Development of a Platform for Surface Enhanced Raman Scattering Endoscopy

by

Patrick Zachary McVeigh

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Department of Medical Biophysics
University of Toronto

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Abstract

Surgical resection or ablation remains the primary curative treatment strategy for most early-stage solid cancers and, as such, an enormous amount of effort has been dedicated to technologies that augment the vision of the surgeon to better ‘see’ the margins of the tumor being removed. With our ever-expanding knowledge of the genetic changes which underpin malignancies, the idea of adding ‘biochemical vision’ to the surgeon’s decision making process should allow for clearer identification of margins, more complete resections with no residual tissue left in-situ, and consequent improvement in local recurrence rates. By performing such early-stage resections endoscopically, patients may benefit from lower morbidity rates as compared to traditional open approaches.

Molecular imaging using surface enhanced Raman scattering (SERS) nanoparticles represents a platform which is well-suited for cancer detection in-vivo. It has already been established that SERS offers the ability to carry out multiplex assays far in excess of what is possible with fluorescence, that the SERS signal is detectable at concentrations far below what is resolvable with similar amounts of fluorophores, and that the SERS signal is temporally stable and does not photobleach over time. The work reported here demonstrates: 1) a method for reliably generating
molecularly-targeted SERS nanoparticles suitable for in-vivo use, 2) a framework for detecting these SERS nanoparticles in-vivo, not using slow point-by-point spectroscopic screening, but rather by fast large-area widefield imaging, 3) results from widefield quantitative, multiplexed SERS imaging in-vivo using phantom models, 4) the first reported study showing widefield SERS imaging of antibody-targeted SERS nanoparticles in a murine xenograft tumour model and 5) proof-of-concept data showing widefield SERS imaging in-vitro using two clinically available endoscopy platforms. Together these results have demonstrated the potential utility of SERS endoscopy in cancer imaging and have established a roadmap to future clinical translation in specific oncologic fields.
Acknowledgments

‘I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.’

- Sir Isaac Newton (1642-1727)

To my family, old and new, for their years of support and encouragement.

To the giants of research who have guided this work and my development as a scientist.

To my wife, Ana, for the unconditional love and understanding which underpins my every day and night.

‘Better is possible. It does not take genius. It takes diligence. It takes moral clarity. It takes ingenuity. And above all, it takes a willingness to try.’

-Atul Gawande
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Abbreviations

AFB – Autofluorescence Bronchoscopy
CCD – Charge Coupled Device
CFE – Coherent Fiber Endoscope
CFI – Canada Foundation for Innovation
CIS – Carcinoma in-situ
CRC – Colorectal Carcinoma
CT – Computed Tomography
Da – Dalton
EGFR – Epidermal Growth Factor Receptor
GMP – Good Manufacturing Practices
HER2 – Human Epidermal Growth Factor Receptor 2
NIR – Near Infrared
NSCLC – Non-Small Cell Lung Cancer
PDGFR – Platelet Derived Growth Factor Receptor
PEG – Polyethylene Glycol
SCLC – Small Cell Lung Cancer
SERS – Surface Enhanced Raman Scattering
SFE – Scanning Fiber Endoscope
VEGFR – Vascular Endothelial Growth Factor Receptor
QD – Quantum Dot
1 Introduction

1.1 Motivation

As our understanding of the molecular origins of cancer has grown exponentially in the last 20 years there has been a paradigm shift in the approach to treatment, away from demographic-based generic approaches to personalized regimens based on the genetic composition of an individual’s particular disease. In the case of lung cancer, numerous subtypes have been identified that are driven by distinct genetic mutations and novel drugs have been developed to target these unique pathways in the hope of improving the dismal overall 5-year survival rate which continues to hover below 20%\(^1\). A focus on only personalized therapy faces an uphill battle in lung cancer, however, as almost 75% of cases have evidence of metastatic spread to regional or distant sites at the time of diagnosis. In contrast, those patients diagnosed with early, stage I disease have a 5 year survival rate exceeding 60%. However, less than 16% of all lung cancers are diagnosed at this early stage\(^1\). It follows then that the most significant improvements in lung cancer survival rates in the near future may come not from personalized drug therapy, but simply from earlier detection before the disease has begun diffuse invasion of the body.

The challenge of screening for early stage lung cancer is certainly not trivial: such cancers are difficult to distinguish from normal lung tissue on macroscopic examination, and the molecular profiling techniques used to identify neoplasia in the pathology lab work only with isolated cells and are not compatible with screening the lung in-situ. The focus of this thesis is the development of a platform to allow the translation of molecular profiling of cells from the laboratory bench directly into the lungs of a patient being screened for cancer. This section provides a general introduction to lung cancer pathogenesis and screening, reviews existing and emerging techniques for molecular profiling of cells to detect early malignancy, and gives an overview of work to date in molecular endoscopy for cancer detection.
1.2 Lung cancer as a model system

1.2.1 Epidemiological overview of lung cancer

Smoking, the primary causative agent in lung cancer, has a prevalence of approximately 20% at present in Canada and the United States\(^2\). This percentage has been decreasing in males since the US Surgeon General’s report on smoking and health in 1964, but only began to decrease in women in the 1980s\(^1\). There is a long latency period between exposure and disease development, and as such there are at least 100 million current or former smokers in North America who are at an increased risk of developing lung cancer\(^3\). The prevalence of tobacco consumption is not homogeneous globally, and is estimated to be above 60% in men in countries such as China, Indonesia, Greece, and Jordan\(^2\) and is generally increasing throughout the developing world. Despite it already being the leading cause of cancer deaths among men and the second leading cause in women worldwide, the global burden of lung cancer is likely to increase dramatically in the near future\(^2\).

In Canada lung cancer kills more individuals each year than breast, colorectal, and prostate cancer combined, and will be diagnosed in over 25,000 patients in 2013\(^1\). The most recent overall 5 year relative survival ratio (RSR) in Canada (excluding Quebec) for all lung cancers is 18%, as compared to 88% in breast and 65% in colorectal cancers. The 5 year RSR for lung cancer has increased only slightly (4%) since 1992-94, and this improvement in outcomes has not kept pace with those seen other cancers such as colorectal (9%), prostate (9%), kidney (7%), and leukemia (14%) in the same time interval\(^1\).

1.2.2 Lung cancer pathogenesis

In the standard model of malignant progression, cells which harbor some spontaneous or induced mutation expand into dysregulated clonal patches, followed by dysplasia, carcinoma in situ (CIS), locally advanced disease, and eventually metastatic spread. Lung cancer has been observed to follow this progression and at the time of pathological diagnosis has been classically
divided into 2 primary groups based on histological appearance; small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). Small cell lung cancer, comprising approximately 15% of cases, is primarily a disease of the large airways and is strongly linked to smoking exposure. Non-small-cell lung cancer (NSCLC), comprising the remaining 85% of cases includes squamous cell carcinomas which also tend to originate in central bronchi, and adenocarcinomas which are the most common subtype amongst non-smokers.

A large part of the challenge in detecting, diagnosing, and treating lung cancer is the heterogeneity of the disease itself. The earliest clonal patches are thought to arise from a combination of factors unrelated to smoking such as germline mutations in EGFR, p53 or specific single-nucleotide polymorphisms (SNPs) which then interact with the dominant causative agent, tobacco smoke, which has been shown to induce chronic inflammation in lung tissue leading to mutations, loss of heterozygosity, and increased inflammatory and apoptotic pathway activation. As the clonal patches become increasingly proliferative and dysregulated with respect to apoptosis, angiogenic and other growth and invasion pathways may become activated. There is, as previously alluded to, a great diversity in the mechanisms used by different tumors to achieve this dysregulated growth, and distinct driving mutations may occur within subpopulations of a single tumor. p53 is mutated in more than 50% of all NSCLC cases, while KRAS is mutated in (10-30%) of adenocarcinomas but is rarely altered in other forms of NSCLC. EGFR, a target of many lung cancer therapies in recent years, is amplified in 30% of squamous cell carcinomas, but only 15% of adenocarcinomas. By contrast, the kinase domain of EGFR is mutated in (10-40%) of adenocarcinomas, but is rarely changed in squamous cell cases. Other genetic abnormalities commonly reported include HER2 amplification or kinase domain mutation, MET mutation or amplification, and PIK3CA amplification.

1.2.3 Lung cancer screening with computed tomography
Since early detection is key to improving outcomes in lung cancer, a significant body of work has been carried out on developing screening techniques, primarily based on computed tomography (CT) scans. In 2002 the US National Cancer Institute (NCI) sponsored the National Lung Screening Trial (NLST), a randomized trial spanning 33 sites and involving 53,000 patients to directly test the utility of low-dose spiral CT scanning as compared to standard chest x-ray (CXR) for the detection of lung cancer. Patients received 3 annual low-dose CT or CXR exams and followed for a median of 6.5 years – an example of typical screening images from the low-dose CT arm are shown in Figure 1.1.

![Figure 1.1](image-url)

Figure 1.1. Evolution of a small 6.5mm ground glass opacity in the right lower lobe by CT at intake into the NLST, then repeat screening images at 2.5 years (B) and finally at 8 years (C) where the nodule has grown to 8mm diameter. At biopsy the diagnosis was adenocarcinoma. Reprinted (adapted) with permission from Aberle DR et al.

The NLST was stopped in late 2010, before the planned end date, when interim data analysis showed a significant reduction in cancer-specific mortality in the low-dose CT arm (247 deaths per 100,000 person-years) as compared to the CXR arm (309 deaths per 100,000 person-years). While these results were encouraging, a major limitation of low-dose CT was immediately identified: of the 75,126 CT scans studied by radiologists over the course of the study, 18,146...
(24.2%) were deemed to be positive for possible lung cancer. Following follow-up testing, however, only 649 cases (3.6%) were deemed to be actual cancer, with the remainder being false positives. The NLST results were also notable for the fact that, while low-dose CT discovered more than 50% of adenocarcinomas at stage I, it found only 6.8% of small-cell carcinomas at this early timepoint (54.1% of SCCs were discovered only at stage IV)\textsuperscript{20}. Adenocarcinomas, which typically arise deep in the lung parenchyma may provide more contrast on CT imaging as compared to SCCs, which typically begin in the epithelial lining of large airways. As the conducting airway walls are relatively radiopaque as compared to the air-filled respiratory spaces, small-cell carcinomas may remain radiologically occult on both CXR and CT until their size begins to influence the contour of the airway at a later stage. As a result of the high false discovery rate, lesions identified on low-dose CT require a follow-up diagnostic procedure with a higher specificity, which generally has a higher degree of invasiveness such as bronchoscopy or surgical exploration. Lam et al. report\textsuperscript{21} that in the NLST 25% of patients who underwent a surgical procedure as a result of their screening CT had lesions which were pathologically determined to be benign, underscoring the need for an improved specificity prior to surgery.

1.2.4 Bronchoscopic detection of lung cancer

First-line invasive imaging in the lung usually in the form of bronchoscopy, whereby a flexible shaft containing a camera and biopsy tool are passed into the conduction airways in the lung and a physician examines the tissues under white light reflection imaging. The color images obtained can allow for diagnosis of states such as inflammation or fibrosis that may be undetectable on CT, and any grossly abnormal tissues can be biopsied on the spot. These capabilities have led to an interest in screening high-risk individuals using bronchoscopy, however the location of carcinoma in-situ, let alone metaplasia or dysplasia, is technically challenging using conventional white light examination as these early dysplastic states do not differ macroscopically from normal tissue.
The use of fluorescence-based techniques to improve the sensitivity and specificity of bronchoscopy, specifically autofluorescence bronchoscopy (AFB), has been the focus of a large amount of interest since its introduction in the early 1990s. AFB relies on a change in the chemical composition and structure of dysplastic or CIS cells as compared to normal epithelial cells, which will generate a difference in fluorescence emission spectra when excited with intense blue light, shown in Figure 1.2. The majority of this contrast results from a thickening of the epithelium in abnormal regions, which decreases the emission signal component from the underlying connective tissue. As such AFB can be easily misled by acute inflammation or chronic lung damage and remodeling, both of which are commonly found in smokers, leading to a varying specificity of 25-50%. AFB remains under investigation as a possible screening methodology, but the low specificity will continue to limit its application as bronchoscopy remains relatively expensive in comparison to CT for similarly inconclusive results.

Figure 1.2. (A) White light and (B) blue excitation light fluorescence images acquired during bronchoscopic examination. Normal tissue fluorescence is green, while the absence of green and appearance of darker red signal (center of image) on AFB is indicative of disease. Reprinted (adapted) with permission from Short MA et al.
In order to improve the sensitivity of bronchoscopy to premalignant and malignant lesions, methylene blue (MB) has been used as a topically-applied nonspecific contrast agent\textsuperscript{29-31}. While MB has shown to be useful for mucosal lesion delineation in GI endoscopy\textsuperscript{32}, so-called ‘chromobronchoscopy’ was shown to have no benefit over standard white light screening in the central airways\textsuperscript{30}. In a related study, intravenously administered fluorescein sodium (FS) was used along with confocal endomicroscopy (CEM) for detection of premalignant and malignant transformation of the bronchial epithelium\textsuperscript{33}. This approach was shown to provide a microscopic image of the fluorophore in the lung interstitium and alveoli, but the contrast agent is nonspecific and relies on differential uptake between healthy and abnormal tissue, which was not observed. These findings are unsurprising given that structural changes associated with premalignancy in the lung epithelium are subtle and, as in the case of AFB, may be overshadowed by the chronic inflammatory state of the lung in some smokers.

As white light, autofluorescence, and chromobronchoscopy are primarily reliant on a structural reorganization, rather than a molecular mechanism for signal generation, then by definition they cannot be used to detect the early genetic changes associated with lung cancer. What is needed is an alternative method of molecular imaging which would be specific to the genetic fingerprint of dysplasia, rather than to its downstream physical effects.

### 1.3 Raman Spectroscopy

#### 1.3.1 Inelastic light scattering

“...in every case in which light is scattered by the molecules in dust-free liquids or gases, the diffuse radiation of the ordinary kind, having the same wavelength as the incident beam, is accompanied by a modified scattered radiation of degraded frequency.”
In his letter to Nature in 1928, C.V. Raman described the first experimental demonstration of the scattering effect that would later bear his name. It was only 5 years earlier that Compton published his finding that x-rays could scatter inelastically in collisions with electrons, providing concrete evidence to support the theory of the quantum nature of electromagnetic radiation, which at the time was still somewhat controversial. Raman’s experiments extended the quantum nature of light into the visible region, and just as Compton was awarded the Nobel prize in 1927, Raman received his Nobel in 1930.

![Jablonski diagram showing the transitions undergone in elastic (Rayleigh) and Stokes/Anti-Stokes Raman scattering of an incoming photon with energy $h\nu_0$.](image)

Figure 1.3. Jablonski diagram showing the transitions undergone in elastic (Rayleigh) and Stokes/Anti-Stokes Raman scattering of an incoming photon with energy $h\nu_0$.

When a photon with energy $h\nu$ scatters off a molecule, elastic (Rayleigh) scattering with equal outgoing photon energy $h\nu$ is the most probable event. Inelastic (Raman) scattering, in which the
light quantum exchanges some vibrational energy with the molecule and scatters with energy $\hbar \nu \pm \Delta E$ is far less likely. As shown in Figure 1.3, the scattered photon may receive some energy from the molecule and leave at a shorter wavelength (anti-Stokes shifted), but from thermodynamics we know that in standard conditions the majority of molecules will be in the ground vibrational state, and thus the more likely scattering event sees the outgoing photon having donated some energy to the molecule and being shifted to a longer wavelength (Stokes shifted).

All molecules, built from atoms of varying mass which are joined by flexible electronic bonds will undergo vibrations that will be characteristic of the atoms involved, the strength of the bonds between them, and their 3d arrangement in space. This vibrational ‘fingerprint’ will be altered by differences in atomic composition such as isotopic substitution, altered structural configuration or conformation, or changes in the chemical environment. Raman scattering probes this vibrational fingerprint by exposing the molecule to an oscillating electric field in the form of monochromatic light, which forces the nuclei and electrons to move in opposite directions, inducing an instantaneous dipole moment $\mu$ which is classically given by:

$$
\mu = \alpha E
$$

where $E$ is the electric field strength and $\alpha$ the molecular polarizability, a measure of the lability of the electrons relative to the nuclei within the molecule. Stated explicitly, a vibration will only be Raman active when:

$$
\left( \frac{\partial \alpha}{\partial q} \right) \neq 0
$$

where $q$ is the simplified coordinate describing the atomic motion during a vibration. Isolated individual atoms have isotropic polarizability and thus they simply re-radiate at the original frequency (Rayleigh scattering) but, in molecules where atoms participating in a bond are separated by larger distances, the polarizability may be anisotropic depending on the vibrational coordinates. In this case the re-radiated light will be at frequency shifted from the incoming radiation by $\nu_R$, the vibrational frequency.
1.3.2 Raman spectroscopy

Raman spectroscopy records the amplitude of the frequency-shifted scattered light relative to the excitation frequency, allowing for the determination of the vibrational fingerprint of the molecules being studied. The frequency shift of the outgoing light is generally reported in wavenumbers relative to the frequency of the excitation source, since the magnitude of the shift is independent of the actual wavelength of light used. Figure 1.4 shows the Stokes Raman spectrum for gold-adsorbed 4,4’-dipyridyl as well as that of its deuterated substitution, demonstrating the shift in both intensity and frequency of the Raman-active vibrations following isotopic substitution.

