support certain distributions of light called modes. The number and types of modes that a fiber will support are closely related to the \( V \) number defined as
In the case where $V < 2.405$ the fiber is said to be single mode. Under this condition, the fiber maintains coherence and produces the smallest and smoothest illumination profile. However, it is inherently difficult to efficiently couple light into a single mode fiber. Often a fiber with a $V$ number slightly greater than 2.405 provides the best compromise between resolution and throughput.

The $NA$ and optical mode structure of an imaging fiber bundle are identical to that of the individual fibers in the bundle. However, the lateral resolution and throughput are largely dependent on the properties of the bundle itself. Generally the lateral resolution of a fiber bundle, as measured by the spatial size of the PSF, is equal to the center-to-center spacing of the individual fiber cores and has very little to do with the core diameters. However, the larger the ratio of core diameter to core spacing, the larger the throughput of the fiber. This ratio is often referred to as the fill ratio and it is commonly in the range of 30% to 50%. Figure 2.10 shows a small region of the fiber-optic imaging bundle used in the MCME. The light regions in the figure correspond to the individual fiber cores. Figure 2.10 clearly depicts the hexagonal fiber packing and the throughput effects of the fill ratio.
Figure 2.10 Close up of a small portion of an imaging fiber bundle. The individual fiber cores are white and the cladding is black.

When coupling light into imaging fiber bundles it is crucial to properly fill the $NA$ of the fiber. Under filling the $NA$ of a fiber used in confocal microscopy will cause a significant loss in lateral and axial resolution. Over filling the $NA$ will cause light to escape the cores of the individual fibers. This leads to cladding modes and crosstalk between adjacent cores that will ultimately degrade the optical performance of the system.
CHAPTER 3: MULTI-SPECTRAL IMAGING THEORY

One of the primary novel aspects to this dissertation is the MCME's ability to collect spectral information from a sample in addition to grayscale spatial data. In effect, this enables the user to collect a four-dimensional data set comprised of a traditional three-dimensional voxel of spatial information with the additional knowledge of spectra at each location. The many uses for spatially resolved spectral information in the study of pathology and physiology are well documented. The applications range from enhancements in image contrast and clarity to more complex measurements of functional changes in structures labeled with fluorescent dyes. Various potential applications for the MCME will be discussed in Chapter 6.

The majority of imaging spectrometers fall into one of three categories: filter systems, scanning systems, and interferometric systems. Each of these categories is discussed in the subsequent sections. Extra detail is given to the section on scanning systems because this technology is pertinent to the MCME.

3.1 Filter Techniques

The most common method used to combine the technologies of microscopy and spectroscopy involves carefully selected optical filters and/or beamsplitters, which sort the spectral information collected from the sample into two or more spectral bands. Such
an approach works well if the spectral information is largely contained within distinct spectral bands. However, this is rarely the case. Fluorophors usually have broad emission spectra and most dyes used in multi-dye experiments have some degree of spectral overlap in their emission profiles. Figure 3.1 shows the emission spectra for two common exogenous fluorophors, Alexa Fluor 488 and FM-43. The darker line represents an example of a spectral signal that might be created by the coexistence of both dyes at the same location in the object. The two distinct maxima in the collected signal represent the individual peaks in the emission characteristics of each fluorophor. Superimposed on the spectra in Figure 3.1 are ideal pass bands for two filters that one might use to collect the two responses. While these dyes are considered well separated, there is still a significant amount of FM-43 fluorescence that is mapped into the Alexa Fluor 488 channel and vice versa.
Figure 3.1 Example spectrum of Alexa 488, and FM-43 in a dual dye experiment. Collected signal is overlaid with the emission spectra for each dye. Dotted lines represent filter pass bands.

To help alleviate crosstalk between spectral channels, fluorophors are specifically chosen to minimize spectral overlap. Unfortunately, this significantly reduces the availability of suitable fluorophors. In addition, the number of different dyes that one can use in a given experiment is usually limited to 2 or 3. The addition of more dyes will cause an increase in spectral overlap making spectral separation more problematic.

Another drawback to the filter technique is the inherent time delay between spectral measurements. One must sequentially collect a full two-dimensional spatial image for each spectral band. Filter wheels or liquid crystal tunable filters are sometimes used to
increase the ease and flexibility of acquiring the spectral data. Despite these drawbacks, the filter technique remains the most common method of gaining spectral information from microscopy due to the low cost and design simplicity.

3.2 Scanning Techniques

Another option for gathering spectra from an imaging system is to use a dispersing element to map spectral information into a spatial dimension. The dispersing element is commonly a prism or a grating, both of which will be discussed in detail in this section. Such a system must, in general, employ either a point or line scanning assembly in order to read out spectral and spatial information. For example, a point-scanning confocal microscope might disperse the fluorescence signal passed by the collection pinhole onto a one-dimensional detector array. For a fixed position of the scanning assembly, the detection records a spectral profile from a specific location in the object. The necessity to build up an image point-by-point or line-by-line may add significant complexity to the optical system. However, the dispersion method greatly increases the number of spectral bands in the final data set.

Figure 3.2 shows the same spectral information presented in Figure 3.1. The collected signal is a summation of the emission profiles for Alexa Fluor 488 and FM-43. The dotted lines represent a typical sampling pattern for multi-spectral collection. In this example 16 spectral samples have been taken over a wavelength range of 150nm. In practice, the data collected from a filter system may be thought of as a multi-spectral
dispersion system with a limited number of spectral samples. Clearly the finer the
spectral sampling, the easier it will be to unmix the two profiles. This is especially true if
one knows the spectral signatures of each fluorophor. In such a case, the measured signal
\( g(\lambda) \) is comprised of \( n \) individual fluorophors each contributing a signal \( f_n(\lambda) \) or

\[
g(\lambda) = c_0 + \sum_{n} c_n f_n(\lambda),
\]

(3.1)

where \( c_n \) represents the relative amount of each fluorophor and \( c_0 \) is a background term.
If one knows the functions \( f_n(\lambda) \), the spectral unmixing process becomes a relatively
straightforward inversion of equation (3.1). Such a technique is commonly referred to
spectral deconvolution.
In general, the primary drawback to employing a dispersion method in a spectral imaging system is the need to scan the object. Such systems are usually more complex than filter systems and often require more time to collect an image. However, all confocal microscopes are inherently scanning systems and, therefore, naturally lend themselves to a multi-spectral dispersion method. Since the object is being scanned, it is relatively straightforward to acquire spectral data. Since starting the work for this dissertation, Carl Zeiss\textsuperscript{35} introduced the first commercial multi-spectral confocal...
microscope, the LSM 510 META. The system is a modern bench-top point-scanning confocal microscope with an added META camera module capable of multi-spectral acquisition. Since the LSM 510 is a point scanning system, it is very likely that the META module simply uses a prism or grating to disperse the collected energy onto a line scan detector.

The MCME employs a dispersive element in a slit-scanning configuration. The details of this configuration will be presented in Chapter 4. The remainder of this section will discuss the important aspects of multi-spectral imaging using a dispersive element in a scanning system.

3.2.1 Gratings as Dispersion Elements

The most common method of dispersing light to record and evaluate spectra uses a diffraction grating. A diffraction grating is made up of a repetitive pattern of very small apertures. Gratings are designed to work in either reflection or transmission and the periodic structure may introduce a variation in amplitude and/or phase to the incident wavefront.

Most spectroscopy instruments employ either ruled or holographic reflective phase gratings. In the case of ruled gratings, the periodic structure is cut into the substrate using a precise diamond tool. In holographic gratings, the periodic structure is etched into a photosensitive substrate after exposure to a holographic representation of the grating pattern. In each case, the manufactured gratings are generally used as masters to stamp out replica gratings for sale and general use. The major difference between the two types is that a holographic grating generates significantly less stray light, which
yields higher spectral resolution while the ruled method offers higher reflectivity and sensitivity.

3.2.1.1 The Grating Equation

Regardless of the manufacturing process, the final grating is simply a fine periodic structure that is designed to introduce a periodic amplitude and/or phase delay to a collimated incident wavefront. The performance metrics for a grating are commonly derived for a simple binary amplitude transmission grating. The math is straightforward and the results are common to most grating types. A superposition of waves technique may be used to calculate the irradiance pattern transmitted by a simple binary grating of groove spacing \( a \), with \( N \) individual features each of width \( b \). The irradiance as a function of angle is expressed as

\[
I(\theta_i) \propto \left( \frac{\sin \beta}{\beta} \right)^2 \left( \frac{\sin(N\alpha)}{\sin \alpha} \right)^2,
\]  

(3.2)

where

\[
\alpha = \frac{\pi a (\sin \theta_i - \sin \theta_t)}{\lambda},
\]

(3.3)

\[
\beta = \frac{\pi b (\sin \theta_i - \sin \theta_t)}{\lambda}.
\]

In this equation, \( \theta_i \) and \( \theta_t \) are angles of incidence and transmission, respectively.

Equation (3.2) is plotted in Figure 3.3. The functional form may be described by a series of sharp peaks modulated by a sinc function envelope. The peaks are commonly called
orders and they will occur when $\alpha = 0, \pm \pi, \pm 2\pi, \ldots$, which when entered into equation (3.3) yields

$$a(\sin \theta_m - \sin \theta_i) = m\lambda \quad m = 0, \pm 1, \pm 2, \ldots,$$

(3.4)

where $\theta_i$ has been renamed $\theta_m$, the transmitted angle of the $m$th diffraction order.

Equation (3.4) is commonly referred to as the grating equation and may also be derived by calculating the optical path difference between light diffracted from two adjacent features on the grating. The resultant Fraunhofer diffraction pattern will exhibit maxima for all combinations of angles that produce path differences equal to an integer multiple of the wavelength. The case when $m = 0$ is said to be the zeroth order of the grating. For transmission gratings the zeroth order corresponds to the light that passes through the neuron activity. ADDIN EN.CITE

Ion grating the zeroth order corresponds to the geometrical angle of specular reflection with respect to the plane of the grating. For values of $m \neq 0$ the angle of reflection $\theta_m$ depends on $\lambda$ and hence the grating is able to separate the various colors of the source into plane waves of slightly different propagation angles $\theta_m$. A properly placed lens will image this angular dispersion to spatial coordinates on a detector and record the spectrum of the source. Systems employing gratings are generally aligned to view the 1st diffraction order.
Typical Grating Irradiance Distribution

Figure 3.3 Irradiance profile for a small grating. Plot of equation (3.2) with $N = 8$ and $a = 4b$. Each peak represents a diffraction order. The shape of each peak is the spectral linewidth for this grating.

3.2.1.2 Angular Dispersion

The angular dispersion of a grating is defined as the change in diffraction angle with respect to wavelength or

$$
\Delta \theta = \frac{d\theta_m}{d\lambda}. 
$$

(3.5)

Assuming that the angle of incidence is constant with respect to wavelength, differentiating equation (3.4) yields

$$
\Delta \theta = \frac{m}{a \cos \theta_m}. 
$$

(3.6)
This result reveals that the angular separation between two wavelengths will increase for higher diffraction orders and finer groove spacing. The angular dispersion is also a function of wavelength since $\theta_\omega$ is a function of $\lambda$ according to the grating equation. This introduces a slight nonlinearity with respect to wavelength in equation (3.6). However, gratings are typically operated at small angles so that the relationship between wavelength and detector position is roughly linear. This will not be the case for prism dispersers discussed in Section 3.2.2.

3.2.1.3 Spectral Linewidth

The spectral linewidth of a grating is defined as the broadened profile that the spectrometer imparts on an infinitely small monochromatic source. For a given grating, the transmitted irradiance profile will exhibit local maxima according to the grating equation described by (3.4) and plotted in Figure 3.3. From equation (3.2) it may be shown that the first zero immediately adjacent to each local maximum is separated by $\Delta \alpha = 2\pi / N$. From the definition of $\alpha$ one can derive

$$\frac{d\alpha}{d\theta_t} = \frac{\pi a \cos \theta_t}{\lambda},$$

(3.7)

which yields

$$\Delta \theta_t = \frac{2\lambda}{aN \cos \theta_t}$$

(3.8)

for the angular separation between the peak and the first zero. Equation (3.8) describes the resolution of a grating and reveals that angular resolution is inversely proportional to $aN$, the physical size of the device.
3.2.1.4 Resolving Power

Another important performance metric for spectral systems is the chromatic resolving power defined as

\[ R \equiv \frac{\lambda}{(\Delta \lambda)_{\text{min}}} , \]  

(3.9)

where \((\Delta \lambda)_{\text{min}}\) is the minimum resolvable wavelength difference using Rayleigh’s criteria. It follows from equation (3.8) that

\[ (\Delta \theta_j)_{\text{min}} = \frac{\lambda}{N \cos \theta_m} . \]  

(3.10)

The dispersion equation gives a relation between \(\Delta \theta_j\) and \(\Delta \lambda\), which in conjunction with equation (3.10) yields

\[ R = mN . \]  

(3.11)

Thus the resolving power of a grating is a function of the diffraction order and the number of grooves in the grating.

3.2.1.5 Free Spectral Range

The left side of equation (3.4) will remain constant for many combinations of \(m, \lambda\). In other words, at the position corresponding to a specific wavelength \(\lambda\), one will also find energy contributed from the 2\(^{\text{nd}}\) order of \(\lambda/2\), the 3\(^{\text{rd}}\) order of \(\lambda/3\), and so on. This phenomenon effectively limits the available spectral range of a given order to a quantity called the free spectral range. Assume that wavelength \(\lambda\) in order \(m+1\) precisely coincides with wavelength \(\lambda + (\Delta \lambda)_{\text{FSR}}\) in order \(m\). According to the grating equation
Thus the larger the diffraction order used in a spectrometer, the lower the free spectral range available for measurements.

### 3.2.1.6 Blazed Gratings

The peak irradiance of the signal returned from a reflection grating will follow the path of the specular reflection. For a basic flat reflective phase grating the specular reflection is located at \( \theta_m = \theta_l \), which is the condition for the zero\(^{th} \) order. Unfortunately, the zero\(^{th} \) diffraction order is of little interest in spectroscopy since \( \theta_0 \) is not dependent on wavelength. To control the location of the peak in the diffraction pattern efficiency, most manufactures blaze their gratings to a given angle \( \gamma \). Figure 3.4 shows an example of a blazed grating. The specular reflection is determined by

\[
\theta_{\text{specular}} = \theta_l - 2\gamma. \tag{3.14}
\]

In the manufacturing process, the blaze angle \( \gamma \) is chosen such that the specular reflection is coincident with the order and wavelength that is of most importance to the application. In other words, \( \gamma \) is chosen such that \( \theta_{\text{specular}} = \theta_m \) for a specific order and wavelength. Most diffraction gratings are blazed to maximize the signal in the 1\(^{st} \) diffraction order.
3.2.2 Prisms as Dispersion elements

In addition to gratings, prisms are another common optical element used to disperse light into its spectral components. A prism is a simple triangular or wedge shaped piece of optically clear material. As light travels through a prism it undergoes refraction at air-glass interfaces. In general, the amount of deviation introduced to a collimated beam will be related to the index of refraction of the prism, which in turn is related to the wavelength of the light passing through the device. Therefore, a prism
disperses an incoming wavefront into its spectral components. As with a grating, a lens can be used to map this angular dispersion to a spatial separation.

3.2.2.1 Angular Deviation

Dispersion prisms typically operate in transmission. In this configuration, the optical path of the light undergoes an angular deviation that depends on various parameters of the prism. Figure 3.5 shows a simple prism of wedge angle $\alpha$. Light enters one face of the prism at an angle of incidence $\theta$ and undergoes an overall angular deviation equal to $\delta$.

