Development and Evaluation of Whole Slide Hyperspectral Confocal Fluorescence and Brightfield Macroscopy

by

Paul Constantinou

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Physics
University of Toronto

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2009

Abstract

Microscopic imaging in the biomedical sciences allows for detailed study of the structure and function of normal and abnormal (i.e., diseased) states of cells and tissues. The expression patterns of proteins and/or physiological parameters within these specimens can be related to disease progression and prognosis, and are often heterogeneously spread throughout the entire specimen. With conventional microscopy, a large number of individual image ‘tiles’ must be captured and subsequently combined into a mosaic of the entire specimen. This has the potential to introduce artefacts at the image seams, as well as introducing non-uniform illumination of the entire specimen.

A further limitation often encountered in biomedical fluorescence microscopy is the high background due to the autofluorescence (AF) of endogenous compounds within cells and tissues. Often, AF can prevent the detection and/or accurate quantification in fluorescently-labelled tissues and, in general, can reduce the reliability of results obtained from such specimens. AF spectra are relatively broad and so can be present across a large number of image spectral channels. The intensity of AF also increases as the excitation wavelength is decreased, causing
increasing amounts of autofluorescence when exciting in the blue and near-UV range of the spectrum (400 - 500 nm).

This thesis reports the development of hyperspectral, fluorescence and brightfield imaging of entire, paraffin-embedded, formalin-fixed (PEFF) tissue slides using a prototype confocal scanner with a large field of view (FOV). This technology addresses the challenges of imaging large tissue sections through the use of a telecentric f-theta laser scan lens thus allowing an entire microscope slide (22x70 mm) to be imaged in a single scan at resolution equivalent to a 10x microscope objective. The development and optimization of brightfield and single-channel fluorescence imaging modes are discussed in the first half of this thesis, while the second half and appendices concentrate on the spectral properties of the system and removal of AF from PEFF tissue sections. The hyperspectral imaging mode designed for this system allows the fluorescence emission spectrum of each image pixel to be sampled at 6.7 nm/channel over a spectral range of 500-700 nm. This results in the ability to separate distinct fluorescence signatures from each other, and enables quantification even in situations where the AF completely masks the signal from the applied labels.
Acknowledgments

I wish to dedicate this thesis to my immediate family. To my father, who I get my scientific curiosity from, my mother who is the most loving person I know, and my sister who always took care of her kid brother. The task of completing the work and studies in this thesis would have been impossible without the scientific, emotional and moral guidance of the many people whom I have come to know over my life, and am honoured to call friends. To my best and closest friends: I wished to recognize each of you individually here, but I found it impossible to keep these acknowledgements brief when I tried. I have failed as a scientist in that I have yet to come up with a way to effectively shrink the ten pages I require into a single one.

To my graduate supervisory committee of Dr. David Hedley, Dr. Alex Vitkin and Dr. Ted Dixon, I sincerely thank you for the time you invested as well as the guidance and support you offered over my graduate research. The balance of knowledge present in the room at committee meetings was often overwhelming and I am a better scientist for it. It is safe to say that without the understanding and patience of my supervisor Dr. Brian Wilson, the completion of this thesis would not have been possible. Brian’s ability to manage a seemingly insurmountable number of projects and commitments while maintaining a full battery of graduate students has proved to be an invaluable resource and inspiration. Under his guidance I was able to freely explore research side-projects and activities that broadened my knowledge far beyond the scope of this thesis. With my future career goals now in mind, I reflect back and see quite clearly that without Brian as my supervisor I would not be as ready for the ‘real world’. I wish to thank him deeply for helping me through the graduate experience.

I must also recognize Dr. Savvas Damaskinos for his significant impact on my academic and professional career. The sheer volume of work I have seen him undertake on his own at BPI serves as a constant motivation for me. Without his friendship, numerous discussions, and overwhelming support during the writing of this thesis, the road to completion would have been considerably rougher.
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<th>Description</th>
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<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>AF</td>
<td>autofluorescence</td>
</tr>
<tr>
<td>AOMF</td>
<td>advanced optical microscopy facility</td>
</tr>
<tr>
<td>Ar:K</td>
<td>argon krypton</td>
</tr>
<tr>
<td>BPI</td>
<td>biomedical photometrics incorporated</td>
</tr>
<tr>
<td>BS</td>
<td>beamsplitter</td>
</tr>
<tr>
<td>CCD</td>
<td>charge coupled detector</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFM</td>
<td>confocal fluorescence microscope</td>
</tr>
<tr>
<td>CPU</td>
<td>computer</td>
</tr>
<tr>
<td>CRI</td>
<td>cambridge research and instrumentation</td>
</tr>
<tr>
<td>CSLM</td>
<td>confocal scanning laser microscope</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine 3</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPC</td>
<td>differential phase contrast</td>
</tr>
<tr>
<td>EEM</td>
<td>excitation emission matrix</td>
</tr>
<tr>
<td>F/AF</td>
<td>fluorescence/autofluorescence ratio</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>f</td>
<td>focal length</td>
</tr>
<tr>
<td>FL</td>
<td>fluorescence</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FOV</td>
<td>field of view</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width half maximum</td>
</tr>
<tr>
<td>GB</td>
<td>gigabyte</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>He:Ne</td>
<td>helium neon</td>
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<table>
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<tr>
<th>Term</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>HIF1-α</td>
<td>hypoxia inducible transcription factor 1-α</td>
</tr>
<tr>
<td>HSM</td>
<td>hyperspectral macroscope</td>
</tr>
<tr>
<td>HSI</td>
<td>hyperspectral imaging</td>
</tr>
<tr>
<td>IBIDI</td>
<td>integrated biodiagnostics</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>LSL</td>
<td>laser scan lens</td>
</tr>
<tr>
<td>M</td>
<td>magnification</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MB</td>
<td>megabyte</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>OFM</td>
<td>open frame macroscope</td>
</tr>
<tr>
<td>OBIC</td>
<td>optical beam induced current</td>
</tr>
<tr>
<td>OO</td>
<td>ocean optics</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PH</td>
<td>pinhole</td>
</tr>
<tr>
<td>QDot</td>
<td>quantum dot</td>
</tr>
<tr>
<td>RL</td>
<td>reflected light</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>SNR</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>TAG72</td>
<td>tumour associated glycoprotein 72</td>
</tr>
<tr>
<td>TL</td>
<td>transmitted light</td>
</tr>
<tr>
<td>T2B2</td>
<td>tissuescope 2 beta 2</td>
</tr>
<tr>
<td>TS4000</td>
<td>tissuescope 4000</td>
</tr>
<tr>
<td>USB</td>
<td>universal serial bus</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1

1  Background and Thesis Overview

1.1  Introduction

The use of digital fluorescence confocal microscopy in biological sciences has grown in recent decades due to the versatility of fluorescence imaging and the increased power of hardware and software for data acquisition and processing. The ability to selectively label specific morphological features, proteins, genetic mutations and/or chemical micro-environmental changes with discreet fluorescent labels allows a better understanding of the complex systems that regulate cellular processes, and thus sheds light on the function of the most basic unit of life: the cell (Hibbs 2004). Microscopy specimens can range in size from single cells to full tissue sections and tissue arrays (Moch, Kononen et al. 2001; Al Kuraya, Simon et al. 2004; Hassan, Ferrario et al. 2008); the latter two can be much larger than the field of view of a typical microscope objective. This makes the study of heterogeneous signals within the specimen difficult on a traditional microscope, since a large number of image ‘tiles’ must be acquired and subsequently digitally stitched together to form a composite image of the entire specimen. Another roadblock often encountered in fluorescence imaging arises from the existence of endogenous autofluorescence from the specimen, which can often overwhelm the signal from administered fluorescent labels and confound quantitative imaging (Schnell, Staines et al. 1999; Swenson, Price et al. 2007).

This thesis reports on a prototype confocal fluorescence imaging system designed to address these challenges by enabling 1) transmitted light and fluorescence imaging of an entire 25 x 75 mm microscope slide in a single field of view (FOV) and 2) high resolution spectral sampling of the fluorescence emission from the specimen, coupled with spectral image processing tools, to
minimize the effects of tissue autofluorescence. The organization of this thesis is as follows. *Chapter 1* contains an introduction to relevant concepts in optical, confocal and fluorescence microscopy, in addition to a review of the challenges posed by tissue autofluorescence in biomedical fluorescence imaging. *Chapter 2* contains an initial feasibility study evaluating the core optical technology and performance of a modified cDNA (complementary DNA) reader for fluorescent whole-slide imaging and analysis. From these initial studies, improvements to the cDNA system were identified and implemented in the design of a prototype Hyperspectral Macroscope (HSM). *Chapter 3* describes this instrument, and also presents calibration and validation data for the transmission, fluorescence and hyperspectral imaging modes. *Chapter 4* presents a study on the use of the hyperspectral imaging mode in whole-slide images of a fluorescently labelled human tumour xenograft. The extraction of a weak fluorescence signal from the strong autofluorescent background in these samples demonstrated the advantages of hyperspectral imaging over traditional single channel fluorescence imaging. As the instrument developed is still at a proof of concept phase, a discussion on future directions is found in *Chapter 5*. Appendices contain a discussion of the mathematics of the spectral unmixing algorithm (*Appendix 1*), as well as additional examples of hyperspectral imaging and linear unmixing in phantoms (*Appendices 2-4*). *Appendix 5* contains unpublished results showing the effect of formalin fixation and antigen retrieval on tissue autofluorescence intensity.
1.2 Microscopy Basics

The following section contains an introduction to the basic optical principles in microscopy. Only a general discussion of selected fundamental concepts is given here, for more detailed discussion see (Hibbs 2004; Pawley 2006; Pedrotti, Pedrotti et al. 2007).

1.2.1 Imaging Properties of a Thin Lens

The simplest way to produce a magnified image is with a single lens of given focal length, f (figure 1.1). In figure 1.1, the object distance (d_{obj}) is the distance from the center of the lens to the object and the image distance (d_{img}) is the corresponding distance from the lens to the image. If the focal length and object distance are known, the location of the image can be calculated via the lens-maker’s formula (Pedrotti, Pedrotti et al. 2007) and the size of the image is calculated by the magnification ratio (M). When the object lies at a distance of 2f, the image is also formed at 2f and the magnification is -1 (i.e., the image is inverted). As the lens is moved closer to the object (decreasing d_{obj}), the image distance and magnification increase.
1.2.2 Numerical Aperture and Optical Resolution

A relevant property of a lens is its Numerical Aperture (NA). The NA is a measure of the light collection volume of a lens; a higher NA indicates more efficient light collection and a smaller focused spot size, which translates into better image contrast and resolution. A diagram of the NA measure is shown in figure 1.2. Light is focused to a point which is one focal length from the lens centre. If the lens diameter is defined as D, then the maximum collection angle of the lens for an on-axis point can be calculated as: \( \theta = \tan^{-1}(2f / D) \). The NA of a lens is defined as \( NA = n \sin(\theta) \), where \( n \) is the index of refraction of the medium between the lens and the focal point.
As the NA is increased, the size of the focused spot decreases and the optical resolution increases. Strictly speaking, however, the distribution of the focused spot is not actually a ‘spot’, but an Airy pattern, named after George Biddell Airy. This pattern arises due to diffraction from the circular aperture of the lens. The physical implication of this is that a single point will be imaged not to a single point, but into a distribution described by the Airy pattern (figure 1.3). The width of the central peak in the Airy pattern can be calculated via the equation in the lower right portion of figure 1.3, where $\lambda$ is the wavelength of light transmitted through the lens.

**Figure 1.2** – Numerical Aperture (NA) is a measurement of the collection volume of a thin lens. In the figure, D represents the lens diameter, f the focal length, n the index of refraction of the surrounding medium, and $\theta$ is the maximum collection angle of the lens.
The Rayleigh Criterion (Pedrotti, Pedrotti et al. 2007) defines resolution in terms of the Airy radius, stating that two objects separated by at least the radius of the Airy disc will be resolved if the system is operating at the diffraction limit. Figure 1.3d shows the overlapping Airy patterns of two well-resolved points (left), two points at the Rayleigh limit (middle) and two unresolved points that will not be optically distinguished (right). In general, lenses with a high NA will collect more light and produce sharper images than a lower NA lens.

\[
D_{\text{Airy}} = 1.22 \frac{\lambda}{NA}
\]

Figure 1.3 – Airy diffraction pattern produced by diffraction from a circular aperture (A-C). Optical resolution can be defined in terms of the width of the Airy pattern; two points separated by >1 Airy radius will be resolved as distinct objects, while objects closer than one Airy unit will blend together (D).

1.2.3 Compound (Simple) Microscopes

The first compound microscope was essentially a system of two lenses (eyepiece and objective) housed in a tube. The objective lens is used to create a real magnified image of the specimen at a
location inside of the tube, while the eyepiece focuses and magnifies this image through the observer’s eye (figure 1.4).

![Diagram of a simple compound microscope](image)

**Figure 1.4** – A simple compound microscope that can be used to produce a magnified image of the specimen. The dotted line represents the apparent image size as seen by an observer.

Modern microscopes are much more complex than the system shown above; however, their basic principle of operation is similar. An objective lens is still used to produce an image of the specimen, which is then relayed to the eye or a light detector through the eyepiece. From the basic principles in the discussion above, it can be seen that an ideal microscope objective would have a very high NA and large FOV. Recall from figure 1.2 that the NA depends on $n \cdot \sin(\theta)$, which is maximum when $\theta = 90^\circ$. While it is physically impossible to achieve this collection angle in practice, there are so called ‘dry’ objectives with an NA of 0.95 ($\theta \approx 72^\circ$). To further increase the NA beyond 0.95, a ‘wet’ or immersion objective is required, since increasing the index of refraction ($n$) of the region between the lens and the specimen also increases the NA. Air has an index of refraction of approximately 1.0, while for water and oil it is 1.33 and 1.55, respectively. Thus, an oil immersion objective will have roughly 1.5x the NA of a dry objective.
Typically, to achieve a magnification greater than 40x/0.75NA, a water or oil immersion objective is used.

1.3 Biomedical Microscopy Applications

The following section describes some basic principles in biomedical microscopy, including staining and labelling of specimens for transmitted light and fluorescence detection. The hyperspectral instrument described in later chapters has similarities to a confocal microscope, thus a brief introduction to confocal microscopy and hyperspectral imaging is also presented. A valuable resource for further discussion on confocal microscopy can be found in (Hibbs 2004; Pawley 2006), as well as the interactive tutorials on the Nikon Microscopy U website (www.microscopyu.com).

1.3.1 Brightfield Microscopy

The optical resolution provided by the objective lens is only part of the solution in biomedical imaging, as specimens generally contain very little intrinsic contrast under broadband illumination. A brightfield (or transmitted light) microscope is the most common contrast method used in the life sciences. The specimen (usually a tissue section) is stained with dye(s) that impart additional contrast and enable discrimination of morphologic features based on their differential colour absorption across red, green and blue colour channels. A familiar example of this is shown in figure 1.5, which is of a cervical carcinoma cell line (me180) grown as a xenograft in a mouse host and stained with Haematoxylin and Eosin (H&E). H&E staining is routinely used to allow imaging of tissue morphology for pathological evaluation of diseases
such as cancer (Cormack 2001). Haematoxylin stains basophilic structures such as DNA in the nucleus dark purple, while Eosin generally stains the cytoplasm and intra/extra-cellular regions varying shades of pink.

1.3.2 Fluorescence Microscopy

Fluorescence is a phenomenon first discovered by British scientist Sir George Stokes in 1852 (Stokes, Angstrom et al. 1852). The process involves the absorption of an incident photon of light by a molecule, referred to as a fluorophore, and subsequent emission of a second photon a short time later (figure 1.6). The energy transferred to the fluorophore after absorption causes the molecule to enter an excited energy state that is short-lived, typically on the order of $10^{-9}$ seconds ($1/e$ value). During this time, some of the incident energy is lost to vibrational/rotational motion and heat, among other things. When the molecule returns to the ground state, a second fluorescence photon is emitted at a longer wavelength. This fluorescence can be detected with a
colour filter that passes the fluorescence emission and blocks the shorter wavelength excitation light. The difference in wavelength between the incident and outgoing photons is referred to as the Stokes shift, and it is this phenomenon that is the basis for all fluorescence imaging.

Fluorescent stains such as 4’-6-Diamidino-2-phenylindole (DAPI) are commonly used in fluorescence microscopy (Kapuscinski 1995). Upon binding to DNA, the fluorescence emission of DAPI increases from its unbound state, which enables detection of cell nuclei when excited in the ultra-violet (UV) wavelength range, approximately 350-400 nm. Immunofluorescence microscopy also allows labelling of specific antibodies or antigens with fluorescent molecules (Hibbs 2004). Antibodies can be very specific in their binding affinities and this can be exploited to deliver a target to a specific region within the cell or tissue. While stains such as DAPI stain DNA fragments yielding morphological information of the specimen, antibody labelling imparts the ability to visualize the sub-cellular distribution of a wide range of biomolecules and markers (Opas 1999; Goldman 2000; Miyashita 2004; Riccio, Dembic et al. 2004).

**Figure 1.6** – Schematic representation of the process of fluorescence. Light absorbed by a molecule causes it to move into an electronic excited state. Some of the absorbed energy is lost to heat and vibrational/rotational motion of the molecule and a short time later a second photon of lower energy is emitted. The wavelength difference between the excitation and emission photon is the Stokes shift.
Figure 1.7 demonstrates the concept of fluorescent antibody labelling using a two-stage process. A primary antibody is first incubated and allowed to label the antigen of interest (left). In a second step, a fluorescent antibody recognizing the primary antibody labels the cell (middle). After washing any unbound labels, fluorescence from the bound fluorescent antibody can be detected and quantified (right).

First, a primary antibody that is specific to a particular antigen expressed on a cell is incubated with the specimen and binds to the target (left). Next, a secondary antibody linked to a fluorescent molecule is incubated with the sample and allowed to bind to the primary antibody (middle). Detection can then proceed by excitation with the appropriate wavelength of light, combined with efficient collection and filtering of the generated fluorescence. Multiple biomarkers can be localized in a single specimen through the use of secondary antibodies that excite/emit at different wavelengths. The two-step antibody labelling method allows for flexibility in sample preparation and study; a single primary antibody label can be combined with a large number of secondary antibodies possessing fluorescent, absorptive, chemotherapeutic or...
radiological properties. An example of this is the TAG72 primary antibody used for fluorescence labelling in *Chapter 4*. Since the TAG72 antigen is over-expressed in tumour tissue, it can be used to localize tumour regions when combined with a fluorescent secondary antibody, or alternatively be combined with radioactive isotopes for targeted radiotherapy (Buchsbaum, Rogers et al. 1999; Mohsin, Jia et al. 2006), or combined with chemotherapeutic agents such as doxorubicin (Johnson, Briggs et al. 1995).