Figure 1.4. Raman spectrum of 4,4’-dipyridyl (black) and its deuterated analogue (red), demonstrating the sensitivity of Raman spectroscopy to even subtle changes in the ‘fingerprint’ of the scattering molecule. By convention throughout this thesis spectra are displayed in a biological, not spectroscopic, format with increasing Stokes shift shown on the positive x-axis unless otherwise noted.
Since the intensity of the bands in a Raman spectrum are proportional to the concentration of molecules which give rise to them, mixtures can be analyzed and the relative amounts of different compounds deduced from the distribution of peak frequencies and intensities. Raman spectroscopy provides a molecular analysis which does not require any special sample preparation, nor does it result in the destruction of the sample itself, both of which make it particularly appealing for use in biological applications.

1.3.3 Raman spectroscopy in biological diagnostics

Because of its high degree of molecular sensitivity, Raman spectroscopy has found a number of applications in biomedical science: the identification of different bacterial strains\textsuperscript{37}, detecting changes in bone structure\textsuperscript{38-40}, and monitoring drug-cell interaction\textsuperscript{41} have all been demonstrated. Of particular interest is the demonstrated ability of Raman spectroscopy to identify malignant changes from normal tissues in cancers of the skin\textsuperscript{42,43}, bladder\textsuperscript{44}, breast\textsuperscript{45,46}, and esophagus\textsuperscript{47} among others.

Endogenous Raman signals arising from the lung epithelium have been sampled using fiber optic Raman probes operating in the near infrared (NIR) which are passed through the accessory channel of a white light endoscope to perform an ‘optical biopsy’ of an area of interest during routine clinical examination\textsuperscript{28,48,49}. Differences in the Raman signals from DNA, hemoglobin, phenylalanine, and collagen type I have been observed between dysplastic lesions and normal epithelium, and the ability of Raman to differentiate moderately dysplastic or worse lesions from low grade or normal was reported with a sensitivity of 90% and a specificity of 91%\textsuperscript{28}. 
Figure 1.5. (A) Unprocessed Raman spectra from lung pathologies taken in-vivo with excitation at 785nm (inset) Raw, unscaled spectra. (B) Spectra processed with (intensity calibration, fluorescence background fitting and subtraction, normalization) for regions identified as squamous cell carcinoma (SCC), carcinoma in-situ (CIS), dysplasia, and normal lung epithelial tissue. Reprinted (adapted) with permission from Short MA et al.48

Figure 1.5 shows pre/post-processed Raman spectra acquired from different lung pathological states including dysplasia, carcinoma in-situ, and squamous cell carcinoma as compared to normal lung epithelium48. It is immediately apparent from the raw data that the endogenous Raman signal represents only a small perturbation on a broad background which arises primarily from tissue autofluorescence. Even with excitation in the NIR, the magnitude of the background fluorescence dwarfs the Raman signal, so that spectral processing is necessary to extract only the Raman features of interest. The processed Raman spectra display differences between pathologic states which are subtle at best, and multivariate statistical analysis, primarily with principal components analysis (PCA), is necessary to differentiate reliably between the tissue types28. These studies were further limited by the long times necessary to acquire an endogenous Raman
spectrum with sufficient signal to process (typically seconds per point), which precludes the possibility of using endogenous Raman in a bronchoscopic screening application. This is true of all endomicroscopic approaches which have a field of view or sampling volume of only 100’s of microns, which restricts their application to ‘optical biopsy’ applications and does not address the more critical issue of where the biopsy should be taken in the first place. Raman spectroscopy may be able to provide the molecular specificity necessary to identify early dysplastic changes in the lung, but it cannot be applied to large-area surveys such as would be necessary in lung screening without a significant increase in signal intensity.

1.4 Surface enhanced Raman scattering

1.4.1 Physical basis of surface enhancement

As previously alluded to, for all the richness of molecular information one can obtain from Raman spectroscopy there is a large price to be paid in regards to signal levels: only approximately 1 in every $10^7$ photons will be scattered inelastically, which makes the study of dilute solutions or mixtures containing even small amounts of fluorescent contaminants extremely challenging. In 1974, Fleischmann et al. observed intensity increases in the Raman signal from pyridine when adsorbed onto a roughened silver electrode, attributing it to an increased concentration of the pyridine on the larger surface area, but it was not until 1977 that Jeanmaire and Albrecht conclusively demonstrated that the enhancement was actually $10^4$-$10^6$ times higher on a per-molecule basis when adsorbed onto the metal surface. This effect, surface enhanced Raman scattering (SERS), opened the door to the study of an enormous number of adsorbed molecules at concentrations far below what had previously been possible with conventional Raman spectroscopy.

The origin of the signal enhancement lies with the fact that the surface features on the roughened silver electrode were smaller than the wavelength of the light used for illumination. In such cases the conduction electrons in the metal can be driven into collective oscillations, referred to as surface plasmons and, in the case in which the driving oscillatory field is in resonance with the
plasmon frequency of the metal, significant local field enhancement will arise in the near field. Using the tensor-free simplified description developed by Moscovits\textsuperscript{56}, the average magnitude of the near field, $E_{\text{near}}$, can be expressed by:

$$E_{\text{near}} = gE_0$$

where $E_0$ is the magnitude of the incident field and $g$ the average ‘field enhancement factor’, which depends on the metal geometry and composition. If a molecule were located sufficiently close to the surface of the metal, it would experience this enhanced field and the Raman scattered electric field would have a consequent magnitude given by:

$$E_{\text{Raman}} \propto \alpha_R gE_0$$

where $\alpha_R$ is taken to be a simplified version of the full treatment of the Raman tensor, taking into account the effects of vector combination of the wave and polarization states. It is important to note that the emitted field will also be scattered by the enhanced metal field; the enhancement factor may be slightly different from that for the exciting frequency light and is thus denoted $g'$, although for low wavenumber Raman bands $g \approx g'$ for all practical purposes. Since what is detected is the intensity of the electric field, proportional to the square of the field magnitude, we arrive at:

$$I_{\text{SERS}} \propto |\alpha_R|^2|gg'|^2I_{\text{incident}}$$

It is now clear where the dramatically increased signals reported by Fleischmann arose from: the $g$ factor for silver has been experimentally determined\textsuperscript{56} to be $\sim 30$, so that the intensity of the Raman signal would be enhanced by a factor of more than $10^5$. The results of Fleischmann, which demonstrated a redistribution of intensities of the dye peaks as compared to the bulk Raman spectrum, also present an important distinction in SERS: the $\alpha_R$ polarizability factor is for the molecule on the surface of the metal, and may be different than that of the free molecule. Depending on how the electronic field of the molecule interacts with the metal, charge transfer may occur\textsuperscript{36} leading to greatly increased or decreased polarizability which is commonly explained as a ‘chemical’ component of SERS enhancement\textsuperscript{56-58}.

1.4.2 Typical SERS substrates

The initial substrates used for SERS observation were metallic electrodes which had been roughened by repetitive electrochemical oxidation-reduction cycling which results in a random distribution of metal clusters on the electrode surface\(^5^9\). To create more precise surface features, top-down nanofabrication techniques such as photolithography and electron beam lithography have been applied to create highly SERS-active, uniform substrates consisting of periodic arrays of metallic pillars with different aspect ratios and cross-sectional profiles (circular, square, triangular etc.)\(^6^0\), which all support plasmon oscillations and give rise to very large local fields for sensitive SERS detection. Such designs have been used for the detection of organic molecules such as rhodamine 6G\(^6^1\) and biomolecules such as glucose\(^6^2\) at concentrations far below what is achievable with conventional Raman spectroscopy – in the case of glucose measurement, an increased sensitivity factor of \(~10^{11}\) was reported as compared to bulk Raman detection\(^6^2\).

At present, the most commonly reported SERS substrates are colloidal suspensions of nanoscale metallic particles. Silver and gold are the most frequently used starting materials, and the chemistry used can be tuned to produce colloids which range in size from a few nm to over 100 nm with relatively narrow size distributions\(^6^3-6^5\). Nanoparticles can also be directly formed by pulsed laser ablation\(^6^6\), an approach which also supports the generation of alloy nanoparticles with unique photonic properties. The nanoparticles may be simple spheres\(^6^4\) or may take on more complicated shapes such as rods\(^6^7-6^9\), stars\(^7^0, 7^1\), urchins\(^7^2\), and any number of other shapes to allow for tuning of the plasmon resonant frequency and SERS enhancement factor.

1.4.3 SERS enhancement of endogenous biological Raman signals

By bringing a biomolecule of interest near the surface of a SERS substrate, the endogenous Raman signals can be enhanced to the point that they are detectable above background autofluorescence. As examples of this approach, by introducing 60 nm gold nanoparticles into the cell culture medium of osteosarcoma cells, the Raman fingerprint spectra of nuclear and
cytoplasmic contents have been measured with micron-level resolution using brief integration times\textsuperscript{73, 74}. After mixing serum samples from normal and colorectal cancer patients with 40nm gold nanospheres, the resulting SERS spectra showed biomolecular changes which were capable of differentiating the two groups\textsuperscript{75}.

As an alternative to solution-based enhancement, endogenous Raman signals can be amplified by contact with a miniaturized SERS surface on the tip of a fiber optic probe: the SERS tip may be produced lithographically\textsuperscript{76} or through the binding of nanoparticles on the fiber surface\textsuperscript{77}. An intracellular SERS probe based on a nanopipette tip functionalized with gold nanoparticles was recently described\textsuperscript{78, 79}: such tips can be mounted to standard cell micromanipulators and be used to acquire SERS spectra from different regions of the cell under microscopic visualization, while also allowing for the injection of solutions through the pipette tip to allow for dynamic SERS acquisition from cell compartments responding to stresses such as osmotic shock.

While the analysis of endogenous Raman spectra to study biological mechanisms is appealing in principle, it remains technically challenging: even with SERS enhancement the differences between the spectra of diseased and normal cells often amounts to minute changes in peak ratios, which require substantial amounts of data processing to differentiate. Consequently a major focus has been on the development of SERS probes which leverage the unique spectral properties of Raman scattering to replace conventional optical probes in cell biology by using exogenous, rather than endogenous, Raman reporter molecules.

1.5 Contrast agents for multiplex molecular imaging

1.5.1 Fluorescence-based contrast agents

Molecular imaging, which may be broadly defined as the imaging of specific biological molecules or processes in the intact organism (as compared to structural imaging) originated with radiological techniques including PET and SPECT, but has seen enormous growth in the
optical domain. A large part of optical imaging’s appeal lies in the ability for multiplex detection; while PET/SPECT are essentially monochromatic and in general require separate scans for every target to be imaged, optical imaging allows for the simultaneous imaging of different ‘colors’ which may be assigned to distinct biological targets. The workhorse agents in this role to date have been organic fluorophores which are available spanning the visible to near infrared (NIR) in excitation/emission wavelengths. However, these dyes are far from ideal especially in the setting of in-vivo imaging. A significant number of endogenous biomolecules such as NADH and collagen are fluorescent when excited in the UV/blue/green range, which provides a large background signal which the organic fluorophore must overcome to be detectable. This difficulty in working in the visible spectrum is compounded by the strong absorption of light below approximately 600nm by hemoglobin, which by necessity is pervasive in all in-vivo imaging experiments but ultimately limits the amount of light which can reach the fluorophore to excite it, as well as absorbing a significant amount of the emitted light. Conventional fluorophores may also be photobleached, a process through which their emission can be permanently quenched by continuous exposure to the illuminating light, rendering them useless for imaging.

To counteract the autofluorescence and absorption issues when imaging in-vivo, it is preferable to choose fluorophores that absorb/emit in the ‘tissue optical window’ where hemoglobin and water absorption are minimized and the wavelength is long enough to avoid stimulating significant autofluorescence, which in practice spans approximately (650-900nm). This presents an immediate limitation on the multiplex imaging capability of organic fluorophores in this region, as they have emission peaks which are typically 100nm FWHM or more in the NIR, so that at most 2-3 colors could be imaged simultaneously and not without significant cross-talk between channels which limits the quantification of results. These fluorophores also require a separate excitation light source for each dye to be imaged, which implies that the ‘multiplex’ imaging would still need to be acquired in a serial fashion, not simultaneously.
Fluorescent semiconductor nanocrystals or quantum dots (QDs) have significant photophysical advantages over conventional fluorophores including improved brightness, narrower emission peaks (typically 40-60nm FWHM in the visible), broad excitation spectra, and resistance to photobleaching\textsuperscript{82}. While these features make QDs appealing for DNA and cell-based assays\textsuperscript{83, 84}, in the NIR tissue optical window the emission peaks of QDs begin to broaden significantly, and the excitation efficiency drops precipitously. There are also potential concerns about toxicity from the QD core materials which are typically alloyed from heavy metals such as cadmium and selenium\textsuperscript{85, 86}.

### 1.5.2 SERS nanoparticle-based contrast agents

By adsorbing a molecule with a known Raman spectrum onto the surface of a nanoparticle capable of supporting SERS enhancement, a Raman analogue to the quantum dot can be created\textsuperscript{87-94}. A primary advantage of this probe design is the richness of the spectroscopic signal it provides, as compared to the single broad peak of fluorescence emission. As shown in Figure 1.6, SERS peaks have a FWHM on the order of (0.5-2nm) when excited in the NIR, significantly narrower than a quantum dot emission peak, allowing for the possibility of multiplexing beyond 10x even in the narrow bandwidth of the tissue optical window.
A further advantage of SERS-based contrast agents is the necessity for only a single excitation wavelength to image multiple reporter molecules simultaneously as compared to organic fluorophores that require an excitation source for every probe to be imaged, frequently overlapping with the emission bands of other fluorophores and precluding the possibility of simultaneous imaging. The materials used for SERS nanoparticle cores also provide improvements over QDs: silver generally gives excellent SERS enhancement factors, but has been shown to have potent in-vitro toxicity.96, 97. Gold, however, has been well-established as biocompatible when used in bulk (as in dental implants, for example) and colloidal gold nanoparticles have shown very little toxicity when used in-vitro/vivo98-102, and even in human exposure trials103.
Just as with organic fluorophores or quantum dots, SERS nanoparticles can be targeted to specific molecules of interest using targeting moieties such as antibodies, peptides, or affibodies and SERS NPs have been used to image components of some canonical oncologic pathways both in-vitro and in-vivo. Epidermal growth factor receptor (EGFR/HER1), known to be overexpressed in some head and neck, breast, lung, bladder, and colon cancers\textsuperscript{104}, has been imaged in-vitro using SERS-active nanorods and spheres targeted with antibodies/antibody fragments\textsuperscript{87,88,94}, as well as affibodies and peptides\textsuperscript{89}. EGFR overexpression in xenograft tumors has also been successfully imaged in-vivo using nanoparticles injected intravenously\textsuperscript{87,105} or applied topically\textsuperscript{89}. HER2/neu, a proto-oncogene in the same family as EGFR, has been found to be amplified in many breast, stomach, uterine, and lung cancers\textsuperscript{106}. HER2 has been imaged directly using antibody-targeted SERS nanorods\textsuperscript{107} and hollow spheres\textsuperscript{90} in-vitro. Folate receptor (FR), the natural receptor for vitamin B9 has been shown to be up-regulated in ovarian, lung, breast, and kidney malignancy\textsuperscript{108}. SERS nanorods, directly conjugated to folic acid as a targeting agent, have been used for imaging of FR in a cervical cancer cell line\textsuperscript{109}. Prostate-specific antigen (PSA, KLK3), a commonly used biomarker of prostate tissue which is expressed variably in prostate cancers\textsuperscript{110} has been imaged using antibody-targeted SERS gold nanoshells\textsuperscript{111} as well as gold/silver nanoshell assemblies\textsuperscript{112}.

1.6 Development of a platform for SERS endoscopy

As our understanding of the genetic underpinning of complex diseases such as lung cancer grows, it has become clear that detecting a single molecular marker alone is insufficient to differentiate malignancy from normal tissue, especially in the early stages of dysplastic progression. Optical detection, as compared to nuclear or other imaging modalities, has the potential for multiplex biomarker imaging, but in the in-vivo setting conventional fluorophores cannot provide the necessary sensitivity or degree of multiplexing. Endogenous tissue Raman spectroscopy, with or without SERS enhancement, may provide the necessary sensitivity to early neoplasia but is limited to point-by-point sampling with long integration times which makes it unsuitable for screening an entire organ for possible disease.
Molecularly-targeted SERS-based contrast agents have been shown to possess the biomarker sensitivity and multiplexing ability required for in-vivo diagnostics, but these prior works have used microscopic imaging arrangements that are not compatible with endoscopic imaging. It has been suggested\textsuperscript{113} that a SERS-enabled endoscope could be constructed with a rotating-spot/pullback geometry similar to what has been used in optical coherence tomography\textsuperscript{114,115} and fluorescence\textsuperscript{116} but, as this approach still constructs an image by scanning point-by-point, the relatively long exposure times necessary for dilute SERS probe detection may render the endoscope clinically unusable.