![Figure 3.5 Angular deviation of a prism. The light path is designated by the thick solid line. $\delta$ is the overall deviation in path.](image)

To derive a formula for $\delta$ we start with the following four formulas based on Snell's law and the angle relationships of Figure 3.5.

$$\sin \theta = n \sin a$$  \hspace{1cm} (3.15)

$$n \sin b = \sin c$$  \hspace{1cm} (3.16)
\[
\alpha = a + b \quad (3.17)
\]

\[
\delta = (\theta - a) + (c - b) , \quad (3.18)
\]

where the prism is assumed to reside in air. Equations (3.17) and (3.18) yield

\[
\delta = \theta + c - \alpha . \quad (3.19)
\]

Solving equation (3.16) and (3.17) for \( c \) gives

\[
c = \sin^{-1}\left[ n \sin(\alpha - a) \right]. \quad (3.20)
\]

Expanding \( \sin(\alpha - a) \) we have

\[
c = \sin^{-1}\left( \sin \alpha \sqrt{n^2 - n^2 \sin^2 a - n \sin a \cos \alpha} \right). \quad (3.21)
\]

Substituting equation (3.15) for \( \sin a \) gives

\[
c = \sin^{-1}\left( \sin \alpha \sqrt{n^2 - \sin^2 \theta - \sin \theta \cos \alpha} \right), \quad (3.22)
\]

and finally equations (3.19) and (3.22) yield a final result for the angular deviation

\[
\delta = \theta + \sin^{-1}\left( \sin \alpha \sqrt{n^2 - \sin^2 \theta - \sin \theta \cos \alpha} \right) - \alpha . \quad (3.23)
\]

Therefore, the angular deviation of a prism is a function of the angle of incidence, the wedge angle, and the index of refraction of the prism. The angular deviation of a monochromatic beam is plotted with respect to incident angle in Figure 3.6 for a 40° prism of refractive index 1.5. The minimum deviation condition is of importance to certain applications and occurs when \( \theta = c \). In other words, a prism imparts minimum deviation when it is operated in a symmetrical situation with the angle of incidence equal to the angle of transmission.
3.2.2.2 Angular Dispersion

Returning to equation (3.23) it is evident that the angular deviation of a beam is dependent on the index of refraction of the prism. This is of particular importance because the index itself is dependent on wavelength. In the visible spectrum all dielectric material exhibits *normal dispersion* in that the index of refraction decreases with increasing wavelength. Under these conditions, the dispersive qualities of a dielectric may be approximated by the Sellmeier formula:

\[
n^2(\lambda) = 1 + \sum K_j \frac{\lambda^2}{\lambda^2 - L_j},
\]  

(3.24)
where $K_j$ and $L_j$ are constants that fit this equation for a specific material. In general, only a few terms are necessary to accurately characterize a dielectric. The Sellmeier constants for the glass BK7 are shown in Table 3.1 and the resultant dispersion is plotted in Figure 3.7.

$$K_1 = 1.03961212$$
$$K_2 = 0.23179234$$
$$K_3 = 1.01046945$$
$$L_1 = 6.00069867 \times 10^{-3}$$
$$L_2 = 2.00179144 \times 10^{-2}$$
$$L_3 = 1.03560653 \times 10^{-3}$$

<table>
<thead>
<tr>
<th>$K_1$</th>
<th>$K_2$</th>
<th>$K_3$</th>
<th>$L_1$</th>
<th>$L_2$</th>
<th>$L_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.03961212</td>
<td>0.23179234</td>
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<td>2.00179144 $\times 10^{-2}$</td>
<td>1.03560653 $\times 10^{-3}$</td>
</tr>
</tbody>
</table>

**Table 3.1** Sellmeier constants for BK7

**Figure 3.7** Normal dispersion of BK7 glass using the Sellmeier approximation.
From Figure 3.7 and Equation (3.24) it is clear that the index of refraction of BK7 and other dielectrics decreases with increasing wavelength. It is important to elaborate on the difference between deviation and dispersion in the context of prisms. Angular deviation corresponds to the angular change a wavelength will undergo after refraction through a prism. Angular dispersion refers to the local spread in color caused by subtle changes in the angular deviation with respect to wavelength. The angular dispersion of a prism is closely related to the index dispersion of glass as depicted in Figure 3.7. The level of index dispersion in a specific glass is usually characterized by the Abbe number or

\[ V = \frac{n_d - 1}{n_F - n_C}, \quad (3.25) \]

where \( n_d, n_F, \) and \( n_C \) are the refractive indices of the glass at the three primary Fraunhofer wavelengths (\( \lambda_d = 587.6\text{nm}, \lambda_F = 486.1\text{nm}, \lambda_C = 656.3\text{nm} \)).

As with a grating, the angular dispersion of a prism is defined as a change in angle with respect to wavelength or

\[ \mathcal{D} = \frac{d\delta}{d\lambda} = \frac{d\delta}{dn} \frac{dn}{d\lambda}, \quad (3.26) \]

where \( \delta \) and \( n \) are defined in Equations (3.23) and (3.24) respectively. Unfortunately there is no simple analytical solution to this equation. However, mathematical software may be employed to generate and plot numerical data for specific optical systems. Figure 3.8 shows \( \mathcal{D} \) for a BK7 prism with a wedge angle of 40° assuming an angle of incidence of 30°. Note that \( \mathcal{D} \) is a function of wavelength and decreases in a non-linear fashion as...
\( \lambda \) increases. When imaged onto a detector this will produce a non-linear mapping between pixel position and wavelength, which will need to be accounted for through careful calibration of the spectrometer. This is in contrast to the angular dispersion of a grating, which is roughly constant with respect to \( \lambda \) and produces a nearly linear mapping on a detector.

![Angular Dispersion of a Prism](image)

**Figure 3.8** Angular dispersion of a BK7 prism with \( \alpha = 40 \) and \( \theta = 30 \).

### 3.2.2.3 Spectral Linewidth

A monochromatic plane wave incident on a prism will undergo a certain amount of angular broadening due solely to the diffraction effects of the prism aperture. Figure
3.9 shows a broad monochromatic plane wave completely filling a prism such that the exiting wavefront has a full width of $d$. The Fraunhofer diffraction pattern of this wavefront at a distance $z$ is

$$I(\theta_i) \propto \text{sinc}^2\left(\frac{d}{\lambda z} x\right) = \text{sinc}^2\left(\frac{d}{\lambda} \theta_i\right),$$

(3.27)

where the small angle approximation

$$\theta_i \approx \frac{x}{z}$$

(3.28)

has been used to introduce $\theta_i$ as the angular coordinate of the light transmitted by the prism. To maintain common notation between gratings and prisms, the spectral linewidth is defined as the full angular separation between the principal minima of equation (3.27) or

$$\Delta \theta_i = \frac{2\lambda}{d}$$

(3.29)

Thus, the spectral linewidth of a prism is proportional to the wavelength under observation and inversely proportional to the size of the limiting aperture in the system.
3.2.2.4 Resolving Power

As with gratings, the resolving power of a prism is defined as

\[ R = \frac{\lambda}{(\Delta \lambda)_{\text{min}}} \]  
(3.30)

where \((\Delta \lambda)_{\text{min}}\) is the minimum resolvable wavelength difference using Rayleigh's criteria. It follows from equation (3.29) that

\[ (\Delta \theta_{\gamma})_{\text{min}} = \frac{\lambda}{d} \]  
(3.31)

Ideally equations (3.23) and (3.24) would be used to relate \((\Delta \theta_{\gamma})_{\text{min}}\) to \((\Delta \lambda)_{\text{min}}\).

Unfortunately there is no simple analytical solution to these equations. However, Fermat’s principle may be used to relate \((\Delta \theta_{\gamma})_{\text{min}}\) to \((\Delta \lambda)_{\text{min}}\) by equating various optical path lengths. Referring to Figure 3.10 we see that

\[ FTW_1 = GX = n_1 t \]  
(3.32)
where \( t \) is the length of the prism base and \( n_1 \) is the index of the prism for wavelength \( \lambda_1 \). Assume that another wavefront of wavelength \( \lambda_2 = \lambda_1 + \Delta\lambda \) associated with index \( n_2 = n_1 - \Delta n \) is also incident and fully filling the prism aperture. Then

\[
\overline{FTW_2} = GX = n_2 t = (n_1 - \Delta n) t .
\] (3.33)

But

\[
\overline{FTW_1} = \overline{FTW_2} + \Delta s .
\] (3.34)

Solving Equations (3.32) through (3.34) for \( \Delta s \) yields

\[
\Delta s = t \Delta n ,
\] (3.35)

or

\[
\Delta \theta_i = \frac{\Delta s}{d} = \frac{t}{d} \Delta n .
\] (3.36)

Finally, the glass dispersion may be introduced by

\[
\Delta \theta_i = \frac{t}{d} \left( \frac{dn}{d\lambda} \right) \Delta \lambda .
\] (3.37)

Equation (3.37) now relates a small change in wavelength to a small change in transmission angle.

Combining equations (3.31) and (3.37) yields

\[
(\Delta \lambda)_{\text{min}} = \frac{\lambda}{t} \left( \frac{dn}{d\lambda} \right) ,
\] (3.38)

which describes the minimum resolvable wavelength separation for a given prism.

Substituting this into Equation (3.30) gives
\[ \mathcal{R} = t \left( \frac{dn}{d\lambda} \right). \]  

This result reveals that the resolving power of a prism is approximately equal to the dispersion of the prism material multiplied by the physical length of the longest path that light travels through the prism.

**Figure 3.10** Path of two plane waves of color \( \lambda_1 \) and \( \lambda_2 \) through a prism.

Fermat’s principle states that the optical path length \( FTW \) must equal \( GX \).

### 3.2.2.5 Free Spectral Range

One of the distinct advantages of using a prism over a grating in a spectrometer is free spectral range. Based on the definition introduced in Section 3.2.1.5 the free spectral range of a prism is effectively infinite because there are no orders to overlap. Practically, the free spectral range of a prism is limited by the spectral transmission characteristics of the optical system.
3.2.3 Sampling, Speed, and SNR

Whether to use a grating or a prism in a spectrometer is largely dependent on the application and personal preference. Once properly calibrated the end result is quite similar in that spectral information is mapped into a spatial position on a detector. In general, the specific properties of a grating or a prism will be tailored to fit the characteristics of an application. For instance, if higher spectral sampling is required, one would use a prism with a large wedge angle or a grating with a high number of grooves. However, the trade off to higher spectral sampling will be lower signal to noise ratio (SNR) since a lower amount of energy would be collected per sample. One possible solution to this decrease in SNR would be to slow down the acquisition rate. For a given detection system, there is a fundamental relationship between sampling rate, SNR, and detection speed. In general, a system may be tailored to increase performance in one or two of these areas at the expense of an inherent decrease in performance in the third area.

3.3 Interferometric Techniques

Another technique used to measures spatially resolved spectra employs interferometric techniques. In general, light collected from a sample is read by a two beam interferometer such as a Michelson interferometer. As the path length of one arm of the interferometer is varied, the intereferogram is modulated with respect to time and the frequency of this modulation is directly related to the spectral properties of the source. If the source is monochromatic, the intereferogram plotted with respect to time will have a pure sinusoidal form, the frequency of which is directly related to the
wavelength of the source and the velocity of the reference mirror. For a polychromatic 
source, the plotted function will be a sum of sinusoidal functions, which may be treated 
as a Fourier series. The Fourier transform of this function will yield the relative 
composition of each frequency, which is the spectral signature of the source.

One of the major advantages to Fourier transform spectroscopy is that the 
complete spectrum is measured simultaneously and with high SNR. Imaging 
spectrometers built upon this technology are traditionally transmission systems and are 
normally more complex than filter and dispersion systems.

3.4 Multiplexed Techniques

In addition to the three primary categories of imaging spectrometers discussed so 
far, there are several novel configurations that are able to multiplex spectral information 
in the same acquisition frame as the spatial information. In other words, these systems 
are able to encode a three-dimensional data set comprised of spatial and spectral 
information on a single two-dimensional CCD.

One such system, the Dual-View Micro-Imager, is made by Optical Insights[^1]. 
This system attaches to the camera port of a conventional microscope and splits the 
ingoing image into two separately filtered images. The system then directs one image 
onto the left half of the CCD and the other image onto the right half of the CCD. Field of 
view is maintained in one-dimension but reduces by half in the other dimension. The 
Micro-Imager is excellent for applications that require simultaneous measurement of two
spectral bands. Optical Insights also makes a Quad-View Micro-Imager with four spectral channels. Such a system reduces the field of view by half if both dimensions.

Another multiplexed imaging spectrometer was developed by Descour. This non-scanning device, called the CTIS, uses a novel computer generated hologram to disperse a limited field of view of the sample and display a 7 x 7 array of diffraction orders on the CCD. A computer algorithm is then used to calculate spatially resolved spectral information from the blur patterns in the diffraction orders.

Both the Micro-Imager and the CTIS are designed to collect spectral information and spatial information in one integration of the CCD. This is important for applications that require precise measurements of quickly varying spectral changes. In such dynamic applications, it is crucial that all spatial and spectral information be collected simultaneously.
CHAPTER 4: SYSTEM DESIGN

The MCME consists of a fiber-optic catheter coupled to a custom built bench-top confocal microscope and a custom built imaging spectrometer. Figure 4.1 depicts the general layout and operation of the system. The two items that set the MCME apart from a traditional bench-top confocal microscope are the catheter and the multi-spectral collection subassembly. Each of these items will be described in detail in subsequent sections.

Figure 4.1 Functional components of the MCME
It is interesting to note that the MCME’s catheter may be easily removed and coupled to a commercial bench-top confocal system. A custom mount would have to be manufactured to position the proximal face of the fiber within the field-of-view of the microscope. A microscope objective would be selected to appropriately match the characteristics of the fiber. Such a union effectively turns a commercial bench-top confocal microscope into an in-vivo imaging system. When coupled to the Zeiss LSM 510 META, the combined system would be capable of in-vivo multi-spectral imaging. Such systems would not be portable due to the size and alignment requirements of commercial confocal microscopes. However, a commercial system modified for in-vivo use is certainly an exciting potential application for the MCME’s novel catheter assembly.

In its current state the MCME is a second generation clinical prototype. It is largely based on a first generation clinical prototype developed by Aziz\textsuperscript{20,21} and Sabharwal\textsuperscript{22,23}. The original prototype employed a 7mm diameter catheter with a hydraulic focus mechanism. The system collected high resolution grayscale images from living tissue. The second generation system, now called the MCME, has the ability to collect multi-spectral data. The new system employs a redesigned 3mm diameter catheter with a choice of either a mechanical or pneumatic focus mechanism. The most notable change to the bench-top optical system is the new multi-spectral collection subassembly. A few additional improvements dramatically increase the stability of the MCME. The remainder of this chapter briefly reviews the important aspects of the bench-top system and presents details of items new to the MCME.
4.1 Bench-top Optical System

Figure 4.2 shows a detailed schematic of the MCME’s bench-top components. Table 4.1 lists the specification for each lens in the system. The system consists of an illumination arm, scanning optics, a fiber-optic catheter, and a detection arm.

Figure 4.2 Bench-top components of the MCME
### Table 4.1 Specification of the MCME’s bench-top optical system

<table>
<thead>
<tr>
<th>Lens</th>
<th>Focal Length (mm)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>Expand and collimate laser</td>
</tr>
<tr>
<td>2</td>
<td>175</td>
<td>Focus</td>
</tr>
<tr>
<td>3</td>
<td>-150</td>
<td>Create line illumination (cylindrical)</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>Collimate</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>Afocal relay</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>Afocal relay</td>
</tr>
<tr>
<td>7</td>
<td>16.4</td>
<td>Couple to fiber (10x Zeiss objective)</td>
</tr>
<tr>
<td>8</td>
<td>140</td>
<td>Focus onto slit</td>
</tr>
<tr>
<td>9</td>
<td>140</td>
<td>Collimate</td>
</tr>
<tr>
<td>10</td>
<td>75-300</td>
<td>Couple to camera (Nikon zoom lens)</td>
</tr>
<tr>
<td>11</td>
<td>75-300</td>
<td>Couple to camera (Nikon zoom lens)</td>
</tr>
</tbody>
</table>

#### 4.1.1 Illumination Arm and Scanning Optics

The illumination arm incorporates argon ion and krypton ion lasers, which provide a variety of excitation wavelengths throughout the UV and visible spectrum. The 488nm argon ion and 647.1nm krypton ion laser lines are the most commonly used by the MCME. The lasers are individually fiber coupled to the bench-top optical system. Since the entire optical system is aligned to the laser sources, the fiber coupling greatly increases the stability of the MCME relative to the first prototype, which used an optical beam ladder.

Each laser beam expands as it exits the fiber and is then collimated by a lens $L_1$. Dichroic beamsplitter $BS_1$ co-aligns the two sources. This beamsplitter transmits the 488nm argon laser line and reflects the 647.1nm krypton laser line. $BS_1$ would be changed if the lasers are tuned to different wavelengths. Lens $L_2$ creates an intermediate image of the source at location $A$ and the negative cylindrical lens $L_3$ expands the beam along the vertical axis such that the intermediate image at $A$ is a line rather than a point. Moving lens $L_3$ along the optical axis increases or decreases the size of the illumination
line at location $A$. Lens $L_3$ is typically adjusted so that the slit illumination overfills the active area of the fiber bundle. This helps decrease Gaussian falloff inherent to laser sources. By design, the illumination profile is roughly telecentric in the vertical direction (along the slit) at location $A$ and at the proximal face of the fiber. Lens $L_4$ re-collimates the beam in the horizontal plane. Lenses $L_5$ and $L_6$ together form an afocal system that images the scan mirror into the entrance pupil of lens $L_7$. This afocal system insures uniform illumination with respect to scan position. Lens $L_7$ is a high quality infinity-corrected microscope objective that focuses the illumination profile onto the proximal end of the fiber-optic catheter.

4.1.2 Catheter and Detection Arm

The catheter incorporates a fiber-optic imaging bundle from Sumitomo Electric$^{43}$ (IGN 08/30) that transfers the scanned illumination profile to the distal (in-vivo) end of the catheter. The fiber is composed of 30,000 individual optical elements with $2\mu m$ diameter cores at $3\mu m$ center-to-center spacing. The fiber bundle is $1mm$ in overall diameter with an active image diameter of $720\mu m$. A miniature achromatic objective images the distal end of the fiber-bundle into the tissue. A miniature focusing mechanism allows for focus control to $200\mu m$ below the surface of the tissue. Sections 4.2 through 4.4 will concentrate on the specific details of the distal opto-mechanical assembly.