In general, the fluorescence intensity emitted by a stained specimen is linearly related to the light intensity used to excite the fluorescence. However, the process of fluorescence is inherently destructive and there is a limit to how much light a given molecule can emit before it loses its ability to fluoresce. This process is called photobleaching, and is generally the result when too much excitation power is placed on the specimen for extended periods of time (Pawley 2006). A typical example of photobleaching is shown below in figure 1.8. The specimen is a fibroblast cell, fluorescently labelled with two commercial fluorescent staining kits (Invitrogen, Carlsbad, CA, USA), Alexa 488-actin (stains the cytoskeleton) and Hoescht 33258 (stains nuclei). Images were acquired at 2-min intervals, and the level of fluorescence from the nuclear Hoescht stain can be seen to drop rapidly with exposure to the excitation light, while the signal from the Alexa dye drops only slightly. Photobleaching complicates the routine use of fluorescence for quantitative studies, and care must be taken to minimize its effects. Typically, fluorescently labelled specimens are imaged in a dark environment and are stored in a fridge or freezer protected from ambient light.
1.3.3 Confocal Microscopy

A significant development in microscopy came in 1961, when Dr. Marvin Minsky received a patent for the invention of the confocal microscope (Minsky 1961). A confocal microscope has one major advantage over a conventional microscope; it will only allow light from a thin optical ‘slice’ of the sample to reach the detector, which can significantly enhance fluorescence detection. Furthermore, the point-scanning nature of confocal imaging can reduce the effects of photobleaching over a widefield (broad illumination) microscope. Confocal microscopes image a sample by quickly raster-scanning a diffraction limited spot across the sample, either by translating the sample under a fixed beam with an XYZ stage, or by using a system of mirrors to scan the beam across a stationary sample (Pawley 2006). As a result, the region directly surrounding the scanning spot is being illuminated for only a few microseconds. In a widefield microscope, the illumination is applied to the whole sample and, typically, the region illuminated is much larger than the actual region detected, which serves to further increase the effects of photobleaching. A general representation of a confocal microscope is shown in figure 1.9 below.
Light from an excitation source, typically a laser, is focused through a very small pinhole which lies at the focus of a lens. The collimated beam of light emerging from this pinhole then passes through a beamsplitter and is focused on the sample through a microscope objective. Light emitted from the sample (green line) is then re-collimated by the objective lens, reflected off the beamsplitter and focused by a detector lens through a second pinhole placed in front of the detector. Light emitted from out-of-focus planes (red and blue lines) is blocked by the detector pinhole; a smaller pinhole in front of the detector allows for a smaller depth volume to be sampled. The result is a much sharper image with reduced background signal. The Nikon Microscopy U website (www.microscopyu.com) contains interactive tutorials on confocal microscopy that demonstrate the confocal slicing of a wide variety of samples. Shown in figure
1.10 is a typical comparison from the website of a confocal and non-confocal (widefield) microscope.

![Widefield Image](image1.png) ![Confocal Image](image2.png)

**Figure 1.10** - Widefield (A) versus confocal (B) images of a mouse kidney obtained from the Microscopy U website. Images captured with a 100x objective on a Nikon PCM 2000 laser scanning confocal microscope.

The specimen is a 16 µm thick mouse kidney section, imaged at high resolution (100x). The images show a glomerulus that has been fluorescently labelled with wheat germ agglutinin (green) and filamentous actin (red). Due to the relatively thick nature of the specimen and the small depth of focus of the objective used, figure 1.10a, contains a significant amount of defocused light, which presents itself as a haze washing out the signal from in-focus light. The confocal pinhole was reduced in size for figure 1.10b, and has the effect of rejecting the out of focus haze.

It is also possible to use this confocal effect to generate a 3D ‘stack’ of specimen images by moving the specimen closer or further away from the objective lens, and thus acquiring images at different focal depths within the specimen. Figure 1.11 shows an example of a confocal stack through a fluorescent bead 5 µm in diameter. Each image represents a 1 µm thick optical slice and the apparent diameter of the bead is seen to change as the focal plane is moved through its
volume. Typical thick biomedical specimens are highly scattering and, as a result, the practical depth at which confocal imaging can be performed is limited to a few hundred microns (Pawley 2006).

A two-photon confocal microscope (Pawley, 2006) is another confocal system often used for bio-imaging, and can offer some advantages over more traditional confocal microscopes. A two-photon microscope uses an infrared (IR) laser producing high power pulses of excitation light. If these light pulses are short enough (typically ~10^{-10} s) and focused through a high NA objective (>0.5), it is possible for the energy of two IR excitation photons to combine and excite a single fluorescent molecule. Since the energy from two photons is combined, dyes typically excited by UV and blue light (350-500 nm) can undergo two-photon excitation in the IR (700-900 nm) range. These systems achieve confocality without the use of a pinhole or aperture because the two-photon effect only occurs where the excitation light is tightly focused. This also has the added benefit of minimizing photobleaching since areas outside the focal plane do not absorb the IR light. The longer wavelengths used for two-photon microscopes also penetrate further into tissue, allowing for imaging at depths beyond the capabilities of single-photon confocal systems.

Figure 1.11 – Confocal stack through a 5 micron fluorescent bead. The optical slice thickness was approximately 1 micron, with images acquired at z (depth) increments of 0.5 microns.
Two photon systems are typically more expensive and difficult to operate than more conventional confocal microscopes, mainly due to the complexity of the pulsed laser excitation source.

Nipkow disc confocal microscopes are another variant of confocal instrument that is quite different from the system developed in this work. They employ a disc aperture with many pinholes arranged in a pattern such that as the disc spins, the entire FOV is imaged confocally onto a CCD chip. This effectively turns the traditional process of acquiring confocal images one pixel at a time into a parallel process, allowing confocal images to be captured at video rate and beyond (Nakano 2002; Pawley 2006). Due to this, spinning disc confocals are particularly well suited to imaging live samples, though their sensitivity is typically less than that of a point scanning confocal due to the fast frame rate and reduced light collection efficiency of the spinning disc configuration.

### 1.3.4 Multi/Hyperspectral Microscopy

Fluorescence microscopes generally detect light by filtering a narrow range of wavelengths with an emission filter before detection. In hyperspectral imaging (HSI), the entire spectral range of light collected from the sample is passed through or reflected off a grating (Pawley 2006; Pedrotti, Pedrotti et al. 2007), which disperses the light according to wavelength across a linear array detector. Each detector channel/element in the linear array corresponds to a narrow wavelength range (~ 5-10 nm), enabling measurement of the fluorescence emission spectrum at a given spatial location. When this method is used for image acquisition on a 2D tissue section a 3D ‘hyperspectral’ data cube, depicted schematically in figure 1.12, is acquired. In a hyperspectral cube, two dimensions represent space (x,y), while the third (λ) represents the pixel
intensity at a given wavelength. By looking ‘down’ the stack of wavelength images, a spectral profile of a specific (x,y) location in the image can be generated.

![Hyperspectral Data Stack Diagram](image)

**Figure 1.12** – Representation of a hyperspectral data stack. Two dimensions of the stack represent spatial measures (x and y), while the third dimension represents wavelength ($\lambda$). Looking ‘down’ the stack at a single (x,y) position allows visualization of the emission spectrum of that pixel.

Single channel (traditional) fluorescence imaging uses optical filters, typically with ~40-60 nm wide passbands. This limits studies to the analysis of only the intensity of the fluorescence detected from a sample, while HSI adds a further dimension by enabling scientists to study the entire fluorescence spectrum from a sample. One example where HSI is of great use is in Forster Resonance Energy Transfer (FRET) microscopy (Jares-Erijman and Jovin 2003). The process of FRET requires two fluorophores, donor and acceptor. If the donor fluorophore is excited, and the acceptor fluorophore is within the Forster distance (1-10 nm), energy can be transferred from the donor to the acceptor fluorophore. Thus, exciting a sample with a wavelength optimal for the donor and detecting fluorescence emission from the acceptor fluorophore is the ultimate goal. The combinations of donor-acceptor fluorophores used typically have widely overlapping emission spectra, which requires very narrow emission filtering to avoid cross-talk between
image channels in traditional fluorescence microscopy. However, a technique such as HSI can make use of the spectral information captured, and can detect emission from the acceptor fluorophore on the basis of both fluorescence intensity and spectral shape. Combined with spectral processing tools to extract pixels containing the acceptor fluorescence spectrum, hyperspectral FRET is much more sensitive than traditional FRET (Ecker, de Martin et al. 2004; Thaler, Koushik et al. 2005).

A major portion of this thesis is devoted to the design, construction and implementation of a HSI mode in a large FOV confocal macroscope, details of which can be found in Chapters 2-4. A discussion of the spectral processing mathematics used for this work can also be found in Appendix 1 and further examples of spectral unmixing can be found in Appendices 2-4 as well as (Dickinson, Bearman et al. 2001; Zimmermann 2005). The techniques presented here have been in use for a number of years in the field of remote sensing (Huntington 1996; Akgun, Altunbasak et al. 2005; Craig, Lohrenz et al. 2006), but the use of hyperspectral instruments in biomedicine to date has been limited to laboratories and pre-clinical studies (Tsurui, Nishimura et al. 2000; Sinclair, Timlin et al. 2004; Chen, Zhuo et al. 2006; Dicker, Lerner et al. 2007).

1.4 Cellular and Tissue Autofluorescence
1.4.1 Sources of Autofluorescence
While confocal microscopes are good at rejecting out-of-focus light as seen in figures 1.10 and 1.11, little can be done about unwanted light that originates from within the confocal slice region. A common problem in fluorescence microscopy is the influence of background signals, particularly tissue autofluorescence (AF); that is, fluorescence from endogenous molecules such as proteins within the tissue. The major source of autofluorescence in cells originates from the
mitochondria and lysosomes, most specifically coenzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) and flavins such as flavin adenine dinucleotide (FAD) (Monici 2005). These fluorescent molecules have very broad emission spectra that can overlap much of the visible spectrum, making it almost impossible to eliminate their influence in fluorescence images (Benson, Meyer et al. 1979; Wu and Qu 2006). In tissues, collagen and elastin contribute greatly to autofluorescence and generally express more endogenous fluorescence than cells in culture (Monici 2005).

A useful method for quantifying the spectral characteristics of AF components within cells and tissue is the construction of an excitation emission matrix (EEM) (DaCosta, Andersson et al. 2003; Li and Xie 2005). EEMs are generated by recording the fluorescence emission spectra at a large number of excitation wavelengths, thus producing a 2D matrix of fluorescence emission plotted against excitation wavelength. There is an online EEM database containing data on 35 potential endogenous fluorophores within human colonic tissue, available through the Photochemistry and Photobiology website. Shown in figure 1.13 are the EEMs for two major sources of autofluorescence excited in the violet-blue spectral range; elastin, a protein abundant in connective tissue, and flavins such as FAD, a redox cofactor involved in cellular metabolism. Two sources of autofluorescence excited in the UV spectral range are also shown; collagen is a major component of the extracellular matrix, and the reduced form of nicotinamide adenine dinucleotide (NADH). These data sets were reproduced from the online EEM database (www.aspjournal.com).
A number of observations are evident from the figures above. Excitation at UV wavelengths will produce AF from ~300-550 nm due to endogenous compounds such as collagen, elastin and NADH, while excitation wavelengths in the violet and blue produces strong green AF from FAD and elastin. Typically, minimal autofluorescence is generated by wavelengths beyond 600 nm.

The autofluorescence of cells and tissues is generally considered a source of background noise or interference, in some instances, the autofluorescence can be sufficiently intense that it becomes virtually impossible to detect the emission from the relevant labelled fluorophore (Swenson, Price et al. 2007).

Figure 1.13 - EEM data for elastin (A), FAD (B), collagen IV (C) and NADH (D). Autofluorescence generated by these endogenous compounds increases as the excitation wavelength is reduced. Data obtained from the online EEM database.
In particular, the autofluorescence from paraffin-embedded, formalin-fixed (PEFF) tissue is generally higher than that of fresh or frozen sections, yet it is routine practice to fix tissues in formalin and archive them in paraffin blocks in order to prevent autolysis and preserve tissue morphology. Fresh tissue is difficult to cut into thin sections suitable for microscopic imaging, so that tissues are typically embedded in paraffin wax before having sections cut in a microtome. This gives the tissue block enough mechanical rigidity to cut a thin slice, typically < 10 µm, for staining. After being cut, the sections are de-waxed and antigens within the tissue that have been cross-linked by the fixation process are unmasked through heat-induced antigen retrieval or enzymatic digestion (Leong and Leong 2007). After antigen unmasking, the archived tissue can be processed for immunofluorescence labelling.

Tissue autofluorescence increases after fixation, due to the cross-linking of structural proteins induced by the fixative (Collins and Goldsmith 1981), and again after antigen retrieval, where many of the endogenous fluorescent compounds in the tissue are unmasked along with the antigens to be labelled (see Appendix 5). This poses a problem for fluorescence studies, since it ultimately means that weakly-expressed signals are difficult to detect in most PEFF tissues.

### 1.4.2 Autofluorescence in Microscopy

An example of images from a highly autofluorescent tissue sample is shown below in figure 1.14. The image on the left is of a stain intended for brightfield microscopy, showing the location of a phosphorylated protein (S6) within a sample of lung tissue obtained from Dr. David Hedley. The fluorescence image from a serial section excited at 488 nm (right) shows additional autofluorescence contributions from the surrounding tissue. In the absence of autofluorescence, figure 1.14b would only contain signal from regions corresponding to the brown staining in the
brightfield image, and the high AF background makes quantification of the true fluorescence signal difficult.

As seen in the EEM plots in figure 1.13, tissue autofluorescence increases at lower excitation wavelengths, with the least autofluorescence being generated by red and near-infrared (NIR) light (> 600 nm). While there are a number of molecules excited by NIR light commercially available from companies like Invitrogen (Carlsbad, CA, USA), e.g. Cy5 and Alexa635/647, there are many more that are excited at wavelengths in the violet/blue range of the visible spectrum, e.g. FITC, Cy2, Alexa488, where autofluorescence excitation becomes a major problem. An excellent online resource containing interactive viewing of the excitation and emission spectra from the entire Invitrogen catalogue of fluorescent probes can be found at (probes.invitrogen.com/resources/spectraviewer/). In figure 1.15, the autofluorescence spectrum from FAD is compared with the emission from three common fluorophores, Alexa488, FITC, and Alexa514. The broad emission spectrum of FAD significantly overlaps emission from probes often used in the blue range of the visible spectrum, where FAD is also optimally excited.
As a result, use of these dyes in PEFF tissues is routinely hindered by flavin autofluorescence (Benson, Meyer et al. 1979).

1.4.3 Suppression of Autofluorescence

The tissue autofluorescence intensity can be altered by chemical treatment of certain tissue types. For example, work by Baschong et al. showed that it is possible to chemically effect the amount of autofluorescence in glutaraldehyde- and formaldehyde-fixed human bone marrow, myocardium and bovine cartilage by treatment with combinations of ammonia-ethanol, Sudan Black B and sodium borohydrate (Baschong, Suetterlin et al. 2001). Clancy and Cauller showed that the autofluorescence of aldehyde-fixed brain sections could be reduced by treatment with sodium borohydride (Clancy and Cauller 1998). Schnell et al also reported the use of copper sulphate and Sudan Black B to reduce autofluorescence from lipofuscin in human, monkey and rat neural tissues (Schnell, Staines et al. 1999). Attempts have also been made to reduce autofluorescence by pre-bleaching the sectioned tissues with UV light before fluorescence labelling (Kingsley, Carroll et al. 2001; Neumann and Gabel 2002; Maggiano, Dupras et al.)

Figure 1.15 - Comparison of FAD autofluorescence emission (orange) with Alexa 488 (green), FITC (blue) and Alexa514 (red). Spectra were obtained from the molecular probes website and the EEM database. The broad FAD spectrum significantly overlaps the spectra from the probes.
and UV pre-bleaching combined with Sudan Black B treatment was investigated in a recent article by Viegas et al. (Viegas, Martins et al. 2007).

While these studies have demonstrated a reduction in the autofluorescence detected from treated sections, two potential problems with the chemical treatment of autofluorescence remain: treatment could alter the staining characteristics of the subsequently applied fluorescent labels, and more importantly, there is no general protocol to reduce autofluorescence in all types of tissue. In contrast, by taking advantage of the spectral information captured in HSI, differences between autofluorescence and antibody fluorescence can be detected. These difference can then be used to remove the contributions of autofluorescence without the need for additional chemical processing.

1.4.4 Spectral Approaches for Autofluorescence Removal

HSI microscopes such as the Zeiss LSM 710 Meta (Carl Zeiss, Thornwood, NY, USA), and the Leica SP2 (Leica Microsystems, Wetzlar, DE) allow spectral imaging on a micro-scale (~ 0.5 \( \mu \text{m} \) resolution), but are not equipped to acquire spectral images of very large specimens such as whole tissue sections (25 mm\(^2\) and larger). Autofluorescence removal from whole animal fluorescence images is possible with non-confocal instruments on a macro-scale (~1-2 mm resolution), such as the Maestro (Cambridge Research & Instrumentation, Woburn, MA, USA), and an example from the CRI website demonstrating spectral unmixing of autofluorescence is shown below in figure 1.16.
Inset in figure 1.16 is a conventional colour fluorescence image of two mice captured on a Maestro whole animal imaging system. The top mouse has been injected with a fluorescent contrast agent producing red light, but the signal is barely visible amidst the bright green autofluorescence of the skin and hair of the animal. By applying spectral unmixing to the hyperspectral data cube, the signal from the fluorescent agent can be extracted and quantified. The un-injected control mouse (bottom) does not contain any significant indication of the target spectrum in the unmixed image.

The ability to remove background fluorescence sources such as autofluorescence, or cross-talk between fluorophores, enables more sensitive detection in fluorescence imaging studies. To date however, none of the HSI systems available are well suited to imaging of large tissue specimens.
at high resolution, and a major portion of this thesis is intended to directly address this through the development of a confocal hyperspectral macroscope, capable of imaging areas up to 22x70 mm at a 1-2 µm resolution, comparable to a typical 10x microscope objective. The large FOV of this instrument is well suited to imaging and hyperspectral analysis of whole tissue sections and/or microarrays, with specific emphasis on autofluorescence removal from PEFF tissue sections without the need for chemical treatments or image tiling. Furthermore, since the autofluorescence of any tissue type can be measured quite easily by preparing a fluorescence negative control section, HSI can potentially be used as a general method for autofluorescence removal from PEFF tissue sections.

1.5 Author Contributions

The work in Chapter 2 was published in the Journal of Biomedical Optics 6(3) p326-31, 2001. The author set up a modified macroscope, including image acquisition. Data analysis was performed in collaboration with Dr. Vojislav Vukovic. The xenograft sections used for analysis were provided by Dr. David Hedley and tissue staining was performed by Dr. Hans-Kristian Haugland. Images from the tiling system used were acquired by Ms. Trudey Nicklee.

The work in Chapter 3 was published in the Journal of Selected Topics in Quantum Electronics 11(4) p 766-777, 2005. The author was responsible for the complete re-design of the system used in the initial work and subsequently the construction of a new prototype system in order to adapt the macroscope technology to tissue-section imaging. Major additions included the integration of higher quality objective lens, changes to the optical layout of the system to further improve image quality, and the addition of transmitted light and hyperspectral fluorescence imaging modes. The brightfield and phase contrast modes were implemented under the guidance
of Dr. Savvas Damaskinos, and the author was responsible for the evaluation and subsequent improvement of these imaging modes. The construction of the hyperspectral imaging mode was the responsibility of the author, and integration of the detector into the system software was performed by Mr. Reg Martin under the direction of the author. Specimens that were used for this work were provided by Dr. David Hedley and Ms. Trudey Nicklee.

The work in Chapter 4 has been accepted into the Journal of Microscopy, but not yet published at the time of writing. The prototype spectral imaging mode was evaluated and compared with single-channel fluorescence imaging results. Implementation of spectral unmixing software and algorithm development for the spectral unmixing process was also the responsibility of the author, as were the experiment design, system setup, image acquisition and data processing. Specimens were provided by Dr. Ralph DaCosta.

In Chapter 5, the data presented were acquired by the author on a beta-development system at Biomedical Photometrics Inc. (BPI), Waterloo, ON. The second-generation spectral detector discussed in the future directions of this work was designed by the author, and will be implemented into a system similar to the beta instrument used at BPI.

Data presented in Appendices 1-5 are from unpublished by the author over the progress of this research, and serve as supplementary information to the published papers that constitute the body of this thesis.