An alternative approach to SERS endoscopy is similar to what is commonly performed in fluorescence endoscopy; as shown in Figure 1.7 the entire FOV is illuminated with excitation light and a bandpass filter is placed in front of the endoscope’s camera to allow only the light from a particular SERS band of interest to be detected. The filter is switched to another SERS band from a different reporter molecule and a series of images are constructed which convey the spatial distribution of each SERS reporter molecule in the FOV. This approach, commonly referred to as widefield, direct, or global Raman imaging has rarely been demonstrated and only ever in a microscopic arrangement\textsuperscript{117-119}. 
Figure 1.7. Schematic illustration of a widefield SERS endoscope with global illumination and a tunable filter module to resolve distinct Raman bands onto a detector.

1.7 Thesis objectives and overview

The overall goal of this thesis is the development of multiplex SERS-based nanoparticle contrast agents which are molecularly targeted against lung cancer, and to develop the novel widefield endoscopic imaging hardware to detect these nanoparticles in-vivo. While much foundational work has been done with respect to demonstrating molecular imaging with SERS nanoparticles
for single biomarker targets, at present truly multiplexed targeted imaging remains a ‘future
direction’ in the in-vivo setting. Similarly widefield Raman imaging has only ever previously
been demonstrated using a microscope as an optical base, not an endoscope, and attempts at in-
vivo widefield Raman imaging have not been reported to date.

Chapter 2 of this thesis describes the synthesis and characterization of molecularly-targeted
Multiplex detection of 3 distinct cell surface proteins commonly overexpressed in lung cancer is
demonstrated. Chapter 3 describes a framework for widefield imaging and image processing
with SERS nanoparticles and demonstrates the first example of widefield SERS imaging in-vivo.
Building upon the approach in chapter 3, Chapter 4 describes the development of a tunable filter
module suitable for multiplex SERS imaging and demonstrates widefield 4-plex imaging both in
phantoms and in-vivo, both of which were scientific firsts. Chapter 5 details the first use of
widefield SERS imaging to detect molecularly-targeted SERS nanoparticles from lung cancer
cells and xenografted tumors, both ex-vivo and in-vivo, which previously has only been shown
using microscopic detection. Chapter 6 contains a description of the first endoscopic widefield
Raman imaging of molecularly-targeted SERS nanoparticles in phantom and in-vitro test
systems. Chapter 7 presents a summary and discussion of future directions.
2 Molecularly-targeted SERS nanoparticle development

2.1 Acknowledgements

The work in this chapter describes the protocol developed for the conjugation of targeting antibodies and fluorophores to the surface of commercially available SERS nanoparticles. The development of this procedure was central to the ability to carry out in-vitro and in-vivo tests of the Raman imaging system which follow, and was hard-won considering the paucity of technical details reported in most early SERS bio-imaging reports. In large part this chapter is a reformatted version of the manuscript entitled “Optimization of multimodal silica-encapsulated surface enhanced Raman scattering nanoparticles for in-vitro diagnostics”, which is in preparation for submission to PLOS One. My contribution to this work included designing all experiments, carrying out the initial synthetic reactions, performing all the analytic tests, analyzing the data, and writing the first draft of the manuscript. I would like to thank Dr. Benjamin Scott for his tireless help with the synthetic chemistry troubleshooting, Darryl Fong for much of the optimization work, and Carl Fisher for assisting with all the in-vitro sample preparation.

2.2 Introduction

Contrast agents for molecular imaging based on surface enhanced Raman scattering (SERS) have been the subject of numerous studies in recent years. Typical SERS probes are based on a colloidal metallic nanoparticle (NP) core with an adsorbed reporter molecule that gives rise to the characteristic SERS spectrum, which can be used as a readout in a number of bioassays. Silica encapsulation of the dye-NP complex has been shown to be effective in stabilizing the SERS signal by preventing dye desorption or core-core aggregation induced by changes in the chemical environment\(^9\). If the shell is sufficiently thick to avoid near-field quenching, fluorophores may be added to the surface of the NPs to allow for dual-modality imaging and processing using high-throughput fluorescence techniques such as flow cytometry\(^8,10\). The silica surface also provides a convenient substrate for the coordination of targeting and passivation ligands for use in biological systems.
A primary advantage of SERS biosensing is the multiplexing ability it affords as compared to fluorescence: typical SERS peak widths are 1-2 orders of magnitude narrower than fluorescence emission peaks, especially in the NIR where background signals from cells are minimized. Prior works using silica-encapsulated AuNPs have demonstrated in-vitro SERS molecular imaging of EGFR, HER2, folate receptor, and CD24/44 as examples. These studies have not, however, demonstrated targeted multiplex imaging beyond 2x reporters and contain primarily proof-of-principle formulations which may not be optimal for their intended applications.

Quantitative immunostaining analysis, such as the In-Cell Western Assay (Li-Cor Biosciences, Lincoln NE) or in-cell ELISA (ICE), allows for the quantification of target proteins in a microplate format using NIR fluorescence-based probes. While useful, ICE is limited by the necessity for the fluorophores to be completely separated spectrally which has resulted in the majority of applications testing only 2 proteins at a time. A SERS-based ICE assay would overcome this limitation and allow for significantly higher throughput in applications such as phosphorylation screens and surface receptor profiling.

We have systematically examined the synthesis of multi-modality, molecularly-targeted SERS nanoparticles starting with commercially available substrates for in-vitro diagnostics and have applied these particles to an in-cell ELISA assay to demonstrate multiplex molecular imaging in a high-throughput format.

2.3 Materials and Methods

Reagents. SERS-active nanoparticles were purchased from Cabot Security Materials Inc. (Mountain View, CA, USA) and consist of a 60nm spherical gold core, an adsorbed layer of the Raman-active molecules optimized for use at 785nm, and a 30nm thick thiolated silica
encapsulant layer that stabilizes the reporter molecule on the AuNP surface and provides a reactive substrate for further functionalization. The 4 reporter molecules used were designated S420 (4,4’-dipyridyl), S421 (d8-4,4’-dipyridyl), S440 (trans-1,2-Bis(4-pyridyl)-ethylene), and S481 (4-Azobis(pyridine)). Targeting monoclonal antibodies (HER2, EPCAM) were purchased in carrier-free format (Fisher, Fremont CA). Panitumumab, a fully human anti-EGFR IgG2, was used in clinical formulation as provided by Amgen (Thousand Oaks, CA). Defined chain length heterobifunctional polyethylene glycol (PEG) linker (SM(PEG)12) and methoxy-terminated passivation ligands (MM(PEG)12/24/36) were purchased from Pierce Bio (Rockford, IL). The maleimide-activated fluorophore Cyto647 was purchased from Cytodiagnostics (Burlington, ON). All other chemicals used were of reagent grade.

**Fluorescent labeling of SERS nanoparticles.** Stock SERS AuNPs (3.8nM) were added to 3x volume of freshly prepared and vigorously argon-degassed 10mM MOPS buffer (pH 7.25) in a thoroughly rinsed amber borosilicate glass vial. TCEP-HCl was added at a molar ratio of (60,000 TCEP:1 AuNP) and allowed to react for 20 minutes at room temperature. Cyto647, prepared in anhydrous DMSO, was added in a molar ratio varying from (100-5x10^6 Cyto647:1 AuNP) and the reaction was allowed to proceed for 1 hour at room temperature. Conjugates were purified by 3 rounds of centrifugation (1000g, 10min) and resuspension in MOPS buffer and the relative fluorescence intensity for each condition was then determined using a plate reader (SpectraMax, Molecular Devices, Sunnyvale CA), and the signal normalized by the gold plasmon absorbance measured at 545nm.

**Antibody conjugation and methoxy-PEG surface passivation.** Antibody solutions were diluted to 2mg/mL and buffer exchanged into 10mM MOPS using a desalt column (Zeba 7kMWCO, Pierce). Antibody was added to SERS AuNPs in molar ratios varying from (10-1000 mAb:1 AuNP), followed by SM(PEG)12, prepared in anhydrous DMSO to a final ratio of (50 SM(PEG)12:1 mAb), and the reaction was allowed to proceed for 2 hours at room temperature while shaking. MM(PEG), prepared in anhydrous DMSO, was added to the reaction vessel in a
final ratio of (650,000 mPEG:1 AuNP) and transferred to 4°C for overnight reaction while shaking.

**Multimodality nanoparticle purification.** Sodium 2-mercaptoethanesulfonate (MESA) in MOPS storage buffer (20mM MOPS, 0.1% BSA, 0.05% NaN₃, pH 7.5) was added to the crude conjugates at a molar ratio of (1x10⁶ MESA:1 AuNP) and allowed to react for 30min at room temperature while shaking. Centrifugal purification of the final AuNP conjugates was by 4 rounds of centrifugation in storage buffer at 1000g x10min. Size exclusion purification was carried out on a glass column (1.0x20 cm, Bio-Rad, Mississauga ON) packed with Superose 6 PG media(GE Healthcare Life Sciences, Baie d’Urfe QC) and eluted with MOPS buffer of varying ionic strength (10-50mM), salinity (0-125mM NaCl), and pH (6.5-7.5).

**Nanoparticle conjugate characterization.** Nanoparticle concentration and aggregation status were evaluated by UV/VIS spectra obtained on a Cary 50 spectrophotometer (Agilent, Santa Clara CA). Nanoparticle size distribution was assessed by nanoparticle tracking analysis (NTA) using a NanoSight instrument (NanoSight Ltd, Amesbury, UK) with MOPS buffer as a diluent. Raman spectra were acquired using a spectrometer operating at 785 nm excitation (Advantage Series, DeltaNu, Laramie WY) in a quartz cuvette. Probe stability was assessed by gel shift assays carried out in 0.4% high-strength agarose on Gelbond support film (Lonza, Allendale NJ) run at 80V in 0.25X TBE buffer for 3 hours.

**Cell culture, flow cytometry, and cytotoxicity assays.** A549, Calu-3, and H520 cell lines were acquired from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON). For flow cytometry, cells were washed with PBS and detached with Accutase (ICT, San Diego, CA) for counting in cold PBS. 1x10⁶ cells in 100µL per condition were blocked using 3% BSA in PBS for 30min at room temperature, then AuNP probes were added to a final concentration of 20pM and incubated for 60min at room temperature with shaking. Cells were washed 5x by
centrifugation at (200g, 3min) using flow cytometry wash buffer (PBS, 2% FBS, 0.05% NaN₃) and filtered through a 30µm filter prior to analysis. Data for 20,000 cells were collected on a BD FACSCalibur (San Jose, CA) cytometer using 633nm fluorescence excitation. To assess cytotoxicity, cells were seeded to a density of 20,000 cells per well in clear bottom 96 well plates and allowed to attach overnight. AuNP conjugates were diluted in culture medium and introduced into the wells for 2, 4, 8, and 24 hours. Viability was then assessed using the MTT assay (Invitrogen, Burlington, ON) according to the manufacturer’s instructions.

**Raman in-cell ELISA.** Cells were plated at a density of 10,000 cells per well into clear-bottom assay plates, and stained/washed as in the above flow cytometry experiments. Following the final wash, cells were counterstained with Draq5 (BioStatus, Shepshed UK) for nuclear enumeration. Raman imaging was carried out on using a Renishaw inVia confocal Raman microscope (Hoffman Estates, IL) configured for biological imaging. The system used an inverted microscope base (Leica DMI6000B, Concord, ON) with Raman excitation light at 785nm. HTS plate data were acquired using a 20x long working distance objective (NA=0.4), line-focused laser spot and Streamline image acquisition mode, in which the stage movement is coordinated with the CCD readout such that the shutter is not closed and data is read out continuously; the step size was 36µm and a pixel integration time was 60ms. 676 spectra for each well were co-added and normalized by the nuclear Draq5 signal, and distinct peak areas were quantified to separate the relative amounts of each of the 4 reporter molecules.

### 2.4 Results and Discussion

The overall reaction scheme used in this study is shown in Figure 2.1. The choice of buffer system is dependent on the chemistry to be used for the downstream coupling of the targeting molecule onto the AuNP, which in this study is via antibody primary amines. The maleimide reaction with sulphhydryl groups on the AuNP surface is best carried out between pH (6.5-7.5), however the NHS ester reaction with primary amines is most efficient between pH (7-9). 2-(N-morpholino) ethanesulfonic acid (MES) has previously been used in this application. However,
provides virtually no buffering capacity in the necessary pH range and thus must be made immediately prior to use and is not suitable for long term storage. Both phosphate and MOPS buffers provide good buffering capacity between pH (7-7.5), but MOPS was found to be more suitable for AuNP purification by size exclusion chromatography and so was used throughout.

The first step in the one-pot synthesis is the reaction of the maleimide-activated fluorophore with the SERS AuNPs, which has previously been shown to have a low overall reaction efficiency\(^89\). As shown in Figure 2.2 the fluorophore labeling reaches saturation at approximately (500,000 fluorophore:1 AuNP), with the actual number of fluorophores per nanoparticle at approximately 1,500\(^89\). This corresponds to approximately 5% of the 30,000 thiol groups typically available on the surface of these specific AuNPs for conjugation. As is also shown in Figure 2.2, reduction of the AuNPs by tris(2-carboxyethyl)phosphine (TCEP) prior to fluorophore addition did not have any influence on the labeling reaction efficiency, indicating that disulfide bonds between sulfhydryl groups on the surface of AuNPs is not a contributing factor to the low reaction yield.
Figure 2.1. Multimodality nanoparticle reaction scheme: i. Fluorescent labeling of SERS AuNPs with maleimide-activated NIR fluorophore, ii. Conjugation of targeting antibody to SERS AuNPs using a flexible heterobifunctional crosslinker, iii. Surface passivation of unreacted thiol groups using methoxy-PEG.

The conjugation of targeting moieties to the thiolated nanoparticle surface using a heterobifunctional crosslinker targeting amine groups on the antibody lends itself to a one-pot synthesis, as there is little chance of nanoparticle-nanoparticle or antibody-antibody cross reaction under standard conditions. We have previously made use of a short chain rigid
crosslinker (Sulfo-SMCC, 8.3Å spacer arm length), however the large size of the IgG antibodies used here presents a steric concern: as the reaction is not site directed towards the Fc region it is possible that a significant number of bound antibodies would have their Fab regions bound too close to the surface of the AuNP to be able to interact with their targets. We have instead used a longer, flexible crosslinker (SM(PEG)12, 53.4Å spacer arm length) which allows the antibody to sit further from the surface of the AuNP and permits more conformational flexibility for binding to the target site. While orientation concerns of short crosslinkers are likely to be less significant for other targeting molecules such as peptides/affibodies where the antigen binding region may be engineered to be distinct from the nanoparticle linking region, the additional flexibility conferred by the PEG chain may still allow for enhanced binding as compared to more rigid linkers which would require reorientation of the whole nanoparticle to allow for tight binding to a mis-oriented receptor.

As shown in Figure 2.3, the ability of functionalized AuNPs to bind target cells begins to plateau beyond approximately 200 IgG:NP. This is in keeping with prior reports which have achieved saturation at approximately 400 IgG:NP89, although in these previous studies the NPs were

Figure 2.2. (left) Titration of fluorophore/AuNP ratio for the creation of multi-modality SERS AuNPs. Error bars represent ±1 standard deviation of 3 independent measurements. (right) Comparison of fluorescence labeling efficiency with and without TCEP reduction of the AuNPs.

As shown in Figure 2.3, the ability of functionalized AuNPs to bind target cells begins to plateau beyond approximately 200 IgG:NP. This is in keeping with prior reports which have achieved saturation at approximately 400 IgG:NP89, although in these previous studies the NPs were
labeled with a secondary antibody, not the primary detection moiety, so the results may not be directly comparable. Specific binding of the particles is seen with as few as 10 IgG molecules per nanoparticle, but the staining is not as intense and homogeneous as is seen at higher antibody density. This may be a result of an avidity effect (multiple bonds between an individual AuNP and numerous receptors on the cell surface), especially in the setting of the dilute AuNP concentrations used for cell staining: at 200 IgG:AuNP the concentration of antibody on the surface of the AuNPs used here is approximately 0.06µg/100µL, and so is reduced to roughly 3ng/100µL at 10 IgG:AuNP. This is drastically reduced from the 1µg/100uL concentration necessary in antibody-alone staining to achieve saturation of all available receptors, and as such we expect that the on/off rate of the antibody-AuNPs in solution will reduce the number of antibodies bound to receptors at any given time and that this will be more apparent at lower IgG concentrations, as observed.

In contrast to the findings of Jokerst\textsuperscript{89}, we did not experience any technical difficulties in conjugating anti-EGFR antibodies to the AuNPs using the aforementioned strategy, nor have we encountered any particular subtype of antibody that could not be made to work successfully. To date we have successfully conjugated IgG and IgM antibodies from human, mouse, rabbit, goat, and mouse/human chimeric host species with a variety of cellular targets. We have observed, however, rapid precipitation of the AuNPs from solution similar to those described previously in cases where the IgG:AuNP ratio exceeds 1000:1, and as such we have found a starting ratio of 350:1 strikes an acceptable balance between probe stability and avidity, although optimization is certainly possible for each individual ligand. It is worth noting that the most common cause of reaction failure we have observed is contamination of the antibody solution; with detergents such as Tween, preservatives such as sodium azide, or stabilizing agents such as BSA, glycerol, or gelatin. Failure to remove all of the aforementioned interfering compounds or to correctly buffer exchange the mAb into salt-free MOPS prior to addition to the AuNPs can result in significant aggregation of the solution and no useable probe generation.
In general we prefer to order carrier-free formulated antibodies supplied in PBS alone, to which a small amount of sodium azide is added for long term storage, and which is easily removed by the desalting step. We have also observed in the case of IgM targeting that multiple AuNPs may bind a single IgM, resulting in dimer and trimer populations which are discernible by NTA analysis. While the frequency of multiple-binding can be reduced by adjusting the reaction stoichiometry, we have never observed the formation of a significant number of multimers when using IgG antibodies and so use them almost exclusively.