Induced sample fluorescence is collected by the distal optics and relayed back through the catheter to the bench-top optical system. The scan mirror descans the
returned light, and dichroic beamsplitter $BS_2$ directs the fluorescence emission into the detection arm. $BS_2$ is interchangeable allowing the user to tailor the system to the specific excitation and emission characteristics of the fluorescent probe. The beamsplitters used at this location are conventionally called 45° band pass filters. Such filters reflect a desired wavelength range while passing one or more spectral bands. In the current dual laser configuration, the MCME uses a 45° band pass filter that reflects the visible spectrum while passing a very narrow region at 488 nm and 647 nm. Therefore, $BS_2$ passes the laser excitation while simultaneously directing the fluorescence signal into the detection arm. Lens $L_8$ focuses the fluorescent light onto the confocal slit aperture, which rejects the majority of light collected from out-of-focus planes. Energy passed by the slit aperture is collimated by lens $L_9$ and then directed into either the grayscale or multi-spectral collection systems.

4.1.2.1 Grayscale Collection

In grayscale imaging mode, the light transmitted by the stationary confocal slit aperture is re-scanned by the scan mirror and imaged onto a thermoelectrically cooled Photometrics Quantix 57 CCD. The Quantix is a 535 x 512, back-illuminated, frame-transfer CCD with a 3 MHz readout speed. Zoom lens $L_{10}$, mounted on the camera, is adjusted such that the image of the fiber bundle fills the active area of the CCD. While the camera reads out a frame from the transfer buffer, the scan mirror sweeps a full two-dimensional image on the active area of the CCD. This eliminates the need for a shutter and provides repeated scanning with very little dead time between successive scans.
4.1.2.2 Multi-Spectral Collection

In multi-spectral imaging mode the light transmitted by the confocal aperture is reflected by a flip mirror into the multi-spectral detection arm. The flip mirror allows the user to quickly switch between grayscale and multi-spectral imaging modes without the need for realignment. Figure 4.3 shows the concept behind the multi-spectral collection system. The collimated fluorescence signal is dispersed by a prism and then imaged by zoom lens $L_{ij}$ onto a 535 x 512, frame-transfer, thermoelectrically cooled Photometrics Quantix™ 57 CCD. The prism is oriented such that it provides dispersion in a direction perpendicular to the collection aperture. Therefore, for a fixed position of the scan mirror, the two-dimensional light distribution on the CCD represents one (vertical) dimension of spatial information (along the slit direction) and one (horizontal) dimension of spectral information. Typically, the zoom lens $L_{ij}$ is adjusted such that the spatial extent of the image covers 256 pixels in the vertical dimension of the CCD. At this magnification, the image of the slit aperture on the CCD is approximately 1 pixel wide. An 18° wedge prism is currently used to produce a smear in the spectral dimension of 26 pixels over a 286nm range centered at 600nm. The 256 x 26 pixel region of interest is read out of the CCD in 5.9ms using a 3Mhz 12 bit digitizer. The scan rate of the mirror is adjusted so that the illumination moves one spatial resolution element during the 5.9ms integration period. As the mirror is scanned, 256 frames of data are read out in 1.5sec to produce the full 3D data set (256 x 256 spatial x 26 spectral).
Various macros were written in the camera software (V++) to enable efficient operation of the MCME in multi-spectral collection mode. These macros include a *focus mode*, which rapidly acquires an image with fully binned spectra, and an *acquire mode*, which obtains a full three-dimensional data cube. In *acquire mode* the data set is saved as a multi-page TIFF file. This file is then read into IDL for data processing and presentation (see Chapter 6). Before each experiment the spectral dispersion is registered on the CCD by illuminating the distal end of the catheter with narrow band light and recording the exact pixel location of the dispersed light on the detector. Two or three measurements at separate wavelengths are sufficient to generate a functional relationship between wavelength and pixel location. The exact multi-spectral registration and calibration process will be presented in Chapter 5.

### 4.2 Catheter: Design goals

The first-generation catheter employed a miniature F/1 achromatic objective that was 7mm in diameter and 30mm in length. It provided a magnification of 1.67 from the...
tissue to the fiber and achieved nearly diffraction-limited performance between 488nm and 550nm. Attached to the objective was a hydraulic focus mechanism that controlled the position of the fiber bundle with respect to the miniature objective. This system allowed for focus control up to 200μm below the surface of the tissue. The objective and focus mechanism screwed together to form a rigid opto-mechanical assembly that was 7mm in diameter and 60mm in length. The flexible portion of the catheter consisted of the 1mm diameter fiber-optic imaging bundle described in Section 4.1.2 and two flexible hydraulic feed and bleed tubes for the distal focus mechanism.

This 7mm diameter first-generation catheter was small and flexible enough for remote imaging; however it was felt that further miniaturization of the catheter would dramatically increase the MCME's clinical utility. Clearly, a smaller catheter enables imaging of remote locations not accessible the first generation design. The development of a sufficiently small and flexible catheter also allows the MCME to be used as a daughter scope to clinical endoscopes. For example, an Olympus CF-100L colonoscope has an open 3.2mm diameter instrument channel. To insure smooth operation of the MCME in this instrument channel, the diameter of the catheter must not exceed 3.0mm and the rigid opto-mechanical distal assembly must be shorter than 25mm.

In addition to the size constraints stated above, the catheter for the MCME must also adhere to some requirements for multi-spectral imaging. The 7mm first generation catheter was designed for grayscale imaging with the 488nm argon ion laser line as the primary illumination wavelength. The tissue was typically stained with one fluorophor with peak emission below 550nm. Therefore, the 7mm objective was corrected for
chromatic aberrations over the spectral range of 488nm to 550nm. Multi-spectral detection commonly uses an assortment of fluorophors in one experiment to label specific features of the tissue with dyes of different spectral signatures. It is vital that the measured spectra accurately correlate to a spatial location in the tissue, a property that depends on appropriately correcting chromatic aberrations in the miniature objective. The new miniature objective is therefore corrected for chromatic aberrations over the spectral range from 480nm to 660nm. The better color correction also allows operation at longer excitation wavelengths to increase the penetration depth of the system. The MCME incorporates a krypton ion laser operating at 647.1nm for this purpose.

### 4.3 Catheter: 3mm Miniature Objective

#### 4.3.1 Lens Specifications

The miniature objective serves two purposes. First, it images illumination from the distal tip of the fiber bundle into the tissue. Second, it collects the fluorescence excited in the tissue and images this energy back onto the distal tip of the fiber bundle. Since light travels both ways though the lens, the terms *tissue space* and *fiber space* will be used in place of the usual object and image space nomenclature.

The general optical properties and the requirements on the imaging performance for the lens are summarized in Table 4.2. Many of the optical properties of the lens were chosen to match specific properties of the fiber-optic imaging bundle. In order to reduce the likelihood of cross-talk between individual pixels of the fiber bundle, a fiber side $NA$ of 0.29 was chosen, which slightly under fills the fiber bundle $NA$ of 0.35. The lens has a
nominal magnification of 1.6 from tissue to fiber. The $NA$ and field-of-view in the tissue are determined by this magnification and the characteristics of the fiber bundle.

One of the primary design goals for the lens was to achieve a MTF contrast at the fiber plane greater than 50% at 166 lp/mm. This corresponds to the maximum fiber-plane spatial frequency that will not be aliased by a Nyquist sampling rate equal to 1/3 $\mu$m$^{-1}$ (inverse of the fiber bundle’s center-to-center spacing). A minimum contrast of roughly 50% at this frequency ensures that the lateral resolution of the system is not limited by the performance of the miniature objective, but rather by the inherent resolution characteristics of the fiber bundle.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnification (tissue to fiber)</td>
<td>1.6</td>
</tr>
<tr>
<td>Full field-of-view in tissue</td>
<td>450 $\mu$m</td>
</tr>
<tr>
<td>$NA$ in fiber space</td>
<td>0.29</td>
</tr>
<tr>
<td>$NA$ in tissue space</td>
<td>0.46</td>
</tr>
<tr>
<td>Refractive index in tissue space</td>
<td>Water</td>
</tr>
<tr>
<td>MTF at 166 lp/mm at fiber</td>
<td>$\geq$ 50%</td>
</tr>
<tr>
<td>RMS spot size at fiber</td>
<td>$\leq$ 3 $\mu$m diameter</td>
</tr>
<tr>
<td>Telecentric in tissue space</td>
<td>Yes</td>
</tr>
<tr>
<td>Telecentric in fiber space</td>
<td>Yes</td>
</tr>
<tr>
<td>Nominal focal distance in tissue</td>
<td>25 $\mu$m</td>
</tr>
<tr>
<td>Range of focus in tissue</td>
<td>0 to 200 $\mu$m</td>
</tr>
<tr>
<td>Focus compensator</td>
<td>fiber position</td>
</tr>
<tr>
<td>Achromatic range</td>
<td>480 - 660 nm</td>
</tr>
<tr>
<td>Packaged Diameter</td>
<td>$&lt;3$ mm</td>
</tr>
<tr>
<td>Packaged Length</td>
<td>$&lt;13$ mm</td>
</tr>
</tbody>
</table>

**Table 4.2** Specifications of the 3mm diameter objective.

As with most microscope objectives, the lens system must be roughly telecentric in tissue space in order to minimize the change in magnification with respect to focal position. Unlike traditional microscope objectives, the lens must also be telecentric in fiber space. If the lens is not telecentric in fiber space, some light incident on the fiber
near the edge of the field would exceed the acceptance angle \((NA)\) of the fiber bundle. Telecentricity in fiber space ensures uniform fiber coupling efficiency across the entire field-of-view.

The miniature focus mechanism described in Section 4.4 mounts directly to the miniature objective to form a seamless opto-mechanical assembly. The focus mechanism moves the position of the fiber relative to the objective, and therefore provides the ability to adjust the focal depth in the tissue. When assembled, the objective and focus mechanisms focus to a depth of 200\(\mu\)m in the tissue. Therefore, the objective must be designed to perform adequately for conjugates throughout this focal range.

In addition to the specifications discussed in this section, the miniature objective must also adhere to the physical size and chromatic aberration constraints presented in the preceding section. A final lens, assembled to the specification listed in Table 4.2 should provide the MCME with the optical and physical performance necessary for a broad range of applications.

4.3.2 Lens Design

Extensive work was done using ZEMAX optical design software to design a lens that meets the specifications listed in Table 4.2. Through many attempts and several meetings with Jose Sasian a final solution depicted in Figure 4.4 was developed. The design consists of 6 elements with 13 surfaces imparting optical power. In Figure 4.4, the thick grey line surrounding the lens represents the metal housing in which the individual elements are mounted. Element 1 is a microscope cover slip glued to the exterior face of the housing. The cover slip protects the lens from moisture and abuse and is easy to
replace when excessively dirty or scratched. The physical length of the fabricated lens is slightly less than 13mm and the outside diameter of the housing is exactly 3mm. Table 4.3 presents the specification of the miniature objective. The surface numbers correspond to each surface, including the aperture stop, light encounters on its path though the objective. The zeroth surface is the nominal imaging plane in the tissue.

Figure 4.4 Final design for the 3mm miniature objective. The tissue plane is on the left.
### Table 4.3

<table>
<thead>
<tr>
<th>Surface</th>
<th>Element</th>
<th>Radius</th>
<th>Thickness</th>
<th>Glass</th>
<th>Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tissue</td>
<td>4.000</td>
<td>0.050</td>
<td>Water</td>
<td>0.45</td>
</tr>
<tr>
<td>1</td>
<td>Coverslip (1)</td>
<td>Infinity</td>
<td>0.150</td>
<td>1.523, 56.00</td>
<td>2.50</td>
</tr>
<tr>
<td>2</td>
<td>Infinity</td>
<td>0.318</td>
<td></td>
<td></td>
<td>2.50</td>
</tr>
<tr>
<td>3</td>
<td>Singlet (2)</td>
<td>-1.190</td>
<td>0.893</td>
<td>SFL6</td>
<td>0.93</td>
</tr>
<tr>
<td>4</td>
<td>Singlet (3)</td>
<td>-1.412</td>
<td>0.096</td>
<td></td>
<td>1.63</td>
</tr>
<tr>
<td>5</td>
<td>Singlet (3)</td>
<td>-4.795</td>
<td>0.400</td>
<td>SFL6</td>
<td>1.80</td>
</tr>
<tr>
<td>6</td>
<td>Singlet (3)</td>
<td>-2.000</td>
<td>0.100</td>
<td></td>
<td>1.93</td>
</tr>
<tr>
<td>7</td>
<td>Triplet (4)</td>
<td>4.947</td>
<td>0.400</td>
<td>SFL6</td>
<td>1.96</td>
</tr>
<tr>
<td>8</td>
<td>Triplet (4)</td>
<td>1.523</td>
<td>0.887</td>
<td>N-PSK53</td>
<td>1.88</td>
</tr>
<tr>
<td>9</td>
<td>-1.190</td>
<td>2.232</td>
<td></td>
<td>F2</td>
<td>1.88</td>
</tr>
<tr>
<td>10</td>
<td>-2.766</td>
<td>0.099</td>
<td></td>
<td></td>
<td>1.96</td>
</tr>
<tr>
<td>11</td>
<td>Stop</td>
<td>Infinity</td>
<td>1.578</td>
<td></td>
<td>1.84</td>
</tr>
<tr>
<td>12</td>
<td>Doublet (5)</td>
<td>4.628</td>
<td>1.158</td>
<td>N-PSK53</td>
<td>1.93</td>
</tr>
<tr>
<td>13</td>
<td>-1.604</td>
<td>2.841</td>
<td></td>
<td>SFL6</td>
<td>1.83</td>
</tr>
<tr>
<td>14</td>
<td>-4.775</td>
<td>0.100</td>
<td></td>
<td></td>
<td>1.96</td>
</tr>
<tr>
<td>15</td>
<td>Singlet (6)</td>
<td>1.672</td>
<td>1.271</td>
<td>SFL6</td>
<td>1.83</td>
</tr>
<tr>
<td>16</td>
<td>Focus Space</td>
<td>1.190</td>
<td>0.597</td>
<td></td>
<td>1.04</td>
</tr>
<tr>
<td>17</td>
<td>Fiber</td>
<td>Infinity</td>
<td></td>
<td>Silica</td>
<td>0.72</td>
</tr>
</tbody>
</table>

The concept of buried achromatizing surfaces was used to arrive at the solution depicted in Figure 4.4. This technique is based on the selection of a pair of glasses that have nearly identical refractive indices but significantly different dispersions. Since the index of refraction does not change across the buried surface, the designer is able to vary the curvature and position of the surface without altering the monochromatic performance of the lens.

Figure 4.5 shows a simple example of how one might use a buried surface in an optical design. Figure 4.5(a) shows a typical doublet made from BK7 and SFL6, which have dramatically different dispersions and indices of refraction. If one were to alter the radius of curvature of any of the three surfaces in this doublet one would immediately
change the first order properties of the lens as well as the monochromatic and polychromatic performance. In contrast, Figure 4.5(b) shows a typical buried surface doublet made from N-PSK53 and F2. Since the index of refraction of the two glasses is essentially the same, the designer can alter the curvature and location of the cemented surface without changing the first order properties of the lens. More importantly, a change in the cemented surface will not affect the monochromatic performance of the lens.

![Figure 4.5](image)

**Figure 4.5** Buried surface technique. Doublet (a) is made of BK7 and SFL6, which have dramatically different indices and dispersion. Doublet (b) is made of N-PSK53 and F2, which have very similar indices but different dispersion characteristics.

In theory, the use of a buried surface allows a well-corrected monochromatic design to be achromatized with no effect on the original monochromatic performance. In practice, it is difficult to attain sufficient achromatization without altering the monochromatic solution slightly. However, the technique is still a useful tool to help reduce the complexity of the initial design process. N-PSK53 and F2 were used as the buried surface glasses for the miniature objective.
To further simplify the design process, the higher NA tissue-half of the lens (elements 1-4) was initially designed independently of the fiber-half of the lens (elements 5-6). The starting condition for the tissue-half of the lens was modeled after a Petzval objective and consisted of two singlets and a field flattening lens near the image plane. The final monochromatic solution for the tissue-half of the lens was composed of a coverslip (element 1), two singlets (elements 2 and 3), and a doublet (element 4). In contrast to what is shown in Figure 4.4, element 4 was only a doublet in the monochromatic design since the buried surface had not yet been inserted. Once a monochromic solution to the tissue-half of the lens was realized, a starting condition for the fiber-half of the system was obtained by simply flipping and scaling the tissue-half solution. The two sections were then placed on either side of an aperture stop and further optimized to achieve a final monochromatic solution for the objective.

To achromatize the lens, each of the N-PSK53 glass spaces was split into N-PSK53 and F2 regions. This formed a triplet on either side of the aperture stop, each incorporating one buried surface. The lens system was then re-optimized over the full spectral range. As a final solution was achieved, the fiber-half of the lens was reduced to a doublet and one singlet without sacrificing image quality.

During the design process, field curvature was one of the aberrations that significantly limited the optical performance of the lens. The physical result of using an objective with field curvature is that a planar object is mapped to a spherical image. For the miniature objective, the planar fiber face will be mapped to a spherical surface inside the tissue. Therefore, the optical sections created by the MCME will be slightly curved.
rather than perfectly flat. Since the system will be used to image thick biological tissue
samples, a small amount of curvature in the optical sections will not be a problem.