Except as noted, all experiments were performed in Dr. Brian Wilson’s laboratory at the Ontario Cancer Institute/University of Toronto.
Chapter 2

2 Analysis of Core Macroscope Imaging Technology for Fluorescence Imaging

2.1 Preface

The macroscope systems were initially conceived as cDNA genechip readers, and the work described in this chapter is the first use of the macroscope technology for fluorescence imaging of stained tissue sections. The initial pilot study involved comparing the performance of the cDNA macroscope to that of a commercially available fluorescence tiling microscope. It was found that the system did not possess as high a spatial resolution as the tiling system, but produced images with sufficient detail for analysis without the need for image tiling, and at an acquisition rate 8x faster than the tiling system that was available at the time. From the experience gained using this system, improvements were identified and implemented into the instrument used for the work in subsequent chapters.

This chapter is presented as published, except for the section numbering and some minor re-formatting to be consistent with the other chapters.
2.2 Imaging of Tumour Xenografts using a Novel Confocal Fluorescence Macroscope

Paul Constantinou¹, Vojislav Vukovic¹, Hans-Kristian Haugland¹, Trudey Nicklee¹, David W. Hedley¹,², Brian C. Wilson¹

¹ Department of Medical Physics, Ontario Cancer Institute/University of Toronto, Toronto, Ontario, Canada.

² Department of Medical Oncology, Ontario Cancer Institute/University of Toronto, Toronto, Ontario, Canada.

2.2.1 Abstract

Hypoxia caused by inadequate structure and function of the tumour vasculature has been found to negatively determine the prognosis of cancer patients. Hence, understanding the biological basis of tumour hypoxia is of significant clinical interest. To study solid tumour microenvironments in sufficient detail, large areas (several mm in diameter) need to be imaged at μm resolutions. A novel confocal scanning laser microscope (CSLM) was used to acquire images over fields of view up to 2x2 cm. To demonstrate its performance, frozen sections from a cervical carcinoma xenograft were triple-labelled for tissue hypoxia, blood vessels and hypoxia-inducible transcription factor 1 alpha (HIF-1α), imaged using the CSLM and compared to images obtained using a standard epifluorescence microscope imaging system. The results indicate that the CSLM is a useful instrument for imaging tissue-based fluorescence at resolutions comparable to standard low-power microscope objectives.

2.2.2 Introduction

The biology and pathology of cancers can vary widely, which is indicative of the complexity of malignant disease. Solid tumours are characterized by a heterogeneous arrangement of blood vessels and intermittent fluctuations in blood flow, which can result in inadequate supply of oxygen and nutrients to tumour cells (DewHirst 1998). Consequently, the microenvironment of solid tumours is often characterized by the presence of intermittent and chronic hypoxia (lack of, or chronically low oxygen levels). Tumour hypoxia has been linked to biologically aggressive malignant disease and overall poor patient outcome in the clinical setting (Hockel, Schlenger et al. 1996; Fyles, Milosevic et al. 1998). Understanding the physiology of tumour circulation and
the responses of tumour cells to the effects of hypoxia is, therefore, of significant biological and clinical interest.

Molecular events that occur in response to tumour hypoxia (e.g. upregulation of the hypoxia-inducible transcription factor HIF-1α and subsequent stimulation of angiogenesis) can be characterized by analyzing their spatial relation to other parameters that determine tissue oxygenation, such as the presence of blood vessels and perfusion, on a microscopic scale. Histological sections from tumours can be stained with multiple fluorochrome-labelled monoclonal antibodies (MAb), so that tissue structure and function can be analyzed in their spatial context. Hence, to study the complex physiology and biology of solid tumours in sufficient detail and accuracy requires imaging of large areas/volumes with high resolution.

Commercially-available microscopes are suitable for imaging small fields of view (FOV) with resolutions in the sub-micron range. However, since biopsies from human tumours are often >1cm across, they cannot be imaged in a single microscopic FOV. Traditionally, this problem has been solved by using a computer-controlled stage to acquire individual FOVs and then ‘tiling’ these as image arrays to form a composite of the object (see figure 2.1). The tiling process is time consuming and requires accurate tile alignment and flat-fielding to produce an artefact-free image.
The Confocal Scanning Laser MACROscope (CSLM), designed and patented at the University of Waterloo, Ontario, Canada (Dixon and Damaskinos 1995), is a raster-scanning confocal fluorescence imaging system using galvanometric mirrors to scan a focused laser spot across the specimen. The CSLM was initially designed for quality control reflection and optical beam induced current imaging (OBIC) of solar cells and porous silicon (Ribes, Damaskinos et al. 1995; Ribes, Damaskinos et al. 1996). Subsequent modifications included design of a chromatically-corrected objective lens for fluorescence imaging and further adaptation into a cDNA reader (Seto, Damaskinos et al. 1995).

In this initial study the CSLM was used to image triple-labelled sections from a cervical carcinoma xenograft (me180) in order to obtain spatially co-registered images of blood vessels, tissue hypoxia and the expression of HIF-1α. These images were compared to tiled images obtained using a standard epifluorescence microscope equipped with a computerized microscope stage and digital CCD camera.

**Figure 2.1 - Schematic diagram of the generation of a tiled microscopic image.**
2.2.3 Materials and Methods

2.2.3.1 CSLM Overview

The component that distinguishes the MACROscope from its microscopic counterparts is the objective lens. To be able to scan over wide areas and obtain a fluorescence image of a tissue section, an equally wide objective lens must be used. The laser scan lens (LSL) used in this work has a casing of approximately 8 cm in diameter and 15 cm in length, containing 7 large lens elements, making it much larger than conventional microscope objectives. The LSL is a telecentric f-theta scan lens with a numerical aperture (NA) of 0.2. The focused spot diameter is 4 \( \mu \text{m} \) at 1/\( e^2 \), corresponding to a maximum lateral (x-y) resolution of 2 \( \mu \text{m} \), and an axial (z) resolution of 6 \( \mu \text{m} \). By adjusting the angular sweep of a galvanometer scanning mirror, the size of the scan area can be adjusted from 1 x 1 mm to 20 x 20 mm, thus allowing the user to zoom in on areas of interest and scan them at higher resolution.

The property of the LSL that enables the system to achieve its large FOV is that it is an f-theta lens. In microscope objectives that are not designed for f-theta operation, a beam pivoted about the objective’s entrance pupil will be translated by \( (f) \cdot [\tan(\theta)] \) in the focal plane, where \( f \) is the focal length of the objective, and \( \theta \) is the scan angle with respect to the optic axis of the lens (see figure 2.2). However, to scan the small FOV of a microscope objective, \( \theta \) is generally small enough so that \( (f) \cdot [\tan(\theta)] \approx (f) \cdot (\theta) \). Thus a constant scan rate will produce a constant pixel size and sensitivity in the image. The largest scan angle used in the macroscope system is \( \pm 10^\circ \), so the small angle approximation does not hold, and if the LSL did not follow a strict f-theta dependence there would be a significant amount of geometric distortion across the FOV when a fixed pixel acquisition rate is used, as it is with all point-scanning systems.
To raster scan the excitation laser across the specimen, a single mirror placed at the entrance pupil of the LSL and pivoting in both the x and y directions would work ideally. Unfortunately, it is difficult to control the motion of such a mirror accurately (Ribes 1997). A simple solution to this is to use two closely-spaced mirrors, with the LSL entrance pupil located between them. This approximates the single mirror configuration well and is easier to implement.

A schematic diagram of the cDNA macroscope is shown in figure 2.3 and photographs of the actual instrument with the case removed are shown in figure 2.4. There are three excitation wavelengths, currently provided by a Helium-Neon laser (633 nm), a frequency-doubled Nd:YAG laser (532 nm) and an argon-ion laser (488 nm). The laser energy incident on the specimen is $\cong 0.5$ mW for each wavelength. The selected laser beam is expanded and collimated to a diameter of 1 cm by beam expanders just after the lasers (not shown). The light is then transmitted along a common optical path by dichroic beam splitters (DBS), which reflect or transmit light based on wavelength. The excitation beam is directed onto a 70% transmitting, 30% reflecting beam splitter (BS) and then passed through a 2x beam expander (BE) where the full 20 mm diameter collimated beam emerges. The beam then strikes the two scanning mirrors, passes through the LSL and is focused on the sample.
**Figure 2.3** – Schematic setup of the confocal macroscope. M: Mirror, DBS: Dichroic Beam Splitter, SM: Scanning Mirror, BS: Beamsplitter, BE: Beam Expander, SM: Scanning Mirror, FW: Filter Wheel, PMT: Photomultiplier Tube, LSL: Laser Scan Lens.

**Figure 2.4** – Photographs of the cDNA based macroscope with the covers removed and optical components exposed. The positions of the excitation lasers, scanning mirrors, filter wheel, LSL and PMT are indicated. During normal operation the upper portion of the system is closed.
Fluorescence emitted by the sample is collected and re-collimated by the LSL and passed through the main beam splitter. The light passes through a fluorescence emission filter mounted on a filter wheel, which transmits the fluorescence and blocks any unwanted back-reflections. Finally, the beam is focused through a pinhole to produce the confocal effect, then into a photomultiplier tube. The use of a PMT for light detection provides extremely sensitive imaging capabilities with a fast response. The output of the PMT is fed into a frame grabber in a computer, which also controls the motion of the scanning mirrors. The scan rate, scan size and sampling frequency are all selectable by the user through the interface program. For example, if one chooses a 4 x 4 mm area, with a resolution of 2048 x 2048 pixels, the pixel side length would correspond to approximately 2 µm. The pixel dwell time is also selectable by the user and is generally ~ 20 µs/pixel, so a 2048 x 2048 pixel scan can be completed in approximately 160 s.

### 2.2.3.2 Tumour Identification and Sample Preparation

ME180 cervical carcinoma cells, obtained from the American Type Culture Collection, were injected into the gastrocnemius muscle of an 8-9 week old female severe combined immunodeficient (SCID) mouse. After the tumour reached a size of approximately 9 mm in diameter, the nitroimidazole hypoxia marker EF5 (Lord, Harwell et al. 1993) was injected via a lateral tail vein (200 µl of a 10 mM stock solution, to give a total body concentration of 100 µM). Three hours later, the tumour was excised and placed in a vial containing tissue embedding medium (Sakura Finetek, Torrance, CA) and immediately frozen in liquid nitrogen. Whole sections were then cut using a cryostat and fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 5 minutes. All incubations were done at room temperature, except where stated otherwise. Next, the anti-HIF-1α monoclonal antibody (Affinity Bioreagents,
Golden, CO) at 1:600 dilution, was incubated overnight at 4°C, followed by incubation with a donkey-anti-mouse Cy3 conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). Blood vessels were labelled using an anti-CD31 antibody (BD Pharmingen, San Diego, CA) at 1:500 dilution for 1 hour, followed by incubation with a donkey-anti-rat Cy2 conjugated secondary antibody (Jackson). Finally, tissue-bound EF5 was labelled using a 1:120 dilution of the ELK3-51 MAb directly labelled with Cy5 (generously provided by Dr. Koch, University of Pennsylvania) for 1 hour. The sections were washed in PBS 3 times for 3 minutes between labelling steps.

2.2.3.3 Image Acquisition and Processing

Triple-labelled sections from the ME180 cervical carcinoma xenografts were imaged using the CSLM and a standard epifluorescence microscope imaging system. The scan area of the CSLM was set to 8 x 8 mm with a resolution of 2048 x 2048 pixels. Focusing the CSLM was done manually by adjusting the z distance to the LSL. A continuous x-axis scan produced a real time fluorescence emission histogram. The optimal focus was found by minimizing the full width at half maximum (FWHM) of the emission peaks (see figure 2.5). To compensate for the relatively weak HIF-1α signal, the scan rate was slowed from 20 µs/pixel to 30 µs/pixel for Cy3 excitation (532 nm). Cy2, Cy3 and Cy5 fluorescence was collected using bandpass filters centered at 545 ± 45 nm, 605 ± 45 nm and 705 ± 30 nm, respectively.
To compare the quality of images created using the CSLM, the same slides were imaged using a commercial epifluorescence imaging system (MCID 5+, Imaging Research, St. Catharines, ON, Canada). This system consisted of an Olympus BX50 fluorescence microscope linked to a Xillix MicroImager (Xillix Corp, Richmond, BC, Canada) and a motorized stage (Ludl Biopoint). Using a 10x, 0.3 NA apochromat objective lens, sections were imaged and the emitted fluorescence collected using appropriate filter cubes. Image integration times were set to 3 seconds per field and the field of view was 0.78 x 0.78 mm, for a total of 84 tiled images. The image of the vasculature was obtained by exciting the Cy2-tagged anti-CD31 MAb at 480 ± 10 nm: fluorescence emission was collected using a 520 nm longpass filter. HIF-1α expression was imaged by exciting the Cy3-tagged anti-HIF-1α MAb at 535 ± 25 nm, with fluorescence collection using a 610 ± 38 nm bandpass filter. Tissue hypoxia (EF5) was visualized by exciting the Cy5-tagged ELK3-51 MAb at 620 ± 30 nm and detected by a 700 ± 38 nm bandpass filter.

Images captured using the MCID and the CSLM software were exported as 8-bit TIFF files. Processing was done using Adobe PhotoShop 5.0 (Adobe Systems Inc., San Jose, CA). Images obtained using the MCID system were contrast enhanced for presentation. Subsequently, images

Figure 2.5 - Defocused (A) and properly focused (B) line profiles across the x-axis of the CSLM image. Optimal focus is obtained when the FWHM of the peaks is minimized.
from both systems were converted to RGB format and false-coloured. Selected regions of the HIF-1α (Cy3) fluorescence images were converted to binary images to extract nuclear structures from the background. Images were binarized by setting a brightness threshold at a level corresponding to three times the average background, as determined in four corner regions of the image. This was followed by median filtering (filter width set to 2 pixels) to remove non-specific single-pixel fluorescence.

2.2.4 Results and Discussion

Frozen and fixed sections from a ME180 cervical carcinoma xenograft were triple-stained for blood vessels, tissue hypoxia and the transcription factor HIF-1α. Figure 2.6 contains a representative set of images of the vasculature (2.6a), tissue hypoxia (2.6b) and the transcription factor HIF-1α (2.6c), generated using the MCID system. Panels 2.6d-f contain the corresponding images obtained using the CSLM. The MCID software allows for tiling with and without automated image alignment that compensates for microscope stage positioning artifacts. At high resolution, this feature is essential for proper alignment of image features. Without alignment, tiling of the entire specimen took approximately 12 min per fluorophore; with alignment, this was increased to 28 min per fluorophore. Image acquisition on the CSLM took approximately 3 min for Cy2 and Cy5. For Cy3, the pixel dwell time was increased by 50%, resulting in an acquisition time of approximately 4.5 min. Hence, imaging of a typical tumour section, as shown in figure 2.6, was approximately 3-8 times faster using the CSLM compared to the MCID system.
One of the reasons for the choice of imaging tissue hypoxia, blood vessels and a nuclear transcription factor was to compare the resolving ability of the CSLM to that of a standard microscope. The hypoxia marker EF5 is a cell-based stain that labels entire areas within the specimen. On cross-section, blood vessels are essentially structures composed of either a single or multiple cells, while HIF-1α is expressed almost exclusively in the cell nucleus. Visual inspection of the blood vessel images (2.6a and 2.6d) and the images of tissue hypoxia (2.6b and 2.6e) obtained using the CSLM and the MCID systems indicates that images of cellular and tissue-based features are of comparable quality in terms of resolution and contrast. At low

Figure 2.6 - Fluorescence images of the ME180 tumour vasculature (A) & (D), hypoxia (B) & (E) and HIF-1α (C) & (F) generated using the epifluorescence microscope (A-C) and the CSLM (D-F).
magnification, images of the HIF-1α fluorescence (2.6c and 2.6f) obtained with the MCID and the CSLM also appear to be of comparable visual quality (see 2.6c and 2.6f).

To study the sub cellular localization of HIF-1α fluorescence, the CSLM scan area was decreased to 4 x 4 mm, while maintaining the scanning at 2048 x 2048 pixels (figure 2.7c). Figure 2.7a shows the same region in the MCID-generated HIF-1α image, generated using a 10x objective. It is obvious that the MCID image has higher resolution and captures more detail than the CSLM-generated image. This is mainly due to the increased numerical aperture of the MCID

**Figure 2.7 - HIF-1α expression imaged using the epifluorescence microscope (A) and the CSLM (C). (B) and (D): binary images of HIF-1α fluorescence generated by brightness thresholding and median filtering of (A) and (C), respectively.**
objective, as well as smaller pixel size. Further processing of images, with the purpose of obtaining quantitative information, involved generation of binary images by setting a threshold on pixel brightness. The resulting binary images (2.7b and 2.7d) can be analyzed to determine the fraction of HIF-1α positive cells and the spatial HIF-1α expression patterns. Despite the lower spatial resolution, the number and location of HIF-1α positive nuclei in the binary HIF-1α image obtained with the CSLM and MCID systems were similar.

2.2.5 Conclusions

In this study, a modified confocal fluorescence cDNA reader was used to generate images of entire frozen tumour sections labelled with multiple fluorescence markers. The image acquisition speed of the CSLM and its ability to resolve tissue features were compared to that of the MCID epifluorescence microscope imaging system. In the current configuration, the CSLM can generate images up to 8 times faster than the MCID system and resolve features at the size scale of individual cells. Thus, the CSLM is a useful optical instrument for multi-parameter fluorescence imaging of cells and tissues.

The ability to image multiple parameters quickly is important, since it can significantly increase throughput, especially in applications where large areas of tissue sections are analyzed, as in 3-D reconstruction of entire tumours. In the MACROscope three separate excitation wavelengths are available but, at present, there is only one PMT detector, so that individual fluorophores are imaged sequentially. By adding two more PMTs, this system can be converted to allow simultaneous acquisition in three fluorescence channels. This modification would further decrease the acquisition times, so that a triple-stained section could be imaged in 3-4 minutes. By comparison, the MCID system takes approximately 26 minutes to obtain a three-color image.
(without tile alignment, thus allowing for image registration artifacts), and 86 minutes with tile alignment.

The system used here was also limited to exciting a single fluorescent dye for each laser source. A new system currently under development will incorporate multispectral imaging to allow imaging of two or more fluorophores with a single laser source and allow the simultaneous detection of more than 3 parameters in a single section. Two methods were considered for multispectral imaging in the range from 450-750 nm. The first involves the use of a transmission or reflection grating to generate the spectrum of a single pixel on a back-thinned linear CCD array. This configuration would give a spectral resolution of 1-2 nm (CCD pixel size X spectral dispersion of the grating). However, longer pixel integration time (i.e., pixel dwell time) required and slow readout rate of CCDs (as compared with PMTs) make it difficult to accumulate enough signal with the CCD in a reasonable amount of time, thus significantly increasing image acquisition time. A spectral detection design better suited to the point-scanning architecture of these instruments uses a grating and a linear PMT array, which has far fewer elements (32 as opposed to 1024), yet is capable of acquiring a useable signal at readout rates 100x faster than CCD based spectral detection. This setup is expected to retain the image acquisition speed and high sensitivity of a PMT, with a spectral resolution of 10 nm. The costs of implementing these techniques are similar for each method, although PMTs are better suited to the CSLM architecture due to the increased acquisition time that a linear CCD array would require.
2.2.6 Acknowledgements

This work was supported by the National Cancer Institute of Canada, using funds raised by the Terry Fox run. The MACROscope® was funded by the Canadian Institute for Photonics Innovations. The author would like to thank Drs. A.E. Dixon and S. Damaskinos from the University of Waterloo and Biomedical Photometrics for access to the MACROscope® technology and advice.