Figure 2.3. (left) Flow cytometry histograms showing the signal from A549 cells stained with SERS AuNPs with varying amounts of antibodies/nanoparticle, (right) Quantification of the mean fluorescence intensity from A549 cells stained with SERS AuNPs with varying amounts of antibody/nanoparticle; data are collected from 20,000 cells ±1 SEM of the fluorescence signal.
The most common techniques used to purify functionalized AuNPs from excess antibody and crosslinker are centrifugation, dialysis, and size exclusion chromatography (SEC). Given the large size difference between the AuNPs and other molecules, SEC can efficiently separate the final product while also allowing for in-line post-column analysis and further separation. We have tested a number of elution conditions for Superose 6 PG SEC media and determined that 10mM MOPS buffer at pH 6.5 produced the narrowest bands and highest recovery yields for crude AuNP conjugates prepared as previously described. As shown in Figure 2.4, purification using SEC results in a more narrow size distribution in the final conjugates than centrifugal purification. Importantly, the addition of a quenching step with high concentration MESA prior to purification further narrows the size distribution of the resultant probes and dramatically improves their long term stability. MESA-quenched, SEC-purified conjugates stored in a (BSA+NaN₃)-containing buffer have continued to give equivalent results at 1 year following their initial preparation.

Figure 2.4. (left) NTA size distribution histograms from IgG-functionalized SERS AuNPs purified using centrifugal or size exclusion approaches as compared to unmodified AuNPs, (right) NTA size distribution width (in nm) for IgG modified SERS AuNPs 7 and 365 days following purification using either centrifugation or size exclusion chromatography, as compared to unmodified AuNPs stored in identical conditions.
In order to modify the solubility and stability properties of nanoparticle probes, as well as to reduce their nonspecific binding in biological systems, surface passivation with PEG is common. Methoxy-terminated PEG with molecular weight in the range of (1-10kDa) has commonly been used and it has been suggested that smaller MW PEG may be advantageous in some applications. As shown in Figure 2.5, AuNPs with a coverage of moderate MW (2400 Da) mPEG are less susceptible to modification in biological solutions as compared to smaller (1200/700 Da) PEG chains. It is notable that the surface modification by proteins detected with the gel shift assay used here is not apparent in the UV/VIS spectrum of the AuNPs (Figure 2.5, inset), which is commonly used as a measure of stability. This illustrates the importance of matching the characterization assay to the particle in question, as the thick silica shell of the AuNPs used here likely reduces the sensitivity to core-core coupling to which the UV/VIS spectrum is most sensitive as a measure of aggregation or modification.
Figure 2.5. Agarose gel shift assay for SERS AuNPs with different mPEG surface coverage after 24 hours incubation in (I) PBS, (II) MOPS, (III) RPMI with 10% FBS, (IV) 50% FBS in PBS. Failure to migrate evenly is indicative of modification of the AuNPs by the solution. (Below) UV/VIS Spectra showing the AuNP plasmon peak absorbance after 24 hour incubation in biological solution. Only the low (700 Da) PEG coverage shows any significant changes as compared to storage in MOPS buffer alone.

The performance of the different MW PEG surface coatings was also investigated in the in-vitro setting. As shown in Figure 2.6, there was no significant cell toxicity associated with any MW PEG coatings at ratios of (10-10,000) AuNP:cell. When the various mPEG molecules were used to stabilize AuNPs functionalized with an anti-EGFR antibody, the binding of the AuNPs to control (H520, EGFR-negative) cells was minimized in the case of the largest PEG molecule, as shown in Figure 2.7. When the same AuNPs were used to stain target (A549, EGFR-positive)
cells, the ratio of target:control binding was again highest in the case of the large PEG surface coverage, also shown in Figure 2.7. Given the improvements in stability and nonspecific binding conferred by passivation with moderate length (2400 Da) mPEG, this size was used for all further in-vitro work.

Figure 2.6. Cytotoxicity of SERS AuNPs with different surface mPEG ligands on A549 cells as determined by MTT assay. Relative cell viability was normalized for each timepoint and concentration to cells treated with vehicle (MOPS buffer) control. Error bars represent the standard deviation of 3 independent experiments, each with 3 technical replicates for every condition.
Figure 2.7. (left) Flow cytometry fluorescence intensity from H520 (EGFR negative) cells stained with anti-EGFR SERS AuNPs with surface passivation from varying length mPEG chains, demonstrating the least non-specific binding with the highest molecular weight mPEG chain, (right) A549/H520 intensity ratio for SERS AuNPs with different length mPEG molecules for surface passivation: the highest signal:background ratio was achieved with the highest MW PEG molecule.

The performance of these optimized SERS NPs in an ICE assay are shown in Figure 2.8. The results of quadruplex SERS staining (EGFR, HER2, EPCAM, untargeted (PEG-only) control) for the Calu-3 cell line are presented as compared to their individual staining intensities determined using flow cytometry with the targeting antibodies alone. There is qualitative agreement between the two techniques in terms of the markers known to be abundant in these cells (HER2 and EPCAM), however the SERS imaging showed a higher background staining level than was observed using antibody alone in flow cytometry. As a result, the level of EGFR present in the cells could not be quantified above baseline using SERS as was done with flow. This is most likely due to the difficulty in efficiently washing unbound tag from a HTS well without dislodging the attached cells, unlike the relatively simple and aggressive washing permitted when staining cells in solution as in flow cytometry. Work is underway to optimize this washing step through the addition of agitation and detergents to reduce the background level to be comparable between the two techniques.
As the degree of multiplexing in the assay is increased, spectral unmixing algorithms such as direct classical least squares (DCLS) may be employed to accurately determine the reporter abundances as single SERS peaks begin to overlap. As the SERS tags used here are stable at room temperature, cannot be photobleached, and do not show spectral shifts upon drying, storage and handling of the HTS plates was found to be greatly simplified as compared to conventional fluorescence or colorimetric ELISA assays.

![Comparison of Calu-3 surface receptor staining using conventional flow cytometry (left) and Raman ICE (right).](image)

Figure 2.8. Comparison of Calu-3 surface receptor staining using conventional flow cytometry (left) and Raman ICE (right). Flow data were acquired from 20,000 cells, while the SERS signal was acquired over an area of approximately 1mm² in a high-throughput plate. The SERS signal was normalized to the number of nuclei in the scan area.

### 2.5 Conclusions

We have demonstrated a reliable conjugation protocol for the functionalization of commercially-available SERS nanoparticles using a variety of antibodies for in-vitro diagnostics. These particles have been shown to resist agglomeration or signal degradation in commonly encountered biological conditions, and were shown to have no effect on cell viability over 48h. The utility of these particles in multiplex biomarker identification assays was shown in a quadruplex screen with quantitative results that were in good agreement with flow cytometry
analysis. As these SERS particles possess numerous optical advantages over classical colorimetric or fluorescence reporter molecules, this work has demonstrated one possible application in which SERS may become the standard of practice, namely in high-throughput, high-sensitivity in-vitro signal detection.

With a reliable protocol for creating molecularly-targeted SERS nanoparticles established, the focus of the thesis will now shift to the development of an imaging system capable of widefield Raman imaging of these nanoparticles with sufficient speed to be used in-vivo, as compared to the relatively slow microscopic acquisition used in these in-vitro studies.
3  Bandpass SERS imaging in-vivo

3.1  Acknowledgements

The work in this chapter addresses the optical challenge of imaging SERS nanoparticles in a widefield configuration and describes an approach to leverage the narrow peak width from Raman reporter molecules to estimate and remove the autofluorescence background arising from tissue. In large part chapter 3 is a reformatted version of the manuscript entitled “Filter-based method for background removal in high-sensitivity wide-field surface enhanced Raman scattering imaging in-vivo” published in the Journal of Biomedical Optics in 2012. My contributions to this work included designing and constructing the imaging system, carrying out the phantom and in-vivo imaging studies, analyzing the data, and writing the manuscript text. I would like to thank Israel Veilleux for the assistance with the optical design and Rupananda Mallia for assisting with the animal imaging and manuscript writing.

3.2  Introduction

Molecular imaging, based on both optical and non-optical signals, is evolving rapidly not only in terms of the range of potential biomarkers for targeting contrast agents, but also in the variety of these agents and corresponding imaging techniques and instruments, for both preclinical research and clinical applications. Optical nanoparticle (NP)-based contrast agents, including fluorescent quantum dots (QD) and surface enhanced Raman scattering (SERS)-active NPs, have been a focus of much recent research. SERS-active NPs based on colloidal gold cores provide amplification of the Raman signal from the reporter molecule(s) on the surface (due to plasmonic effects) to a large enough extent (>10^6-fold) to make them competitive with fluorophores in terms of the optical signal strength. If sufficient SERS signal can be generated, then the advantage of the extremely narrow (~1 to 3 nm) spectral features becomes very important compared with organic fluorophores or even quantum dots to enable high level multiplexing, as illustrated in Fig. 3.1.
SERS imaging is usually performed by full-spectrum raster scanning, point-by-point across the image, as, e.g., in confocal Raman microscopy\textsuperscript{127}. In every pixel, each contributing Raman active molecule can then be isolated, based on their unique Raman signatures, typically applying polynomial curve fitting to subtract the background spectrum. This requires long integration times to acquire sufficient signal-to-noise in each pixel across the field of view\textsuperscript{119,127}. In the case of imaging the intrinsic NIR Raman signature of tissues or cells, the alternative would be to use an expensive focal plane array and a fast tunable filter. However, for imaging SERS contrast agents, since the location of the narrow SERS peaks of the reporter molecule(s) is known a priori, rapid background-free wide-field imaging should be possible using narrow-bandpass (BP) filters centered on these peaks. Then, for each peak, over sufficiently small spectral windows, all non-SERS contributions are approximately linear functions of wavenumber. Hence, the background can be accurately determined and subtracted by using narrow BP filters immediately on each side of the SERS peak. Since this requires at most three images per SERS line of interest, the time to form a complete SERS contrast image is reduced by orders of magnitude over complete spectral mapping at equivalent optical resolution\textsuperscript{119}. Here, we present a proof-of-principle demonstration of this approach.

3.3 Materials and Methods

3.3.1 SERS-active gold nanoparticles

SERS-active gold nanoparticles (AuNPs) were purchased from Cabot Security Materials Inc. (Mountain View, CA, USA) and consist of a 60-nm colloidal gold core, an adsorbed layer of the Raman-active molecule S440 [Trans-1,2-Bis(4-pyridyl)-ethylene],\textsuperscript{7} and a 30-nm thick thiolated silica encapsulant layer that stabilizes the reporter molecule on the AuNP surface and provides a reactive substrate for further conjugation steps. For in vivo use, the AuNPs are further coated with polyethylene glycol (PEG) to improve their biostability. A schematic diagram of structure and the corresponding SERS spectrum are shown in Fig. 3.1. These NPs can be targeted by
attaching suitable tumor specific agents, e.g., antibodies, affibodies, or peptides, to the silica, either directly or through flexible linker molecules.

Figure 3.1. (a) Comparison of the SERS spectrum from the S440 reporter molecule (inset) and 800-nm peak-emission quantum dots, both excited at 785 nm, and (b) schematic of SERS AuNPs with 60-nm colloidal gold core, adsorbed SERS reporter molecule, 30-nm thick silica encapsulant, and polyethylene glycol surface coating. Reprinted (adapted) with permission from McVeigh PZ et al.

3.3.2 Instrumentation and image processing

Wide-field SERS BP imaging was performed using an instrument built in-house, comprised of a virtual-phase EM-CCD camera (Andor, Belfast, UK) with motorized interference filters to select the SERS bands of interest and to reject Rayleigh scattered light, together with suitable relay optics to provide a 2.2-cm diameter uniform field of view at a working distance of 15 cm. For the proof-of-principle studies, a single SERS line at 900 nm (1630 cm$^{-1}$) was selected. The corresponding BP filters (900, 910, and 890 nm) were selected to be relatively inexpensive and have a bandwidth of approximately 10 nm (FB10 series, Thorlabs, Newton, NJ, USA), with a peak transmission of ca. 50%. Excitation over a 1 cm area of the sample was provided by a 400
mW 785 nm fiber-coupled diode laser (BWTek, Newark, DE, USA), so that the power density was below the ANSI skin exposure limit for 3 s continuous-wave laser exposure\textsuperscript{127}.

By considering a sufficiently narrow spectral window, we assume that the non-SERS background spectrum is a linear function of wavelength, so that in each image pixel it may be approximated by linear interpolation of the corresponding off-peak pixel intensities. Here, to simplify the procedure, we have used BP filters that have symmetric in-band transmission spectra, are equally spaced on either side of the SERS peak, and do not have significant spectral overlap, and have used a spectral range where the quantum efficiency of the detector is linear over the spectral window. However, the approach can easily be extended to the more general case by including appropriate scaling factors, if required.

3.3.3 In-vivo imaging
All animal procedures were carried out under institutional approval (University Health Network, Toronto, Canada), using eight-week old female nude mice (Taconic, Hudson, NY, USA). The objective was to determine the lowest NP concentration that could be detected with the imaging system. Both SERS-active and control NPs without the S440 reporter molecule were used, at concentrations of 20 to 360 pM, and mixed 1:1 with Matrigel (BD, Mississauga, Ontario, Canada). Under general anesthesia, 10 µL of each concentration was injected subcutaneously at different locations along the dorsum of an individual mouse immediately prior to imaging.

3.4 Results and discussion
Figure 3.2 shows a typical full-spectrum point measurement taken on a confocal Raman microscope with a 40 pM SERS AuNP sample placed on ex vivo mouse skin, showing the expected SERS signal superimposed on the broad tissue autofluorescence background. Even though the tissue autofluorescence is greatly reduced with NIR (785 nm) excitation compared to visible excitation\textsuperscript{128}, it still overwhelms the SERS signal at these pM nanoparticle concentrations.
In other in vivo point-spectroscopy studies of intrinsic tissue Raman, post-processing of the spectrum has often been used to subtract the autofluorescence background \(^{129}\) but, as mentioned above, this cannot directly be applied in wide-field imaging mode.

![Graph showing Raman spectra](image)

Figure 3.2. Raman spectra of 40pM control gold nanoparticles (no reporter molecule: dotted line) and SERS-active nanoparticles (solid line) measured on a background of ex-vivo mouse skin; SERS signals are typically a small perturbation (<<5\%) on the broad tissue background. Reprinted (adapted) with permission from McVeigh PZ et al.\(^ {95}\)

Figure 3.3 shows BP images of a 40 pM sample taken at 900 nm (SERS peak) and the two adjacent wavelengths (890 and 910 nm). The on-peak image is not appreciably brighter than the adjacent off-peak images. Furthermore, this background varies significantly for every sample, depending on composition, illumination conditions, and acquisition parameters, so that it cannot simply be subtracted by calibration measurements.
Figure 3.3. SERS bandpass images of 40pM SERS-active AuNPs in solution. (a) White light image showing the laser irradiation volume, and (b) to (d) SERS bandpass images centered at 890, 900, and 910nm, respectively; excitation at 785nm with 1s integration time per image. Circle indicates the laser illumination spot. Reprinted (adapted) with permission from McVeigh PZ et al.95

Figure 3.4(a) shows the details of the 900 nm SERS peak and the 3 BP filters, while Fig. 3.4(b) shows images of SERS-active and control NPs on a non-tissue background. The image intensities are not significantly different when considering only the peak signal, even in this simple case. However, Fig. 3.4(c) and 3.4(d) shows significant enhancement of the signal-to-background ratio (SBR) in the SERS images (from 1.7 to 9.1) after applying the linear background subtraction, even at very short total exposure times.

The in vivo imaging test results are shown in Fig. 3.5; as expected, there was significant tissue autofluorescence that camouflaged the SERS signal, such that the lower NP concentrations were indistinguishable from control AuNPs in the single-band images. However, following the filter-based linear background removal, images with excellent SBRs (356 and 9.3 for 40 and 20 pM, respectively) could be acquired at low pM concentrations using image integration time of only a few seconds. Similar to QDs and other organic fluorophores, we observed an ultimate decrease in the limit of detection as a function of depth in tissue. However, the use of SERS nanoparticles excited in the NIR should give markedly greater in vivo penetration depth than QDs excited in the UV-visible range. It is also worth noting that, in the case of topically applied SERS AuNPs for endoscopic detection of early cancer89, the optical penetration depth is not likely to be the limiting factor, but rather the penetration of the NPs into the tissue.
Figure 3.4. (left) SERS spectrum illustrating the filter passbands used to isolate the SERS signal (solid) from background (dashed). (right) (a) white light image of 40pM SERS-active and control AuNPs on filter paper, (b) 900nm image showing signal from both samples, (c) linear background-subtracted image demonstrating improved SBR (1.7 to 9.1), and (d) background-subtracted signal shown in false color superimposed on the white light image; excited simultaneously at 785nm with 1s image integration time. Reprinted (adapted) with permission from McVeigh PZ et al.95

From these results it is evident that this rapid imaging capability is critically important for translation, especially for clinical applications, where the typical point-by-point full spectrum mapping to form SERS contrast images93 can take minutes to hours for comparable spatial resolution. Thus, for example, at a working distance of 120 mm, our system has an approximate CCD pixel resolution of 25 μm and required 9 s to acquire the three BP images for a 1 cm diameter illumination spot, whereas point-by-point spectral scanning with equal integration time per pixel and spatial resolution would require in excess of 125 h.
Currently, the main limitations of this technique are the time delay for filter switching and the relatively broad transmission profile of the filters used, which limits the achievable level of multiplexing. In practical terms, the degree of achievable multiplexing depends partly on the spacing between peaks of the different SERS reporter molecules selected. If major peaks between the reporter molecules are spectrally isolated, i.e., the spectra do not interfere, then the detection limit should be similar to that demonstrated here. If there is spectral overlap even with
the best reporters available, then there will be loss of sensitivity. In such cases, it will likely be necessary to apply a more rigorous linear spectral-unmixing algorithm, as we have used previously in hyperspectral fluorescence imaging\textsuperscript{130}. We are currently investigating this for SERS imaging, using multiple discrete BP filter bands for each reporter. We are also currently implementing a modified version of the instrument, using a fast tunable narrow BP filter with high transmission efficiency. This should both further reduce the total imaging time and allow programmable multiplexed imaging to match any “cocktail” of selected SERS reporters.