In the design process, the correction for field curvature was relaxed by optimizing
and evaluating the lens assuming a slightly curved tissue plane. From Table 4.3 we see
that the image or tissue plane has a radius of curvature of 4mm. This number was fixed
throughout the design process and corresponds to a sag of approximately 6μm at the edge
of the field in tissue space. A sag of 6μm is much less than the predicted axial resolution
of the MCME and should not present a problem for in-vivo imaging.

4.3.3 Theoretical Performance

Various metrics were used to evaluate the final miniature objective design and the
most important of these metrics will be discussed in the following sections. First
however, a few important details about the design process will be discussed to help
explain the upcoming data.

In the optical design software, the objective was oriented such that the tissue was
located in object space and the fiber was located in image space. Unless otherwise stated,
the performance metrics discussed in this section are in fiber-plane coordinates. To
convert a lateral spatial quantity from fiber to tissue space coordinates one simply divides
by the lateral magnification of the objective or

\[
x_{\text{tissue}} = \frac{x_{\text{fiber}}}{m_{\text{objective}}} = \frac{x_{\text{fiber}}}{1.6}.
\]  

(4.1)
To convert an axial spatial quantity from fiber to tissue space coordinates one must divide by the square of the lateral magnification and then scale the result by the tissue space index or

\[ z_{\text{tissue}} = \frac{z_{\text{fiber}}}{(m_{\text{objective}})^2 n_{\text{tissue}}} = \frac{z_{\text{fiber}}}{2.56^{1.33}}. \]  

(4.2)

It should also be noted that the miniature objective was optimized and evaluated over five field positions, five wavelengths, and four focus configurations. However, to simplify the presentation of the following data, the design was reduced to three field positions, three wavelength values, and four focus configurations. Throughout the design process, significantly more weight was given to the shallow focal positions since the confocal microendoscope will primarily be used to image the first 50\( \mu \text{m} \) of tissue. This non-uniform weight factor manifests itself in a slight degradation in optical performance for the deeper focal positions, particularly the deepest focal position of 200\( \mu \text{m} \).

4.3.3.1 RMS Spot Diameter

One of the most common metrics used to characterize an optical system is root mean square (RMS) spot size. Table 4.4 shows the predicted RMS spot diameters for the miniature objective at three different field locations and four separate focus depths. The data are presented in fiber space coordinates and are averaged over all design wavelengths. The fiber space RMS spot size specification for the lens was a diameter of less than or equal to 3\( \mu \text{m} \). The miniature objective outperforms this specification considerably in all but the most extreme case of maximum field and maximum focus.
depth. The reason for the degradation in the high field and deep focal positions is partially due to the weighting factor that was applied to the merit function during optimization. However, it is also due to a significant amount of lateral color and coma, which will be discussed in the next section.

<table>
<thead>
<tr>
<th>Focal position</th>
<th>On-axis</th>
<th>70% Off-axis</th>
<th>100% Off-axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.18</td>
<td>1.98</td>
<td>2.26</td>
</tr>
<tr>
<td>50</td>
<td>1.94</td>
<td>1.92</td>
<td>2.32</td>
</tr>
<tr>
<td>100</td>
<td>1.82</td>
<td>1.92</td>
<td>2.48</td>
</tr>
<tr>
<td>200</td>
<td>1.82</td>
<td>2.06</td>
<td>3.00</td>
</tr>
</tbody>
</table>

Table 4.4 Predicted RMS spot size diameters in fiber space for the miniature objective. Spot sizes are averaged over all design wavelengths. All values in (μm).

4.3.3.2 Third-Order Aberrations and OPD Fans

The residual aberrations in an optical design are often characterized by the third-order wavefront aberration coefficients. These coefficients describe the maximum optical path length (OPD) difference between a perfectly spherical reference wavefront and the aberrated wavefront. Table 4.5 summarizes the individual coefficients in terms of maximum waves of OPD. The table suggests that the performance of the miniature objective is limited by spherical aberration, Petzval field curvature, and lateral color. It is important to note that the third-order aberrations coefficients presented in Table 4.5 are a mathematical estimate to the actual aberrations in the optical system. Of specific interest to the miniature objective is the fact that these coefficients assume a planar object and image space and are not affected by a change in the curvature of these planes.
Table 4.5 Predicted third-order wavefront aberrations in waves for the miniature objective. The listed coefficients are averaged over all four focus test configurations and assume planar object and image surfaces.

<table>
<thead>
<tr>
<th>Aberration</th>
<th>Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical</td>
<td>1.39</td>
</tr>
<tr>
<td>Coma</td>
<td>-0.31</td>
</tr>
<tr>
<td>Astigmatism</td>
<td>0.08</td>
</tr>
<tr>
<td>Petzval field curvature</td>
<td>1.52</td>
</tr>
<tr>
<td>Distortion</td>
<td>0.15</td>
</tr>
<tr>
<td>Axial color</td>
<td>-0.79</td>
</tr>
<tr>
<td>Lateral color</td>
<td>-1.58</td>
</tr>
</tbody>
</table>

The specific shape of the aberrated wavefront may be explored by viewing the OPD fans\(^{47}\) presented in Figures 4.6 and 4.7. This collection of plots describes the physical shape of the aberrated wavefront with respect to a reference sphere. A perfect unaberrated optical system will produce flat OPD fans that overlay the abscissas. Overall the OPD fans show that the design is adequately corrected for most aberrations. In the on-axis positions there is a small amount of third and higher order spherical aberration. For deeper focal positions, there is an increased asymmetry in the tangential OPD plots at the central wavelength of 580nm due to coma. For deeper focal positions there is also an increased asymmetrical separation between the tangential OPD plots for the three primary wavelengths due to lateral color. Both coma and lateral color are odd aberrations that greatly depend on the symmetry of an optical system. As focal position increases, the symmetry of the miniature objective decreases and hence odd aberrations like coma and lateral color increase. It is important to note that while these aberrations seem threatening, the vertical scale in the wavefront OPD plots has a maximum value of ±\(\frac{1}{2}\) waves. In general, a combined residual wavefront error of less than ±\(\frac{1}{2}\) waves peak to valley is sufficient for nearly diffraction-limited performance.
Figure 4.6 Predicted wavefront OPD fans for the miniature objective at focal depths of 0\(\mu m\) and 50\(\mu m\). Plots are presented for tangential (left) and sagittal (right) orientations at three field locations.
Figure 4.7 Predicted wavefront OPD fans for the miniature objective at focal depths of 100μm and 200μm. Plots are presented for tangential (left) and sagittal (right) orientations at three field locations.
4.3.3.3 Astigmatism and Distortion

Field and distortion plots provide an excellent way to investigate the amount of astigmatism, field curvature, and distortion in an optical system. While astigmatism and field curvature may be determined to some degree by viewing wavefront OPD fans, their effect is significantly more isolated in field curvature plots. Distortion does not appear in wavefront OPD fans.

Figure 4.8 shows field and distortion plots for each of the four focal positions. At each focal position, the figure presents a pair of plots. The field plot on the left shows the shape of the sagittal and tangential astigmatic focal planes. The vertical axis is normalized field position and the horizontal axis is axial position in terms of defocus from the paraxial focus. The field curves have an overall spherical shape with a split in sagittal and tangential surfaces for large field. The overall spherical shape roughly corresponds to residual field curvature while the split in the sagittal and tangential surfaces corresponds to astigmatism. The field curves presented in Figure 4.8 suggest a maximum sag at the edge of the field due to field curvature of roughly 7\(\mu\)m and a maximum split in the sagittal and tangential fields of approximately 3\(\mu\)m. Converting from fiber space to tissue space coordinates using Equation (4.2) we find a tissue space field curvature of approximately 3.5\(\mu\)m and an astigmatic split of roughly 1.5\(\mu\)m. Each of these values is well within the predicted axial resolution of the MCME.

For each focal position, the right hand distortion curve shows the amount of distortion with respect to field. The vertical axis is normalized field position and the percentage of distortion is given as
\[ \% Distortion = \left( \frac{\Delta h}{h} \right) \times 100, \]  

where \( h \) is the paraxial field position and \( \Delta h \) is the error between the paraxial and real field locations. In general, the human eye can not discern less than 2\% distortion. The plots in Figure 4.8 predict less than 0.5\% distortion.

![Figure 4.8 Predicted astigmatic field and distortion plots in fiber space for each of the four focal positions. For each pair of plots, the astigmatic field is shown in microns on the left and the distortion is shown as a percentage on the right. A wavelength of 570nm was used for these data.](image)
4.3.3.4 Polychromatic MTF

One of the primary metrics used in the design of the miniature objective was MTF. As stated in Section 4.3.1, the lens needs to have an MTF contrast of greater than 50% at 166 lp/mm in fiber space. Figure 4.9 shows the predicted fiber space MTFs for the 50μm and 200μm focal position. The predicted MTFs for the 0μm and 100μm focal depths were omitted because they were virtually identical to the 50μm case. The markings on the plots label the tangential and sagittal lines for the three different fields. The topmost line in each plot represents diffraction-limited performance. The top plot shows greater than 80% contrast at 166 lp/mm. At the edge of the field for the 200μm focal depth the contrast drops to 70% at 166 lp/mm. These results show that the lens significantly outperforms the MTF design metric. This excellent performance should ensure greater than 50% contrast in the final manufactured product.
Focal Position = 50μm

Focal Position = 200μm

Figure 4.9 Predicted polychromatic fiber space MTF for the miniature objective.
4.3.3.5 Chromatic focal shift

The chromatic properties of the miniature objective are explained using a chromatic focal shift plot. Figure 4.10 shows such a plot for the 50μm focal position. A chromatic focal shift plot shows the axial image location in terms of wavelength. Figure 4.10 reveals a nearly linear relationship between wavelength and object location. The predicted maximum shift at the 50μm focal location is 10.72μm measured in fiber space. The plots for the other three focal locations were quite similar in functional form to Figure 4.10. Table 4.6 summarizes the predicted maximum focal shift for all four tissue depth locations. The maximum chromatic focal shift, measured in tissue space, is 6.1μm and occurs at the 0μm depth position. This value is well within the axial resolution of the MCME and should not pose a significant problem to the overall spatial or spectral resolution of the system.
Figure 4.10 Predicted fiber space chromatic focal shift for the miniature objective at 50μm focal depth.

<table>
<thead>
<tr>
<th>Focal position</th>
<th>Fiber Space Shift</th>
<th>Tissue Space Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.7</td>
<td>6.1</td>
</tr>
<tr>
<td>50</td>
<td>10.7</td>
<td>5.6</td>
</tr>
<tr>
<td>100</td>
<td>9.9</td>
<td>5.1</td>
</tr>
<tr>
<td>200</td>
<td>8.8</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Table 4.6 Predicted maximum chromatic focal shift in fiber and tissue space. All values in (μm).

4.3.4 Tolerancing

The data in the preceding section show that the design for the miniature objective outperforms the stated specifications. However, these data on their own are not sufficient to verify a design. No manufacturing process is perfect and any deviation from the exact optical design during the course of manufacturing degrades the final performance of the lens system. It is up to the lens designer to provide the manufacturer with a realistic and
cost effective list of tolerances. In order to do that, the designer must explore the individual effects of manufacturing and assembly errors on the performance of the lens and show that the combined effects of all fabrication errors will produce the desired result.

ZEMAX has three primary methods for exploring the effect of manufacturing errors on the optical performance of a system. The user may perform a Sensitivity Analysis, an Inverse Sensitivity Analysis or a Monte Carlo Analysis. In sensitivity mode, the user assigns specific tolerances to the system and ZEMAX computes the maximum change in a given metric for each tolerance individually. In inverse sensitivity mode, the user enters a maximum acceptable change in a given metric and ZEMAX individually computes the tolerances that will cause such a change. In each case, the software only explores one tolerance at a time. The results from either of these analyses help determine which individual tolerances cause the largest degradation in performance. A Monte Carlo Analysis estimates the combined effects of all the fabrication and alignment tolerances. In this mode, the user enters a specific tolerance set and ZEMAX generates a random configuration that meets the specified tolerances. A specified metric is computed to evaluate the performance of each lens. The process is repeated hundreds or thousands of times to generate the statistics on the merit of choice.

A sensitivity analysis was performed to confirm that the miniature objective was not overly sensitive to specific manufacturing and/or alignment errors. Out of this analysis came a reasonable and cost effective list of tolerances for the miniature objective. A Monte Carlo analysis was used to simulate the combined effects of all of the
tolerances. The simulations predicted, with sufficient confidence, that a lens with adequate performance could be fabricated to our specifications. Table 4.7 shows the final tolerance data for the miniature objective. Refer to Figure 4.4 and Table 4.3 to match specific surface numbers to physical elements.

<table>
<thead>
<tr>
<th>Tolerance</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius</td>
<td>12.5μm on surfaces 8,10,13 and 25μm on all others</td>
</tr>
<tr>
<td>Thickness</td>
<td>25μm after surface 2,3,4,5 and 50μm after all others</td>
</tr>
<tr>
<td>Decenter</td>
<td>10μm</td>
</tr>
<tr>
<td>Tilt</td>
<td>0.005 degrees</td>
</tr>
<tr>
<td>Irregularity</td>
<td>¼ fringe</td>
</tr>
<tr>
<td>Index and abbe</td>
<td>normal Schott melt tolerances</td>
</tr>
</tbody>
</table>

Table 4.7 Final tolerance data for the miniature objective

4.3.5 Fabrication

Four miniature objectives were fabricated and assembled by Optics Technology. They specialize in custom microscope objectives and have extensive experience in the field of miniature optics. Optics Technology was consulted throughout the design and tolerance process to insure that the physical properties of the lens remained within the scope of their manufacturing expertise.

Roughly eight copies of each individual optical element were made to help guarantee at least four spec versions. At the end of the polishing process, Optics Technology measured the radii of curvature and thickness of each element. ZEMAX was used to systematically replace the ideal elements in the design with real lens data. Optics Technology was then given a new prescription for each final lens with re-optimized air spacings. This process effectively maximized the final performance of each of the four miniature objectives.
4.4 Catheter: 3mm Miniature Focus mechanisms

The catheter for the MCME employs a focusing system that is capable of accurately controlling the axial position of the fiber bundle with respect to the miniature objective. This enables the operator to change the imaging conditions for the objective and, in effect, scan the focal position in the sample to a tissue depth of 200 μm.

The hydraulic focusing system used in the first generation confocal microendoscope was more than adequate for the 7mm diameter catheter but a simple reduction in size was not practical for the new assembly. Accounting for the size of the fiber bundle and the minimum wall thickness of the mechanical housing, there is less than 3/4 mm of available space on either side of the fiber in which to connect the hydraulic source and bleed lines. There was also a general desire to move away from the use of hydraulic fluid and develop a system less susceptible to fluid leaks and air bubbles.

Various alternatives for focusing mechanisms were considered and evaluated including piezo-electric crystals, magnetic voice coils, and electronic motor drives. However, each system had specific limitations that ultimately made the technology impractical for such a small device. Eventually two promising technologies were chosen, one pneumatic and the other mechanical. Each has advantages and disadvantages.

4.4.1 Pneumatic Focus Mechanism

Figure 4.11 shows the layout of the miniature pneumatic focus mechanism. The mechanism uses pressurized air to move the fiber with respect to the miniature objective. The mechanism consists of a miniature distal subassembly and a somewhat larger
proximal subassembly. The focus system is based on a novel design that integrates the imaging fiber and pneumatic supply line into a single concentric unit. The fiber bundle is routed through semi-flexible high-pressure tubing with an inside diameter of 1.6mm and an outside diameter of 2mm. This tubing runs the entire length of the catheter and delivers air to the distal opto-mechanical assembly while simultaneously providing an additional layer of protection to the fiber bundle.

The proximal subassembly introduces air into the pneumatic tubing and simultaneously allows the fiber to move freely with respect to the tubing and proximal subassembly housing. A small cylindrical piece of latex, custom manufactured by North American Latex\(^\text{49}\), acts as a diaphragm and allows the fiber to move with respect to the housing. The latex diaphragm is fitted tightly over the fiber, wrapped back over the housing, and held in place by a retaining cap. As air pressure is increased, the diaphragm in the proximal portion of the mechanism is pressed against the rigid walls of the end cap and produces no axial (focus) forces on the fiber.
A similar concept is employed in the distal subassembly. A small cylindrical piece of latex acts as a diaphragm and allows the fiber to move with respect to the tubing and distal subassembly housing. The latex diaphragm is placed on the fiber bundle and then folded over the semi-flexible pneumatic tubing. The tubing and latex diaphragm are then inserted into the metal housing of the focus mechanism. The fiber bundle is permanently attached to a piston that is located between the latex diaphragm and the miniature objective. As air is introduced into the feed line via the proximal subassembly, the pressure causes the diaphragm to expand and the piston and fiber are pushed towards the lens. A spring between the lens and the piston provides the restoring force necessary to push the piston and fiber away from the lens as air pressure is released. Once everything is assembled, the axial forces on the fiber originate from the force applied to the piston through the diaphragm and through the restoring spring in the distal assembly. Focusing of the MCME is accomplished by regulating the air pressure in the catheter.
The precision mechanical parts of the miniature focus mechanism were fabricated by Optics Technology. The final distal opto-mechanical assembly, incorporating both the miniature objective and miniature pneumatic focus mechanism, measures 3mm in diameter and 21mm in length.