2.3 Summary & Future Directions

At the conclusion of this study it was determined that the f-theta scanning technology of the macroscope allowed for simplified image acquisition of whole specimens. However, the quality of the images on biological specimens was not optimal when compared to standard, low-power objectives. For this reason, a new prototype incorporating several additions and modifications was designed and constructed to address some of the issues discovered with the modified cDNA system. This new system is built around a new laser scan lens with a higher fluorescence collection NA (0.35 vs 0.2). In addition, the dual axis scanner configuration was replaced with a single scan axis, coupled with a scanning stage. This configuration offers two key advantages over the cDNA system described in this chapter: 1) Scanning about a single axis in the scan lens reduces the effects of off-axis optical aberrations. 2) Use of a stage to translate the specimen under the scanning line produced by the single mirror configuration allows for the entire length of a microscope slide to be scanned. Effectively, this resulted in increasing the field of view of the system, while improving optical quality of images and field flatness. The data acquisition rate was also increased by a factor of 4x over the cDNA based system, enabling rapid image acquisition.
Three new imaging modes were also added to the system, and are discussed in detail in *Chapter 3*. Brightfield and phase contrast imaging modes were added, with the development of a transmitted light 3-channel detector. One major system addition highlighted by the work in Chapter 4 is the hyperspectral fluorescence imaging mode and data processing methods. These new imaging modes are expected to further advance the macroscope technology and could be used to successfully and efficiently simplify biomedical imaging of large tissue sections and specimens.
Chapter 3

3 Prototype Hyperspectral Macroscope Validation and Characterization

3.1 Preface

This chapter contains calibration and validation data on the instrument conceived and constructed at the conclusion of the work presented in Chapter 2. The improvements to the system design and optical performance in this prototype system demonstrated a marked improvement over the modified-cDNA system. The additional imaging modes extended the capabilities of the instrument and allowed a wide range of biomedical specimens to be imaged efficiently in multiple contrast modes on a single platform.

This chapter is presented as published, except for the section numbering and some minor reformatting to be consistent with the other chapters.
3.2 A High Resolution Macroscope with Differential Phase Contrast, Transmitted Light, Confocal Fluorescence and Hyperspectral Capabilities

Paul Constantinou¹, Trudey Nicklee¹, David W. Hedley¹⁻², Savvas Damaskinos³, Brian, C. Wilson¹

¹ Dept. of Medical Physics, Ontario Cancer Institute/University of Toronto, Toronto, Ontario, Canada.
² Dept. of Medical Oncology, Ontario Cancer Institute/University of Toronto, Toronto, Ontario, Canada.
³ Biomedical Photometrics Inc, Waterloo, Ontario, Canada.

3.2.1 Abstract

Recent advances in imaging technology have contributed greatly to biological science. Confocal fluorescence microscopes (CFM) can acquire 2D and 3D images of biological samples such as live or fixed cells and tissues. Specimens that are large (e.g., a 10 x 10 mm tissue section) and overfill the field of view (FOV) of typical microscope objectives require the use of image tiling to cover the entire specimen. This can be time consuming and cause artefacts in the composite image. The MACROscope system (Biomedical Photometrics Inc, Waterloo, Canada) is a confocal device with a 22 x 70 mm FOV, designed for imaging large tissue sections in a single frame. The prototype demonstrated here can obtain images in reflected, transmitted, fluorescence, phase contrast and hyperspectral modes. The new spectral imaging mode is characterized with a series of test targets and sampled spectra are compared to a commercial spectrometer. Fluorescence images of human SiHa tumour xenografts stained with CD31-Cy3, showing blood vessel location, and EF5-Cy5, showing areas of tissue hypoxia, were collected. Differential phase contrast (DPC) images of the same section, as well human epithelial cells were recorded to test the phase contrast mode. Finally, RGB transmitted light images of human tongue were obtained. This new device avoids the need for image tiling and provides simultaneous imaging of multiple fluorescently-labeled, tissue-specific markers in large biological samples. This enables time- and cost-efficient high-throughput screening of (immuno) histopathological samples. This device may also serve in the imaging of high-throughput DNA and tissue arrays.
3.2.2 Introduction

Confocal microscopes have found a permanent home in biological imaging due to advantages over wide-field microscopes in signal-to-noise ratio (SNR), resolution and the ability to reconstruct 3-D representations of specimens (Amos and White 2003). Confocal microscopes employ a pinhole aperture in front of a single element detector. This limits the light reaching the detector to a very thin region centered at the focal plane of the objective (Minsky 1961). Using this confocal effect, 3D images of tissues and live cells can be obtained by moving the sample axially in small increments over a sequence of images. Confocal slicing can also be used to eliminate background signals originating from outside the focal volume, providing an increased Signal to Noise Ratio (SNR) over non-confocal images.

The instrument used in this work is a prototype Open-Frame MACROscope (Dixon and Damaskinos 1995; Ribes 1997) (OFM), designed around a telecentric f-theta Laser Scan Lens (LSL) with a lateral (x-y) spot size of $3.6 \, \mu m \, @ \, 1/e^2$ and a 22 x 70 mm Field Of View (FOV), chosen to match the size of a typical microscope slide (25 x 70 mm). This large field of view facilitates a range of biomedical applications, from histopathology to high-throughput screening. Using laser excitation, this OFM can acquire images in reflected light (RL), transmitted light (TL), fluorescence (FL), hyperspectral (HS) and Differential Phase Contrast (DPC) modes. These modes are also available on many conventional and scanning laser microscopes, but without the large FOV capability of the OFM. To obtain an image of an entire tissue section (100 mm$^2$ or more) with a conventional microscope a tiling process is employed; using a computer-controlled stage to take sequential images of the entire sample, the individual ‘tiles’ then being stitched together (aligned). For imaging histopathological samples that overfill the microscope FOV, the tiling procedure can be cumbersome to set up, and there remains the potential for introducing image artifacts at the seams where tiles are stitched together. The
primary advantage of the OFM is the ability to quickly image an entire sample in a single FOV, which simplifies and speeds up image acquisition (Constantinou, Vukovic et al. 2001).

The hyperspectral imaging mode represents a new imaging modality for a MACROscope system. Here, the hyperspectral system is characterized with a number of test targets, and spectra are compared with those obtained by replacing the OFM spectrometer with a commercial unit (Ocean Optics, Dunedin, FL, USA). In order to demonstrate the capability of the single channel modes, images of SiHa cervical carcinoma tumour xenografts were obtained in fluorescence, transmitted and DPC. Phase contrast images of human cheek epithelial cells were also captured. Finally, 3-color RGB (Red, Green, Blue) transmitted light brightfield images of human tongue stained with Masson’s Trichrome were acquired.

3.2.3 Materials and Methods

3.2.3.1 Open Frame MACROscope System

Arguably, the most important optical component in an imaging system is the objective lens. Since the OFM Laser Scan Lens (LSL) is so different from traditional microscope objectives, it is difficult to select a system for direct comparison. The best match, from previous experience, is to use an image-tiling microscope system equipped with a 10x objective (Constantinou, Vukovic et al. 2001). This selection is made to match the lateral resolution and numerical aperture (NA) of the objective lens in the OFM to the microscope.
Figure 3.1 - Schematic of the OFM. Only the opto-mechanical hardware is shown. Detectors, stages, bandpass filters, laser shutters and a galvanometer are all interfaced to a PC. For DPC in reflected light the detector is placed as shown. For hyperspectral imaging, the mirrors are removed to allow all collected light to enter the spectrometer. The principle of the f-theta operation of the LSL is shown inset.

Figure 3.1 shows a schematic of the OFM system. Excitation is provided from a single, multi-line Ar:Kr laser (Melles Griot, Carlsbad, CA, USA). The main excitation lines used for imaging are at 488, 568 and 647 nm. Laser bandpass filters are used to select the excitation wavelength, and laser power is adjustable from 1-15 mW for 488 and 568 nm, and 0-25 mW for the 647 nm line. Using a single laser eliminates the need to couple multiple sources into the same optical path, as in previous systems. Excitation light is passed through a spatial filter/beam expander combination; the output is a collimated beam 10 mm in diameter. This beam is then reflected off a 30% reflecting/70% transmitting beamsplitter. Next, the beam passes through a 2x beam expander, producing a 20 mm beam. The beam is then deflected by a galvanometer scanning mirror into the LSL that focuses the beam onto the sample. The sample is placed on a stage,
which moves along the y-axis. Fluorescence and reflected light are collected by the LSL, de-scanned by the galvanometric mirror and passed through the confocal pinhole sub-assembly.

Previous MACROscope versions, such as the system used for the work in Chapter 2, used a pinhole in front of each single channel PMT (only one PMT is used in this system, although simultaneous acquisition from 3 PMTs is possible with the appropriate additional hardware). Furthermore, the OFM spectrometer requires a collimated light input with many additional optical elements and potential for misalignment if each detection channel has its own pinhole. The opto-mechanical system configuration is made simpler by the use of a single pinhole for all detectors. For this reason, the confocal pinhole sub-assembly consists of a 2x beam expander with a 50 µm pinhole at the focus of the lenses. This setup spatially filters the detected light, while maintaining a collimated beam for input to the spectrometer. For hyperspectral imaging, the removable mirrors are detached from their mounts and collected light is allowed to pass through to the OFM spectrometer, which consists of a concave grating used to disperse a 213 nm band of wavelengths across a 32 element PMT.

For transmitted light (TL) and DPC imaging modes, the detection optics lie below the sample stage. A Fresnel lens is used to focus the transmitted light onto the detectors. A confocal pinhole is not used in the TL modes. In TL, image quality degrades slightly from the theoretical lateral resolution of the LSL, due to the use of the Fresnel lens and the small active area of the detector. Fresnel lenses are essentially concentric prisms, making them thinner than a convex lens of the same focal length, but with increased light collection and reduced resolution. More importantly, Fresnel lenses have f-theta properties. This means that a beam rotated through an angle \( \theta \) at the entrance pupil of the lens will be displaced a lateral distance \( f \cdot \theta \) in the focal plane (figure 3.1 inset). Since the LSL is an f-theta lens, another f-theta lens must be used to de-scan
the beam. The image resolution is reduced slightly, but only at 1 μm pixel resolution. For many transmission imaging applications, a 2 μm pixel resolution is adequate.

The LSL used here has a lateral (x-y) spot size of 2 μm, and the FWHM along the z-axis is 20 μm with the 50 μm pinhole. A smaller pinhole would allow a smaller optical slice thickness, but at the expense of signal. The NA is 0.20 in transmitted, reflected and DPC modes. The collection NA for fluorescence and hyperspectral mode is 0.35.

3.2.3.2 Fluorescence Imaging

The section imaged here was obtained by the following method: SiHa cervical carcinoma cells (American Type Culture Collection, Manassas, VA, USA), were injected into the hind leg of a 6 week old female severe combined immunodeficient (SCID) mouse. After the tumour reached approximately 9 mm diameter, it was excised, cubed and placed in a vial containing Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) and immediately snap frozen in liquid nitrogen. 3 h prior to sacrifice the mouse was injected with the hypoxia probe EF 5. Using a Leica CM3050S Cryostat, a 5 μm thick section was cut from this block. The section was fixed for 10 min in 2% methanol-free formaldehyde. It was then rinsed 3 times with phosphate buffered saline (PBS), incubated for 1 h with 1/500 purified anti-mouse CD31 (Pharmingen, San Diego, CA, USA), rinsed 3 times with PBS and incubated 1 h with 1/100 goat anti-rat IgG-Cy3 (Jackson ImmunoResearch, West Grove, PA, USA). Following 3 more rinses with PBS, the slide was incubated with 1/50 EF5 directly conjugated to Cy5 (kindly provided by Dr. Cameron Koch, University of Pennsylvania) for 1 h. Again, after rinsing 3 times with PBS, the stained section was imaged with the OFM.
Phase contrast imaging can be used to image stained or unstained transparent samples. It was first described by Frits Zernike (Zernike 1942; Zernike 1942). Contrast is generated by measuring minute phase variations in light transmitted through the sample. These can be the result of variations in specimen thickness ($\Delta L$) and/or index of refraction ($\Delta n$); commonly referred to as optical path differences. Since the tissue section imaged here was cut to a constant (~5 µm) thickness, $\Delta L \approx 0$. Thus, the change in index of refraction ($\Delta n$) between cellular components is largely responsible for the contrast generated through DPC. Normally, unstained tissue sections provide little contrast when viewed under bright-field illumination. These tissues must be stained with fluorescent dyes, antibody tagged fluorophores or other chromogenic stains to obtain contrast. In DPC the contrast is intrinsic to the specimen, so that tissues or live cells can be imaged without the need to introduce extrinsic contrast agents or to fix the specimen.

3.2.3.3 Differential Phase Contrast (DPC) Imaging

The method of DPC image formation in the OFM is slightly different from traditional DIC (Differential Interference Contrast) microscopy. In the OFM, DPC images can be obtained in reflected and transmitted modes using a split-detector photodiode (Amos, Reichelt et al. 2003). In transmitted mode, a single laser source is passed through the specimen to a detector placed underneath the sample stage. A Fresnel lens is used to focus the light onto the detector as the image is scanned (figure 3.2a). The transmitted beam is centered on the detector (figure 3.2b). As the beam is scanned across the sample, minute variations in index of refraction ($\Delta n$) cause the centered beam to ‘wobble’ in the detector plane. The detector is split into four quadrants, with three discrete outputs. One output is from the sum of all quadrants, the other two are differential outputs performing the arithmetic: 1. Left minus Right and 2. Top minus Bottom. These
differential outputs give a measure of how much the beam is wobbling in the detector plane. Combining the L–R and T–B images into a single image produces the final phase contrast image and the Sum channel provides a transmitted light image. In the final images, the T-B and L-R images are combined into a single image (executed as a post-processing step in Adobe Photoshop). In reflected mode, the same detector is placed as shown in figure 3.1 and the same imaging principle applies to reflected and transmitted modes.

![Figure 3.2](image)

**Figure 3.2 – (A)** Schematic of detector and lens system for OFM DPC transmission mode. The Fresnel lens acts as an f-theta lens, bringing the laterally scanning beam to a stationary pivoting beam at the detector plane. **(B)** DPC image formation. Left; beam centered on detector, differential outputs read zero. Right; beam translated on detector due to $\Delta n$ within the sample causing refraction of the transmitted beam.

This method of imaging can provide gross morphological information on thin, transparent specimens. DPC can complement fluorescence images by locating cells in a tissue section; images of nuclei can then be overlaid with an image of tissue specific markers obtained in fluorescence. Setting up the DPC detector involves placing an empty slide in the sample holder and positioning the Fresnel lens such that the beam remains centered on the detector as the laser is scanned across the FOV. A bias of roughly 32,000 greyscale values is added to the two differential outputs, since the L-R and T-B outputs can be either positive or negative. DPC images were obtained with 568 nm light, but the other laser lines could also have been used.
3.2.3.4 Brightfield Transmitted Light Imaging

Histological stains such as H & E (Hematoxylin & Eosin) are used routinely to evaluate tissue morphology. These samples would normally be imaged in a brightfield microscope by tiling individual fields together. The OFM uses laser excitation, which complicates the image acquisition, since there is no broadband laser source easily available. However, it is possible to obtain a pseudo-brightfield image in transmission mode with the OFM, since the 3 lasers used constitute an RGB (Red, Green, Blue) light source. For these images, a custom-made detector mount (figure 3.3) is placed underneath the sample.

![Figure 3.3 - Schematic for the brightfield RGB detector.](image)

**Figure 3.3** - Schematic for the brightfield RGB detector. Light transmitted through the sample is split by the dichroics and directed onto the detectors. Each detector is read out, forming 3 transmitted light images that are combined digitally into the final RGB image. Two field points are shown to illustrate the beam path as it scans across the sample.

A Fresnel lens is used to de-scan the transmitted light onto the detectors, and beam splitters are used to direct the light from each laser to the respective detector (figure 3.3). The output from each detector is maximized to roughly 60,000 greyscale units, giving three images; transmitted red, green and blue light. When overlaid, the individual red, green and blue images form the
colour white in regions where no absorption of the transmitted light has occurred (i.e., regions without a specimen), while the differential absorption of the transmitted light within the specimen combine to form a true colour image of the sample. These RGB images are combined into a final brightfield image using Photoshop. Future enhancements to the system control software will incorporate direct overlay of the RGB channels during acquisition, eliminating the need for third-party software to merge the individual image channels into a brightfield image.

3.2.3.5 Hyperspectral Apparatus

Fluorescence microscopy has proved an invaluable tool in biological research over the past few decades. One of the limitations of fluorescence images is that they are formed as a single channel image; the collected fluorescence from the objective lens is passed through a set of optical filters that allows a discrete range of wavelengths to be ‘seen’ by the monochromatic detector. This poses a problem when multiple fluorescent species are present within the sample, e.g. two fluorophores with closely spaced (< 20nm) emission peaks. The ability to visualize multiple specific labels in an image allows tracking/monitoring of specific populations or types of cells and their micro-environments (Schultz, Nielsen et al. 2001; Zimmermann, Rietdorf et al. 2003; Ecker, de Martin et al. 2004). This information is essential to further understanding of the complex nature of biological processes.

The goal in spectral imaging is to record the emission spectrum for each image pixel, rather than combining all the light into a single channel. The resulting data set is typically referred to as a hyperspectral data cube, with two axes representing spatial dimensions, and the third axis being the spectral intensity at each pixel. There are a number of methods by which hyperspectral data can be obtained; in wide field instruments, a tuneable liquid or acousto-optic filter placed in front
of a CCD detector allows individual images of very small ($\Delta \lambda = 5$ nm) bandwidths to be acquired (Gebhart, Lin et al. 2003; Gupta and Voloshinov 2004). In point- and line-scanning instruments, a grating or prism can be used to disperse the light along a 1D or 2D detector array (Huebschman, Schultz et al. 2002). The individual detector elements then correspond to specific wavelength ranges, determined by the grating and method of focusing onto the detector active area.

The smallest pixel size selectable with the current LSL is $1 \mu$m. Thus, a 10 x 10 mm area requires $10^8$ pixels. As a result, one of the main design considerations was to maximize the pixel acquisition rate in hyperspectral mode. A grating/CCD based spectrometer is not practical in this case, since the readout rate of the CCD would be a major limiting factor. In single-channel mode, the OFM acquires data at 5 $\mu$s/pixel. From previous experience a CCD-based spectrometer requires a 20-500 ms integration time to detect typical fluorescence intensities seen in the OFM. This $\sim 10^3$-5 increase in single pixel detection time means that a hyperspectral image would require impractical scan times. A detector capable of sampling much faster is required.

The fastest detector available at the time of the system design was a 32 element linear PMT array (Hamamatsu, Bridgewater, NJ, USA). This allows all 32 channels to be read out in 200 $\mu$s, so that, while spectral imaging times still increase over steady state fluorescence images, they are realistic for proof-of-principle studies.

The spectrometer designed and implemented into the MACROscope is based on a concave reflectance grating (Richardson Grating Labs, Rochester, NY), producing a linear dispersion of 6.7 nm/mm at its focus, as shown in figure 3.4. The linear PMT array is placed at the plane of focus to detect the diffracted light. Each of its 32 channels is 1 mm in width, so that a total bandpass of $\sim 215$ nm is available, with 6.7 nm/channel spacing. The range of wavelengths
sampled can be adjusted by rotating the grating, so that the entire visual spectrum can be sampled if desired. The PMT photocathode will detect light up to 900 nm.

![Schematic of the spectrometer designed for the OFM. The PMT lies directly over incoming beam, with ~7° vertical tilt. The grating can be rotated to allow sampling of the visible spectrum at 7 nm/channel over a 225 nm bandwidth.](image)

**Figure 3.4** - Schematic of the spectrometer designed for the OFM. The PMT lies directly over incoming beam, with ~7° vertical tilt. The grating can be rotated to allow sampling of the visible spectrum at 7 nm/channel over a 225 nm bandwidth.