3.5 Conclusions

In summary, we have shown that a simple and economical filter based technique can markedly reduce the intrinsic background signals in wide-field SERS imaging, enabling fast imaging with pM nanoparticle sensitivity, which is below the typical AuNP concentrations reported in vivo to date\textsuperscript{87, 93}. The proof-of-concept results clearly demonstrate significant improvement in the signal-to-background ratio as compared to direct SERS peak imaging at biologically realistic AuNP concentrations. This background suppression approach should facilitate the further development and deployment of SERS-based contrast agents for prospective molecular imaging in vivo.

With the feasibility of widefield SERS imaging from a single spectral peak established, the focus now shifts to the development of a tunable filter to allow for imaging of multiple SERS peaks from different reporter molecules within a sample.
4  Quantitative Multiplex SERS imaging in-vivo

4.1 Acknowledgements

The work in this chapter builds upon the prior demonstration of single-channel widefield SERS imaging (Chapter 3) through the addition of a tunable filter module suitable for imaging multiple distinct SERS peaks in-vivo. Chapter 4 represents a reformatted version of the manuscript entitled “Widefield quantitative multiplex surface enhanced Raman scattering imaging in-vivo” published in the Journal of Biomedical Optics in 2013131. My contributions to this work included constructing the imaging system and control software, designing and carrying out the experiments, analyzing the data and writing the manuscript. I would like to thank Israel Veilleux for his assistance in the optical design and system alignment.

4.2 Introduction

As biomedical research has vastly expanded our knowledge of biomarkers of diseases such as cancer in recent years, it has become clear than single markers alone will be insufficient to completely detect and diagnose most complex disease processes. While many tools exist to image single biomarkers in in-vitro and in-vivo model systems, the molecular imaging tools available for multiplex imaging are significantly more limited especially in the complex in-vivo setting. In-vivo optical imaging is best carried out in the NIR tissue ‘optical window’ from 750-900nm, where background tissue absorption and fluorescence are minimal allowing for improved depth of detection and overall sensitivity. Conventional fluorophores which emit in the NIR have found numerous applications132-136 but are inherently limited by their relatively broad emission profiles and quick photobleaching. Semiconductor quantum dots have improved brightness and photostability as compared to conventional dyes, but their optimum excitation in the UV significantly limits applicability in deep tissue imaging, as well as having relatively broad emission spectra in the NIR137 and potential concerns regarding heavy metal toxicity from the core material. Using the example of multiplex tumour detection, the number of biomarkers
necessary to differentiate early dysplastic changes from normal tissue may be >5; to image 5 distinct probes in the 150nm wide NIR optical window in tissue quantitatively would require emission peaks on the order of 30nm FWHM to avoid significant overlap between biomarkers. However commercial 800nm quantum dots have an emission peak width of >100nm. Ultimately any approach based on fluorescence will face interference from the autofluorescence of the background tissues *in-vivo*, which can significantly impact the sensitivity limits of an imaging technique.

Imaging agents that produce signal from surface enhanced Raman scattering (SERS) are most commonly based on colloidal metallic nanoparticle (NP) cores that have reporter dyes adsorbed to the surface which give rise to a characteristic SERS spectrum. By changing the adsorbed dye, multiple ‘colors’ of NPs may be generated and, as Raman peak widths are generally <5nm FWHM, their potential for multiplexing greatly outstrips that of any other current imaging technique. The choice of core material, typically gold for *in-vivo* use, may reduce toxicity concerns as compared to quantum dots. SERS NPs have been successfully used in ex-vivo/serum based assays, *in-vitro* diagnostics, and *in-vivo* imaging. Critically, however, all these studies have used either point-measurement or microscopic imaging approaches whereby complete Raman spectra are acquired pixel-by-pixel and later combined to form a complete image. This is pivotal as, at the long integration times needed for detection of dilute Raman agents (as compared to fluorescence), sensitive *in-vivo* imaging of large areas with these approaches may require unacceptably long imaging times, during which the ability to capture any kinetic information is lost.

Widefield or global Raman imaging, where bandpass techniques are used to image distinct Raman bands directly, has been applied far less frequently than complete spectral acquisition approaches and almost exclusively in a microscopic imaging format. We have recently outlined an approach to extend widefield Raman imaging to detect SERS NPs using significantly larger FOVs than are possible in a microscopic configuration for *in-vivo* imaging, but this prior work was restricted to a single spectral channel.
4.3 Materials and Methods

4.3.1 Experimental reagents

SERS-active gold nanoparticles were purchased from Cabot Security Materials Inc. (Mountain View, CA, USA) and consist of a 60nm colloidal gold core, an adsorbed layer of the Raman-active molecules, and a 30nm thick thiolated silica encapsulant layer that stabilizes the reporter molecule on the AuNP surface and provides a reactive substrate for bioconjugation steps. The 4 reporter molecules used were S420 (4,4’-dipyridyl), S421 (d8-4,4’-dipyridyl), S440 (trans-1,2-Bis(4-pyridyl)-ethylene), and S481(4-Azobis(pyridine)). Peaks selected for imaging were located at: 1295cm\(^{-1}\)(S420), 1578cm\(^{-1}\)(S421), 1339cm\(^{-1}\)(S440), 1164cm\(^{-1}\)(S481).

Figure 4.1 785nm excitation surface enhanced Raman scattering (SERS) spectra for the four reporter molecules used in this study – the peaks used to image each reporter in multiplex experiments are indicated by (*). Reprinted (adapted) with permission from McVeigh PZ et al.\(^{131}\)
4.3.2 Imaging system design

The widefield Raman imaging system was designed for small animal imaging and consists of a high-power, single transverse mode laser source (CleanLaze 785, BWTek, Newark, DE) which is collimated and reflected onto the sample stage by a dichroic mirror (RazorEdge, Semrock, Rochester NY). Scattered light is collected by an objective and relay lens arrangement and laser light is removed by a notch filter (StopLine, Semrock) prior to entering the tunable filter module. The tunable filter is comprised of bandpass filters designed for operation at non-normal incidence (Versachrome 900/11, Semrock), which are offset so as to produce a transmission FWHM of approximately 4nm from 810-890nm. One of the filters was requested from red-shifted stock so as to maximize the tuning range of the composite design (to minimize the broadening of FWHM at near-normal angles of incidence). The filters are mounted on a precision encoded rotary stage (PRM1Z8, Thorlabs, Newton NJ), which has a bidirectional repeatability of +/-0.1° which is equivalent to approximately 3.95cm⁻¹ in Raman shift. The filtered light is imaged onto a cooled EMCCD (iXon DV885, Andor, Belfast UK) which is air cooled to -70°C and operated in baseline-clamped mode. The field of view (FOV) of the system was 2.2cm² at a working distance of 12cm, with a pixel resolution of 50µm.
4.3.3 Raman image processing

As previously described\textsuperscript{95}, background removal was applied to all Raman images in a standardized fashion; images were acquired at spectral locations immediately blue/red shifted from the peak of interest in which there was no expected spectral components from any of the other SERS reporter molecules. The change in intensity between the two reference images was assumed to be linear and an estimated background signal level was interpolated on a per-pixel basis and subtracted from the measured peak image. In the case of multiplex experiments, images were also corrected by a compensation matrix to account for inter-reporter signal overlap.
The compensation values were determined by imaging pure reporter molecule solutions in each bandpass channel and were fixed for each particular bandpass definition set – as the reporter spectra were stable over time, the compensation was only calculated once and applied automatically for all later imaging sessions.

As certain key determinants of image intensity (such as working distance and CCD gain) were not absolutely calibrated in the system due to the components used, the images produced cannot be quantified in terms of an absolute number of photons/solid angle/unit time. Images are displayed scaled by the integration time to present a relative number counts for a given instrument setup, similar to what is done with comparable fluorescence imaging systems.

4.3.4 Linearity, multiplexing, depth of detection imaging assays

SERS AuNPs were diluted in water and 200uL aliquots of each concentration were plated in triplicate in 96 well clear-walled assay plates (Sarstedt, Montreal QC). Raman bandpass images were acquired in each well for each reporter peak with an integration time of 3s, averaged 5 times. Intensity data was recorded from an ROI corresponding to the known laser illumination area. To estimate the depth imaging capability of the system, a capillary tube filled with a dilute solution of S421 SERS NPs in various depths of a tissue-mimicking solution (1% Intralipid) having a reduced scattering coefficient of approximately $\mu_s'=1\text{mm}^{-1}$ similar to that of soft tissue in the NIR with negligible absorption\textsuperscript{142}. Images were acquired at each depth with an integration time of 5s, averaged 5 times. The sensitivity limit was estimated as the point at which a linear fit of the signal/background curve equaled unity.

4.3.5 In-vivo Raman imaging

All animal procedures were carried out with institutional approval (University Health Network, Toronto, Canada), using 8-week old female nude mice (Taconic, Hudson, NY, USA). SERS-active or control (reporter-free) AuNPs were diluted to the desired concentration in acrylamide
monomer solution (Sigma-Aldrich, Oakville ON) which was then polymerized to form an optically-clear matrix that prevented the diffusion of the AuNPs over time in-vivo. Mice were anesthetized using a mixture of ketamine/xylazine and placed on a heated stage under the Raman imaging system. 25μL AuNP-acrylamide discs were implanted in triplicate subcutaneously along the dorsum of the mouse and imaged directly. The laser spot size and power level were adjusted to remain at or below the ANSI skin exposure limit for CW 785nm laser light. Images were acquired with an integration time of 5s, averaged 5 times.

4.4 Results

4.4.1 Nanoparticle design

The nanoparticles employed consisted of a colloidal gold core having a mean diameter of 60nm with an adsorbed layer of Raman reporter molecules, followed by a 30nm thick silica shell. The Raman spectra of the 4 dyes used in this study are shown in Figure 4.1. The silica encapsulant ensures that there is no desorption of the dye from the colloidal core (leading to a signal decrease), nor any core-core aggregation of the gold nanoparticles (leading to a possible signal increase) as a result of changes in the NP’s chemical environment. This physical stability ensures that SERS peak intensities measured prior to use remain unchanged once the NPs are placed in a complex biological milieu, allowing for quantitative multiplex imaging.

The relative SERS signal intensities of these NPs have been found to be stable over time in biological media, and show no signs of photobleaching/degradation over extended periods of continuous imaging. These findings contrast starkly with fluorescence-based imaging agents whose signal can be greatly influenced by small changes in pH or through processes such as chemisorption, and where photobleaching can reduce signals to undetectable levels in only a few minutes of continuous monitoring.
4.4.2 Widefield Raman imager design

In order to permit rapid imaging of multiple SERS peaks it was necessary to implement a tunable filter with a sufficiently narrow bandpass to prevent cross-talk between probes, while still maintaining high overall transmission and fast switching between wavelengths of interest. The system uses excitation light at 785nm to maximize tissue penetration in the ‘tissue optical window’, and at this wavelength the majority of SERS peaks from the SERS NPs used are ~2nm FWHM. Acousto-optical tunable filters (AOTFs) and liquid crystal tunable filters (LCTFs) are available with bandpass widths of <5nm FWHM which would be suitable for Raman imaging. However, since both designs recover only one polarization of light, when imaging diffusely scattered light in-vivo their overall transmission decreases to typically <30%. The cost of large-format, imaging-quality AOTFs or LCTFs with a suitably narrow bandpass for Raman imaging is generally in excess of $20,000 USD.

Figure 4.3. Measured full width half maximum (FWHM) of the tunable filter as a function of center wavelength; the FWHM is fixed at approximately 4nm from 810 to 890nm, which corresponds to 400 to 1500cm\(^{-1}\) at 785nm excitation. Reprinted (adapted) with permission from McVeigh PZ et al.\textsuperscript{131}
The design of our system is shown in Figure 4.2, and is based on two relatively inexpensive offset bandpass filters which are designed to be used in angle-tuning applications – by varying the angle of the filters with respect to the incoming light the center wavelength of transmission shifts proportionately. The offset between the filters sets the overall composite FWHM of the filter system, which is constant over a linear tuning range of 400-1500cm\(^{-1}\) (Figure 4.3 and 4.4) while maintaining a transmission >90%. The filters are housed on an optically encoded rotation stage which has an effective positional accuracy of 3.95cm\(^{-1}\) and changes between adjacent SERS peak locations in <1s. The total cost of the two filters and rotation stage is approximately $3,000 USD.

Figure 4.4. Measured center wavelength of the tunable filter as a function of the angle of incidence from the optical axis. This is in good agreement with the predicted value based on the effective index of refraction for the filter. Reprinted (adapted) with permission from McVeigh PZ et al.\(^{131}\)

The SERS imaging system automatically applies our previously described background minimization approach\(^{95}\) by capturing pre-defined anti-Stokes/Stokes images for each SERS peak and interpolating the pixel intensities to determine the approximate fluorescence background contribution to each peak image, which is then subtracted. Crosstalk between
channels is generally <5% and can be compensated for exactly using the a priori known spectral features of each SERS dye used and the measured instrument response as a function of wavelength. The degree of crosstalk between SERS channels is a function of the filter bandpass and the inter-peak spacing of the different reporter molecules and, by selecting imaging peaks that are well separated, spectral overlap is generally reduced to <1% such that compensation is not applied.

4.4.3 Widefield SERS imaging performance

The system response was extremely linear over a range of (1–100)pM of nanoparticle concentration as shown in Figure 4.5, demonstrating a sensitivity which is two orders of magnitude better than commercial in-vivo imaging systems with quantum dots\textsuperscript{142}. At present the limit of detection (LOD) is set by the combination of the fluorescence background signal level of specimen and the instrument noise, which for SERS NPs diluted in a 96-well high-throughput assay plate results in an LOD less than 0.01 pM. In the case of the assay plate there is little detectable fluorescence signal and the sensitivity is primarily limited by stray light in the imaging system – the tunable filter operates by reflecting light outside its bandpass and, for certain SERS peaks, the angle of incidence of the filter may reflect off-band light from the stage enclosure and into the CCD, which quickly leads to saturation. At concentrations above 50pM the optical density of the SERS NP solution begins to limit the amount of light detected by absorption of both the excitation laser light and the Raman scattered signal photons.
Figure 4.5. Widefield Raman image intensity as a function of SERS nanoparticle (NP) concentration (S421).

Triplicate 200µL aliquots of each concentration were imaged in a clear-wall 96 well plate, error bars represent +/- standard deviation of the three replicates.

As shown in Figure 4.6, the system response *in-vivo* is also extremely linear (R²=0.995) and with a fixed integration time of 5s has a limit of detection below 2.5pM. At this concentration the signal to background ratio (as compared to 40pM reporter-free control nanoparticles) is reduced to 1.20:1, primarily as a result of the skin fluorescence which leads to CCD saturation prior to collecting a significant number of Raman scattered photons.
Figure 4.6. (left) Widefield Raman image of 80pM SERS AuNPs injected subcutaneously on a nude mouse dorsum, superimposed in a white light structural image. (right) Results of serial dilution of S481 measured in-vivo, in triplicate, demonstrating a limit of detection (LOD) below 2.5pM using an acquisition time of 5s. Reprinted (adapted) with permission from McVeigh PZ et al.131

4.4.4 Multiplex SERS imaging

To demonstrate the multiplex imaging capability of the system, drops of 4 spectrally distinct SERS NPs were placed on filter paper and illuminated simultaneously while the system imaged at each of the 4 individual SERS peaks highlighted in Figure 4.1. As shown in Figure 4.7, the individual dyes were easily distinguished based on their unique spectral peaks, with <5% overlap between image channels. As each SERS peak being imaged produces a different intensity (area under curve in Figure 4.1) that varies depending on how much of the peak is captured by the bandpass filter position, it is necessary to correct for this intensity difference prior to image quantification. However, as the silica-encapsulated dye spectra are extremely stable over time, it is only necessary to perform this calibration once for any selected set of SERS peaks.
To test this multiplex quantification accuracy a triplex mixture of varying amounts of 3 SERS reporter molecules was imaged in a 96 well plate: one reporter remained constant at 30pM for all conditions, one increased from (0-50)pM, and the third reporter decreased from (50-0)pM over 6 discrete wells. SERS images were taken at all 3 peak wavelengths and the relative amounts of each were calculated from the known spectral intensities for each reporter. As shown in Figure 4.8, the reconstructed amounts of each SERS reporter are in excellent agreement with the actual amounts in each well ($R^2>0.99$). As the cross-channel overlap is generally $<5\%$ for the SERS reporters used here (e.g. S420 has a 4% spectral overlap with S421 and 2% with S481), the overall system sensitivity is not significantly different in the multiplex imaging case than when imaging a single reporter molecule.