4.4.2 Mechanical Focus Mechanism

The MCME may also be focused by a miniature mechanical focus mechanism. This system works on a principle similar to that of a brake cable on a bicycle. Figure 4.12 shows the layout of the system. The fiber is routed through semi-rigid tubing with an outside diameter of 2mm. Focus is achieved by mechanically moving the fiber with respect to the outer tubing. The proximal subassembly consists of a catheter end-cap and a precision micrometer. The end-cap is glued to the external tubing and is then fixed to the optical bench. The bare fiber bundle is then mounted to a translation stage controlled by a micrometer, which allows the user to move the fiber bundle with respect to the external tubing and proximal end-cap.

In the distal subassembly, the external tubing is fixed to the housing of the focus mechanism. Therefore, the external tubing provides a rigid axial bond between the proximal and distal subassemblies. As the fiber is retracted by the proximal micrometer, the fiber and piston move away from the lens and compress a spring that rests on a ridge inside the distal housing. As tension on the fiber is relaxed, the spring provides the restoring force necessary to push the piston and fiber towards the miniature objective.

Since the focus system relies on mechanical forces, there is no need for the complex diaphragms incorporated in the pneumatic design. However, the system is very
sensitive to frictional forces between the fiber bundle and the semi-rigid external tubing. To help alleviate this friction, the fiber is covered with a length of thin internal Teflon tubing. The internal tubing is not attached to the end cap or the distal housing. The internal Teflon tubing floats back and forth much like a bearing and helps lessen the frictional forces in the system. The precision mechanical parts of the miniature mechanical focus mechanism were fabricated by Optics Technology. The final distal opto-mechanical assembly, incorporating both the miniature objective and miniature mechanical focus mechanism, measures 3mm in diameter and 21mm in length.

Figure 4.12 Mechanical focus assembly. Distal subassembly is shown attached to the lens barrel. The fiber extends to the right and enters the bench-top optical system. (Drawing not to scale)
CHAPTER 5: SYSTEM CHARACTERIZATION

Once the precision components of new 3mm diameter catheter were fabricated, a series of experiments were carried out to characterize the performance of the distal opto-mechanical assembly. These experiments included spatial and spectral resolution measurements for each of the four miniature objectives and characterization of the focal plane movement provided by the pneumatic and mechanical focus mechanisms. Once the performance of the opto-mechanical system was verified, the new 3mm catheter was assembled and the grayscale imaging performance of the MCME was tested. Finally, the multi-spectral detection system was characterized through various calibration and resolution experiments. These experiments are described in detail in the subsequent sections.

5.1 Miniature Objective

The first step to characterizing the performance of the MCME was to verify the capabilities of the four miniature objectives. Figure 5.1 depicts the setup of the optical system used to test the distal objectives. A roughly collimated white light source was filtered to provide uniform illumination over a specific spectral band. Two diffusers were used to insure a uniform illumination profile across the entire $NA$ of the miniature objective. An ultra high resolution air force bar target was placed in the tissue space of
the miniature objective which produced an image of this bar target in fiber space. A high quality infinity correct 20x microscope objective relayed the image of the bar target onto a scientific grade CCD camera fitted with a 75mm to 300mm zoom lens. The miniature objective and the bar target were mounted on separate precision xyz-stages to allow for accurate positioning.

![Diagram of optical setup](image)

**Figure 5.1** Setup used to test the resolution of the miniature objective. The objective is oriented such that the resolution target is in the tissue plane.

To ensure proper centration an additional light source temporarily illuminated the side of the miniature objective closest to the camera (fiber space). The miniature objective was then translated while looking at the active area of its final surface until the objective was properly centered on the optical axis of the test system.

The miniature objective is intended for use in contact with tissue. During the design process, the index of refraction of water (1.33) was used to approximate the true index of tissue. However, the measurements described in the subsequent sections required frequent repositioning of the bar target, which made water coupling impractical.
Since the miniature objective was designed for water immersion, the air space between
the bar target and the objective introduces a small amount of additional spherical
aberration. However, if the miniature objective performs adequately in air, it should
perform better when in contact with tissue. This was verified by taking several contrast
measurements with and without water coupling and noting a slight increase in contrast
for the water coupled images.

It is important to note that a given bundle of rays traveling though the miniature
objective from fiber space to tissue space will focus at a deeper position in a tissue space
comprised of water versus air. Since the miniature objective was designed to operate to a
depth of 200µm in water, it would be inaccurate to test the objective at a depth of 200µm
in air. The relationship between an axial distance in air, \(z_{\text{air}}\), and an axial distance in
water, \(z_{\text{water}}\), is given by

\[
\frac{z_{\text{air}}}{n_{\text{water}}} = \frac{n_{\text{air}}}{z_{\text{water}}} = \frac{1}{1.33} z_{\text{water}},
\]

where \(n_{\text{air}}\) and \(n_{\text{water}}\) are the index of refractions of air and water, respectively. Therefore,
a depth of 200µm in water corresponds to a depth of 150µm in air.

Optics Technology manufactured four copies of the miniature objective. It took
them significantly longer than expected to grind and polish the individual elements. The
first miniature objective (lens 1) was rushed through the assembly process in order to
deliver the lens in a timely manner. The other miniature objectives (lenses 2-4) were
assembled after the first one was delivered. As a result, the mounting threads on the first
lens did not exactly match the mounting threads on the other three lenses. Lens 1 is
currently being rebuilt by Optics Technology and is unavailable for testing. Therefore, the resolution data provided in the subsequent sections are only for lenses 2 through 4.

5.1.1 Magnification

The first and most basic test was to verify the lateral magnification of the miniature objective. For this test the miniature objective was positioned on-axis while a known spatial frequency was recorded from the bar target. Data were taken with the bar target at various axial locations to check the telecentricity of the miniature objective. These data were labeled $m_{\text{total}}$. A similar method was used to measure the lateral magnification of the test optical system (camera, zoom lens, 20x objective). These data were labeled $m_{\text{test}}$. The lateral magnification of the miniature objective $m_{\text{objective}}$ is related to these two measurements by

$$m_{\text{objective}} = \frac{m_{\text{total}}}{m_{\text{test}}} \quad (5.2)$$

Table 5.1 summarizes the results for this experiment and includes the magnification predicted by ZEMAX. The magnification data for lenses 3 and 4 closely match predicted values. However, lens 2 has significant differences. The magnification predicted by ZEMAX is only a paraxial approximation to the true magnification and does not take into account any aberrations in the optical system. In addition, the tissue space is not perfectly telecentric. Therefore, the magnification of the miniature objective is affected by the thickness of the epoxy layer used to attach the coverslip (element 1) to the lens barrel. It is unclear if either of these factors effect the magnification of the miniature
objective enough to account for the discrepancies in Table 5.1. Subsequent sections will show that the three lenses are relatively equivalent in performance.

<table>
<thead>
<tr>
<th></th>
<th>38 µm Depth</th>
<th>75 µm Depth</th>
<th>150 µm Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Lens 2</td>
<td>1.4</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Lens 3</td>
<td>1.6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Lens 4</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 5.1** Predicted and measured lateral magnification of the miniature objective

5.1.2 MTF Contrast Test

MTF was one of the primary metrics used to design the miniature objective. The configuration depicted in Figure 5.1 can sample the MTF of the miniature objective by measuring the degradation in the spatial frequency patterns of the bar target. The MTF of an optical system may be thought of as the ability of the system to pass specific spatial frequencies. This ability can be measured by calculating the contrast in an image of a bar target pattern of a given spatial frequency as

\[
\text{Contrast} = \frac{\text{max} - \text{min}}{\text{max} + \text{min}},
\]

where \text{max} and \text{min} are the maximum and minimum irradiance (or digital value) in the image of the target pattern.

For this test the illumination was spectrally limited by an 80nm band-pass filter centered at 560nm. This filter provided appreciable transmission over the majority of the operating spectral range of the miniature objective. The air force bar target was placed 19µm away from the exterior surface of the miniature objective's cover slip (element 1).
According to equation (5.1) this corresponds to a tissue depth of 25\(\mu\)m, which is a typical focal depth for the MCME.

The MTF of the entire optical system (lens plus test optics) was sampled by measuring the contrast in the image of the bar target for a variety of spatial frequencies. An identical procedure was used to calculate the MTF of the optical test system alone without the miniature objective. The true MTF of the miniature objective was obtained by dividing the MTF of the whole system by that of the optical measurement system.

This MTF test was very labor intensive and was, therefore, performed on lens 2 alone. Figure 5.2 shows the results for three field positions: on-axis, 70% off-axis, and at full field. The contrast measurements are the average of the horizontal and vertical bar target patterns for each frequency. The thick solid line shows the on-axis diffraction-limited performance for the miniature objective. The thin solid line is the predicted MTF of the lens, averaged over all fields, assuming no fabrication or assembly errors. The data are presented in terms of fiber-plane spatial frequency. The vertical dashed line represents a spatial frequency of 166 lp/mm, which corresponds to the maximum test spatial frequency, stated in Table 4.2. These results show a minimum contrast of greater than 50% over the entire field at the test frequency.
Figure 5.2 Measured MTF of Lens 2. Data were taken in air at a focal depth of 19μm (25μm equivalent in tissue).

At very low spatial frequencies, the MTF curves in Figure 5.2 converge to a common average MTF for all fields. The low spatial frequency features of the bar target are quite large with respect to the field-of-view of the miniature objective. This makes it difficult to measure the contrast at a particular field position. Therefore, the first data point in Figure 5.2, which corresponds to the lowest spatial frequency that could be sampled by the test setup, should be viewed as an average MTF over all fields. The contrast is slightly lower than one might expect at the lowest spatial frequencies, presumably due to imperfect coatings that introduce a small amount of diffuse stray light in the images. However, this performance reduction is not noticeable under normal operating conditions.
5.1.3 PSF Knife Edge Test

The method used to sample the MTF of lens 2 in the preceding section was labor intensive and was impractical for more than one lens at one focal depth. Therefore, a simple edge response test (knife edge test) was used as an additional verification of lateral resolution. The light source in Figure 5.1 was spectrally limited by a 500nm long pass filter and a 650nm short pass filter. This produced roughly uniform illumination over the majority of the operating spectral range of the miniature objective. The bar target was positioned such that a large on/off region was centered in the field-of-view of the miniature objective. Data were taken for focal positions of 38\textmu m, 75\textmu m, and 150\textmu m in air. According to equation (5.1) these positions correspond to focal depths of 50\textmu m, 100\textmu m, 200\textmu m in tissue. The test was performed on-axis and at the 70% off-axis field location.

A one-dimensional profile of the edge response for lens 3 is plotted in Figure 5.3(a). These data were taken on-axis at a focal depth of 38\textmu m in air. The data are shown fit to a cumulative Lorentzian distribution. The derivative of this profile corresponds to the lateral point spread function (PSF) for the lens\textsuperscript{27,29} and is plotted in Figure 5.3(b). The Lorentzian shape of the PSF is only an estimate since the cumulative distribution used to fit the profile data is only an approximation. However, its overall form and width are adequate for lateral resolution estimates. The lateral resolution of the miniature objective may be approximated by the full-width-half-maximum (FWHM) of the PSF. The data presented in Figure 5.3 indicates a FWHM of 1.4\textmu m in tissue space.
Accounting for magnification, this value corresponds to 2.2μm in fiber space, which implies that the lens will be able to resolve the individual elements of the fiber bundle.

Figure 5.3 Measured lateral resolution of the miniature objective (knife edge test). (a) Edge response with cumulative Lorentzian distribution fit and (b) the corresponding Lorentzian approximation to the PSF.

The procedure described above was repeated for all three miniature objectives at each focal and field position. Table 5.2 summarizes the results in terms of fiber space coordinates. These data should be compared to the predicted RMS spot size diameters presented in Table 4.4.

<table>
<thead>
<tr>
<th></th>
<th>38μm Depth</th>
<th>75μm Depth</th>
<th>150μm Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens2 on-axis</td>
<td>1.8</td>
<td>1.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Lens2 off-axis</td>
<td>2.0</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Lens3 on-axis</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Lens3 off-axis</td>
<td>2.6</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Lens4 on-axis</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Lens4 off-axis</td>
<td>1.4</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 5.2 Measured lateral resolution of the miniature objective (knife edge test) in fiber space. All numbers in μm.
Overall, the lateral resolution measurements presented here and in the previous section suggest that the new 3mm diameter miniature objectives will perform in accordance with the resolution specifications listed in Table 4.2.

5.1.4 Axial Chromatic Aberration

The spectral performance of lens 2 was investigated by measuring the axial shift in the image plane for a fixed object of varying color. The high-resolution bar target was illuminated using a series of 10nm wide band pass filters. The resultant axial shift in image plane (fiber space) location was measured. These data were then divided by the square of the magnification of the miniature objective and scaled by the index of refraction of water to obtain the chromatic focal shift in tissue space. The results are compared to the predicted lens performance in Figure 5.4. The data show a maximum image plane focal shift of 6.5μm over the full spectral range of the design. This is in close agreement with the predicted performance. Most fluorophors excite and emit within an 80nm wide spectral bandwidth. The chromatic focal shift data for the miniature objective indicate less than 3μm of axial color over this spectral range.
Chromatic Focal Shift in Tissue Space

\[ \text{Wavelength (nm)} \]

- Predicted
- Measured

Focal Shift (\(\mu\text{m in tissue plane}\))

Figure 5.4 Measured and predicted axial color of the miniature objective (lens 2). Data are presented in terms of tissue-plane axial position assuming an index of refraction of 1.33 for tissue.

5.1.5 Throughput

To test the throughput of the miniature objective the optical diffusers depicted in Figure 5.1 were replaced with a lens specifically chosen to fill the NA of the miniature objective. The illumination was spectrally limited by an 80nm band-pass filter centered at 560nm. Power measurements recorded before and after the miniature objective yielded a throughput of 78%. Elements 1 through 6 in the miniature objective (Figure 4.4) have 11 air-glass interfaces treated with single layer MgF\(_2\) antireflection coatings. Such coatings reduce the traditional 4% loss at an air-glass interface to roughly 1.5%. Therefore, the predicted transmittance for the miniature objective is 85%. The miniature
objective slightly underperforms this prediction due to problems with the coatings as mentioned in Section 5.1.2.

5.2 Pneumatic Focus Mechanism

The assembled pneumatic focus mechanism was tested to characterize its functionality and performance. Figure 5.5 shows the tissue space imaging location vs. air pressure. To collect these data, the air pressure was slowly increased from 0 to 80 psi and then decreased back to 0 psi while tracking the imaging position in tissue space. The focal plane measurements were taken in air and then scaled by the index of water (1.33) to estimate the depth of focus control in tissue. While the mechanism exhibits significant hysteresis, focal plane positioning is fairly linear and quite repeatable. The backlash in the focus mechanism makes absolute positioning difficult. However, in practice a trained user can quickly scan through the hysteresis region and obtain reliable relative focus control. The mechanism is unaffected by small air leaks because the position of the piston is based on pressure in the tube, not by the quantity of air introduced into the system. The focal position is stable over long periods of time and unaffected by small movements of the catheter. The lateral position of the image is extremely stable with respect to focus due to the tight tolerance between the piston and the interior wall of the housing. The pneumatic focus mechanism has been used successfully to image biological samples.
Figure 5.5 Measured performance of the pneumatic focus mechanism.

5.3 Mechanical Focus Mechanism

The mechanical focus mechanism was tested in a similar fashion to that of the pneumatic design described in the preceding section. The micrometer in the proximal portion of the focus mechanism was adjusted while tracking the image of the fiber in tissue space. Figure 5.6 shows the results for this experiment. The tissue space measurements were taken in air and then scaled by the refractive index of water to estimate the penetration depth in tissue. It is clear from the data that the focus extent of the mechanical assembly exceeds the imaging depth specification of the MCME, which is designed to operate to a depth of 200μm in the tissue.

The mechanical focus mechanism suffers from significant hysteresis. However, motion is fairly linear and repeatable. As with the pneumatic design, a trained user can
quickly scan past the flat hysteresis regions and achieve reliable relative focus control. Fairly accurate absolute measurements of depth may be achieved by starting at the tissue surface and scanning in one direction into the tissue. The focal position in the tissue is stable with respect to time but does move slightly as the catheter is repositioned significantly. However, this should not be a problem since the vast majority of the focusing will likely be performed after the catheter is positioned. As with the pneumatic design, the lateral position of the image is extremely stable with respect to focus. The mechanical focus version of the MCME has been successfully and reliably used to image various animal and human tissue samples.