Image acquisition is performed with MACROview control software, modified to accept input from the 32 element PMT through a USB 1.0 readout interface. This readout is the limiting factor in data acquisition. With custom readout electronics, it would be possible to sample much faster than 200 μs per spectrum. Before a spectral scan, the FOV is selected and the channels to display as the image is acquired are also chosen. Using this method, the resulting displayed image can be made to simulate a single channel image passed through optical filters. Further data processing is done off line, after the data cube has been saved.
3.2.3.6 Hyperspectral Tests and Characterization

Confocal spectral imaging systems are sensitive to misalignments, even with two microscopes from the same manufacturer (Lerner and Zucker 2004). It is, therefore, necessary to use a series of test targets to characterize and track changes in the OFM hyperspectral mode performance. For this purpose, light collected and passed through the confocal pinhole sub-assembly was passed into a commercial fiber-optic spectrometer (Ocean Optics, Dunedin, FL, USA). This setup will see the same light distribution as the OFM spectrometer, and any differences between the recorded spectra should then be the result of the difference in system spectral responses. The Ocean Optics spectrometer was not integrated with the MACROscope data acquisition; rather MACROview software was used to control opto-mechanical hardware only. OOIBase32 spectrometer operating software was used to collect these data. For this reason, only point spectra could be compared and the spectral imaging performances could not be compared.

To ensure that the same spectral signature (ie, spatial location) was being measured using each detection scheme, a series of test specimens with a uniform intensity and large fluorescent features were selected to compare the spectral response from the OFM and Ocean Optics spectrometers. Two fluorescent plastic calibration slides from Applied Precision Life Products (Issaquah, WA, USA) and two from Chroma Technology (Rockingham, VT, USA) were sampled with both spectrometers. A slide of 15.5 μm fluorescent rainbow microspheres (Spherotech Inc, Libertyville, IL, USA) was sampled with both spectrometers. Four different colors of fluorescent highlighters were used to mark standard glass microscope slides and were also sampled. As a biological specimen, a section of unstained, paraffin-embedded, formalin-fixed mouse heart tissue was imaged on the OFM spectrometer alone.
One attractive feature of hyperspectral imaging is the potential to separate distinct fluorescence signals; for example, to subtract background signal such as autofluorescence from glass in a hyperspectral microarray reader (Sinclair, Timlin et al. 2004). In single-channel fluorescence tissue imaging, autofluorescence background can inhibit detection of a weak fluorescence signal. Capturing the entire spectrum at each image pixel should allow separation of the fluorescence and autofluorescence signals, providing increased SNR in the resulting images. Before imaging a stained specimen, an unstained tissue section with relatively bright autofluorescence was imaged. Chapter 4 will focus on the unmixing/subtraction of this autofluorescence background from a variety of stained specimens with varying levels of tissue autofluorescence.

3.2.4 Data Acquisition

3.2.4.1 Fluorescence Images

CD31-Cy3 and EF5-Cy5 fluorescence were collected using bandpass filters (Omega Optical) centered at 600 ± 25 nm and 700 ± 35 nm, respectively. Excitation was provided by the green and red laser lines of the Ar:Kr laser. For the SiHa section, the scan area was set to 9 x 7 mm and image acquisition took 2 min at 2 µm/pixel. The depth-of-focus of this particular setup was ~10 µm, corresponding to the thickness of the tissue sections. Using a SR570 current-to-voltage amplifier (Stanford Research Systems, Sunnyvale CA, USA), the gain of each detection channel was set independently to maximize the signal dynamic range over 16 bits (65,536 greyscale values). Images were saved as 8-bit uncompressed tiff files to minimize the final image size. These greyscale images were then false coloured (red for Ef5-Cy5, green for CD31-Cy3) and overlaid in Adobe Photoshop.
3.2.4.2 DPC Images

The DPC images were taken here to complement the fluorescence images of the SiHa xenograft and to assess imaging of an unstained specimen. Any fluorescence staining is subject to a degree of non-specific background, so it is useful to obtain morphological information via DPC imaging in order to help discriminate the ‘true’ and ‘background’ fluorescence signals. Brightfield imaging can also be used to obtain this information, but requires chromogenic staining. Similarly, it also may be necessary to distinguish between cells in tumour and adjacent normal tissue.

In addition to the SiHa xenograft, a slide of human cheek epithelial cells was imaged. These cells represent a sensitive test of the DPC imaging capabilities of the split detector scheme. They are approximately 50 µm in diameter and are fairly transparent under brightfield illumination. Cells were obtained by soft abrasion of the inner cheek with a toothpick; the cells were then transferred to a glass slide and cover-slipped before imaging. A 10 x 10 mm image was acquired at 2 µm/pixel, with a 1 x 1 mm sub-section shown below. For the SiHa section, the scan was set to 9 x 7 mm at 2 µm/pixel. Image acquisition time was 2 min. For the cheek cells, a smaller field of 4 x 4 mm was imaged; acquisition time was 20 s at 2 µm/pixel.

3.2.4.3 Brightfield Images

The section imaged was a sample of human tongue stained with Masson’s Trichrome. This sample was chosen due to the bright contrast observed between muscle and collagen under traditional brightfield illumination. Masson’s Trichrome is a 3-color stain; nuclei stain black, cytoplasm and muscle stain red and collagen stains blue. It is used routinely to differentiate between muscle and collagen in tumours. For each sample, the signal strength at the detector
was maximized for light passing straight through the glass slide. By adjusting the amplification factor on the current-to-voltage amplifier until the maximum intensity was roughly 60,000 greyscale values (~16 bits), the signal dynamic range was maximized. This was performed once at the beginning of the imaging process and the same laser intensity and detector gain were then used for all images. In order to save disk space and make the large images easier to handle, the RGB channel images were saved as 8-bit uncompressed greyscale tiff files for combination in Adobe Photoshop. Before combination, the images were scaled to an average greyscale value of 255 for regions where light passes through the glass slide alone, and then each image was specified as the red, green and blue channels of an RGB image and saved as a 24-bit color tiff. The tongue section imaged was 7 x 15 mm, the pixel resolution was set to 2 µm and image acquisition took less than 3 min.

3.2.4.4 Spectral Data and Imaging

In the system tests, it was noticed that adjacent PMT anodes did not have equal sensitivities, so that a correction was derived to compensate for this. The correction factors used for each channel were obtained by passing a 568 nm laser line through a cylindrical optic placed in front of the multi-anode PMT. This produced a 50 mm line of uniform intensity, which was aligned perpendicular to the 32 channels. The average of 1,000 samples from each of the 32 channels was then normalized to create channel specific correction factors. These correction factors are divided into the channel counts from the hyperspectral data set.

All spectra and images were recorded using 488 nm excitation. A 488 nm holographic super-notch filter was available to eliminate detection of backscattered excitation, but the Ar:Kr laser also outputs a low intensity (< 5 mW) 483 nm line that passes through this filter. Hence, a 515
nm longpass filter was used instead to block the excitation light from entering the spectrometer. The same filter was used with the Ocean Optics spectrometer. Both the multi-anode PMT and Ocean Optics CCD operate at 12 bit resolution. For the PMT spectra, the gain of the detector was adjusted so that the fluorescence peak was roughly 4,000 greyscale units (~12 bits). For the Ocean Optics spectrometer, the CCD integration time was varied until approximately the same greyscale peak was reached.

With the fluorescent plastic slides the OFM hyperspectral mode captured spectra at 200 µs/spectrum, while the integration time for the Ocean Optics spectrometer varied from ~25 to 500 ms, with 500 ms required for the red fluorescent slides. The fluorescent marker spectra were obtained by marking a standard glass microscope slide with a different color of fluorescent highlighter, and exciting with the 488 nm line from the excitation laser. Integration times were similar to those used for the plastic slides, with the OFM spectra acquired roughly 100 times faster than with the Ocean Optics spectrometer. For the slide of fluorescent beads, a 5 x 5 mm field was imaged in fluorescence mode, with a 900 x 900 µm region selected for a spectral scan. The imaging time for this field, at 2 µm/pixel in spectral mode, was less than 2 min. The Ocean Optics spectrum for these beads was obtained by slowly scanning the slide under a stationary beam until a bead was found; 11 spectra were then averaged at 60 ms/spectrum. The tissue sample used here was an unstained section of mouse heart tissue with a moderate amount of autofluorescence when viewed under a standard epifluorescence microscope. The image FOV was 7 x 10 mm, with a pixel resolution of 2 µm.
3.2.5 Results and Discussion

3.2.5.1 Imaging Speed Breakdown

A summary of the imaging parameters for each of the OFM modes is listed in table 3.1. The difference in imaging time between hyperspectral and the other modes is somewhat misleading. The hyperspectral mode captures data from 32 channels at every pixel, so that 32 images are actually produced in a spectral scan. The hyperspectral imaging time is still too long; custom-designed readout electronics will be required to go faster. There is currently enough signal to go significantly faster than the current 200 µs/pixel with the multi-anode PMT, but custom-built readout electronics, based on a BUS faster than USB 1.0, would be required for this. The OFM spectrometer PMT is similar to the detector used in the Zeiss LSM 510 META confocal microscopes, a commercial hyperspectral microscope. From previous experience with one of these systems, acquisition times of < 2 µs/pixel can be used for bright samples. It is, therefore, reasonable to ultimately achieve hyperspectral macroscopic images at the same speeds as fluorescence, DPC and TL imaging.

<table>
<thead>
<tr>
<th>Imaging Mode</th>
<th>Resolution (µm)</th>
<th>Numerical Aperture (NA)</th>
<th>Pixel Acquisition Rate (µs/pixel)</th>
<th>ΔT for 10 x 10 mm (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>1</td>
<td>0.35</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>TL-DPC</td>
<td>2</td>
<td>~0.14</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>TL-RGB</td>
<td>2</td>
<td>~0.14</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>RL-DPC</td>
<td>1</td>
<td>0.20</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>HSI</td>
<td>2</td>
<td>0.35</td>
<td>200</td>
<td>~80</td>
</tr>
</tbody>
</table>

Table 3.1 – Image speed breakdown of the HSM. Resolution depends on the NA of a given mode, and higher resolution scans require longer acquisition. Readout of the HSI mode is limited by the USB interface used but can be sped up with appropriate hardware.
3.2.5.2 Differential Phase Contrast & Fluorescence Images

The phase contrast images obtained can be seen in figure 3.5a-d. Figure 3.5a shows a small field (1.5x1.5 mm) from the SiHa section in fluorescence mode, false coloured to show red as tissue hypoxia and green as blood vessel location. Tissue hypoxia is a condition existing in tissues that are poorly perfused with blood. Studying the spatial relationship between blood vessel location and regions of hypoxia allows a better understanding of tumour biology and patient outcome (Moreno-Merlo, Nicklee et al. 1999; Hedley, Nicklee et al. 2005). Image 3.5b is the same field taken through a 10x 0.5 NA DPC Nikon microscope objective. Image 3.5b is the result of tiling 14 individual fields with the Nikon objective. Images 3.5c and 3.5d are from the DPC transmitted and reflected OFM modes, respectively. The 14 fields for the 1.5 mm x 1.5 mm Nikon tiled image were acquired in roughly 3 min, while the imaging time for the entire section (9x7 mm) in the OFM DPC mode was 2 min. The entire section in reflected DPC mode is shown in figure 3.6. The imaging time for the small field shown in the OFM was 5s, although these are simply cropped versions of the full section image.
Figure 3.5 – (A) Two channel fluorescence image of SiHa section. Red (Cy5-EF5) indicates regions of tissue hypoxia, and green (Cy3-CD31) denotes blood vessel location. Autofluorescence from collagen fibers is also seen in the green channel. Field size is 1.5 mm x 1.5 mm. (B) Nikon 10x, 0.5 NA DPC tiled image of SiHa section. This image is the result of 14 individual image ‘tiles’ which were combined into the final mosaic. Total image acquisition took 3 min. (C) Transmitted OFM DPC. The NA of the LSL is reduced due to detector overfilling. (D) Reflected OFM DPC. A 2x beam reduction is used in reflected mode to allow all the light to hit the detector active area. The resulting image looks much closer to the one obtained with the 0.5 NA Nikon tiling system.
Image 3.5d gives a closer representation of the Nikon DPC image than 3.5c. The reason for this is that the transmitted DPC mode is operating at less than the 0.2 NA of the LSL. The Fresnel lens used has a focal length of 38 mm, while the LSL has an effective focal length of 50 mm. This gives a magnification ratio of 0.76, so that the 20 mm diameter collimated beam input to the LSL entrance pupil is reduced to 14.4 mm at the focus of the Fresnel lens. The split photodiode detector is 10 mm across, so that only about 60% of the LSL NA is being used.

In reflected DPC mode, a beam reduction of 2x is introduced before the beam strikes the detector. This allows the full 0.2 NA of the LSL to be used. For transmitted mode, a beam reduction is not possible because the Fresnel lens only translates the laterally scanning beam into a pivoting beam. In order to reduce the beam size, a second galvanometer scanning mirror (synchronized with the imaging scanning mirror) would be required to translate the pivoting
motion back to a fully stationary beam that would pass through a beam-reducing telescope. A much better solution is to simply use a detector with a larger active area for the transmitted mode. It should also be noted that the Nikon 10x DPC objective operates at 0.5 NA, which is 2.5 times the NA of the LSL in TL mode. This difference is largely responsible for the greater resolution and contrast in image 5b compared to 5d.

Figure 3.7 shows an image of the human cheek epithelial cells in MACRO-DPC mode. These cells provided a sensitive test of the DPC imaging mode, since they resemble a common form of cell that are transparent in transmitted light microscopes. Figure 3.7 represents a field of view of 1 x 1 mm. These cells are roughly 50 µm in diameter; pixel resolution is 2 µm. The high index of refraction in the nuclei produces a sharp contrast in these images. The darker features on cells
in the lower right portion of figure 3.7 are the result of the confocal slicing of the OFM: the confocal optical slice thickness due to the confocal pinhole is rejecting some of the backreflected light in the lower right portion of figure 3.7, which causes the cells in this region to appear dark, when in fact they simply lie at a different z-position relative to the cells in the centre of the FOV. In transmitted mode, the contrast and resolution is slightly worse, due to the reduced NA of the transmitted DPC mode.

3.2.5.3 Brightfield Images

The 3-color images shown in figure 3.8 represent the final processed and combined images from the tongue section. The images give an encouraging demonstration of the OFM’s new ability for brightfield imaging. The reproduction of the red and blue contrast between smooth muscle and collagen can be seen in each image, and compares well with what is seen in a white light transmission microscope. Image 3.8b shows a zoomed-in view, measuring approximately 1 x 2 mm. This is one of the papillae. Three layers are apparent from the differential red staining; the dark red outer layer is keratinized epithelium, the inner layer has slightly less keratin (less red staining) and the collagen-rich area in blue. Figure 3.8c shows another section with collagen and smooth muscle staining in a 2 x 2 mm area. The brightfield mode can be used to complement fluorescence imaging with serial H & E section images, without the need to use image tiling, or multiple systems to capture the images.
3.2.5.4 Hyperspectral Results

Figures 3.9 and 3.10 show a summary of spectra obtained with the Ocean Optics and OFM hyperspectral mode with the fluorescent slides and fluorescent markers, respectively. In figure 3.9, fluorescence emission from four different fluorescent plastic slides is shown. The spectra...
from the OFM hyperspectral mode match well with those from the Ocean Optics system, with significantly faster integration times (200 µs compared to 20-500 ms). Similar results were obtained with the fluorescent marker spectra, shown in figure 3.10. The distortion of the red fluorescence spectrum in figures 3.9 and 3.10 is likely due to the gain of the PMT being saturated. The red highlighter and plastic slides did not fluoresce as intensely as the other samples, because 488 nm light was used to excite them. The optimal excitation wavelength is closer to 550 nm, but would have required a different filter set to block excitation light from entering the spectrometer. Hence, a high gain setting was applied to the PMT and the 488 nm line was used for excitation. It is possible that the channel sensitivity correction factors derived for the PMT were not valid at this high gain, which caused the distortion seen in the red slide and marker spectra.

**Figure 3.9** – Fluorescent slide spectra. The Ocean Optics data is represented by solid lines, dots are data from OFM hyperspectral mode. All data sets are averages of 11 spectra. Integration time was 200 µs/spectrum for OFM and 20-500 ms for the Ocean Optics spectrometer.
Figure 3.11 shows an image of a 900 x 900 µm FOV from a slide of 15.5 µm diameter fluorescent rainbow beads. These beads are filled with a mixture of fluorophores and can be used to test the fluorescence image resolution, as well as the spectral response. The pixel resolution in this image is 2 µm. The closely spaced beads are clearly visible as distinct objects; although this was expected due to their large size relative to the LSL focused spot size. Figure 3.12 shows the bead emission spectra from both the OFM hyperspectral mode and Ocean Optics spectrometer. The fluorescence emission profiles of the beads match. The data sheet supplied with the microspheres indicates that there are two dyes which are excitable by 488 nm light: Spherotech refers to these dyes as Yellow and Nile Red. The Yellow emission is almost fully cut off by the 515 nm longpass filter, however, with a peak emission of 495 nm. Nile Red has a peak emission at 570 nm with a shoulder at 590 nm, and both of these features can be seen in the Ocean Optics and OFM data in figure 3.12.
As mentioned above, one of the reasons for designing a hyperspectral capability in the OFM was to enable spectral separation of tissue autofluorescence from fluorescence due to histochemical stains and dyes. This would benefit pathologists who put great effort into controlling background tissue autofluorescence during tissue processing (Baschong, Suetterlin et al. 2001). Care must still be taken to minimize autofluorescence during processing; even with current techniques it is not possible to prepare a tissue section with zero autofluorescence while maintaining cell and tissue morphology.

Figure 3.11 - 900 x 900 µm field showing 15.5 mm diameter fluorescent rainbow particles. Excitation was at 488 nm.
Figure 3.12 - Emission spectra from a fluorescent bead in figure 3.11. Ocean Optics spectrum is the solid line, dots are the OFM data.

Figure 3.13 shows the unstained mouse heart tissue section that was imaged spectrally. The combined intensity from the 540-620 nm channels was calculated at each pixel, and the resulting image scaled down to an 8-bit greyscale. In a stained specimen, the emission of the fluorophores is ideally much higher than autofluorescence but, when the fluorescence signal is weak, the autofluorescence can overwhelm the fluorophore signal. The strong signal in the center of the image is due to autofluorescence from residual red blood cells within a vessel, not to tissue autofluorescence. The left and right ventricles are on the right hand side of the image.
The image in figure 3.13 took just over 1 h to acquire, which is long considering that the image size was only 7 x 10 mm. The limitation on the readout rate in hyperspectral mode is purely a hardware issue, as described earlier. However, as imaging speeds approach 5-10 µs/pixel, one
has to begin considering another issue: that of data storage. Generating hyperspectral cubes of large tissue sections at high resolution with 32 channels, each with 12 bits of resolution can lead to very large data sets. For example, a 22 x 70 mm slide at 2 µm/pixel would require $3.85 \times 10^8$ pixels. For each pixel, 32 values (each 12 bits in size) need to be stored, so the size of this hyperspectral cube would be $1.48 \times 10^{11}$ bits, or roughly 150 Gigabytes (GB). This is an extreme example; rarely will all 32 channels contain useful information over the entire image and rarely will the entire slide area need to be imaged at 2 µm. Nevertheless, hyperspectral data cubes will still be in the range of 1-5 GB for most whole tissue sections and this poses challenges in data handling for multiple image files. A more practical approach, especially with the slow hyperspectral OFM readout, may be to use the fluorescence, phase contrast or transmitted light OFM modes to select a region of interest, which would then be scanned spectrally.