In the case of a defined imaging geometry, as in the high-throughput assay plate, the system can be calibrated absolutely against a known dilution series of SERS NPs to quantify the amount of
each SERS reporter, allowing for truly quantitative multiplex imaging without the elaborate compensation schemes required in flow cytometry or other fluorescence-based techniques. Leveraging the facile multiplexing in SERS imaging, experimental samples can be spiked with a known quantity of a SERS reporter not used in the assay as an internal standard, compensating for any laser intensity variation over the course of an experiment, further improving the quantification accuracy.

Figure 4.8. (left) Widefield Raman bandpass image intensities from SERS NP mixtures in a 96 well plate: the concentration of S481 was held constant in all cases while the amount of S421 increased from (0 to 50pM) in wells 1-6, and the concentration of S420 decreased from (50 to 0pM) in wells 1-6. (right) SERS image intensities for each mixture as a function of the known concentration of each probe. Error bars represent +/- 1 standard deviation of triplicate wells for each case. Reprinted (adapted) with permission from McVeigh PZ et al.131

4.4.5 Multiplex SERS imaging in-vivo

To demonstrate the multiplex imaging capability of this system in-vivo, 4 distinct SERS reporters were immobilized in an acrylamide polymer matrix and implanted subcutaneously into the dorsum of a nude mouse and images were acquired at each of the individual SERS peak wavelengths indicated in Figure 4.1. There was no Raman spectral contribution from the matrix material at the measured peak locations (Figure 4.9). As shown in Figure 4.7, the SERS signal from 4 distinct reporter molecules were easily detected and separated against the relatively complex background tissue autofluorescence.
As a measure of the quantitative imaging possible with SERS NPs, an additional subcutaneous ‘cocktail’ injection was created with varying amounts of each of the 4 reporter molecules. As shown in Figure 4.10, each probe was detectable above background interference and the measured proportions of each probe based on image intensity is in excellent agreement with the known ratio ($R^2=0.986$). As all the reporter molecules used in this study are structurally related, multiplexing at 4x and beyond does require compensation for cross-talk as many bands begin to overlap within the bandpass of our tunable filter (4nm = 65cm$^{-1}$ at 785nm excitation) – as an example, S421 has a 10% spectral overlap with S420, 0.1% with S440, and 7.5% with S481.
Figure 4.10. White light (a) and S420/421/440/481 bandpass images (b-e) from varying amounts of each reporter molecule injected subcutaneously on the dorsum of a nude mouse. (right) Unmixed Raman bandpass image intensity as a function of the known injected concentration for a quadruplex mixture of SERS reporters in-vivo. Error bars represent +/- 1 standard deviation of three measurements from injections at different locations along the mouse dorsum. Images were acquired using an integration time of 5s averaged 5 times. Reprinted (adapted) with permission from McVeigh PZ et al.131

4.5 Discussion

Previous studies using widefield Raman imaging have exclusively used microscopic imaging arrangements and studied only the intrinsic Raman from solid semiconductor or graphene substrates117-119. Prior in-vivo SERS imaging studies have used only point measurement97 or microscopic mapping93 for quantitative multiplex detection of up to 5 reporter molecules injected simultaneously. The work here demonstrates, to the best of our knowledge, the first example of quantitative 4-plex widefield SERS imaging in-vivo, and is a further demonstration of the potential of SERS imaging for molecular imaging. The distinction between the direct Raman imaging approach taken here and point-by-point spectral acquisition techniques used in prior multiplex imaging studies is most apparent when considering the imaging time for large area mapping: at a working distance of 12cm our system has a pixel resolution of 50µm and requires...
<5s per bandpass image to image low picomolar concentrations of SERS NPs in-vivo using a 1cm² illumination spot with a power density at the ANSI skin exposure limit for 785nm light. To create a spectral image using point rastering with equal pixel resolution and integration time (40,000 pixels at 5s/pixel) while still maintaining the same low power density would require in excess of 50h. An equivalent imaging time would require a pixel dwell time of only 125µs, which is at the limit of what is possible with CCD detectors and would have a significantly lower SNR from such dilute amounts of SERS NPs. Endoscopic point-scanning measurement systems have recently been described144 which sacrifice spatial resolution in order to gain a temporal advantage. Such systems collect complete spectra from large pixel areas (>1mm²) at a time and as such will have spectra which may be dominated by background signal if the SERS NPs are relatively spatially confined as would be the case with early dysplastic lesions. This approach necessitates the use of spectral un-mixing algorithms such as DCLS in order to extract meaningful SERS signals from the large spectral background, which further necessitates a uniform reference signal in order to achieve quantification of spectra from pixel-to-pixel. In contrast to these restrictions, our approach to widefield Raman imaging allows the study of dynamic processes in-vivo with no spatial resolution sacrifices, which may be useful in applications such as cell tracking or tracer biodistribution.

This time advantage comes primarily at the expense of spectral information, which in turn affects the ultimate multiplexing ability of the system; with the current commercially available SERS NPs each reporter molecule produces numerous Raman spectral peaks, some of which overlap or lie directly adjacent to peaks from other reporters. While this overlap can increase the accuracy of linear un-mixing approaches in the case of complete spectral mapping138, it has the opposite effect in the case of direct single-peak imaging as used here. The four reporter molecules used in this study are structurally similar and produce numerous peaks that almost completely occupy the tuning range of the filter (400-1500cm⁻¹). The amount of overlap between reporter peaks is also important in biological imaging applications where the relative amounts of various targeted SERS reporters may differ by several orders of magnitude – it is conceivable that a spectral overlap of a few percent from an overexpressed reporter in a tumor could obfuscate the signal from another marker at 1000x lower abundance. In practice this is can be somewhat avoided by
imaging only those peaks which are well separated spectrally, but the ability to do so diminishes as the degree of multiplexing increases. SERS NPs based on simple dyes with isolated Raman peaks would significantly improve the number of reporters that could be imaged without resorting to a full spectral imaging to quantitatively un-mix the signals.

As a result of spectral overlap, the tunable filter center wavelengths used in 4x and higher-order multiplexing experiments are not simply the center of the SERS peaks shown in Figure 4.1. Rather, the filter position is chosen to minimize the amount of signal from all the other dyes which would be included in the 4nm/65cm\(^{-1}\) passband, while maximizing the signal from the peak of interest. In some cases this can mean that the filter is positioned such that it only recovers 30% of the peak of interest, which limits the sensitivity to that reporter. This could be improved by narrowing the FWHM of the tunable filter (by increasing the offset angle), however, with the currently available commercial filters this would limit the overall transmission: with a passband width of 4nm the overall filter has a transmission >95%, but at 2nm the transmission falls below 60%. This is primarily determined by the steepness of the transition edge of the filter (currently fixed at 2nm to switch from <5% to >95% transmission), which could be improved further by using a custom designed filter with a faster transition.

While the system sensitivity demonstrated here represents a significant improvement over fluorescence-based preclinical imaging systems, we do not believe that it represents the ultimate limit of the technique. As our approach collects the background fluorescence along with the Raman signal of interest and removes the background contribution post-hoc, the noise level and dynamic range of the CCD detector are the key determining factors for the overall system sensitivity. In this study the EMCCD is primarily designed for high-speed video imaging and not long-exposure, low-noise readout as would be optimal in the case of Raman imaging. The shallow gain register wells and 14-bit digitization significantly limit the dynamic range, so that at low SERS signal levels the CCD wells saturate with background signal photons before a significant number of Raman photons can accumulate on the detector. The low-light imaging version of the same EMCCD has active area wells that are 2.25x deeper and gain register wells
with 9.125x higher capacity, which would improve the maximum exposure time prior to saturation by an order of magnitude. At present the EMCCD is thermoelectrically (TE) cooled to -70°C in air, which results in some dark noise in long-exposure images. This could be reduced by an order of magnitude by the addition of chilled water recirculation on the TE cooler, allowing for CCD temperatures of -90°C which is standard on most commercial fluorescence preclinical imaging systems.

We have demonstrated that direct 4-plex SERS imaging is possible in-vivo, and that the relative amounts of each of the reporter molecules can be accurately determined from the resulting images. We found that the primary interfering effect limiting the in-vivo sensitivity is the fluorescence of skin components such as collagen, NADH, and melanin. While relatively minimal when exciting at 785nm, this is comparable in intensity to the measured SERS signals: endogenous compounds are typically present in the skin at micromolar concentrations or higher, while the SERS reporter molecules were used at low picomolar amounts. The ability to accurately subtract the fluorescence background through linear estimation was also limited by the reporters chosen here: there was not sufficient ‘blank’ space between all the peaks to allow Stokes/Anti-Stokes images to be taken directly adjacent to each peak of interest, and in some cases the background images were separated by 10nm or more. The extent to which this would influence the background estimation is entirely dependent on the sample: a 96-well assay plate has a very flat background spectrum which could be estimated from widely separated points, but the shape of the background in-vivo depends on the other tissues present in the imaging volume. Spectral simplification to have sparse probe spectra with well-separated peaks should allow for better separation and allow for >5x multiplex imaging with good background rejection.
As currently implemented, our system has a practical depth of detection limit of approximately 5mm in tissue (Figure 4.11), primarily due to CCD background saturation. While improvements may be necessary to make complete depth mapping of large tumors possible, our primary application is endoscopic surface imaging for early cancer detection. The relatively large encapsulated SERS NPs used here have biodistribution properties ideally suited for topical application in endoscopy\textsuperscript{100}, and the utility is likely to be limited more by the penetration of the NPs into tissue than by the penetration of light. Surface imaging of SERS NPs also avoids the difficulties associated with attempting to quantify the amount of each reporter molecule as a function of depth. Since light attenuation in tissue varies with wavelength, increasing depth in tissue causes ambiguity: e.g., the signal from S481 measured at 864 nm could be 5% lower than that from S421 measured at 896 nm as a result of a 5% difference in NP concentration or to a 5% change in light attenuation. Hence, it becomes necessary to apply an appropriate tissue optical
model: this is certainly possible and has been used in fluorescence applications\textsuperscript{147,148}, but adds complexity to the instrumentation required and to the image analysis.

### 4.6 Conclusion

We have shown that multiplex direct SERS imaging is achievable \textit{in-vivo}, and that the resulting images can be analyzed quantitatively to determine the relative amounts of each reporter molecule with high fidelity. There have been significant recent developments in SERS NP design and targeting in biodiagnostic applications, and the work here is extremely complimentary to this, in that it provides a platform with which to study SERS-based contrast agents \textit{in-vivo} with spatial and temporal resolution exceeding other approaches by orders of magnitude. We are presently adapting this technology onto a clinical endoscope to allow for in-situ SERS imaging to detect precancerous lesions in the esophagus, lung, and colon which have well-characterized biomarker targets in sufficiently low abundance that fluorescent endoscopy cannot detect the signal above the considerable endogenous fluorescence background. In parallel with this, we are revising the optical design to include a more suitable CCD, additional baffling to trap stray light, and moving toward independent control of the angular position of the two tunable filters. The last improvement would allow high-throughput, wide bandpass imaging for sensitive detection of only 1-2 well-separated SERS reporters, which could be quickly transitioned to a much narrower but lower throughput filter for higher-order multiplex imaging of reporter molecules with overlapping spectral features.

This chapter has demonstrated that multiplexed widefield SERS imaging is possible \textit{in-vitro} or using \textit{in-vivo} tumour phantoms, however these arrangements are far more straightforward optically than the eventual clinical tumour imaging goal. The focus of this work will now shift to the application of the imaging system to detect actual tumour tissue which has been directly labeled using molecularly-targeted nanoparticles following topical application.
5 Molecularly-targeted SERS imaging in-vivo

5.1 Acknowledgements

The work in this chapter builds upon the prior results to translate the widefield SERS imaging approach from simple phantom experiments to the actual staining of tumor cells and tissue using targeted nanoparticle probes. In large part Chapter 5 is a reformatted version of the manuscript entitled “Widefield multiplexed imaging of EGFR-targeted cancers using topical application of NIR SERS nanoprobes” published in Nanomedicine in 2014149. My contributions to this work included synthesizing the targeted SERS nanoparticles, carrying out the in-vitro staining and animal imaging studies, analyzing the data and editing the manuscript text. I would like to thank Carl Fisher for his ongoing assistance with the in-vitro sample preparation and Rupananda Mallia for assisting with the animal imaging and manuscript writing.

5.2 Introduction

Non-small cell lung cancers arising from the central airways may be detected by conventional white light bronchoscopy (WLB) surveillance, but by itself this has poor diagnostic accuracy that often results in under-diagnosis150. Improved sensitivity and specificity are achieved by combining WLB with complementary techniques such as autofluorescence imaging151. The same is true in the case of colonoscopy, which despite routine biopsy protocols has been shown to miss up to 57% of flat and early adenomatous lesions152. These examples indicate that relying solely on anatomic imaging of lung or any other hollow organ during endoscopy suffers from limited diagnostic ability, as it lacks any molecular insight.

Coupling the benefits of endoscopy with molecular imaging could overcome the above limitations and facilitate early and accurate diagnosis of cancer, resulting in potentially better ‘personalized’ treatment and outcome. However, this requires identification of over-expressed
biomarkers, against which specific exogenous probes can be combined with sensitive imaging technologies that match the optical signals and yield a high signal-to-background ratio. Like other tumorigenic signal cascades, those mediated by the human epidermal growth factor receptor (EGFR) family are frequently over-expressed\textsuperscript{153-155}, with gene amplification\textsuperscript{156, 157} in a wide variety of tumors including NSCLCs and colorectal cancers. Hence, EGFR represents a rational candidate for tumour imaging applications using either single or multiple anti-EGFR targeted molecules.

Recently, Maiti et al reported a proof-of-concept study of multiplexed sensing of cancer in a live mouse model using anti-EGFR nanoprobes\textsuperscript{105}. However, this and similar studies\textsuperscript{89, 127} have been restricted to point-based microscopic imaging in which full Raman spectra are acquired pixel-by-pixel and later combined to form a complete high resolution map for which long acquisition times are required to achieve low detection limits. As a consequence of this approach, in\textsuperscript{-}vivo imaging of substantial areas (\textasciicircum cm\textsuperscript{2}) may require unacceptably long integration times (hours), and any dynamic information will be lost. An alternative approach for rapid, wide-field imaging is to use specific band-pass filters to select Raman peaks of interest. However, ultimately any such approach faces interference from the background tissue autofluorescence, which can significantly impact the sensitivity. In order to address this limitation, we recently introduced a filter-based approach to separate SERS signals from the background autofluorescence by linearly interpolating the sideband signals to estimate and subtract the background fluorescence from SERS signals\textsuperscript{158}. We have demonstrated a high-throughput tunable-filter design that is suitable for imaging several individual SERS peaks. The resulting images enable quantitative analysis at sub-picomolar concentrations of SERS nanoparticles in\textit{vivo}\textsuperscript{131}.

To date, however, ‘true’ in\textit{vivo} molecular targeting or multiplexing of tumor tissue with biomarker-targeted SERS nanoparticles has not been reported using wide-field Raman imaging, which is an essential step in order to validate and optimize this approach for human studies. Hence, as an extension to our previous work, we report here for the first time systematic in\textit{vitro} wide-field image multiplexing using nanoprobes targeted against tumor cells, followed by
corresponding in vivo studies for targeted lung cancer xenograft detection in a mouse model with topical administration of EGFR-targeted nanoprobes. Further, we report a ratiometric method to improve the imaging contrast by compensating the signal arising from non-specific binding of the nanoprobes.

5.3 Materials and Methods

5.3.1 Experimental reagents

Commercial SERS-active gold nanoparticles (Cabot Security Materials Inc., Mountain View, CA) were used. These are optimized for 785 nm excitation and consist of an adsorbed layer of the Raman-active reporter molecules on a 60 nm colloidal gold core and a 30 nm thick thiolated silica encapsulant layer that stabilizes the reporter molecule on the AuNP surface and provides an active surface for bioconjugation. For in vivo use, the AuNPs are further coated with polyethylene glycol (PEG) (MMPEG12: Pierce Bio, Rockford, IL) to improve the bio-stability. The three reporter molecules were designated 4,4’-dipyridyl (S240), d8-4,4’-dipyridyl (S241)) and 4-Azobis(pyridine (S481). Peaks selected for imaging were located at 1295 cm$^{-1}$ (S420), 1578 cm$^{-1}$ (S421) and 1164 cm$^{-1}$ (S481). The SERS stability and reproducibility of these nanoprobes have been reported elsewhere. A maleimide-activated fluorophore Cyto647 (Cytodiagnostics, (Burlington, ON, Canada) was used for the correlative flow cytometry measurements. All other major reagents and solvents were obtained from Sigma-Aldrich (Oakville, ON, Canada). The chimeric anti-EGFR antibody Cetuximab (Bristol-Myers Squibb, Montreal, QC, Canada) and fully humanized anti-EGFR antibody Panitumumab (Amgen, Thousand Oaks, CA) were used in their clinical formulations. Both antibodies target the extracellular domain of EGFR but differ in their affinity due to their isotype differences.