**Figure 5.6** Measured performance of the mechanical focus mechanism.
5.4 Spatial Resolution of the MCME

5.4.1 Lateral Resolution

The overall lateral resolution of the MCME was tested using a fluorescent version of the traditional knife edge test. A thin fluorescent target was made by mixing a highly fluorescent dye with optical cement and spreading a thin layer of the mixture on a microscope slide. After the target was fully cured it was broken into several small pieces and a traditional fluorescent microscope was used to locate a sharp and uniform edge. Figure 5.7(a) shows the profile of this fluorescent edge imaged with the MCME. Figure 5.7(b) shows the corresponding lateral PSF of the MCME determined by differentiating the edge response. The FWHM of the PSF is roughly 3μm. Assuming a perfectly diffraction limited optical system the lateral resolution of the MCME would be governed by the pixel spacing of the fiber bundle in the catheter. The 3μm center-to-center pixel spacing and the miniature objective magnification of 1.6 predict a theoretical tissue space lateral resolution of 1.9μm. The measured data were taken in air, which likely caused a slight degradation in optical performance. This decreased performance may be partially to blame for the discrepancy between the theoretical and measured lateral resolution. It is also quite likely that the MCME’s optical system is not perfectly diffraction limited.
Figure 5.7 Measured lateral resolution of the miniature objective (knife edge test). (a) Edge response and (b) the corresponding PSF. The FWHM of the PSF is approximately 3 microns.

5.4.2 Axial Resolution

The overall axial resolution of the MCME was tested in a similar way used to characterize the lateral resolution in the preceding section. The distal tip of the catheter was placed in contact with a thick, uniformly fluorescent plastic target. The target was then moved away from the catheter in 5μm increments. Figure 5.8(a) shows the axial profile of the fluorescent target. Figure 5.8(b) shows the corresponding axial PSF for the MCME. The FWHM of the PSF is roughly 30μm. This value is close to the 25μm axial resolution measured and justified by Sabharwal\textsuperscript{22} for the first generation system. The discrepancy between the two findings is likely due to a slight NA difference between the first and second generation miniature objectives.
Figure 5.8 Measured axial resolution of the miniature objective (axial knife edge test). (a) Edge response and (b) the corresponding PSF. The FWHM of the PSF is approximately 30μm.

5.5 Spectral Characterization of the MCME

5.5.1 Dispersion Map

To accurately characterize the multi-spectral aspects of the MCME one must first determine the relationship between position on the CCD and wavelength. Equations (3.23) and (3.24) describe the angular deviation $\delta$ introduced by a prism. When focused by a lens of focal length $f$ this angular deviation will produce a lateral shift at the image plane defined as

$$\Delta x(\lambda) = f \tan(\delta(\lambda)). \quad (5.4)$$

If this lens is used to focus the dispersed light onto a CCD, the relationship between the individual pixels of the CCD and wavelength may be written as
\[ \text{pix} (\lambda) = \frac{f \tan (\delta (\lambda))}{a}, \] (5.5)

where \( a \) is the center-to-center spacing of the CCD pixels. Equations (3.23), (3.24), and (5.5) fully describe the relationship between wavelength and pixel position on a CCD in terms of the properties of the CCD, camera lens, and prism. The term dispersion map will be used to describe this functional relationship.

Currently the MCME employs an 18.13° BK7 prism positioned such that the angle of incidence is roughly 14°. The camera in the multi-spectral detection arm has 15\(\mu\)m pixels with a fill ratio of 100%. The camera is mounted with a zoom lens set to a focal length of 175mm. Figure 5.9 show the dispersion map for the MCME assuming the physical constants stated above. The ordinate of this plot has been shifted so that pixel 0 is located at the origin. In practice, the ordinate of Figure 5.9 will be shifted to match the exact location of the spectra on the CCD.
The exact dispersion characteristics of the MCME were measured by illuminating the distal end of the catheter with a white light source spectrally limited by a series of 10nm wide band-pass filters. With the scan mirror fixed in the center position, the illumination from the catheter formed a thin line of light on the CCD. The pixel location of this line in the spectral dimension was noted for various band-pass filters. Figure 5.10 shows the results from this experiment overlaid with the data predicted by equation (5.5). The measured and predicted data are in very close agreement. This implies that the user of the MCME will not need to repeat the entire dispersion map calibration (with the
10nm filters) before each experiment. The only unknown quantity in the functional form of the dispersion map is the DC offset term that shifts the function to the exact position on the CCD. This quantity may be calculated from a single 10nm filter test. In other words, once the pixel location is known for a single wavelength, the entire dispersion map may be extrapolated using equation (5.5).

![Measured Dispersion Map](image)

**Figure 5.10** Measured dispersion map data. The predicted data have been shifted in the y direction to overlay the measured data.

In practice, the user will want to know the wavelength associated with a given pixel on the CCD. In other words, the user needs to know wavelength as a function of pixel location. Equation (5.5) describes pixel location as a function of wavelength but is...
too complex to invert symbolically. However, equation (5.5) may be inverted numerically and then fit to a 6th order polynomial of the form

\[ \lambda(p) = \sum_{n=0}^{6} c_n (p - p_0)^n, \]  

(5.6)

where \( p \) represents a pixel location and \( p_0 \) is the pixel offset that will be used to shift the polynomial to match it to the location of the spectra on the CCD. The constants \( c_n \) for this fit are shown in Table 5.3. Figure 5.11 shows the result of this inversion. A change in the pixel offset term, \( p_0 \), will shift the polynomial plot in the ± pixel direction. For this particular experiment the pixel offset was 211 pixels but this number may be slightly different for each experiment.

| \( C_1 \) | 3.49186x10^4 |
| \( C_2 \) | 1.18747x10^1 |
| \( C_3 \) | 1.15650x10^-2 |
| \( C_4 \) | 1.53427x10^-4 |
| \( C_5 \) | 4.87547x10^-6 |
| \( C_6 \) | 4.94977x10^-8 |
| \( C_7 \) | 2.54478x10^-10 |

Table 5.3 Constants used in the polynomial fit of equation (5.6).
Figure 5.11 Final dispersion map for the MCME. The measured data are the same data presented in Figure 5.10. The polynomial fit is a plot of equation (5.6).

5.5.2 Spectral Sampling Rate

The spectral sampling rate of the MCME, defined as $d\lambda/d\phi$, is plotted in Figure 5.12. This plot was numerically calculated from equation (5.5) and assumes the same system configuration as stated in the previous section. The figure reveals that the sampling rate steadily increases for increasing wavelength, which is one of the drawbacks to using a prism as the dispersive element in the MCME.
5.5.3 Spectral Calibration

A spectral calibration curve describes the spectral transmission characteristics of a system. Such a curve is usually generated by illuminating an optical system with a light source of known spectral emission properties and comparing the spectra transmitted by the system with that of the source. To perform this test on the MCME a spectral irradiance standard from Optronic Laboratories was placed in front of the distal tip of the catheter. A single spectral frame was acquired by the MCME with the scan mirror centered on the field-of-view of the catheter. This produced an image with one dimension of spatial data and one dimension of spectral data. A 40 spatial-pixel wide region was averaged over the spatial dimension and the resultant spectrum is plotted in

**Figure 5.12** Spectral sampling rate for MCME under the current configuration.
Figure 5.13 along with the spectrum of the calibrated source. The dispersion map generated in Section 5.5.1 was used to convert the abscissa of the measured data from pixels to wavelength. The measured spectrum is nearly zero at 490nm because the collection system is designed to reject the 488nm argon ion illumination wavelength. The general trend of increased sensitivity for longer wavelengths is primarily due to the MCME's sampling rate characteristics (see Figure 5.12) that yield more spectral bandwidth per detector element for longer wavelengths. The detector used in the MCME has roughly uniform responsivity from 450nm to 650nm but becomes significantly less sensitive to energy beyond the visible range. Figure 5.13 suggests a drop in sensitivity for wavelengths beyond 690nm which is likely due to the responsivity of the detector.

Calibrated Source and Measured Spectral Response

![Graph showing spectral calibration of the MCME.](image)

**Figure 5.13** Spectral calibration of the MCME.
A calibration curve may be calculated from the data presented in Figure 5.13 by dividing the spectra of the calibrated standard by the measured spectra. The final calibration curve for the MCME is presented in Figure 5.14. The spectral calibration curve for the MCME will be used as a multiplicative factor. In other words, real spectra will be calculated by multiplying measured spectra by the calibration curve. The function rises to infinity near 488nm due to a zero in the spectral response of the MCME.

**Spectral Calibration of the MCME**

![Spectral Calibration Graph](image)

**Figure 5.14** Final spectral sensitivity calibration curve for the MCME.

5.5.4 *Spectral Linewidth*

5.5.4.1 *Measured Linewidth*

The spectral linewidth of the MCME was measured by illuminating the distal end of the catheter with 632.8nm light from a helium-neon laser. For this experiment the slit was 20μm wide and the zoom lens on the camera was adjusted such that the image filled
512 spatial pixels. The focal length setting for the zoom lens was roughly 175mm. A single image was acquired with the scan mirror centered in the field-of-view of the fiber bundle. Figure 5.15 shows a spectrum taken from the central region of this image. The fine sampling of this function was obtained by tilting the CCD camera and collapsing the intensity distribution along the direction of the line image. The FWHM of the distribution is roughly 2 pixels on the CCD or 30µm.

Measured Spectral Linewidth of the MCME

Figure 5.15 Spectral linewidth of the MCME at a wavelength of 632.8nm. The camera pixels are 15µm wide.

5.5.4.2 Theoretical Linewidth

In Section 3.2.2.3 the spectral linewidth of a prism was defined as the full angular separation between the principle minima of equation (3.27). This definition assumes that
the rectangular form of the prism is the limiting aperture of the system. However, the
detection arm of the MCME is designed to pass the fluorescent light collected by the
catheter. Therefore, the size of the beam (NA of the fiber bundle) effectively becomes the
limiting aperture of the system. The discussion in Section 3.2.2.3 also assumed a
monochromatic point source at infinity (plane wave illumination). From the point of
view of the MCME’s prism, the source is the confocal slit aperture. Therefore, the prism
will be illuminated by an angular field of plane waves that depends on the width of the
slit.

The theoretical spectral linewidth of the MCME is, therefore, the physical image
of the slit on the CCD. This result may be modeled as the geometrical image of the slit
on the CCD convolved with the pixel function and the PSF of the detection optics

\[ I_{\text{linewidth}}(x) \propto \text{rect} \left( \frac{x}{d_{\text{slit}}} \right) \ast \text{rect} \left( \frac{x}{d_{\text{pix}}} \right) \ast \text{somb}^2 \left( \frac{2N_A d}{\lambda d} x \right), \]

where \( d_{\text{slit}} \) is the width of the slit image on the CCD, \( d_{\text{pix}} \) is the width of a pixel, \( x \) is the
spatial coordinate on the CCD, and \( \ast \) signifies a convolution. Consult Gaskill\(^{27} \) for the
definition of \( \text{rect}() \). For the data presented in Figure 5.15, the confocal slit aperture was
set to a width of 20\( \mu \)m. With the zoom lens on the camera set to 175mm, the
magnification between the slit and the CCD is roughly 1.25. Therefore, the width of the
slit on the CCD (\( d_{\text{slit}} \)) was approximately 30\( \mu \)m. The camera used in the MCME has
15\( \mu \)m square pixels (\( d_{\text{pix}} \)). The \( NA \) of the MCME at the CCD (\( NA_d \)) is roughly 0.04 when
the zoom lens is set to 175mm. These numbers were used to generate the theoretical
spectral linewidth plot shown in Figure 5.15. The small discrepancy between the
theoretical linewidth and the measured data is likely due to a slight measurement error in
the width of the confocal slit aperture.

It is interesting to note that the spectral linewidth of the MCME is closely
correlated to the width of the confocal slit aperture. Therefore, one could decrease the
spectral linewidth and increase the spectral resolution of the MCME by decreasing the
width of the slit aperture. However, the increased spectral performance would be
balanced by a significant reduction in throughput.

5.5.5 Resolving Power

The resolving power of a prism was defined in section 3.2.2.4 as

\[ R = \frac{\lambda}{(\Delta \lambda)_{\text{min}}} = t \left( \frac{dn}{d\lambda} \right), \quad (5.8) \]

where \((\Delta \lambda)_{\text{min}}\) is the minimum resolvable wavelength difference using Rayleigh’s
criteria and \(t\) is the physical length of the longest path light travels through the prism.
This result assumed that the prism was the limiting system aperture and that the prism
was illuminated by a single monochromatic plane wave. However, neither of these
assumptions is true for the MCME.

Equation (5.7) describes the functional form of the spectral linewidth of the
MCME. Rayleigh’s criteria defines \((\Delta \lambda)_{\text{min}}\) as the spectral separation between the
principle maxima and the first positive minima of this function. It is reasonable to
approximate this quantity as

\[ (\Delta x)_{\text{min}} = \frac{d_{\text{slit}}}{2} + \frac{d_{\text{pix}}}{2} + 1.22\lambda F / \# , \quad (5.9) \]
where the first term is the half-width of the geometrical image of the slit on the CCD, the second term is the half-width of a CCD pixel, and the third term is the half-width of the MCME's detection PSF. In Chapter 3 the quantity \( \theta_i \) was defined as the angular coordinate in the collimated space immediately after the prism. In the MCME, \( \theta_i \) is also the angular coordinate in the collimated space immediately preceding the zoom lens on the camera. Assuming the small angle approximation is valid and a camera lens of focal length \( f \), the relationship between \( \theta_i \) and \( x \) is

\[
\theta_i = \frac{x}{f},
\]  

(5.10)

and the relationship between \( \Delta \theta_i \) and \( \Delta x \) is

\[
\Delta \theta_i = \frac{\Delta x}{f}.
\]

(5.11)

Therefore, equation (5.9) becomes

\[
(\Delta \theta_i)_{\text{min}} = \frac{d_{\text{slit}} + d_{\text{pix}}}{2f} + \frac{1.22\lambda}{D},
\]

(5.12)

where \( D \) is the spatial extent of the collimated beam entering the camera lens. Equation (3.23) and (3.24) may be used to relate \( (\Delta \theta_i)_{\text{min}} \) to \( (\Delta \lambda)_{\text{min}} \) as

\[
(\Delta \lambda)_{\text{min}} = \frac{(\Delta \theta_i)_{\text{min}}}{\frac{d\theta_i}{d\lambda}}.
\]

(5.13)

The symbolic form of the derivative of function (3.23) is too complex to work with, but mathematical software, such as Mathcad, may be employed to numerically calculate \( (\Delta \lambda)_{\text{min}} \) for a specific wavelength. Table 5.4 summarizes the results of such a
calculation. As with the spectral linewidth results, the resolving power of the MCME becomes progressively worse with increasing wavelength due to the non-linear angular dispersion characteristics of the prism.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>(Δλ) min (nm)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>480</td>
<td>6.9</td>
<td>70</td>
</tr>
<tr>
<td>570</td>
<td>11.9</td>
<td>48</td>
</tr>
<tr>
<td>660</td>
<td>18.3</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 5.4 Estimated minimum resolvable wavelength difference and resolving power for the MCME at various wavelengths.

5.6 Acquisition Speed

The acquisition speed of the MCME largely depends on the digitization rate of the CCD. As stated in Section 4.1.2, the grayscale and multi-spectral detection assemblies employ a Quantix 535 x 512 frame transfer CCD camera with a 3MHz digitizer. In grayscale mode the system collects a full 535 x 512 image at a frame rate just under 10 frames-per-second. The fiber bundle in the catheter has 30,000 resolution elements that are greatly over sampled by a 535 x 512 image. If faster frame rates are desired, a 2 x 2 CCD binning (256 x 256 image) may be used to speed up the acquisition rate to roughly 24 frames-per-second with very little loss in image quality.

In multi-spectral collection mode the frame rate is largely dependent on the number of spectral samples required. The current camera takes 0.9 seconds to acquire a 256 x 256 image with 16 spectral samples. The acquisition time is roughly linear with respect to spectral samples. In other words, the MCME takes approximately twice the amount of time to obtain twice the number of spectral samples. The current camera takes roughly 2 seconds to obtain 16 spectral samples and 4 seconds to acquire 32 spectral
samples when configured to acquire a full 512 x 512 image. However, as with grayscale
detection, a 256 x 256 image offers sufficient spatial resolution for most experiments. A
new camera for multi-spectral detection could provide significantly faster frame rates.
However, as discussed in Section 3.2.3, there is an inherent link between frame rate,
spectral samples, and SNR. One can not increase performance in one of these areas
without decreasing performance in one of the other areas.

5.7 Throughput

The radiometric throughput of the first generation confocal microendoscope was
fully characterized by Sabarwal. Many of the MCME’s components remain unchanged
from the first generations design. The two major changes that effect throughput are the
new 3mm diameter miniature objective and new back-illuminated CCD cameras. The
MCME throughput estimates presented in this section rely on recent measurements,
thoretical approximations, and data presented by Sabharwal.

The illumination throughput of the MCME is defined as the transmittance from
the output of the laser fiber couplers to the tissue plane. Sabharwal calculated 50%
transmittance in the bench top optical system between the output of the laser fiber
couplers (spatial filter assembly in first generation system) and the proximal face of the
fiber bundle. The fiber bundle in the catheter has a 50% fill ratio which implies it will
only transmit 50% of the incident energy. In addition it has two air/glass interfaces, one
coated and one uncoated, which will further reduce the overall fiber transmittance to
roughly 47%. The miniature objective throughput is 78% (see Section 5.1.5). The total illumination throughput is 18% not accounting for losses in the laser fiber couplers.