3.2.6 Conclusions

We have demonstrated a confocal imaging system with high speed acquisition of FL, DPC and TL images for large tissue sections. Hyperspectral acquisition is limited by the readout rate of the USB 1.0 electronics. However, as a proof-of-principle, the system meets the performance of a much slower CCD-based commercial spectrometer. The transmitted RGB imaging mode produces images that look comparable to white light tiled images. With appropriate hardware and software updates to the MACROview control software, it is now possible to overlay the RGB channels simultaneously while an image is being acquired. This allows direct viewing of the brightfield image as it is captured.
3.2.7 Acknowledgements

This work was supported by the Ontario Research and Development Challenge Fund, in partnership with Biomedical Photometrics Inc. Core equipment was acquired with support from the Canadian Foundation for Innovation, the Canadian Institute of for Photonics Innovation, and the Princess Margaret Hospital Foundation. Access to the Zeiss LSM META system was provided by the Advanced Optical Microscopy Facility (AOMF) at the Ontario Cancer Institute.

3.3 Summary and Future Directions

This work concluded with the successful demonstration of the transmitted light and spectral imaging modes of the prototype. After the completion of the validation experiments, a spectral processing solution was implemented by the author. A discussion of the linear unmixing mathematics used in this process is presented in Appendix 1. Preliminary unmixing validation work using two different software packages can be found in Appendices 2-4, while Appendix 5 contains unpublished results exploring the origins of tissue autofluorescence using the hyperspectral imaging mode. The work presented in Chapter 4 presents the results of a study designed to quantitatively demonstrate the successful removal of tissue autofluorescence using this instrument.
Chapter 4

4 Removal of Autofluorescence from Whole Tissues with Spectral Unmixing

4.1 Preface

This chapter extends the prior work by incorporating full spectral unmixing to the capabilities of the prototype presented in Chapter 3 and focuses on the fluorescence spectral capabilities of the hyperspectral MACROscope. This work has been accepted for publication in the Journal of Microscopy, but has not been published as of submission of this thesis. The studies were designed to compare the detection limit of single-channel (traditional) fluorescence imaging, with that of hyperspectral imaging in sections stained for clinically-relevant antigens. Further information on the mathematics of spectral unmixing can be found in Appendix 1, and additional examples of spectral imaging using the HSM are presented in Appendix 2-5.
4.2 Extending Immunofluorescence Detection Limits in Whole Paraffin Embedded Formalin Fixed Tissues using Hyperspectral Confocal Fluorescence Imaging

Paul Constantinou¹, Ralph S. DaCosta¹, Brian C. Wilson¹

¹ Department of Medical Physics, Ontario Cancer Institute/University of Toronto, Toronto, Ontario, Canada.

Submitted to Journal of Microscopy, August 2008, Accepted January 2009
4.2.1 Abstract

A major problem in microscopic imaging of ex vivo tissue sections stained with fluorescent agents (e.g., antibodies, peptides, etc.) is the confounding presence of background tissue autofluorescence. Autofluorescence limits i) the accuracy of differentiating background signals from single and multiple fluorescence labels and ii) reliable quantification of fluorescent signals. Advanced techniques such as hyperspectral imaging and spectral unmixing can be applied to essentially remove this autofluorescent signal contribution and this work attempts to quantify the effectiveness of autofluorescence spectral unmixing in a tumour xenograft model. Whole-specimen single channel fluorescence images were acquired using excitation wavelengths of 488 nm (producing high autofluorescence), and 568 nm (producing negligible autofluorescence). These single channel data sets are quantified against hyperspectral images acquired at 488 nm using a prototype whole-slide hyperspectral fluorescence scanner developed in our facility. The development and further refinement of this instrument will improve the quantification of weak fluorescent signals in fluorescence microscopy studies of ex vivo tissues in both preclinical and clinical applications.

4.2.2 Introduction

High resolution fluorescence microscopy of ex vivo tissue samples obtained from biopsy and/or surgical resection is of great clinical importance since it yields diagnostically useful information at the tissue, cell and molecular levels. However, in both clinical and preclinical investigations major problems exist in accurately differentiating background autofluorescence (AF) signals from fluorescent label(s), and reliably quantifying these fluorescent signals. A further challenge often faced in microscopy is the large size of typical specimens relative to the field of view
(FOV) of typical microscopes. Traditional microscope objectives generally have a FOV on the order of 0.5-1 mm², while tissue samples from patients or tumour-bearing animals can be 100 mm² or larger. For such samples, multiple images must be taken (usually automated by software) and tiled together to form a complete image of the entire tissue specimen (Steinberg and Ali 2001; Wang, Shumyatsky et al. 2006). This is time consuming and can introduce ‘stitching’ artefacts in the composite image.

To address these size and sensitivity limitations we have developed a novel confocal scanning macroscope (Constantinou, Vukovic et al. 2001; Dixon and Damaskinos 2001; Mackay, Hedley et al. 2005), integrated with a prototype hyperspectral imaging (HSI) mode (Constantinou, Nicklee et al. 2005). The Hyperspectral Macroscope (HSM) allows image acquisition of entire 1x3” microscope slides in a single field-of-view (FOV), avoiding the need to tile multiple images together. The HSM imaging lens has a focused spot full-width half-maximum (FWHM) of 2 µm, a numerical aperture (NA) of 0.35 and a FOV of 22 x 70 mm². In terms of optical imaging performance the HSM is similar to a 10x microscope objective, but with >100x the FOV. The spectral imaging mode has a 32 channel spectral detector with a resolution of 6.7 nm/channel, and the excitation wavelengths available are 488, 568 and 647 nm.

A major aim of this work has been to develop methods to reduce the confounding contribution of background sample autofluorescence so that imaging of paraffin embedded, formalin fixed (PEFF) tissues stained with exogenous fluorescent agents can be optimized. One technique that has shown great potential for improving the sensitivity and specificity of fluorescence imaging systems is HSI combined with processing methods such as linear unmixing (Tsurui, Nishimura et al. 2000; Ecker, de Martin et al. 2004; Sinclair, Timlin et al. 2004; Zimmermann 2005). In HSI, the emission spectrum of each image pixel is digitally recorded. This spectral information can
then be used to directly study the emission characteristics of the sample, or through the process of linear unmixing overlapping fluorescence spectra from co-localized probes and/or tissue autofluorescence can be isolated. Commercially available epifluorescence instruments used for small animal imaging (e.g., CRI Maestro, Cambridge Research & Instrumentation, Woburn, MA, USA) provide hyperspectral image-based data but with low spatial resolution (~0.5-1 mm). Commercial confocal microscopes have the ability to capture high-resolution spatial and spectral information (e.g., Zeiss LSM 510 Meta, Carl Zeiss Inc, Thornwood, NY, USA and Leica SP2, Leica Microsystems, Wetzlar, DE). However, a major limitation with these systems are the small image FOVs, thus requiring image tiling for tissue section imaging.

We demonstrate here the important capabilities of the HSM prototype instrument by studying the autofluorescence properties from a tumour xenograft model and quantifying the improved sensitivity of HSI versus single channel imaging. The specimens used were obtained from an on-going preclinical study to develop tumour-targeted fluorescence contrast agents in a xenograft mouse model of human colonic adenocarcinoma. A dilution series was used to stain serial tissue sections, producing a set of specimens with varying fluorescence-to-autofluorescence (F/AF) ratios. To further study the origins of autofluorescence in this model, the spectral properties of the autofluorescence measured was also compared to spectra from a database of endogenous fluorescent compounds, and potential sources of the autofluorescence observed in this mouse model are highlighted.
4.2.3 Materials and Methods

4.2.3.1 Antibody Selection

The monoclonal antibody (MAb) against tumor-associated antigen 72 (TAG72) (monoclonal mouse anti-Human-CC49 antibody #ab16838, Abcam Inc., USA) was used to create a fluorescent bioconjugate for detection of human colon cancer. TAG72 is a glycoprotein that is over-expressed in the cell membrane and cytoplasm of most human adenocarcinoma cells, with little or no expression in normal and benign tissues (Safavy, Khazaeli et al. 1999; Tang, Yang et al. 2007).

4.2.3.2 Tumour Tissue Samples

Approximately 2×10^6 LS174T cells were injected subcutaneously in the right hind leg (flank) of 6-8 week old female (~20 g) Swiss athymic (nu/nu) mice (Taconic, Germantown, NY). All animal procedures were approved by University Health Network, Toronto, Canada. Tumours were allowed to reach a diameter of 5-12 mm before excision (12-14 days). During this time, mice were fed low-fluorescence autoclaved rodent diet (Cat. #5010, LabDiet, St. Louis, Missouri, USA) and sterilized water. Resected tumours were fixed in 10% buffered formalin and embedded in paraffin wax for sectioning and staining.

4.2.3.3 Immunofluorescence Staining

Serial 4 μm tissue sections were cut and placed on glass slides. Brightfield stained sections were prepared using a biotinylated secondary antibody coupled to streptavidin-horseradish peroxidase. This was reacted with 3,3′-diaminobenzidine (DAB) to produce a brown staining wherever the
primary CC49 and secondary antibodies are attached. Nuclei were counterstained with Hematoxylin. DAB-stained sections were generated at 1/200, 1/1000 and 1/2000 dilutions. Fluorescence sections were stained at 1/100, 1/200, 1/500, 1/1000, 1/1500 and 1/2000 dilutions using an anti-mouse Cy3 secondary antibody (Jackson Immunoresearch, USA). Negative control slides were generated by omitting the primary CC49 antibody labelling step for each of the DAB and Cy3 tissue sets.

4.2.3.4 Image Acquisition

Images of the DAB stained sections were acquired on a commercial whole-slide brightfield scanner (Scanscope CS, Aperio Technologies, Vista, CA, USA). Hyperspectral images were generated with the HSM using 488 nm excitation, with fluorescence collection over the 500-700 nm spectral range at a spectral resolution of 6.7 nm/channel. Images at 488 and 568 nm excitation wavelengths were acquired with the HSM operating in single-channel mode, with emission filters of 600±50 nm and 610±35 nm, respectively. The pixel resolution for all HSM images was 1 μm, with an image size of 7 x 9 mm.

4.2.3.5 Hyperspectral Unmixing

Linear unmixing was performed in the ENVI software package (ITT Visual Information Solutions, Boulder, CO, USA) to remove tissue autofluorescence and extract the Cy3-CC49 emission from the spectral data cube. Linear unmixing can be described mathematically by the equation below (also see figure 4.1).
A given pixel \((x,y)\) in a spectral image will have a measured spectrum that can be represented by \(S(x,y)\). This spectrum will be, in general, a combination of autofluorescence and/or fluorescence from any exogenous label(s) and can thus be represented as a linear combination of reference, or ‘endmember’, spectra \(R_n\). The scaling factors \(\alpha_n(x,y)\) indicate how much of a given endmember is present in \(S(x,y)\). In the example shown in figure 4.1, the pixel indicated in the lower left image contains the spectrum \(S(x,y)\). Using the two reference spectra shown and solving for the abundance values show that this particular pixel contains a 50/50 mixture of the two reference spectra. The abundance values therefore make up the pixel values in the unmixed images, or in other words linear unmixing produces one image for each reference spectrum. Reference spectra can be read from a spectral library, or extracted from appropriate control samples.

The reference used for unmixing were measured on the following external controls: The fluorescence reference spectrum of the CC49-Cy3 conjugate was measured by filling a microchannel slide (Slide V, Integrated BioDiagnostics, USA) with 10 µl of CC49-Cy3 diluted in phosphate-buffered saline (PBS). This slide was scanned spectrally on the HSM using 488 nm excitation and the emission was saved to a spectral database. The AF reference spectrum from the LS174T tumours was measured in the fluorescence-negative control slide by imaging in the HSM under 488 nm excitation and again extracting the emission spectrum to a database.
Image analysis was performed on the unmixed and single channel images to quantify the F/AF ratio of each data set using ImageJ (IJ) (rsb.info.nih.gov/ij/). Image masks were generated by the following steps: 1) Intensity-based thresholding was performed to locate the positive CC49 regions and remove saturated pixels; 2) overlapping/merged objects were split with a watershed algorithm; 3) any objects less than 4 pixels in size were removed. These image masks were used to calculate the mean fluorescence-to-autofluorescence (F/AF) ratio at each staining concentration.

**Figure 4.1** – Schematic depiction of the linear unmixing process. A measured spectrum $S(x,y)$ can be represented as a linear combination of reference spectra $R_n$. The abundance values ($\alpha_n$) are solved for using linear regression at each $(x,y)$ pixel an image. The resulting abundance values are output to unmixed image channels, which quantitatively represent the abundance of each spectral endmember at a given spatial location.
4.2.4 Results and Discussion

4.2.4.1 DAB Staining

Brightfield images of the DAB stained tissues are shown in figure 4.2; the 1/200 dilution in 4.2A, and the negative control in 4.2B. The red boxed regions are shown in 4.2C and 4.2D, respectively. In these images nuclei are stained blue and the positive CC49-DAB stain is brown in colour. The CC49 regions in the DAB sections stained positively at the 1/200 and 1/1000 dilutions, but no significant positive staining was observed at 1/2000. As a result, fluorescent labelling was carried out over a range of 1/100 – 1/2000.
There is some non-specific staining visible in these images, seen as a lighter shade of brown (black arrows, figure 4.2C) and corresponding to regions of necrosis (black arrows, figure 4.2D).

It is possible that antigen shedding of the necrotic cells in these regions results in ‘leaking’ of the TAG72 mucin into the extracellular matrix, thus: if there are fragments of TAG72 remaining in the necrotic areas that are still recognized by the CC49 antibody, weak labelling can occur.

**Figure 4.2** – Brightfield images of ex-vivo LS174T xenografts stained with DAB (1/200 dilution). (A) Full FOV image showing intense CC49 staining (brown) and haematoxylin counterstain (blue). (B) Negative control slide. (C) Zoom region from A. (D) Zoom region from C, black arrows denote regions of necrosis.
4.2.4.2 Fluorescence Staining

Shown in figure 4.3 are the single-channel 488 nm and 568 nm excitation images at the 1/100 dilution (4.3A & 4.3B), in addition to the negative control slide imaged at 488 nm excitation (4.3C). At 488 nm excitation, the fluorescence intensity in figure 4.3A is comprised of mostly AF, which is significantly lower in figure 4.3B due to the longer excitation wavelength used for this image. The FOV imaged for these specimens was 7x9 mm, which is still only a small fraction of the 22x70 mm full FOV of the HSM. However, at this scale, it is even difficult to see the positive regions clearly in the 568 nm excitation image. The boxed regions indicated in 4.3A-C are therefore shown below each of the full section images at higher zoom (0.5x0.5 mm) to allow better visualization of the staining. The image sets including unmixing results from the 1/100, 1/200, 1/500 and 1/1000 dilution slides can be found in figures 4.5-8.
To determine if non-specific binding of the Cy3 antibody could be a potential source of autofluorescence, the emission from the dilution series was compared with the emission from the negative control slide using the 488 nm excitation wavelength. The autofluorescence spectrum from the LS174T xenografts showed a relatively broad emission across the 500-600 nm range, peaking at ~530 nm. There was no significant change between the emission from the negative control and the stained sections, indicating that bulk of the autofluorescence detected in these samples was due to naturally occurring endogenous compounds within the tissue (figure 4.4A). Previous studies within our group have quantified the excitation and emission properties of a number of endogenous autofluorescent compounds normally found in biological specimens.

Figure 4.3 – 1/100 dilution slide excited at 488 nm (A) and 568 nm (B) as well as the negative control slide excited at 488 nm (C). Boxed regions in macro-images are shown in panels (D-F). There is significant autofluorescence excited at 488 nm, yet comparing (D) to (F) shows there is also positive staining on top of this background.
(DaCosta, Andersson et al. 2003). In 4.4B-E, spectra from this database are compared with the negative control spectrum in attempt to identify the specific compounds responsible for the autofluorescence observed in this mouse model.

Many of the compounds in the database such as collagen and nicotinamide adenine dinucleotide (NADH) are excited minimally at 488 nm, however, the emission from elastin as well as flavins such as flavin adenine dinucleotide (FAD) were identified as potential sources of autofluorescence based on the spectral studies. The emission of elastin (4.4B) and FAD (4.4D) have many characteristics in common with the autofluorescence from the LS174T sections.

Figure 4.4 – Fluorescence spectra from all slides are compared in (A) and show no significant change between the negative and positively stained slides. In (C) the negative fluorescence spectrum is compared with that of two potential endogenous sources for autofluorescence Elastin and FAD, with individual spectra shown in (B, D & E).
Overlaying the normalized spectra in the 500-700 nm range (figure 4.4C) shows that the peak emission and spectral profiles for elastin and FAD are similar to the observed autofluorescence spectrum. Based on this comparison it is suggested that the bulk of the autofluorescence observed in these sections is due a combination of elastin and FAD fluorescence. This conclusion is consistent with the findings of previous studies at this excitation wavelength, as well as from the spectra contained in the EEM database (Benson, Meyer et al. 1979; Izuishi, Tajiri et al. 1999; Silveira, Betiol Filho et al. 2007).

Figure 4.5 shows the unmixing results and single channel images of the 1/100 dilution slide. The boxed region in 4.5E is shown in the adjacent figures. From the top left are the single channel

**Figure 4.5** – 1/100 dilution slide. (A) 488 nm excitation single channel image. (B) 568 nm excitation single channel image. (C) Unmixed 488 nm excitation image. (D) Relative levels of AF (green line) and CC49-Cy3 (yellow line) in the 1/100 dilution slide, integrated over the region indicated in C. (E) Image of entire section (7 x 9 mm), boxed area shows FOV in (A-C).
images taken at 488 nm (4.5A) and 568 nm (4.5B), with the unmixed CC49-Cy3 image in (4.5C). The unmixed image shows a notable reduction in the autofluorescence background generated at 488 nm, even at the starting dilution of 1/100 (compare with 4.5A). The 568 nm excitation image shows negligible autofluorescence, which would be expected since the endogenous compounds mentioned above do not fluoresce significantly this wavelength. The excitation efficiency of Cy3 at 568 nm is also 3x higher than at 488 nm. This results in a higher F/AF ratio for the 568 nm image versus the 488 nm single channel image, producing the marked contrast loss in fig 4.5A compared with fig 4.5B. Shown in figure 4.5D are the relative autofluorescence and CC49-Cy3 emission spectra from a randomly selected region (green outline, fig 4.5C). In these plots the signal from CC49-Cy3 (yellow) is roughly 2x the autofluorescence (green) level in the 550-600 nm spectral range, indicating that one out of three photons detected from positive regions at the 1/100 dilution was due to tissue autofluorescence.
At the 1/200 dilution, the signal in the 488 nm single-channel image (figure 4.6A) is significantly obscured by the tissue autofluorescence and thus, it is difficult to determine the biodistribution of the CC49-Cy3 positive regions reliably. Despite this fact, the unmixed image (4.6C) contains the same positive regions as the 568 nm single channel image (4.6B). When the relative levels of tissue autofluorescence and CC49-Cy3 fluorescence are compared (4.6D), one can see that roughly half of the light detected beyond 550 nm is due to autofluorescence, and the net effect of this is that the CC449-Cy3 signal is essentially undetectable with single channel imaging at 488 nm excitation. The 568 nm excitation image produced strong positive signals, with little detectable autofluorescence.

Figure 4.6 – 1/200 dilution slide. (A) 488 nm excitation single channel image. (B) 568 nm excitation single channel image. (C) Unmixed 488 nm excitation image. (D) Relative levels of AF (green line) and CC49-Cy3 (yellow line) in the 1/200 dilution slide, integrated over the region indicated in C. (E) Image of entire section (7 x 9 mm), boxed area shows FOV in (A-C).
The 1/500 dilution slide results are shown in figure 4.7. The 568 nm image again contains much less autofluorescence than the 488 nm image, and the unmixed 488 nm image has the autofluorescence removed despite the lack of any visible positive signal in figure 4.7A. The spectra in figure 4.7D show that the CC49-Cy3 signal is roughly 3x less than the autofluorescence in the 550–650 nm spectral range. Thus, at the 1/500 dilution and with 488 nm excitation, approximately 3 out of 4 photons detected in the 550–650 nm spectral range are due to autofluorescence, which makes detection of the positive regions impossible. Despite this fact, the unmixed image (figure 4.7C) clearly shows an image comparable to the 568 nm excitation image (figure 7B).