5.3.2 Instrumentation

Reference SERS spectra were acquired in a quartz cuvette using a compact desktop spectrometer operating at 785nm excitation (Advantage Series: DeltaNu, Laramie, WY). For flow cytometry
20,000 cells were measured on a system with 633 nm laser excitation (FACSCalibur: Beckton Dickenson, San Jose, CA). The wide-field Raman imaging system has been described previously elsewhere (28, 29). Briefly, the field of view is illuminated by a high power, single transverse mode 785nm laser (CleanLaze: BWTek, Newark, DE) and specific Raman bands are selected by a tunable filter module comprising interference filters designed for operation at non-normal angles of incidence (Versachrome: Semrock, Rochester, NY) with high throughput. The filter module has a band pass FWHM of about 4 nm, is tunable from 400 to 1500cm\(^{-1}\) and maintains a transmission >90% over this working range. As previously described\(^{158}\), removal of background signal was accomplished by acquiring Stokes/anti-Stokes shifted images for each Raman band and performing a linear interpolation between them to estimate the background signal in each Raman image on a per-pixel basis.

### 5.3.3 Bioconjugation of SERS NPs

To demonstrate molecularly-targeted multiplex imaging *in-vivo*, the SERS nanoparticles with different reporter molecules were conjugated to the different EGFR-targeting antibodies (cetuximab-S421, panitumumab-S481) using a heterobifunctional crosslinker (SMPEG12, Pierce) as previously described\(^{89}\). A third SERS reporter molecule was reserved as a control for non-specific binding and received surface coverage with polyethylene glycol only (mPEG-S420). Conjugates were stored at 4°C in PBS containing 0.1% BSA and 0.05% sodium azide as a preservative and were used within 6 months of synthesis. Additional characterization data including TEM imaging, UV/VIS spectroscopy, and fluorescence emission profiling of the final nanoparticle complexes are shown in Figure 5.2.

### 5.3.4 Cell culture and tumor pellets

A549 and H520 cell lines were acquired from the American Type Culture Collection (Manassas, VA) and propagated in RPMI-1640 media supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada). Labeling was carried out by gently treating aliquots of 1x10\(^6\) cells in suspension either with nanoprobes or a combination of anti-EGFR SERS
nanoprobes (40pM in PBS) and incubated for 30 min at 20°C. For inhibition studies, cells were pre-incubated with a 100x molar excess of free antibody for 30 min before labeling with anti-EGFR nanoprobes. To assess nonspecific binding, an aliquot of cells was incubated with control SERS tags, while another received neither control nor targeted SERS tags, and was then used as a control for evaluating the background autofluorescence signal. After incubation each sample was subjected to five rounds of washing with ice-cold 1% bovine serum albumin (BSA) in PBS to remove nonspecifically bound probe and re-suspended in 2ml PBS/BSA for flow cytometry. Following flow cytometry, each sample was centrifuged to form a pellet which was then used for wide-field Raman imaging.

5.3.5 In-vivo and ex-vivo SERS imaging

All animal procedures were carried out with institutional approval (University Health Network, Toronto, Canada) on 8-week old female nude mice (NCRNU: Taconic, Hudson, NY). The mice were divided in two groups for ex vivo and in vivo targeting studies. 5x10⁶ A549/H520 cells per site, in 50% Matrigel in PBS, were injected subcutaneously in the rear flank (n=3) and allowed to grow (~ 4weeks) until >=5 mm diameter. General anesthesia with a mixture of ketamine/xylazine was used for the surgical and imaging sessions. The explanted tumors (n=3) were divided, washed 5 times with PBS and stored in ice-cold 3% BSA in PBS. For ex vivo targeted studies, one half of each tumor was incubated with 500µL of a 40pM mixture of SERS nanoprobes for 30 min on ice. Matching sections were competitively inhibited by pre-incubation for 30 min with free antibody at 2mg/mL in PBS, and then treated with a mixture of 40pM SERS nanoprobes for 30 min on ice. The incubated tumors were washed 5 times with ice cold 3% BSA in PBS and immediately loaded for widefield Raman imaging. During in vivo labeling, surgically-exposed tumors were washed 3 times with 3% BSA in PBS using a syringe, before and after application of nanoprobes. 100µL of a 40 pM mixture of the nanoprobes was topically administered to the exposed tumor for 30 min before in vivo imaging. The laser spot size and power level for imaging were adjusted to remain below the ANSI skin exposure limit for 785nm CW laser light (1.63W/cm²). Both in vivo and ex vivo images were acquired with an integration time of 5s and averaged 5 times. A one-way ANOVA with Bonferroni correction for multiple
comparisons was used to compare the mean SERS intensity between treatment groups. Following *in vivo* imaging, tumor tissue was excised and prepared for standard EGFR immunohistochemistry.

5.4 Results

5.4.1 Wide-field SERS imaging of tumor cell pellets

To create targeted multimodal nanoprobes for concurrent fluorescence flow cytometry (as an independent measure of uptake and binding) and wide-field Raman imaging, commercially available silica-encapsulated gold nanoparticles (AuNPs) were coated with polyethelene glycol for surface passivation, a far-red fluorophore, and then labeled with one of two clinically-approved anti-EGFR antibodies (Cetuximab and Panitumumab), as shown schematically in Figure 5.1.

![Figure 5.1. SERS nanoprobes used for EGFR targeting.](image)

Figure 5.1. SERS nanoprobes used for EGFR targeting. (Left) Schematic: Raman-active molecules were absorbed on a 60 nm colloidal gold core, encapsulated with 30 nm thick silica and then coated with polyethylene glycol. The outer shell was labelled with both an NIR fluorophore (Cyto647) for flow cytometry and anti-EGFR antibodies for targeting. (Right) Overlay of the near-infrared SERS spectra of the 3 reporter molecules used (785nm excitation). Reprinted (adapted) with permission from McVeigh PZ et al.149
Two different Raman reporters were used here (denoted S481(Pan) and S421(Cetux)). A549 (EGFR+) and H520 (EGFR-) cells were incubated with these two AuNPs separately and then simultaneously, together with a third probe (S420) without antibody that served as a non-targeted control for non-specific binding. Additional samples were incubated with a large molar excess of free antibodies for 30 min before labeling, as a competitive-inhibition control, while cells that were not incubated with any nanoprobe were used to determine the background autofluorescence. After measuring the mean fluorescence intensity (MFI) by flow cytometry (Figure 5.3), each sample was centrifuged to create small tumor pellets. These pellets (targeted, targeted + inhibited, untargeted) of A549 and H520 cells were imaged using the widefield SERS imaging system(Figures 5.4a/b). Negligible SERS signal was detected in either the untargeted or the control pellets that had not been incubated with the nanoparticles. As seen in Figure 5.4c/d, the average Raman intensity (ARI) in the targeted A549 tumor pellet was nearly 10-fold higher for the S481 and S421 probes compared to that in H520 cells (p<0.001), demonstrating antigen expression-specific binding. This was further confirmed by the 9-fold decrease in the Raman
signal observed with competitive inhibition using the targeted nanoprobes in the A549 pellets (p<0.001) and the minimal corresponding effect in the H520 pellets.

Figure 5.3. Fluorescence flow cytometry for EGFR negative (H520) and EGFR overexpressing (A549) cells incubated for 30 min with anti-EGFR labeled dual-modality (fluorescence + SERS) nanoparticles (S481), compared to unstained controls (shaded). Reprinted (adapted) with permission from McVeigh PZ et al.149
Figure 5.4. Wide-field SERS imaging \textit{in vitro}. (a,b) Raman bandpass images (3 s integration) in (a) A549 and (b) H520 cell pellets using targeted nanoprobes, with and without competitive inhibition. (c,d) Corresponding average Raman intensities measured from individual cell tumor pellets. Error bars represent ± 1 standard deviation of triplicate measurements, differences from A549-EGFR signal levels marked with (*) were significant at p<0.05. Reprinted (adapted) with permission from McVeigh PZ et al.\textsuperscript{149}

To demonstrate multiplexed SERS imaging, A549 and H520 cell pellets that were incubated simultaneously with the 3 different nanoprobes were imaged at each of the SERS peaks for each reporter. As shown in Figure 5.5a/b, the SERS signal corresponding to distinct reporter
molecules (targeted S421, targeted S481 and untargeted S420 control) were easily detected and separated against the relatively complex cell autofluorescence background. The ARI values in A549 cells for the two targeted probes were 268 ±13 (S481) and 373 ±21 (S421), whereas the corresponding values in H520 cells were 45 ±3 and 70 ±4. A similar trend was seen in the variation of ARI between A549 and H520 pellets (Figure 5.5c). As a measure of quantitative imaging of the biological target, the measured wide-field ARI with EGFR targeted nanoprobes in the A549 pellets was plotted against the corresponding MFIs determined by flow cytometry (Figure 5.5d), yielding a high correlation (R²=0.995). This serves to validate the quantitative nature of the SERS detection scheme used in the wide-field imager.

Figure 5.5. In vitro targeting and multiplexed detection. a,b) Wide-field Raman images of tumor cell pellets simultaneously targeted with anti-EGFR nanoprobes in a) A549 and b) H520 cells. c) Comparison of corresponding Raman average intensities measured from individual spectral channels. d) Average SERS intensity versus average

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fluorescence intensities measured by flow cytometry. The Raman bandpass images were acquired with 5s integration and averaged 5 times. Error bars represent ± 1 standard deviation of triplicate measurements, differences from A549-EGFR signal levels marked with (*) were significant at p<0.05. Reprinted (adapted) with permission from McVeigh PZ et al.149

5.4.2 Wide-field multiplexed SERS imaging of tumor tissue ex vivo

SERS imaging of intact tumor tissue is more challenging than the above in vitro measurements since, for topical application as envisaged in the ultimate clinical endoscopic technique, the nanoprobes will be largely confined to the tissue surface layer rather than being throughout the whole depth of the tumor. Hence, the signals may be significantly weaker, depending on the density of accessible surface antigens. To investigate the effect on the SERS imaging, A549 and H520 tumors were first grown as subcutaneous xenografts on the flank of nude mice. They were then excised, taking care not to damage the tumor surface, and submerged for 60 min in the same cocktail of the three nanoprobes, S481-EGFR, S421-EGFR and S420-Control. For competitive inhibition, the tissue was pre-incubated with molar excess of anti-EGFR antibodies before the nanoprobe staining. Following staining, the tumors were cut in half, washed 5 times with normal saline and then immediately imaged on the wide-field system.
Typical SERS images, together with corresponding white-light images, are shown in Figure 5.6. Both targeted probes (S481 and S421) were clearly detectable above background in the EGFR+ A549 tumors. After competitive inhibition the signal levels were very low and comparable to the non-specific signal with untargeted AuNPs (S420). In the EGFR- H520 tumors the detected signals were very low under all labeling conditions and were also comparable to the imaging with non-targeted nanoparticles. Quantitatively, the EGFR-targeted nanoparticles gave nearly 4-fold signal increase in A549 tumors over the untargeted probes (p<0.001) and the targeted nanoparticles in the H520 tumors (p<0.001). In order to eliminate the nonspecific contribution to each image, we divided the Raman intensity in each channel by the intensity with the corresponding control probe to form a ratiometric image as previously described. As shown in the Figure 5.7, this enhances the contrast of the targeted nanoparticles in A549 tumors.
5.4.3 Wide-field multiplexed SERS of tumors in vivo

Finally, as a proof-of-principle demonstration of wide-field multiplexed Raman imaging of tumor in vivo, a triplex cocktail of equal amounts of S481-EGFR, S421-EGFR and S420-Ctrl was applied topically to a xenograft tumor in which the overlying skin was carefully removed. The 100 µl volume applied (at 40 pM concentration) was kept in place simply by the ‘well’ created by removing the skin. After 30 min incubation followed by 5 washes, the images in each channel (Figure 5.8) showed significantly more accumulation of S421 and S481, demonstrating selective binding. The absence of any detectable S420 signal indicates that there was minimal nonspecific binding, as was also found in the in vitro cell and ex vivo tissue experiments above. The observed EGFR-SERS signal was found to be heterogeneous across the tumor surface with
widefield Raman imaging, a pattern which was also present when the tissue was stained *ex vivo* with conventional EGFR-immunohistochemistry shown in Figure 5.9.

Figure 5.8. In vivo tumor targeting and multiplex detection of EGFR using wide-field Raman imaging in an A549 xenograft tumor. a) color image showing topical application of the nanoprobe cocktail (40pM) on the surgically-exposed tumor. Wide-field Raman bandpass images shown in false color superimposed on the white light image correspond to b) cetuximab labeling of S421 nanoprobes at 1578 cm$^{-1}$, c) panitumumab labeling of S481 nanoprobes at 1164 cm$^{-1}$ and d) non-specific labeling of m-PEG control S420 nanoprobes at 1295 cm$^{-1}$. Reprinted (adapted) with permission from McVeigh PZ et al. 149
5.5 Discussion

Working in the NIR range in general has several advantages for biomedical applications, including relative insensitivity to tissue fluorophores and deeper tissue penetration depth, while Raman detection has the advantage of very narrow line widths that allow signal multiplexing. In our previous work we showed, by applying a linear background subtraction technique, that wide-field NIR SERS imaging \textit{in vivo} is feasible down to the sub-pM concentration range. We have demonstrated that the present tunable filter design is suitable for imaging numerous individual SERS peaks both in vitro and in vivo quantitatively with the same pM sensitivity. However, these prior works made use of homogeneous simulated-tumor phantoms filled with untargeted SERS nanoparticles, which may not be representative of the conditions found in actual in situ tumor imaging. A significant limitation in using topical application of the nanoparticles as envisaged for endoscopy is the possibility that the signals will be very weak, since the targeted
nanoprobes would not likely have much penetration into tissue. As a result, only a small number of antibody binding sites may exist per image pixel, and as a result the tumor autofluorescence background may overwhelm the SERS signal. Here, we have shown that this may not in fact be a limitation, at least with biomarkers such as EGFR that have high expression levels (e.g. >10,000 copies per cell in A549 cells), and we have successfully demonstrated that images with adequate brightness and contrast can be obtained under somewhat realistic in vivo conditions (topical application of the nanoprobes to an accessible tumor surface). We recognize that the incubation time of the probes on the tumor surface (30 min) is considerably longer than would be convenient in clinical endoscopy and would require a 2-stage procedure: first to administer the nanoprobes and then, after a suitable time delay, to wash off unbound material and image the tumor surface during second endoscopy. This may be further altered by factors such as the presence of mucous, as well as peristaltic and ciliary clearing mechanisms, and clearly any first-in-human study will need to investigate the optimal time interval required for a given tumor type.

The EGFR family plays an important role in the pathogenesis of a wide range of epithelial cancers like lung, colon and esophagus, with high EGFR expression being associated with poor survival and increased risk of invasion/metastasis. The strong correlation here between the SERS signals (ARI) from different targeted tumor pellets with the EGFR level as determined by fluorescence flow cytometry suggests that the wide-field Raman imaging technique could be used to monitor, quantitatively, differences in EGFR status in different types of tumors or tumor stages. The marked (4-fold) increase in the SERS signals of both targeted nanoprobes in A549 tumors compared to H520 tumors clearly shows their high tumor affinity, while the corresponding decrease in the signal from A549 cells during competitive inhibition asserts the in vivo specificity of the nanoprobes.

The hydrodynamic size of the encapsulated nanoparticles is >120 nm, which is well suited to topical application, since there is minimal direct transfer into the blood stream so that the local concentration remains high throughout incubation. Systemic administration would require significantly higher administered dose, with resulting cost and regulatory/toxicity implications.
“Spray-and-image” is a well-established procedure in endoscopy using chromogenic dyes to highlight pathologic lesions, and we envision that topical application of the SERS nanoprobes would fit easily into clinical practice. Topical application does, of course, require that unbound nanoparticles must be thoroughly washed off, and the ease of doing this may vary with tumor factors such as type and location. The effects of residual unbound agent can be reduced using the above ratiometric approach with multiplexed image capture, wherein one of the nanoprobes in the cocktail is used as an internal reference. Similar approaches have been reported in other studies of molecular imaging to improve the detection contrast\textsuperscript{144, 162}. It is noteworthy that with SERS imaging it is straightforward to dedicate an imaging channel to an ‘isotype’ control during \textit{in vivo} imaging as compared to fluorescence, which is afforded by the high degree of multiplexing possible with such narrow Raman linewidths.

The multiplexed SERS imaging reported here demonstrates that our wide-field Raman imager is sensitive enough to measure and separate multiple targeted nanoprobes \textit{in vivo}. As shown in the resultant images, there was considerable heterogeneity in the Raman signal levels in different areas of tumor in both the ex vivo and in vivo studies. This heterogeneity was also observed in the ex vivo immunohistochemical EGFR staining of the tumor tissue and suggests that widefield SERS imaging has the resolution and sensitivity to identify tissue regions for further analysis, such as guiding biopsy site selection at endoscopy. While the present work has focused on clinically-available antibodies to provide tumor targeting, there are many other options including affibodies or peptides which may provide additional advantages for topical application. We have previously demonstrated that this multiplexed imaging technique can be implemented in an endoscopic mode\textsuperscript{163}, and translation into clinical endoscopy should be possible: however, if a conventional fiberscope is used, modification to operate with higher efficiency in the NIR region is required, given that standard clinical endoscopes have antireflection coatings optimized for use in the visible range. Alternatives, including coupling SERS capability into a scanning fiber endoscope\textsuperscript{164} are currently also being developed.
5.6 Conclusions

To the best of our knowledge, this is the first *in vivo* demonstration of a wide-field Raman molecular imaging of tumor biomarkers after simultaneous topical application of multiple targeted SERS nanoprobes. The next stages in development of this method towards clinical SERS endoscopy include both the above mentioned instrumentation development as well as investigation to target a range of cell-surface tumor biomarkers that are overexpressed in the tumors of interest: e.g. for non-small cell lung cancer this may include HER2, VEGFR, and PDGFR. In addition, kinetic studies with the different targeting moieties will be carried out to optimize the application time, evaluating the tumor-to-background contrast, excretion pathways and potential toxicities following topical administration.