The illumination throughput has not been a major issue for the MCME because the laser sources are powerful enough to provide ample excitation signal in the tissue. However, a next generation mobile MCME would greatly benefit from increased illumination throughput because of the limited optical power available from air-cooled laser systems that run off traditional 120V, 60Hz electoral power. With better coatings on the lenses and mirrors in the optical system one might expect roughly 90% transmission in the bench-top optical system and 90% transmission in the miniature objective. This would more than double the overall illumination throughput of the MCME. However, the fill ratio of the fiber bundle will ultimately limit the illumination throughput.

The detection throughput of the MCME is defined as the transmittance from the tissue plane to the CCD plane and includes the quantum efficiency of the CCD. As described above, the miniature objective and fiber have throughputs of 78% and 47%, respectively. Sabharwal calculated 30% transmittance from the proximal face of the fiber bundle to the CCD which includes a loss of 20% at the confocal slit aperture. In addition, the current CCD has a quantum efficiency of 82% in the visible wavelength range. The total detection throughput is 9% which represents a significant loss of signal.

In general, fluorescent dyes have been chosen that provide sufficient quantum yield to overcome the throughput limitations of the MCME. However, increasing the radiometric throughput of the detection arm would facilitate a larger selection of
exogenous dyes and potentially enable the detection of endogenous fluorescent signals. In addition, the multi-spectral collection assembly would greatly benefit from increased detection throughput because it disperses the fluorescence collected from each object location onto 30 to 40 individual CCD pixels. Better coatings in the bench top optical system might yield a throughput of 70% from the proximal face of the fiber bundle to the CCD. Better coatings might also increase the miniature objective throughput to 90%. These changes have the potential to double or triple the radiometric throughput of the detection arm. However, the fiber bundle and confocal aperture will ultimately limit the detection throughput.
CHAPTER 6: IMAGING EXPERIMENTS AND DISCUSSION

As stated in Chapter 4, the physical size of the MCME’s catheter was chosen so it could be used as a daughter scope to a commercial endoscope. Figure 6.1 shows the catheter routed through the instrument channel of an Olympus CF-100L colonoscope. Figure 6.1(a) shows the catheter entering the colonoscope near the control handle and then protruding roughly one inch beyond the distal tip of the colonoscope. Figure 6.1(b) shows an enlarged image of the MCME’s distal opto-mechanical assembly extending beyond the colonoscope. In this image the miniature objective has been unscrewed from the miniature focus mechanism for demonstration purposes. Figure 6.1(c) is a still-frame captured from the video signal of the colonoscope. In this frame, the MCME is seen imaging the intestine of a rat. In practice, the wide field-of-view of the colonoscope can navigate to a suspected site of disease. Then the MCME would be extended and pressed against the tissue to provide a microscopic view of the tissue under observation. Ideally, the live images collected by the MCME will supply the clinician with the information necessary to make a diagnosis. If a traditional biopsy is warranted, the MCME will be removed from the instrument channel and replaced with biopsy forceps. Some
endoscopes have dual instrument channels, which would be ideal for the MCME, as the second instrument channel could deliver dye and/or collect biopsy samples.

![Confocal Microendoscope](image1)

![Lens](image2)

![Distal focus subassembly](image3)

**Figure 6.1** (a) MCME in the instrument channel of an Olympus CF-100L colonoscope. (b) Detail of the opto-mechanical components of the catheter. (c) Still frame captured from video collected by the colonoscope showing the MCME imaging rat intestine.

### 6.1 Grayscale Imaging Results

In grayscale collection mode, the MCME has imaged animal tissues in-vivo and ex-vivo as well as excised human tissues. Initial applications for the system have concentrated on diagnosing diseases of the gastro-intestinal and female reproductive systems. To explore these applications, close collaborations have been developed with physicians who are able to supply human tissue samples. All tissues were collected under protocols approved by the Institutional Review Board of the University of Arizona. To date, the MCME has collected grayscale images of the following excised human tissues: cervix, uterus, ovary, esophagus, stomach, pancreas, and colon. In most cases, the tissue was imaged within one hour of resection. All images presented in this section are of tissues stained with acridine orange (AO), which is a vital nucleic acid
fluorescent dye that intercalates with DNA and RNA. AO is efficiently excited by the 488nm line of an argon ion laser and has dual emission spectra peaks at 525nm and 650nm when bound to DNA and RNA, respectively. In the standard protocol, roughly 100μl of 330μMolar dye is washed over the tissue immediately prior to imaging. AO is inherently cytotoxic because it intercalates with nucleic acids in live cells. However, topical application of extremely small quantities of dye may not be harmful to humans.

Figure 6.2 shows two images obtained with the MCME of excised human ovary from two different patients. Both were diagnosed as normal ovarian tissue via standard histopathology. Figure 6.2(a) shows a regular distribution of densely packed cells characteristic of the surface epithelial layer. Figure 6.2(b) shows the relatively elongated and loosely packed cells indicative of the underlying stroma. The stroma was clearly visible in this case because the epithelial layer had been accidentally scraped off while handling the tissue. The epithelium is a very fragile thin layer of cells that can easily disintegrate if the tissue is not handled carefully. This layer should remain intact when the MCME is used in-vivo because live tissue will be imaged with minimal handling.
Figure 6.2 Excised human ovarian tissue imaged in grayscale mode. (a) Normal epithelial layer and (b) normal stromal layer. The two samples are from separate patients.

Figure 6.3(a) shows diseased human ovary imaged with the MCME. Figure 6.3(b) is a histology image from a similar location on the ovary. The diagnosis for this tissue was cystic papillary carcinoma. An effort was made to match the location of the histology image to the confocal image but exact registration is difficult. In general, histology and MCME images are within 1mm of each other, which is adequate for a qualitative correlation of the results.

The two images presented in Figure 6.3 are orthogonal views of the same sample. In standard histology, tissue is sliced perpendicular to the surface to produce a cross-sectional view. The MCME captures images en-face, and as such, a direct correlation between the images produced by the two systems is impossible. However, it is clear that there are significant differences between the cancerous tissue of Figure 6.3(a) and the
normal tissues presented in Figure 6.2. Matching grayscale MCME images with histology should help determine features in the confocal images that identify pathology.

![Figure 6.3](image)

**Figure 6.3** Excised human ovarian tissue. (a) Grayscale MCME view. (b) Histology image from a nearby region. This patient was diagnosed with cystic papillary carcinoma.

**Figure 6.4** shows an image of excised human cervix. The uniform punctate pattern of nuclei is characteristic of healthy cervical tissue, a finding that was supported by histology. Figure 6.5 shows an example of excised human esophagus. This image shows relatively sparse and punctate nuclei but also depicts the slightly undulated surface of the esophagus. This tissue was excised from a patient diagnosed with Barrett's esophagus. However, this particular tissue sample was extracted from a region in the upper portion of the esophagus and was histologically diagnosed as normal.
Figure 6.4 Healthy excised human cervical tissue imaged with the MCME in grayscale mode.

Figure 6.5 Healthy excised human esophageal tissue imaged with the MCME in grayscale mode.

Figure 6.6 shows two images of excised human colon. The dark regions in Figure 6.6(a) correspond to crypts that extend several hundred microns below the surface of the colon. The regular repetitive nature of these crypts is indicative of healthy tissue. Figure
6.6(b) was taken from a large tumor in the same colon where the normal pattern of crypts is not observed.

Figure 6.6 Excised human colon tissue imaged with the MCME in grayscale mode. (a) Normal tissue and (b) cancerous region from same patient.

Figure 6.7 shows three images of excised human colon sampled from a single patient. Figure 6.7(a) clearly illustrates the crypt pattern indicative of healthy colon tissue. Figures 6.7(b) and (c) are images obtained from a cancerous region and clearly show a breakdown of the structure found in healthy colon tissue. This and other examples suggest that the MCME will be able to differentiate healthy from diseased tissue.
6.2 Multi-spectral Imaging Results

The grayscale data presented in the previous section were collected by a system first introduced in 1993\textsuperscript{20,21}. Since its introduction, the grayscale confocal microendoscope has gone through many modifications including much of the work described in this dissertation. With each modification came an increase in imaging performance and/or functionality. The multi-spectral portion of the MCME is built upon the existing technology of the first generation grayscale confocal microendoscope. However, the multi-spectral aspects of the MCME are still in the early stages of development. The images presented in the remainder of this chapter are intended to give an idea of the types of multi-spectral data the MCME can collect. Potential applications for these images are introduced where appropriate.
6.2.1 Microsphere Phantoms

The first imaging results in multi-spectral collection mode were obtained from a phantom made of a monolayer of 6\(\mu\)m green, 15\(\mu\)m yellow, and 15\(\mu\)m red fluorescent microspheres. The MCME collected, a 256 x 256 spatial image with 32 spectral samples. The full data set was acquired in roughly 2 seconds. This experiment was performed prior to the addition of the krypton laser so the MCME operated with single 488nm illumination. Figure 6.8(a) shows the grayscale projection of the data cube, defined as the raw data averaged over all collected spectra. The grayscale projection is approximately equivalent to an image that might be collected by the MCME in grayscale imaging mode. Figure 6.8(b) shows the spectrum of a representative microsphere of each color. The wavelength labels are estimates since no dispersion map or spectral calibration was performed for this experiment. The plot reveals that the green microspheres have a relatively broad emission spectrum, which significantly overlaps the emission of the yellow and red microspheres.
The goal of this experiment was to sort the microspheres into three color channels. Simply splitting the spectral dimension of the data cube into three regions would yield a significant signal from the green microspheres in the yellow and red channels. This issue was addressed by isolating the green microspheres and subtracting a weighted version of this image from the yellow and red spectral channels. Figure 6.9 shows the outcome of this data processing, which resulted in nearly perfect spectral sorting of the microspheres. Alternatively, the microspheres in this experiment could have been sorted using a spectral deconvolution algorithm. More details on such a technique will be presented in Section 6.2.3.3.
Figure 6.9 Phantom of 6µm green, 15µm yellow, and 15µm red fluorescent microspheres. The microspheres were spectrally sorted into (a) green, (b) yellow, and (c) red channels.

Figure 6.10 shows results from another microsphere experiment. For these data the MCME collected 512 x 512 spatial pixels and 48 spectral samples ranging from roughly 490nm to 710nm. The system was configured for dual excitation but only the 488nm argon laser line was used as a source. It took approximately 6 seconds to acquire the full data set. The sample was a mixture of five different 15µm fluorescent microspheres with excitation/emission maxima of 505/512, 515/534, 540/560, 565/580, and 580/605. Each of the 5 microsphere types provided ample emission signal when excited at 488nm. Figure 6.10 shows a montage of nine discrete spectral bands in the data cube. Each frame is an average over 4 of the 48 spectral samples. The wavelength values superimposed on the montage show the spectral boundaries for each image. Unlike the previous microsphere experiment, these images represent the raw unprocessed MCME data.
Figure 6.10 Phantom of 15\(\mu\)m fluorescent microspheres with excitation/emission maxima of 505/512, 515/534, 540/560, 565/580, and 580/605. This montage shows the raw data cube separated into 9 spectral bands. Each frame contains 4 of the 48 spectral samples. Wavelength labels (nm) show approximate spectral boundaries for each image.

Figure 6.11 shows the spectrum collected by the MCME from a representative microsphere in each of the five categories. The spectral registration (dispersion map) process described in Chapter 5 was used to accurately plot these spectra with respect to
wavelength. The dip near 650nm is due to the dichroic beamsplitter used in the MCME to direct the fluorescent signal onto the confocal slit aperture (BS2 in Figure 4.2). When configured for dual excitation, the MCME requires use of a dichroic beamsplitter that passes both excitation wavelengths and reflects all other wavelengths between 490nm and 750nm. As a result, there is a noticeable rejection of energy near 647.1nm. Attempts to account for this rejection in a spectral calibration curve tend to over amplify the noise in the signal. Ideally, the current fixed dichroic beamsplitter would be replaced with an easily exchangeable filter configuration so that the appropriate beam splitter could be selected prior to each experiment. For this particular experiment, when only 488nm illumination is used, a beam splitter that transmits 488nm and reflects all longer wavelengths would be more appropriate. Such a beamsplitter is currently available but was not used because doing so would have required significant realignment of the bench-top optical system.
6.2.2 Single Excitation Single Dye

6.2.2.1 Cell Culture

Experiments with cell cultures were performed to illustrate the potential of multispectral confocal imaging for the examination of cell morphology. Initially, acridine orange (AO) was used as the fluorophor in these experiments. At proper concentration, acridine orange exhibits dual emission characteristics, staining cell nuclei with a peak emission of 525nm and staining cytoplasm with a peak emission of 650nm. Some publications indicate that the dye has different spectral characteristics when bound to
double stranded DNA vs. single stranded RNA\textsuperscript{26} while others conclude that the dyes' spectral variations are primarily due to its pH and concentration dependence\textsuperscript{19}. The more credible later theory is based on the fact that AO emits green fluorescence at low concentrations and red shifted fluorescence at high concentrations. This property was verified using an ORIEL arc lamp and SPEX spectrometer to measure the spectra of various concentrations of the dye. The results of this experiment are plotted in Figure 6.12 and clearly depict the concentration dependence of AO's fluorescence emission. In addition to its concentration dependent spectral properties, AO is preferentially attracted to low pH environments\textsuperscript{19}. Highly acidic lysosomes result in preferential accumulation of AO in the cytoplasm of cells. Therefore, at proper concentrations the dye yields a green emission in the nucleus and a red emission in the cytoplasm.
Figure 6.12 Concentration dependence of acridine orange. Numeric labels are used to help distinguish the plots.

Figure 6.13 shows a cell culture of rat sinusoidal endothelial (RSE) cells that have been stained with AO. For this experiment the MCME collected a 256 x 256 grayscale image with 30 spectral channels. The MCME was configured for single excitation at 488 nm with a single transmission wavelength beamsplitter such that there was no loss in signal near 647.1 nm. Two spectral bands were selected to isolate the nuclei from the rest of the cell structure based on the spectral differences in fluorescent emission of the dye. Figures 6.13(a) and (b) show the normalized intensity images of the green (nuclei) and red (cytoplasm) channels, respectively. The grayscale image in Figure 6.13(c) was created by summing the data along the spectral dimension and is, therefore, comparable to what might be obtained by the MCME in grayscale imaging mode. The color
reproduction shown in Figure 6.13(d) was created by mapping the two spectrally limited images into the red and green channels of an RGB file format. This data set is intended to show how the multi-spectral aspects of the MCME might be used to enhance the contrast in cellular imaging. The method used to collect data enables the user to quickly tailor the spectral properties of the MCME to the specific experiment. By collecting a full spectrum the user may work with the data cube after the experiment to isolate specific features of the cells.
Figure 6.13 Culture of RSE cells stained with AO. (a) Green channel showing primarily nuclei, (b) red channel showing primarily cytoplasm, (c) grayscale projection, and (d) RGB color reconstruction.

Figure 6.14 shows spectra collected from the nucleus and cytoplasm of one of the RSE cells shown in the previous figure. While there is some overlap between the two signals, there is sufficient separation in the spectra to distinguish nucleus from cytoplasm based on spectra alone. One of the morphological changes that pathologists look for when diagnosing cancer is enlarged nuclei. With the proper fluorescent dye or dyes, it is
possible that spectral information might enable the MCME to aid in the diagnosis and
detection of disease by accurately calculating spatially localized nucleus to cytoplasmic
ratios. One might envision the MCME constantly tracking such a ratio as the catheter is
in use and triggering an audible alert or visual overlay when the nuclei to cytoplasmic
ratio becomes abnormal for the tissue type under observation.

RSE Cell Spectra

Figure 6.14 Spectra from the nucleus and cytoplasm of one of the RSE
cells depicted in the previous figure.

Figure 6.15 shows images obtained from a sample of human lung cancer cells in
culture. These data were processed using a similar technique described for the first
microsphere experiment in this section. The images are presented in the same fashion as
the RSE images of Figure 6.13.
6.2.2.2 Excised Tissue

The experiments described above clearly show the MCME’s ability to enhance the contrast in an image through the use of a multi-spectral dye such as AO. However, cultured cells are generally larger than those found in intact tissue. In addition to their size, cells in culture tend to grow in a very thin monolayer and imaging such a layer does
not take advantage of the confocal nature of the MCME. The true test of the multi-spectral and confocal nature of the MCME is imaging live tissue.