Figure 4.7 – 1/500 dilution slide. (A) 488 nm excitation single channel image. (B) 568 nm excitation single channel image. (C) Unmixed 488 nm excitation image. (D) Relative levels of AF (green line) and CC49-Cy3 (yellow line) in the 1/500 dilution slide, integrated over the region indicated in C. (E) Image of entire section (7 x 9 mm), boxed area shows FOV in (A-C).
The 1/1000 dilution images are shown in figure 4.8, and demonstrate once again the power of the unmixing technique by successfully recovering the positive CC49-Cy3 signal despite the overwhelming autofluorescence emission. The autofluorescence generated at 488 nm with this staining concentration was significant to say the least, since the autofluorescence emission in the 550-600 nm range is almost 6x greater than the signal from CC49-Cy3, which amounts to 6 out of 7 photons in the 488 nm excitation image being due to tissue autofluorescence.

The F/AF ratios generated by calculating the mean signal from positive regions to the mean AF level at each dilution are plotted in figure 4.9. The values are linearly proportional to the staining concentrations for all excitation wavelengths ($R^2>0.99$ in all cases). The error bars

**Figure 4.8** – 1/1000 dilution slide. (A) 488 nm excitation single channel image. (B) 568 nm excitation single channel image. (C) Unmixed 488 nm excitation image. (D) Relative levels of AF (green line) and CC49-Cy3 (yellow line) in the 1/1000 dilution slide, integrated over the region indicated in C. (E) Image of entire section (7 x 9 mm), boxed area shows FOV in (A-C).
represent one standard deviation. At 568 nm (dotted line) there was virtually no autofluorescence produced, and the resulting F/AF plot reflects the increased contrast over the 488 nm (dashed line) excitation wavelength, however the 488 nm unmixed data (solid line) mimicked results generated at 568 nm and essentially produced images free from the effects of autofluorescence background. The limiting dilution in these specimens for single channel imaging at 488 nm was between 1/100 and 1/200, while the unmixing process allowed recovery of the CC49-Cy3 signal at a concentration ~10 times lower than this. The same detection limit was reached in the 568 nm excitation and brightfield images, indicating that the limiting factor in this study was not autofluorescence, but a lack of positive CC49 staining.

One point that should be raised about the process of spectral unmixing is the potential for increasing the noise in the final images. Since the HSM bit depth per pixel is 12 bits (4,905 grey

**Figure 4.9** - F/AF ratio versus stain concentration for single channel and spectrally unmixed data. The 568 nm (dotted line) excitation wavelength produced a significantly better single-channel F/AF ratio than the 488 nm excitation wavelength (dashed line). The unmixed F/AF ratio (solid line) originates from the data excited at 488 nm (dashed line) and represents a significant reduction in the autofluorescence generated at 488 nm.
levels) there is a theoretical and practical limit to how much background can be removed while maintaining an adequate dynamic range with which to analyse results. Linear unmixing is essentially a subtractive process, thus removing the contribution of autofluorescence from the data set inherently reduces the dynamic range of the unmixed images. In other words, if 75% of the signal in the brightest pixels is due to autofluorescence, that leaves ~1,000 grey levels of ‘true’ signal after unmixing.

This rationale assumes perfect, noiseless detection, but when factoring effects of noise such as readout noise and photon statistics into account, the amount of unmixing that could be reliably tolerated would be lower. Even if the noise in the original images was very low (standard deviation of ~5%), that amounts to ~200 grey levels, meaning the unmixed signal in all but the brightest of pixels will be overwhelmed by noise and difficult to reliably quantify. However, it should also be noted that without linear unmixing it would have been impossible to reliably find the positive CC49 regions in this study. Hence, while spectral unmixing can potentially reduce the dynamic range of images, it is suggested that this reduction is outweighed by improving the localization of weak signals and reducing the effects of over-estimating the true fluorescence signal in highly autofluorescent tissue sections.

4.2.5 Conclusions
A clinically-relevant antibody was used to stain human adenocarcinoma xenografts over a large concentration range in an effort to quantify the increased sensitivity of whole-specimen HSI versus single-channel imaging in a prototype fluorescence slide scanner. The emission characteristics of the autofluorescence generated by 488 nm light indicate that the main source of autofluorescence in this mouse model could be due to elastin and/or FAD. The use of HSI for
detection of fluorescently-labelled antibodies and removal of autofluorescence in PEFF tissues has resulted in a significant reduction of tissue autofluorescence from CC49-Cy3 stained LS174T xenografts. The detection limit for single channel imaging using 488 nm excitation was between the 1/100 and 1/200 dilutions, while HSI and linear unmixing allowed detection at much lower staining concentrations. Despite the high AF generated at 488 nm, the spectrally unmixed images demonstrated successful unmixing of positive CC49-Cy3 regions down to the 1/1500 dilution, matching the pattern observed in the 568 nm images at the same dilution. At 1/2000 dilution there was no positive signal in the unmixed CC49-Cy3 channel, which was consistent with our observations on the DAB-stained section at the same dilution.

4.2.6 Acknowledgements

This work was supported by the Ontario Research and Development Challenge Fund, in partnership with Biomedical Photometrics Inc. Core equipment was acquired with support from the Canadian Foundation for Innovation and the Princess Margaret Hospital Foundation. The author would also like to thank Trudey Nicklee and James Ho for their assistance in preparing the paraffin sections.
Chapter 5

5 Conclusions and Future Research

5.1 Summary and Conclusions

The work presented in this thesis addresses two major challenges in biomedical fluorescence microscopy: 1) confocal imaging of large (i.e. > 10 x 10 mm) specimens in fluorescence and brightfield modes without the need for image tiling and 2) hyperspectral image detection and processing to remove the effects of tissue autofluorescence from fluorescently-labelled tissue sections. The prototype system was still at a proof-of-concept phase at the conclusion of this work, with future steps involving further refinement of the spectral detection mode and system automation (discussed in section 5.2).

In Chapter 2 a modified cDNA reader based on the macroscope core technology was evaluated for imaging tissue sections obtained from mel180 cervical carcinoma xenografts. Images from the modified cDNA reader were compared with those obtained using a tiling fluorescence microscope. Tissues were triple-labelled for HIF-1α (nuclear hypoxia marker), CD31 (epithelial blood vessel marker) and EF5 (cytoplasmic hypoxia marker). The hypoxia-induced EF5 expression shows up as large patches of fluorescence and this staining pattern was clearly visualized in the images from the cDNA reader as well as the tiling system. Staining patterns such as these, which are broadly expressed over large tissue sections, can be difficult to visualize accurately on a microscope, and the large FOV of the macroscope system is better suited to study of these patterns. Fluorescent images of the blood vessel marker CD31 also produced similar results between the tiling and macroscope systems, demonstrating the ability to track multiple parameters within the same section. HIF-1α, which is over-expressed in cell nuclei within hypoxic regions, was being investigated for its potential as an intrinsic hypoxia marker.
(Giatromanolaki and Harris 2001; Lee, Bae et al. 2004). While the signal was visible in the macroscope system images, the small size of the nuclei proved to be just beyond its spatial resolution limit, and they were better distinguished from the autofluorescent tissue background in the tiling system.

The experience gained with the cDNA macroscope instrument was then used to design a macroscope system better optimized for combined tissue fluorescence and transmitted light imaging. The system was built around a new f-theta laser scan lens designed with better colour correction and a higher NA. This system also incorporated transmitted light imaging, allowing for brightfield and phase contrast imaging modes. In addition, a prototype hyperspectral imaging mode was designed and integrated into the system hardware and software. This allowed for direct spectral sampling of tissue specimens and subsequent linear unmixing of background tissue autofluorescence.

In Chapter 3 the hyperspectral macroscope and its prototype imaging modes were evaluated and characterized. The brightfield mode proved to be a very useful addition to the system, as it allowed both fluorescence and transmitted light images to be acquired on the same platform, providing excellent co-localization. The system configuration change from a dual-scanning mirror to a scanning mirror-scanning stage configuration also improved image quality by reducing off-axis optical aberrations, which resulted in increasing the uniformity of the signal in fluorescence images and simultaneously increasing the system FOV to that of an entire microscope slide. While the phase-contrast mode did prove useful in visualization of unstained and live specimens, the imaging speed of the system was not optimized for the latter.

The hyperspectral imaging mode was characterized and calibrated by coupling a much slower CCD-based spectrometer into the system and comparing the spectra from a range of fluorescent
dyes and microsphere phantoms. The prototype spectrometer was limited to approximately 20
times slower imaging speeds than the single channel modes (200 versus 5 µs/pixel), but still
operated a factor of 100x faster than the CCD-based spectrometer used for calibration and
validation. Custom electronics would have been required to sample faster in hyperspectral
mode, as a slower commercially-available data readout interface was chosen instead. The scan
speed of the hyperspectral mode fundamentally limited the system to proof-of-concept studies,
with the intention that a second generation hyperspectral detector, discussed in section 5.2.2, will
address the speed issues of the system to allow faster acquisition for all modes.

Initial unmixing work attempted the use of an open-source spectral unmixing plug-in for ImageJ
(http://rsb.info.nih.gov/ij/). While this method demonstrated the successful unmixing of
hyperspectral fluorescence images, the framework of ImageJ was not suited to processing the
large data sets and number of image channels generated by the HSM. A single channel from a
typical section can be between 100 – 500 MB in size, resulting in spectral data cubes on the
order of gigabytes. ImageJ cannot use more than 1 GB for image processing, so that only small
sections and sub-sampled spectral data could be processed through this plug-in. A commercial
spectral processing package designed for working with large image sets was purchased and
installed on the hyperspectral macroscope to address this memory concern and complete the
prototype’s features.

In Chapter 4 the unmixing properties of the system were evaluated with the specific goal of
determining the detection limits of the spectral imaging mode, and its ability to remove tissue
autofluorescence. To this end, a series of serial tissue sections obtained from LS174T colon
cancer xenografts were stained with varying concentrations of the TAG72/CC49 antibody. The
detection limit of single channel fluorescence images taken at 4 different excitation wavelengths
were compared to spectrally-unmixed data taken at an excitation wavelength that produced high autofluorescence signal. The results demonstrated that the detection limit for single channel imaging was approximately 10 times lower than that for linearly-unmixed images obtained at the same excitation wavelength and power. This demonstrated the ability to recover signals reliably even when the autofluorescence completely overwhelmed the fluorescence from the antibody label.

When compared with the chemical autofluorescence removal techniques discussed in Chapter 1, the method employed here offers a novel solution that can effectively eliminate major sources of tissue autofluorescence from entire tissue specimens. Chemical methods are only applicable for certain tissue types and may potentially alter the staining kinetics of any labels that are subsequently applied.

The novel properties of the macroscope f-theta scan lens technology have been extended significantly through the work presented in this thesis. The system constructed has demonstrated its ability to capture brightfield, fluorescence and hyperspectral images of whole tissue sections, without the need for image tiling. The use of HSI in the biological sciences is still in its infancy. However the advantages of HSI over single-channel imaging demonstrated here support its considerable potential. In this regard some of the remaining challenges to be solved in future work are presented in section 5.2, where suggestions for a second-generation hyperspectral prototype system and its potential applications are discussed.
5.2 Future Directions

Two main topics are briefly discussed here; 1) suggestions for a re-designed laser scan lens and 2) details of a second-generation spectral detector. The laser scan lens used for the majority of the work reported above operates at a collection NA of 0.35, and is now considered an obsolete design. Despite the relatively low NA (compared to modern microscope objectives) the colour correction range of the lens is its main limitation, as it was only colour corrected from 500-650 nm. Two commonly-used fluorescent stains for locating nuclei in specimens are DAPI and Hoechst 33442; these are excited by UV wavelengths (around 360 nm) far outside the design range of the HSM laser scan lens. Being unable to image such UV-excited labels severely limits the potential of the macroscope technology in biomedical research and clinical pathology, so that future lens designs should also incorporate colour correction down to 350 nm. The ability to resolve sub-cellular structures with the present lens was limited to objects larger than 1-2 µm. This limits, to some extent, the routine use of this technology in applications requiring a high-NA objective such as fluorescence in-situ hybridization (FISH) (Gozzetti and Le Beau 2000; Levsky and Singer 2003; Halling and Kipp 2007). Redesign of a laser scan lens with a higher NA will potentially address this problem: an ideal system would have an NA (0.75) comparable to that available on current 20-40x dry microscope objectives.

Figures 5.1 and 5.2 show images acquired on a prototype high-resolution system (TS4000; BPI Inc, Canada) which is built around a laser scan lens with an NA of 0.5 and acquires images up to 125x175 mm (5”x7”) in 10x175 mm long strips. The increased NA of this system and its improved automation will serve as the platform on which the hyperspectral work developed in this thesis will continue. For example, figure 5.1 shows a brightfield image of brain tissue stained with a Nissl stain, which stains RNA blue and is used to highlight structural features in neurons (obtained from Dr. Yeni Yucel, St. Mike’s Hospital, Toronto). This image was acquired
at 0.5 µm/pixel over 30x45 mm, resulting in an uncompressed image size of approximately 16 GB when saved as a 24 bit RGB image.

Figure 5.2 shows a fluorescence image of a mouse kidney that has been labelled with Alexa 488-WGA (Wheat Germ Agglutinin), which stains cell membranes, and Cy3-phalloidin, which stains F-actin (fluocells slide #3, Invitrogen). The image was acquired simultaneously in two fluorescence channels, over an area of 7 mm x 9 mm at a resolution of 0.5 µm/pixel.

Figure 5.1 – Brightfield image of a monkey brain stained with Nissl. (A) Entire section imaged in 3 strips on the TS4000. (B) & (C) Boxed regions shown at progressively higher magnification.
The hyperspectral imaging detector constructed for this work was largely based on off-the-shelf technology and, hence, there were some limitations to this first-generation prototype. The main issues that will be addressed with a re-design of the spectrometer are: 1) improving the imaging speed in hyperspectral mode and 2) allowing the number and the width of the detection spectral channels to be varied. The speed of the HSM spectrometer was limited to 200 µs/spectrum, but the architecture of system used to acquire the images in figures 5.1 and 5.2 could, with appropriate modifications, allow for simultaneous acquisition of up to 16 channels at 0.2 µs/spectrum: these systems use a single, 4-channel A/D card operating at an acquisition rate of 8 MHz, 4 of which are linked to produce 16-channel parallel readout at a rate 1000x faster than the hyperspectral mode used in the HSM.

**Figure 5.2** – Fluorescent images of a mouse kidney stained with Alexa 488-WGA (Green) and Cy3-Phalloidin (Red). (A) Entire section imaged in a single FOV. (B) Boxed region shown at higher magnification.
A further development is the use of a holographic transmission grating instead of a reflective grating in the detector arm. This simplifies the light path, allowing for more flexibility and easier alignment. The number of image channels in the spectrometer will also be reduced from 32 to 16, and a number of additional optics that allow the spectral resolution to be changed from 3 nm/channel to 20 nm/channel will be built into the spectral detection path. Thus, when the 20 nm/channel configuration is used, the signal from 2-3 adjacent channels can be combined to produce a pseudo-single channel image. This modification would allow all fluorescence image acquisition to occur in a single detector model that will be more efficient and sensitive than the current method used for multi-channel fluorescence imaging (i.e., the 0.5 NA system used for the images in figures 5.1 and 5.2.)

Spectral image processing (including spectral unmixing) should also be integrated with data readout. In the HSM, all 32 channels are stored to disk whether or not they contain signal. Assuming that the reference spectra for unmixing are known (or read from a library), unmixing the data ‘on the fly’ and storing only the unmixed images will significantly reduce the data storage and post-processing requirements. A typical section that is 10 x 10 mm and captured at 0.5 µm/pixel will require approximately 800 MB per image channel, so unmixing on the fly and/or only storing selected spectral channels would improve performance significantly.

There are also significant advantages offered by novel contrast agents, such as semiconductor quantum dots (qdots). The potential advantages of qdots as fluorescence contrast agents are driving a burst of research in recent years (Geho, Lahar et al. 2005; Hotz 2005). Qdots are extremely bright and photostable. Their fluorescence output can have very narrow (< 20 nm) emission peaks, while the size of the particles (~10^{-9} m) dictates the emission spectrum. In addition, they can all be excited by a single laser operating in the blue (400-500 nm) or near-UV
(350-400 nm) range. Unfortunately, qdots are made of inorganic semiconductor materials and so must be encased in an organic shell to allow antibody labelling (Alivisatos, Gu et al. 2005; Gao, Chung et al. 2007).

Although there is considerable interest in the use of qdots as biological contrast agents, labelling studies are in their infancy but show some promising results (Gao, Chan et al. 2002; Jiang, Papa et al. 2004; Zheng, Ghazani et al. 2006). The advantages of qdots that make them useful for immunofluorescence would also see some benefit from HSI. The narrow emission width and ability to excite multiple qdots with a single wavelength fit very well with the capabilities of a spectral system such as the HSM. The combination of qdots with whole-tissue hyperspectral imaging could enable a large number of simultaneous measurements to be quantified with little or no cross-talk between adjacent channels. Data shown in Appendix 3 demonstrated the successful unmixing of spectra having a 10 nm spacing between their emission maxima, which indicates a great potential for use of the HSM in multiplexing studies. Taking this fact as well as the narrow emission widths of qdots into consideration, it is anticipated that > 10 qdots could be simultaneously imaged and quantified across the visible spectrum utilizing linear unmixing. This assumes a nominal 40 nm wide qdot emission and a 30-40 nm spacing between qdot emission peaks. The main challenge with multiplexing studies of this sort will be in the development of labelling protocols, as it can be difficult to reliably stain even a few simultaneous parameters with organic labels. These challenges will no doubt be addressed, and in the future the use of qdots could become more common than the currently used organic fluorophores. In either case, the development of techniques for quantification of these labels must also proceed in parallel, and to this end, the work presented in this thesis represents a starting point towards clinical application of whole-slide hyperspectral fluorescence imaging.
Appendices

A.1 Linear Unmixing Mathematics

The method of linear unmixing (Chang 2003) is used in this thesis for hyperspectral image processing, and presented here is a general discussion on the principles of linear spectral unmixing. This example and involves the use of two arbitrary detection channels and two fluorescence spectra, shown in figure A.1.1, where emission spectra are shown for two fluorescent dyes. The emission peaks are separated by approximately 40 nm. The red and blue boxed areas represent optical filter bandpasses that could be used to detect each of the dyes individually and, as can be seen, there are contributions from both dyes (cross-talk) present in each channel. This amount of overlap can be treated with fairly straightforward linear algebra, and this example allows a simple understanding of the process of linear unmixing. Consider first two detectors, $S_R$ and $S_B$ that respectively measure the signal contained in the red and blue boxes shown in figure A.1.1. The spectrum measured at a given $(x,y)$ location in an image can be written as a linear combination of the signal due to each fluorophore individually. This can be done for both channels and follows a form similar to equation 1 below:

$$S_R(x, y) = \alpha_{RR} R(x, y) + \alpha_{RB} B(x, y) + D_R(x, y)$$

$$S_B(x, y) = \alpha_{BR} R(x, y) + \alpha_{BB} B(x, y) + D_B(x, y)$$

Figure A.1.1 – Simplified unmixing example using two fluorescent dye spectra and two detection channels.
Here, $S_n(x,y)$ is the signal measured in channel $n$ at spatial position $(x,y)$, while $R(x,y)$ and $B(x,y)$ are the amounts of the red and blue fluorescence (the unknowns to be solved for), and $D(x,y)$ is the dark or background signal. The scaling factor $\alpha_{RR}$ is defined as the proportion of the red fluorophore in the red detection channel, and $\alpha_{RB}$ is defined as the amount of the blue fluorophore in the red channel. Similarly, $\alpha_{BR}$ is the amount of red fluorescence in the blue channel, and $\alpha_{BB}$ is the amount of blue fluorescence in the blue channel. Equation 1 can also be written in the following matrix form.

$$
\begin{pmatrix}
S_R(x,y) \\
S_B(x,y)
\end{pmatrix} =
\begin{pmatrix}
\alpha_{RR} & \alpha_{RB} \\
\alpha_{BR} & \alpha_{BB}
\end{pmatrix}
\begin{pmatrix}
R(x,y) \\
B(x,y)
\end{pmatrix} +
\begin{pmatrix}
D_R(x,y) \\
D_B(x,y)
\end{pmatrix}
$$

(2)

The matrix of $\alpha_{nm}$ coefficients is called the mixing matrix, since it describes how the $R(x,y)$ and $B(x,y)$ signals mix together. To solve equation 2 and determine the values of $R(x,y)$ and $B(x,y)$, the coefficients of the mixing matrix must be known. In this example, they can be estimated from the above plot, though in practice they are generally read from a spectral library or calibration images. Once the mixing matrix is known, its inverse is calculated, and the abundances of the red and blue dyes at pixel $(x,y)$ are calculated via equation 3:

$$
\begin{pmatrix}
R(x,y) \\
B(x,y)
\end{pmatrix} =
\begin{pmatrix}
\alpha_{RR} & \alpha_{RB} \\
\alpha_{BR} & \alpha_{BB}
\end{pmatrix}^{-1}
\begin{pmatrix}
S_R(x,y) \\
S_B(x,y)
\end{pmatrix} -
\begin{pmatrix}
D_R(x,y) \\
D_B(x,y)
\end{pmatrix}
$$

or,

$$
\begin{pmatrix}
R(x,y) \\
B(x,y)
\end{pmatrix} =
\frac{1}{\alpha_{RR}\alpha_{BB} - \alpha_{RB}\alpha_{BR}}
\begin{pmatrix}
\alpha_{BB} & -\alpha_{RB} \\
-\alpha_{BR} & \alpha_{RR}
\end{pmatrix}
\begin{pmatrix}
S_R(x,y) \\
S_B(x,y)
\end{pmatrix} -
\begin{pmatrix}
D_R(x,y) \\
D_B(x,y)
\end{pmatrix}
$$

(3)

To extend this principle to n-dimensional spectra is a relatively straightforward process, as the columns of the unmixing matrix essentially contain the normalized fluorescence spectra of the dyes to be unmixed. The example above assumes two unmixing spectra and two detector
channels, but in practice the number of detection channels should exceed the number of spectra by a factor of two or more depending on the separation of their emission peaks and amount of spectral overlap. Equation 4 shows a general form of equation 3 that has been extended to include an arbitrary number of detection channels:

\[
\begin{bmatrix}
R(x, y) \\
B(x, y)
\end{bmatrix} = \begin{bmatrix}
\alpha_{i,R} & \alpha_{i,B} \\
\vdots & \vdots \\
\alpha_{n,R} & \alpha_{n,B}
\end{bmatrix}^{-1} \begin{bmatrix}
S(x, y) \\
D(x, y)
\end{bmatrix} - \begin{bmatrix}
S(x, y) \\
D(x, y)
\end{bmatrix}
\]

(4)

Solving equation 4 for the spectral abundance values R(x,y) and B(x,y) across an entire image produces two images (one for each reference spectrum) that quantify the fluorescence intensity from the corresponding label.
A.2 Fluorophore Mixture Tests of Linear Unmixing

The most critical factor in successful spectral unmixing is constructing an appropriate mixing matrix. Omitting spectral signatures that are present in the sample will not produce satisfactory results, and care must be taken when defining and measuring the basis spectra. When properly configured, it is possible to separate mixtures of highly overlapping fluorescence spectra with a high degree of accuracy. As an example of the spectral unmixing technique, the average spectra from a series of fluorescence dilutions made from the red and green spectra shown in figure A.1.1 were measured on a commercial spectrofluorimeter located in Dr. Wilson’s laboratory at Princess Margaret Hospital. Excitation was provided by a 488 ± 1 nm bandwidth and data was collected at 1 nm increments from 500-700 nm, producing a 200 point fluorescence spectrum for each combination of dyes.

![Figure A.2.1](image.png)

**Figure A.2.1** – Spectra from a dilution set of two fluorophores. The dyes were mixed in 10% increments from 100:0, to 0:100 generating 11 different mixtures.

The dye solutions were mixed together in 10% increments, from (100% red, 0% blue) to (0% red, 100% blue). These solutions were sampled in the spectrofluorimeter under the instrument settings described above, and the resulting plots from those cuvette mixtures can be found in figure A.2.1. One can see immediately from the spectra how the fluorescence profiles change as
the fluorophore ratio is altered. The 100 % red and 100 % blue spectra were used to spectrally unmix the data from the mixtures back into their relative levels. The results of the unmixing are summarized in figure A.2.2 and table A.2.1.

In figure A.2.2 the unmixed values displayed in table 1 are plotted along two axes (left for red and right for blue). Lines joining the red and blue abundance values are drawn to better visualize the ratios for each solution unmixed. The colours in figure A.2.2 correspond to the colours of the emission curves in figure A.2.1. Unmixing has found the diluted ratio for the entire range sampled, and is capable of clearly differentiating between all of the overlapping dye mixtures. Upon further analysis of the unmixed values in table 1, the red dye abundances are slightly lower than the expected values by 1-3%, and the blue dye abundances are slightly higher by roughly the same amount. It is likely that this is due to some small energy transfer of the red dye to the blue dye molecules in the solution, either by resonant energy transfer, or direct re-absorption of the emitted fluorescence from the red dye molecules.

<table>
<thead>
<tr>
<th>R:B Ratio</th>
<th>% Red</th>
<th>% Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>90:10</td>
<td>0.895</td>
<td>0.105</td>
</tr>
<tr>
<td>80:20</td>
<td>0.775</td>
<td>0.201</td>
</tr>
<tr>
<td>70:30</td>
<td>0.691</td>
<td>0.313</td>
</tr>
<tr>
<td>60:40</td>
<td>0.578</td>
<td>0.419</td>
</tr>
<tr>
<td>50:50</td>
<td>0.473</td>
<td>0.521</td>
</tr>
<tr>
<td>40:60</td>
<td>0.382</td>
<td>0.618</td>
</tr>
<tr>
<td>30:70</td>
<td>0.277</td>
<td>0.729</td>
</tr>
<tr>
<td>20:80</td>
<td>0.178</td>
<td>0.825</td>
</tr>
<tr>
<td>10:90</td>
<td>0.085</td>
<td>0.915</td>
</tr>
<tr>
<td>0:100</td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Figure A.2.2** – Unmixed abundance results for each of the fluorophore dilutions. The left side represents the % of red and the right side represents the % of blue fluorophore in a particular dilution. Lines are plotted between values from each dilution and have a common point of intersection in the centre of the plot.
A.3 Unmixing Calibration Test Slide

A commercial slide designed to test and troubleshoot spectral imaging systems (Focalcheck slide #2, Invitrogen, Carlsbad, CA, USA) was used to demonstrate HSI on a target that is at the optical resolution limit of the LSL, as well as the spectral resolution limit of the HSM spectrometer.

This slide consists of different dye stained polystyrene beads with highly overlapping emission spectra (figure A.3.1). One row of the slide contains a mixture of single stained 6 µm beads, while another row contains a mixture of double stained beads with a ring/core stain; one dye was used to stain the entire bead throughout and a second dye to stain only the outer surface of the bead. These ring/core dyes have emission peaks that are separated by only 13 nm, and cannot be distinguished with conventional single channel imaging.

Image FOVs of the single stained beads, as well as the ring/core beads in areas A1 and A2 of the slide were captured on the HSM under 488 nm excitation at 1 µm/pixel spatial and 6.7 nm/channel spectral resolutions. Unmixing spectra were generated from the single-stained bead.
data cubes. HSI of the beads were also captured using a 1.2 NA 63x water immersion objective on a commercial hyperspectral microscope (Zeiss LSM 510 META) to assess the size of the ring stain on the ring/core beads. META images were sampled at 0.07 μm/pixel over a FOV of 140 x 140 μm, and excited at 488 nm. Fluorescence was collected at 10 nm/channel over the 500 – 660 nm wavelength range, resulting in a 16 channel hyperspectral data cube.

Spectra measured from the single-stained beads in area A1 are shown below in figure A.3.2. These two dyes have highly overlapping spectra, with their emission peaks separated by only 13 nm, making them indistinguishable in conventional single channel imaging. The small size of the beads is also just above the resolution limit for the laser scan lens of the macroscope, thus these beads serve as a test of the spatial and spectral resolving abilities of the HSM.

![Figure A.3.2 – Normalized spectra from the 503/511 nm (Red) and 511/524 nm (Green) dyes used in column A of the Focalcheck slide. The high degree of overlap between the two spectra makes separation of these two signals impossible with single channel imaging.](image)

The results of the spectral unmixing on the ring/core stained beads in the HSM system can be found in figure A.3.3. From the top left are a red false coloured image of the unmixed 503/511 core stain, a green false coloured image of the unmixed 511/524 ring stain, an overlay of the two
unmixed channels, and a zoomed in view of a single bead. Despite the extremely high spatial and spectral co-localization of the 511 nm and 524 nm dyes, it was possible to correctly separate fluorescence from the bead outer ring and the inner core.

Figure A.3.3 – HSM unmixing results from the spectral test slide and the ring/core stained beads. The ring stain dye was false coloured green, and the core stain dye was false coloured red in this figure. The beads are 6 µm in diameter, and despite this small size and the high degree of overlap, the spectral unmixing algorithm successfully distinguished between the two dyes.

Imaging these same beads on a Zeiss LSM 510 META system allowed more detailed study of the ring/core stained beads, since the HSM LSL has a limited resolution when compared to the
immersion objective used on the META system. The LSM 510 images (figure A.3.4) show significantly better resolution due to this.

![Figure A.3.4](image-url)

**Figure A.3.4** – Zeiss LSM 510 META unmixing results from the spectral test slide and the ring/core stained beads. The ring stain dye was false coloured green, and the core stain dye was false coloured red in this figure. The higher NA of the 63x objective used on the META system shows the ring/core staining with much better detail than the HSM system.

The 63x META objective has 4x the NA of the HSM LSL, which translates into 16x greater light collection efficiency, and a diffraction limited spot size of 0.5 µm. As a comparison, the spot size of the laser scan lens in the HSM is approximately 2 µm. Taking this limitation into consideration, the images from the META and HSM can be said to be comparable in terms of their ability to distinguish the ring/core stains from one another, and while the images of the META system are clearly sharper than the HSM images, the FOV of the META objective (140x140 µm) is 10⁴ smaller than the FOV of the HSM (22x70 mm).
A.4 Preliminary Unmixing Results

Data presented in this appendix was from one of the first unmixing tests performed with the HSM on an actual tissue section. A section of human tonsil (obtained from Dr. David Hedley) that was observed to emit a green/yellow autofluorescence when excited with blue light was used. A MiB-1-Cy3 fluorescent antibody (Invitrogen, Carlsbad, CA, USA) was used to label proliferating cells in the section (Gerdes, Schwab et al. 1983; Scholzen and Gerdes 2000). This antibody is often used to assess the mitotic fraction in tumours, as this is linked to tumour recurrence and patient survival in a number of types of cancer including breast, kidney and prostate (Aaltomaa, Lipponen et al. 1997; Aaltomaa, Lipponen et al. 1997; Pirinen, Lipponen et al. 1997; van Diest, van der Wall et al. 2004).

![Figure A.4.1](image)

**Figure A.4.1** – (A) Unmixed Cy3-MiB-1 fluorescence signal, and (B) Unmixed autofluorescence signal from a human tonsil section. Most of the light detected in this spectral imaging cube was due to tissue autofluorescence.

Images were acquired on the HSM under 488 nm excitation in hyperspectral mode. Single channel images were also acquired at the same excitation wavelength and over emission bands of...
575 ± 5 nm (centered on the peak emission of Cy3) and 525 ± 25 nm (detects only autofluorescence). The spectrally unmixed autofluorescence and MiB-1 abundance images are found in figure A.4.1. As can be seen, a significant portion of the signal in the spectral data cube is due to the tissue autofluorescence, but discrete structures are visible in the MiB-1 unmixed image even at this scale. Images of the boxed region in figure A.4.1 from the single channel and unmixed data sets are displayed in figure A.4.2.

Figure A.4.2 – Spectral unmixing compared with single channel imaging in a human tonsil section stained with the proliferation marker MiB-1. (A) Single channel (570-580 nm emission) image. (B) Unmixed Cy3-MiB-1 fluorescence signal. (C) Unmixed autofluorescence signal from a human tonsil section. In single channel images it was impossible to distinguish positive MiB-1 staining (white arrows) from autofluorescent clusters within the tissue (black arrows).

The single channel image (figure A.4.2a) was obtained using a very narrow emission filter (575±5 nm) centered on the emission maximum of Cy3. Since autofluorescence is characterized
by a broad relatively flat emission, this narrow filter minimized the amount of autofluorescence detected in the single channel images. Despite this, there is a considerable amount of autofluorescence present, and it is impossible to obtain an accurate count of the MiB-1 positive cells. Comparing the spectrally unmixed MiB-1 abundance image (figure A.4.2b) with the single channel image shows just how unreliable any statistics based on the single channel data would be. There are clusters of autofluorescent cells (black arrows in A.4.2a) which appear structurally similar to some large clusters of MiB-1 positive cells (white arrows in A.4.2a). Upon spectral analysis and unmixing, it is apparent that many of the clusters that appear to be MiB-1 positive in the single channel image do not contain any Cy3 fluorescence. The location and number of MiB-1 positive cells could be reliably obtained in this sample only by using linear unmixing, as single channel imaging would likely result in the over-estimation of the mitotic fraction.

Figure A.4.3 – Colour overlays of the single channel and spectrally unmixed images of the tonsil section. The Cy3-MiB-1 channels were false coloured yellow and autofluorescence channels coloured blue. (A) Single channel Cy3-MiB-1/AF overlay. (B) Unmixed Cy3-MiB-1/AF overlay.
Shown in figure A.4.3 are false-colour overlays of both the single channel (A.4.3a) and unmixed results (A.4.3b). For both images the MiB-1 channel was coloured yellow, and the autofluorescence channel coloured blue. The intense autofluorescence present in the single channel images washes out the signal from the positive MiB-1 regions.
A.5 Dependence of Autofluorescence on Tissue Processing

A.5.1 Fixation Induced Autofluorescence

As discussed in Chapter 1, fixation and processing generally tends to increase tissue autofluorescence. Hence, limited studies were done to determine if the formalin fixation time could be used to control the level of autofluorescence in a variety of tissue types. Numerous literature references point to fixation as a potential cause of tissue autofluorescence increase (Dowson 1973; Anderson, Paparo et al. 1979; Collins and Goldsmith 1981; Tagliaferro, Tandler et al. 1997; Baschong, Suetterlin et al. 2001), and this study attempted to reproduce this effect in a controlled manner. The potential here is that, if the increase in autofluorescence is proportional to formalin fixation time, this could be used to generate a set of tissues with progressively higher autofluorescence levels, which could then be used to characterize the limits of the spectral unmixing methods in tissue samples of interest.

Tissues cut from previously fixed and paraffin-embedded blocks (mouse liver, gut, heart and pancreas), were fixed in formalin a second time for 24 h, 72 h, 7 days or 30 days. Hyperspectral data were then captured on a Zeiss LSM 510 META system. Illustrative images of the 24 h and 30 day liver and gut section are shown in figures A.5.1 and A.5.2, respectively. All images were acquired under identical excitation light intensity and detector gain settings so that they could be directly and quantitatively compared.
There was no visible change in either the autofluorescence intensity or spectral properties over the time points tested within each tissue type, but the level of autofluorescence did vary slightly between tissue types. While these results did not indicate a link between formalin fixation time and autofluorescence levels, it has been fairly well established in the literature that this link exists. It is likely, therefore, that a saturation point had been reached by the aldehyde-induced

**Figure A.5.1** – Autofluorescence images of serial mouse liver sections. (A) Fixed in formalin for 24h. (B) Fixed in formalin for 30d. No significant increase in the autofluorescence intensity or changes in the spectral profile were observed.

**Figure A.5.2** – Autofluorescence images of serial mouse gut sections. (A) Fixed in formalin for 24h. (B) Fixed in formalin for 30d. No significant increase in the autofluorescence intensity or changes in the spectral profile were observed.
autofluorescence. Hence, the experiment designed in Chapter 4 was modified to simulate the extremely high autofluorescence desired for the spectral validation tests. This was done by varying the antibody staining concentration in a series of serial sections and using an excitation wavelength which was not optimal for the Cy3 fluorophore being used, resulting in poor excitation efficiency coupled with an increased autofluorescence background as the staining concentration was reduced.

A.5.2 Antigen Retrieval Induced Autofluorescence

This limited set of experiments investigated another potential source of tissue autofluorescence increase in paraffin-embedded, formalin-fixed tissues; namely autofluorescence occurring as a result of the process for antigen retrieval for fluorescence staining. Antigen retrieval (Dicker, Lerner et al. 2007; Leong and Leong 2007) essentially unmasks antigens within fixed tissues, allowing for more efficient fluorescence immuno-staining and analysis. Antigen retrieval is not required for all fluorescence stains; however it is a fairly common processing step and is recommended by the manufacturer for both the MiB-1 label used in Appendix 4 and the TAG72/CC49 label used in Chapter 4. Figure A.5.3 shows serial sections from a paraffin-embedded, formalin-fixed pancreatic tumour xenograft (BXPC-9), obtained from Dr. David Hedley. The section in figure A.5.3a has only been de-waxed and placed on a slide, while the section in figure A.5.3b has undergone heat-induced antigen retrieval. Both image sets were acquired under identical excitation intensity and detector gain settings, and the autofluorescence in the section that underwent antigen retrieval has increased noticeably.
Figure A.5.3 – HSM images of serial BXPC9 tumour sections. (A) De-waxed paraffin section excited at 488 nm. (B) Serial section that underwent heat-induced antigen retrieval.

Figure A.5.4 – Spectral plots of the average autofluorescence from the BXPC9 sections shown in figure A.5.3. Red = de-waxed section spectrum, green = antigen retrieval section spectrum, yellow = de-waxed spectrum multiplied by 6.

Figure A.5.4 shows the average autofluorescence spectra from the de-waxed section (red plot) and the section that underwent antigen retrieval (green plot). In the yellow plot, the spectrum from the de-waxed slide has been normalized to the same maximum intensity as the slide that underwent antigen retrieval. While significantly lower in intensity, it appears that the spectrum
from the unprocessed slide is the same as that of the processed section. This suggests that the increase in tissue autofluorescence during antigen retrieval is due to an increase in the fluorescence quantum yield of the endogenous tissue fluorescence, and not the creation or enhancement of a distinct fluorescent species. A likely mechanism for this is as follows. During tissue fixation the structural proteins and antigens within the tissue are cross-linked and preserved by the fixative. As a result, a majority of the endogenous fluorescent species in the tissue are masked, making them non-fluorescent. After antigen retrieval, the majority of the cross-links are removed, resulting in the unmasking of these species and an increase in the tissue autofluorescence is observed.
References