As stated in Section 6.1 the initial applications for the MCME have focused on diseases of the gastro-intestinal and female reproductive systems. Figure 6.16 shows excised human colon tissue imaged with the MCME in multi-spectral collection mode. The tissue was imaged within one hour of extraction and stained with AO immediately prior to the experiment. Figures 6.16(a) and (b) show the normalized intensity images of the green (nuclei) and red (cytoplasm) channels, respectively. The grayscale projection image is presented in Figure 6.16(c) and the RGB color reconstruction is shown in Figure 6.16(d). Figure 6.17 shows a comparison of a grayscale projection and RGB color reconstruction of a colon excised from another human subject. It is clear from each of these image sets that there is additional information in the spectral domain that can successfully be used to enhance the contrast in confocal images.
Figure 6.16 Excised human colon tissue stained with AO. (a) Green channel showing primarily nuclei, (b) red channel showing primarily cytoplasm, (c) grayscale projection, and (d) RGB color reconstruction.
6.2.3 Dual Excitation Dual Dye

The multi-spectral experiments described thus far have all used a single dye (AO) with a single excitation wavelength (488nm). As discussed in Chapter 4, the MCME is equipped with two laser sources, which together provide a variety of choices for excitation. The MCME is currently configured to operate the laser sources at 488nm and 647.1nm. A selection of dyes have been investigated that would potentially increase the contrast between nucleus and cytoplasm in a dual excitation and dual dye configuration. The most promising results have come from a mixture of SYTO 16\textsuperscript{26} and MitoTracker Deep Red\textsuperscript{26}. SYTO dyes are cell-permeant nucleic acid stains. SYTO 16 is a green fluorescent dye with virtually no intrinsic fluorescence. It has high quantum yield when bound to nucleic acid and is more than twice as fluorescent on DNA than RNA. When bound to DNA, SYTO 16 has peak excitation and emission spectra at 488nm and 518nm, respectively, and is, therefore, well suited to the MCME’s 488nm excitation source. The

Figure 6.17 Excised human colon tissue stained with AO. (a) Grayscale projection, and (b) RGB color reconstruction.
MitoTracker series of dyes are cell-permeant stains that are concentrated and well retained by active mitochondria. MitoTracker Deep Red has peak excitation and emission spectra at 644nm and 665nm, respectively, and is therefore well suited to the MCME’s 647.1nm excitation source. For the remainder of this section, the terms SYTO and Mito represent SYTO 16 and MitoTracker Deep Red, respectively.

6.2.3.1 Individual Spectra of SYTO and Mito

The first step in this experiment was to verify that SYTO and Mito could be used individually to stain cells in culture. Figure 6.18 shows two samples from a culture of smooth muscle cells harvested from the aorta of rat embryo. The sample in Figure 6.18(a) was stained with 200µl of 20µM SYTO and excited at 488nm. The sample in Figure 6.18(b) was stained with 200µl of 1µM Mito and excited at 647.1nm. Each image shows the grayscale projection of the collected data cube mapped into an appropriate color channel for display purposes. The dyes were brightly fluorescent and clearly highlighted dramatically different features of the cells. The bright and uniform structures in the SYTO image represent cell nuclei while the structures in the Mito image represent distributed mitochondria in the cytoplasm. Figure 6.19 depicts a representative spectrum collected by the MCME from each of these samples. The plot clearly shows excellent separation of the emission profiles for the two dyes.
Figure 6.18 Two cell cultures of smooth muscle cells stained with (a) SYTO 16 and (b) MitoTracker Deep Red. Each image is a grayscale projection mapped into an appropriate channel of an RGB file format.
Figure 6.19 Spectra from a representative cell in each of the images depicted in the previous figure.

6.2.3.2 Dye Mixture

Figure 6.20 shows an image obtained by the MCME of a culture of smooth muscle cells stained with a mixture of SYTO and Mito. The dye quantities and concentrations used in Section 6.2.3 were applied to a coverslip in sequence: first SYTO, then Mito. The cells were rinsed after the application of each dye. The spectral dimension of the data cube was separated into two spectral bands to highlight the differences between the two dyes. Figure 6.20(a) and (b) show the normalized intensity images of the green (SYTO) and red (Mito) channels, respectively. The grayscale projection image is presented in Figure 6.20(c) and the RGB color reconstruction is show
in Figure 6.20(d). In this example, the SYTO signal was somewhat stronger than the Mito signal, which explains why the cytoplasmic staining of Mito is almost unnoticeable in the grayscale projection image. The images in Figure 6.20 clearly show preferential uptake of SYTO in the nuclei and Mito in the cytoplasm. This experiment shows that the mixture of SYTO and Mito provides a dramatic increase in contrast over the AO data presented earlier.
Figure 6.20 Culture of smooth muscle cells stained with a mixture of SYTO 16 and MitoTracker Deep Red. (a) Green channel showing primarily nuclei, (b) red channel showing primarily cytoplasm, (c) grayscale projection, and (d) RGB color reconstruction.

6.2.3.3 Spectral Deconvolution

In addition to exploring the use of multiple dyes and multiple excitation wavelengths, this experiment also tested a preliminary spectral deconvolution algorithm. The goal of this experiment was to use the spectral data collected from each dye in section 6.2.3.1 to deconvolve the spectra collected from the dye mixture in section
6.2.3.2. As indicated by equation (3.1), the spectra at each spatial location of a multiple dye image may be represented as a linear weighted summation of the spectra of each of the dyes. For a two dye experiment such as this, equation (3.1) reduces to

$$g(\lambda) = c_0 + c_s f_s(\lambda) + c_m f_m(\lambda),$$  \hspace{1cm} (6.1)

where $f_s(\lambda)$ and $f_m(\lambda)$ are the functional forms of the individual spectra of SYTO and Mito, respectively, and $c_0$, $c_s$, and $c_m$ are the spatially dependent weight functions of the background, SYTO, and Mito signals, respectively. Since $f_s(\lambda)$ and $f_m(\lambda)$ are known (Figure 6.19) it is relatively straightforward to invert equation (6.1) and obtain the spectral weight functions $c_s$ and $c_m$ for each spatial position in the data cube. Figure 6.21 shows the result of this spectral deconvolution at two discrete spatial locations in the data cube. The diamond and square markers in the plot represent the spectral signature collected from the nucleus and cytoplasm of a single smooth muscle cell. The solid line overlaying each data set represents $g(\lambda)$ from equation (6.1) for each location. It is clear from these data that the spectral deconvolution algorithm works well for this combination of fluorophors; the algorithm accurately isolates the signal from each dye.
Dual Dye Cell Spectra with Deconvolution Overlay

Figure 6.21 Spectra from the nucleus and cytoplasm of one smooth muscle cell in the dual dye experiment. The solid lines show the spectra generated at the same two locations using spectral deconvolution.

Figure 6.22 shows the spectral deconvolution algorithm applied to the entire data cube. Figure 6.22(a) and (b) show the SYTO and Mito channels, respectively. Figure 6.22(c) shows the grayscale projection of the data cube while Figure 6.22(d) shows a RGB reconstruction of the deconvolved data. The images in this montage should be compared to the spectral band images shown in Figure 6.20. The spectrally deconvolved images show slightly better separation between the nuclei and mitochondria. The resultant RGB reconstruction shows a noticeable improvement in contrast between the nuclei and cytoplasm. These differences are subtle and appear much stronger on a computer monitor than they do in print.
Figure 6.22 Culture of smooth muscle cells stained with a mixture of SYTO 16 and MitoTracker Deep Red. (a) Green channel showing nuclei stained with SYTO 16, (b) red channel showing mitochondria stained with MitoTracker, (c) grayscale projection, and (d) RGB color reconstruction. Images were generated using spectral deconvolution.

The use of spectral deconvolution has slightly improved the images obtained in this experiment. However, this application was not particularly demanding on the deconvolution algorithm since SYTO and Mito have well separated spectral emission characteristics. This exercise was primarily intended to demonstrate the concept of
spectral deconvolution when using the MCME as an imaging device. The technique is primarily used to isolate the signal of two or more dyes that have emission characteristics with significant spectral overlap. The success of this dual dye experiment has prompted the selection of a cell membrane probe to add to the dye mixture. DiA\textsuperscript{26} is a lipophilic tracer that stains the cell membrane of live and fixed cells. DiA is efficiently excited between 440nm and 500nm with maximum excitation at 456nm. The dye has a very broad emission profile with a peak at 590nm. The spectral emission characteristics of DiA should enable the MCME to spectrally resolve individual features of cells stained with a mixture of SYTO 16, MitoTracker Deep Red, and DiA. The triple dye mixture should also be a more rigorous test of the spectral deconvolution algorithm described in this section.

6.3 In-vivo Applications for Multi-spectral Confocal Microscopy

This dissertation introduces the field of in-vivo multi-spectral confocal microendoscopy. As such, the specific applications for the MCME are somewhat unknown. The various applications for imaging and non-imaging spectroscopy as an aid to tissue diagnosis are well documented\textsuperscript{51,52}. These include tracking the metabolic state of tissue by monitoring the naturally occurring fluorescent forms of nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD)\textsuperscript{53-56}. Tissue autofluorescence has been used to diagnosis atherosclerosis by differentiating between fibrous plaque and normal aorta\textsuperscript{57}. The use of endogenous fluorescence has been explored for the diagnosis of cancer and precancer in the colon\textsuperscript{58}, cervix\textsuperscript{59,60}, breast\textsuperscript{61}, oral cavity\textsuperscript{62}, bladder\textsuperscript{63},
bronchus\textsuperscript{64}, and brain\textsuperscript{65}. In its current manifestation, the MCME has relatively low throughput and is generally unable to measure naturally occurring tissue fluorescence. However, it is conceivable that a dedicated system could be designed with sufficient sensitivity to perform multi-spectral imaging of autofluorescence (see Section 5.7).

The investigation of spectral profiles of exogenous fluorescent probes in the context of in-vivo imaging of tissue is a relatively new field. The use of such spectra in the study of disease is currently an area of significant research interest\textsuperscript{51}. Spectral changes in fluorescent dyes such as BCECF\textsuperscript{26} and SNARF\textsuperscript{26} have been used to map extracellular pH\textsuperscript{66}. Tumors have been visualized based on the spectral properties of photodynamic agents such as exogenous porphyrins and Photofrin II\textsuperscript{67}. Indocyanine green has been used to monitor vascular integrity as a result of tumor angiogenesis\textsuperscript{51}. The voltage sensing dye merocyanine oxazolone has been used to study neuronal activity\textsuperscript{68}. Rhodamine 123 has been used to monitor drug target interactions\textsuperscript{17}, and various exogenous dyes have been used to measure the concentrations of Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, and other ions\textsuperscript{26,69}.

In general, each of the areas touched upon above have employed spectroscopy systems capable of acquiring only a single point measurement. In cases where an imaging spectrometer was used the experiments were not performed in-vivo. This is because biological spectroscopy and in-vivo cellular imaging are two distinct fields. The MCME has the potential to combine these fields and provide scientists and clinicians with in-vivo spectroscopic imaging.
CHAPTER 7: CONCLUSION

7.1 Summary of Work

This dissertation introduces the concept of multi-spectral confocal microendoscopy for in-vivo imaging. The various applications for microscopy, spectroscopy, and endoscopy in the field of disease diagnosis are well documented. Commercial systems are available that combine aspects from any two of these three core technologies. The fundamental basis for this dissertation is the belief that there is a natural synergy between microscopy, spectroscopy, and endoscopy and that the key features of each of these core technologies may and should be combined into one seamless unit.

To demonstrate this principle, a multi-spectral confocal microendoscope was designed, built, and tested. The new multi-spectral system was built upon an existing slit-scan confocal microendoscope capable of grayscale image acquisition. The first generation grayscale confocal microendoscope incorporated a single excitation source and a 7mm diameter flexible fiber-optic catheter. In the new design the MCME adds a second excitation source, a multi-spectral collection assembly, and a new 3mm diameter catheter. These major changes and additions greatly extend the scope of medical
applications for the system. The work in this dissertation falls into four categories: design, fabrication, characterization, and preliminary evaluation of applications.

The design stage involved a significant effort devoted to the distal opto-mechanical assembly in the new catheter. A nearly diffraction-limited miniature objective was designed with a final packaged size of 3mm in diameter and 13mm in length. Two miniature focus mechanisms were designed to allow axial positioning of the observation plane in the tissue. A new multi-spectral collection arm incorporating a dispersing prism was designed for the bench-top optical system.

The miniature objective was successfully fabricated and assembled by an outside source. The various parts of the miniature focus mechanism were fabricated by an outside source and assembled in house. The multi-spectral collection arm was assembled in house using catalog optics and a scientific grade CCD. Several additional modifications were added to the bench-top optical system including: a second laser source, a stable fiber coupling for each laser, new dichroic beamsplitters and holographic notch filters, and a more stable method of coupling the catheter to the optical system.

The individual components of the MCME's distal opto-mechanical assembly were tested to insure their compliance with the catheter specifications. The lateral resolution of the miniature objective was measured at roughly 2μm in fiber space, which slightly outperforms the 3μm lateral resolution of the fiber itself. The axial chromatic aberration of the objective was roughly 6.5μm in tissue space, which is in close agreement with the predicted value. Both the mechanical and pneumatic focus mechanisms were tested over their full range of travel. While the motion provided by the
focus mechanisms was fairly linear and repeatable, each system exhibited significant hysteresis. It was found that a trained user could quickly scan through the stationary hysteresis regions and achieve reliable focus control. The overall lateral and axial resolution of the MCME was 3μm and 30μm, respectively. These numbers are slightly larger than the predicted values but the current measurements are in close agreement with characterizations performed by previous authors.

The new multi-spectral collection assembly was fully characterized. The spectral dispersion of the prism-based optical system was determined and plotted. A calibrated light source was used to determine the spectral sensitivity of the MCME. At a wavelength of 632.8nm the measured spectral linewidth of the system was roughly 2 camera pixels FWHM under normal 512 x 512 spatial pixel operation and roughly 1 camera pixel FWHM under normal 256 x 256 spatial pixel operation. The spectral sampling rate varied from 4nm/pixel at 490nm to 8nm/pixel at 660nm and the minimum resolvable wavelength difference varied from 7nm to 18nm over the same spectral range. It was found that the spectral characteristics of the MCME were dictated by the dispersive power of the prism, the width of the confocal slit aperture, and the lateral spatial resolution of the optical system.

Grayscale results with ex-vivo tissue suggest that the confocal microendoscope with the new 3mm diameter catheter will produce images of sufficient quality to assist in disease diagnosis. Grayscale images of the cervix, ovary, esophagus, and colon presented in this dissertation show significant detail that should aid in-vivo identification of pathology. To facilitate multi-spectral collection, macros were written in the camera’s
software package to operate the MCME's focus and capture modes. Extensive software was developed in IDL to help display the spectral data cube in several fashions including: grayscale projection images, spectral band images, and point-by-point spectral plots. To test the multi-spectral collection mode of the MCME, preliminary data were obtained from multi-colored fluorescent microsphere phantoms. High quality images of cell cultures stained with the multi-spectral dye acridine orange were obtained and presented. Multi-spectral images were obtained from several samples of excised human colon tissue. High contrast multi-spectral images were presented from a dual dye, dual excitation experiment using SYTO to stain DNA and Mito to stain mitochondria. The dual dye experiment was used to demonstrate the concept of spectral deconvolution. These preliminary experiments demonstrate the ability of the MCME to obtain high quality multi-spectral images of live cells.

The new 3mm diameter catheter is sufficiently small and flexible to allow access to remote locations in the body. The catheter may be deployed on its own or inserted through the instrument channel of a commercial endoscope. The MCME has the potential to provide real-time grayscale and multi-spectral cellular imaging to the field of medical endoscopy.

7.2 Future Plans

Future work on the MCME will involve increasing the flexibility, stability, and ease of use of the system as a whole. The system would offer significantly more flexibility in dye selection if the fixed mounts that currently hold the dichroic
beamsplitters were replaced with a quick-change filter system or filter wheel. This would allow the user to select 1 or 2 excitation wavelengths and easily place the appropriate beamsplitters in the system. The stability of the MCME could be improved by using sturdier optical mounts that integrate several optical elements into easily aligned subassemblies. The overall ease of use of the system could be increased by adding remotely powered actuators to key alignment elements such as the confocal aperture. In addition to hardware improvements, a dedicated graphical software interface for multi-spectral imaging mode should be written.

The MCME could also benefit from increased sensitivity. Section 5.7 includes a discussion of how and why one might improve the overall throughput of the illumination and detection optical systems. With enhanced coatings on the optical components the throughput of the MCME might be double to triple its current value. However, the fiber bundle will ultimately limit the throughput of the illumination and detection arms. There are currently no fibers that can outperform the imaging bundle employed by the MCME. Nevertheless, future technology may produce advancements in center-to-center pixel spacing and fill ratio, which would further increase the resolution and radiometric throughput of the MCME.

The ultimate test for the MCME will be in human clinical trails. Central to the success of the MCME as a clinical tool is the development of contrast agents suitable for use in humans. Preliminary data suggest that acridine orange causes low morbidity in small topical applications to the colon. However, significant work is necessary to develop a broader range of available fluorophors for both grayscale and multi-spectral
imaging modes. Once suitable contrast agents are developed, the catheter of the MCME will need to be rebuilt to meet the necessary health and safety requirements placed on medical equipment. The bench-top portion of the MCME will need to be redesigned so that it is small and stable enough to easily wheel into the clinical setting. In addition to the size and safety requirements, the limitations to spatial and spectral resolution should be explored. This investigation should include an analysis of the spatial and spectral resolution required by the potential applications for the MCME. The successful completion of these tasks will allow the MCME to add microscopic grayscale and multispectral imaging functionality to clinical endoscopy procedures.
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