The work contained in the previous chapters (2-5) has demonstrated the potential of SERS imaging for multiplex biomarker imaging in both microscopic and widefield configurations, primarily in pre-clinical model systems. Ultimately, translation to the envisioned clinical application in bronchoscopy requires that these approaches be combined with an existing endoscopic imaging platform – this will be the focus of the next chapter.
6 Widefield SERS Endoscopy Instrumentation

6.1 Acknowledgements

The work in this chapter represents the current status of the ongoing work to translate the previously described widefield SERS imaging approach (Chapters 3-5) into an endoscopic imaging platform. My contribution to this work included designing the optical filter system for both the Olympus and SFE endoscopes, the construction and testing of the NIR SFE module, carrying out all in-vitro and in-vivo experiments, and performing all data analysis. I would like to acknowledge Israel Veilleux for his assistance with the optical design and electrical work, as well as Richard Johnston for his expertise in configuring and troubleshooting the scanning fiber endoscope. I would also like to thank Dr Kazuhiro Yasufuku for facilitating the development of a NIR-optimized bronchoscope for our testing with Olympus Medical Systems Inc.

6.2 Introduction

In the most direct approach, a fiber optic SERS probe may be passed down the hollow working channel included in most bronchoscopes to take spectral data directly (either in contact with the tissue or at a fixed stand-off distance). Such experiments have been widely described in human subjects using endogenous Raman\textsuperscript{28, 48, 49} as well as using SERS-based contrast agents\textsuperscript{113}. This approach benefits from the ability to make use of the excellent white light imaging provided by the endoscope to visualize the placement of the probe to take an ‘optical biopsy’ of tissue, but is an extremely low-throughput approach to screening a large area of tissue and suffers from significant sampling bias as to where to make the individual measurements. It has been suggested that an improvement to this approach be made using a rotating-pullback approach such as is commonly used in OCT\textsuperscript{114, 115}. This revised technique would benefit from full-spectral collection at each point being sampled as well as improved surface coverage with automatic scanning, but is ultimately limited by the increased setup time for pullback acquisition and the
limited area covered in each pull, in which data collection is still relatively slow as a result of the point-by-point serial acquisition of data.

In order to apply the previously described widefield imaging methodology\textsuperscript{131, 158, 163} endoscopically, the tunable SERS filter module must be inserted between the imaging detector and the collection optics of the endoscope. This precludes the use of the majority of new clinical endoscopic devices, which have a miniature CCD or CMOS sensor placed at the distal tip of the endoscope directly behind the objective lens without room for any intervening optics. Even if a relay were inserted, the miniature size (1-2mm diameter) and length (<10mm rigid tip) of the tip optics precludes any practical tunable filter designs without obstructing the light guide or working channel components, and is certainly not feasible on a research basis.

Instead, widefield SERS imaging may be possible using a coherent fiber bundle endoscope, in which an array of packed, fused fibers is used to transmit the image collected at the distal objective lens of the endoscope to a proximal eyepiece lens for detection. These endoscopes have fallen out of clinical favor somewhat as a result of their ‘honeycomb’ image appearance and limited spatial resolution, high cost, and mechanical fragility, but their optical layout is well suited for integration into an imaging system. By relaying the eyepiece image through a tunable filter module and providing suitable illumination of the FOV, widefield SERS images could be acquired using all the previously developed hardware and methodologies.

Scanning fiber endoscopes (SFEs) represent a complete departure from the conventional approach to endoscopic image formation\textsuperscript{164}, and may represent an appealing format for molecular endoscopic imaging with SERS. By raster scanning a high intensity laser illumination spot over the FOV at megahertz rates and detecting the emitted light using a high sensitivity photomultiplier tube (PMT), SFEs have a simple optical return path which can easily be extended to include a tunable filter module for SERS detection, as well as providing the possibility for beam ‘parking’ for complete spectral acquisition in areas of high clinical
suspicion, combining the strengths of full spectral detection with the speed of bandpass SERS imaging for screening.

This chapter describes the methods and proof-of-principle results of integrating both a conventional, as well as NIR-optimized fiber optic bronchoscope onto the widefield SERS imaging system in phantom and in-vitro test systems. It also details the design of a hybrid fluorescence/SERS detector system for the SFE and demonstrates the first Raman measurements with the SFE in phantom and in-vitro experiments.

6.3 Materials and Methods

6.3.1 Experimental reagents and in-vitro sample generation

SERS-active gold nanoparticles with the reporter molecule S481(4-Azobis(pyridine)) were obtained from Cabot Security Materials Inc. (Mountain View, CA, USA). For the phantom test experiments, the AuNPs were diluted in water to known concentrations and imaged in 1.5mL polypropylene tubes. Molecularly targeted anti-Her2 AuNPs were generated as described elsewhere (Chapter 2). Her2 overexpressing (Calu3) cells were acquired from the American Type Culture Collection (Manassas, VA) and propagated in RPMI-1640 media supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada). Labeling was carried out by treating aliquots of $1 \times 10^6$ cells in suspension with anti-Her2 AuNPs (40pM in PBS) for 30min at room temperature. After incubation each sample was subjected to five rounds of washing with 3% BSA in PBS to remove nonspecifically bound probe prior to imaging.

6.3.2 Coherent fiber endoscopy

6.3.2.1 SERS bronchoscope and detector design

As previously described, traditional small-diameter endoscopes were constructed using a packed array of optical fibers which have been fused to form a coherent fiber endoscope (CFE). An
objective lens at the distal end relays the image onto one face of the bundle, and an eyepiece is permanently fixed at the proximal end of the bundle to resolve the image. By replacing the objective lens of the widefield SERS imaging system with a suitable mechanical adapter, the image from the CFE eyepiece lens may be re-imaged through the tunable filter module so as to allow for endoscopic SERS imaging with no further revisions to the detection optics. In these tests, a miniature 5.9mm diameter CFE bronchoscope (BF1T60, Olympus) and proprietary optomechanical adapter provided by the manufacturer (A10Y003, Olympus) were used – interestingly it was observed that the adapter changes the position of the eyepiece lens in the bronchoscope handle via an internal helical drive mechanism when it is engaged, to unknown effect. The adapter presents an image at a flange-focal-distance equivalent to the c-mount lens standard (17.5mm) and so was used as a direct substitution for the widefield c-mount objective lens with no further modification.

Initial bench testing showed that the transmission of light in the NIR SERS operating range of (750-900nm) was extremely poor through the CFE (<5%) – discussions with the manufacturer revealed that the optics at either end of the fiber bundle had anti-reflection coatings optimized for visible wavelength imaging, which resulted in significant per-surface reflections in the NIR. Consequently, Olympus provided a prototype NIR-optimized endoscope and c-mount adapter with optics having a VIS-NIR coating that should improve imaging performance. An image of the complete endoscopic widefield imaging arrangement is shown in Figure 6.1.
6.3.2.2 Coherent fiber illumination design

To provide SERS excitation light onto the sample when imaging in endoscopic mode requires significant revision from the macro-scale illumination system. The light source for conventional white light endoscopy is a high-intensity arc lamp, typically a 300W Xenon source in Olympus.
systems, which provides intense illumination of the tissue being studied. This intensity of illumination is necessary, since the low NA of typical endoscope objectives combined with the poor transmission efficiency of the CFE bundle and numerous optical elements in the chain produces a system with very poor throughput. Even with high-intensity illuminators and modern CCD detectors, video rate imaging using a camera on a CFE tends to produce relatively dim images without significant signal amplification and concomitant noise. As the maximum output of the widefield SERS imaging laser source is approximately 500mW, prior to amplified spontaneous emission (ASE) filter and coupling losses which result in approximately 85% transmission, light delivery must be as efficient as possible if SERS imaging is to be realizable.

Typical endoscope illumination is provided by a pair of lightguides which run beside the fiber bundle in the endoscope shaft, but initial testing revealed the transmission of these channels to be extremely poor (<10%) at 785nm. Because the working distance of the bronchoscope is quite small (typically <5mm), it is not possible to provide simple illumination from a collimated external beam as there is insufficient room to pass between the sample and CFE end obliquely while still covering the entire FOV. As a result of these limitations, test illumination was ultimately delivered using a high-NA fiber placed in the accessory channel of the endoscope with the necessary ASE filters placed at the proximal end of the fiber between two collimators. This solution did not illuminate the entire FOV of the endoscope, but delivered sufficient power for initial proof-of-principle testing.

6.3.3 Scanning fiber endoscopy

The specific details of the operation of a scanning fiber endoscope are detailed elsewhere\textsuperscript{164-166}. Briefly, the SFE consists of a singlemode optical fiber which carries RGB excitation light from a base station through a very flexible shaft to the tissue being imaged. The fiber is scanned using a piezoelectric drive mechanism to point the illumination spot at different areas of tissue, and the scattered light is collected by a ring of multimode collection fibers which relay the signal back to the base station where it is converted into an image at video rate. Because the fibers used in the
SFE are not fused as in a coherent fiber endoscope (CFE), they have a significantly smaller minimum safe bend radius allowing for a smaller and more mechanically robust endoscope design. The smooth scanning of the SFE means that the resolution of the image is set by the digitization rate of the system and not by the number of fibers in a bundle, as is the case in a CFE. A comparison of images acquired using the SFE and a standard CFE is shown in Figure 6.2, demonstrating the marked improvements in imaging possible with the SFE. The standard SFE is designed to operate in RGB (442/532/635nm) reflectance mode imaging, but the flexibility of the design allows for additional imaging wavelengths and detectors to be integrated with the closed loop scanning hardware.
6.3.3.1 **SFE fiber selection for NIR SERS**

At the core of the SFE resonant scanner is the cantilevered singlemode fiber which is scanned in a spiral pattern to conduct the excitation light onto the surface being imaged. To operate at video
rates requires that the natural resonance frequency of the fiber be at least 10kHz when mounted in the piezo collar, which in the present scan engine design requires that the outer diameter of the fiber be approximately 80µm or less. A singlemode fiber is used since this ensures that the light emerges as a purely Gaussian distribution which will be projected into a diffraction limited spot by a correctly aligned objective lens system.

The standard fiber used for RGB imaging with the SFE was custom-pulled to have a 3.5µm core and 80µm cladding diameter (StockerYale, Salem NH). This fiber is incompatible with our standard Raman excitation light at 785nm, as the mode field diameter of the 785nm light exceeds the core diameter of the fiber to such an extent that bend losses would be essentially 100% if the fiber were disturbed at all after beam alignment. Considering the number of bends in the shaft of the SFE which are anticipated in clinical applications, as well as the extreme drive angles produced in the scan engine itself, it is imperative that the excitation light be within the design range of the singlemode fiber. To achieve this, we have had a probe constructed with a larger core reduced-diameter fiber, having a core diameter of 4.2µm and cladding diameter of 80µm (SM800G80, Thorlabs). This fiber will still have a resonant frequency above 10kHertz as required for video rate imaging, but has the drawback of multimode conduction of the 442/532nm excitation light for the RGB imaging. This implies that the projected spot of the blue and green spots will be blurred somewhat, which in principle reduces the resolution of the resultant images. A direct imaging comparison of the 3.5µm and 4.2µm fiber probes is shown in Figure 6.3, and shows limited practical differences in RGB image quality in the larger core fiber.
Figure 6.3. Comparison of images acquired using a 3.5µm core RGB fiber (a,c) and 4.5µm core NIR fiber (b,d) using the SFE. The spatial resolution is slightly decreased using the larger core fiber as evidenced by the blurring in the USAF target image and lack of fine vessel resolution in the tympanic membrane image, but these effects are only significant for sub-millimeter scale details which are not the primary focus of endoscopic screening. Note that the white balance was not equalized between the two probes.
6.3.3.2 SFE NIR SERS module design

In order to maintain the white light imaging capability of the SFE probe it is necessary to superimpose the SERS excitation and emission light onto the existing optical paths within the endoscope. A spectrum-stabilized 785nm laser source having a peak FWHM <0.03nm and output power of 50mW at the FC/APC terminal was sourced (BRM-OEM, BWTek, Newark DE) for this application, and was factory aligned to couple into a 5µm core fiber having an NA=0.13. As the RGB laser source in the SFE base station is launched using a FC/PC terminal configured for 3.5µm/0.12NA fiber, a pigtailed wave division multiplexer (WDM) (OZ Optics, Ottawa ON) was used to combine the two beams into a FC/PC terminal aligned using a 4.2µm/0.14NA fiber to match the NIR SFE probe specifications. An ASE blocking filter (Maxline, Semrock, Rochester NY) was installed in the WDM on the 785nm input port to reduce spontaneous laser background signal, as was done in the widefield imaging system.

Both reflected RGB light and SERS signal light is collected by the standard return fibers (50µm/0.66NA) in the endoscope tip and are presented to the end of the probe packed into an SMA connector. A block diagram of the SERS detection module is shown in Figure 6.4 – the tunable filter module is an acousto-optical tunable filter which has a working range of (650-1000nm) and a bandpass FWHM of <2nm. Because the AOTF splits the diffracted beam into ±1-order beams based on their polarization and the output light from the SFE is unpolarized, split-D pickoff mirrors were used to recover both polarizations to increase light recovery.
Figure 6.4. Schematic diagram of the layout of the NIR SERS SFE module. Return light from the SFE probe is split by a dichroic mirror (DM) and the RGB signal is returned to the SFE base station. The NIR light is collimated and passed through the AOTF, and the ±1-order beams are selected by pickoff mirrors and relayed to the NIR PMT. The undiffracted 0-order light is collected by a beam stop.

Images of the NIR module as presently constructed are shown in Figure 6.5. To maximize the utility of the NIR detection module for applications other than SERS, a diversion line which bypasses the AOTF has also been included using kinematic mirrors, which allows the device to operate in standard fluorescence mode for wavelengths within the PMT sensitivity range (400-1200nm).
Figure 6.5. (Top) Photograph showing NIR SERS module beside RGB SFE basestation along with associated connections. (Bottom) Internal optical arrangement of NIR SERS module showing positions of the AOTF, pickoff D-mirrors (DM), PMT, and kinematic fluorescence line filters (FM).
6.3.3.3 NIR detector module and SFE interface

At present, the SFE acquires 600-line images at 30Hz - the actual outward scanning portion of each cycle occupies approximately half of each frame’s time interval, with the balance being used for active braking and passive settling. This implies that, at the outer points in the FOV, the per-pixel integration time is approximately 50ns and the signal is digitized by the base station ADC at 50MHz. These extremely short per-point dwell times and very low light levels necessitate the use of PMT detectors for both the SERS and RGB signals as only they have sufficient gain, transit/reset speeds, and low noise levels to allow efficient detection.

With SERS excitation at 785nm, to cover a Raman shift range of (100-2000cm⁻¹) requires that the PMT be responsive from (790-930nm), which is a spectrum that is not well-covered by most PMT cathode materials. Such NIR PMTs typically also have high dark currents at room temperature as a result of their very low work functions. The PMT used in the NIR SERS module has a cathode made of Ag-O-Cs which is sensitive from (400-1200nm) and is housed in a thermoelectric (TE)-cooled housing which holds the PMT at -25°C during operation to minimize noise (R5108, Hamamatsu). Despite being optimized for NIR operation, the quantum efficiency curve of the cathode is relatively flat between (600-1000nm) at ~0.25%.

The signal from the PMT is amplified into the range of the SFE’s ADC by a high speed, low noise transimpedance amplifier (HCA-100M, Femto, Berlin). Importantly, the amplifier supports a bandwidth of (DC-100MHz) which is necessary to prevent constant signals from disappearing from the image (DC) while still covering the fast scan rate of the SFE at the periphery. An adjustable offset circuit is used to move the signal into the pickup range of the SFE’s ADC, and a snubbing (adjustable impedance dampening) network is placed on the pre-amplifier signal cable from the PMT to minimize ringing due to slight impedance mismatches.
Initial tests of the NIR PMT with the SFE showed a fixed periodic noise with a period on the order of 10ns that overwhelmed any optical signal whenever the PMT cooler was powered on. This manifested as a spiral pattern on the SFE display which rendered the images unusable, as the noise signal occupied the whole of the ADC’s dynamic range. Discussions with the manufacturer (Hamamatsu) indicated that this noise was unavoidable but could be removed by thresholding the signal to the ADC to select pulses above the noise floor – this was achieved in practice by using a high speed diode and adjusting the amplifier offset to move the level of the noise such that it was shorted to ground.

6.4 Results

6.4.1 Coherent fiber endoscope SERS imaging

After ASE filter and fiber coupling losses, the total power output from the illuminating fiber in the accessory channel of the fiber bronchoscope was measured to be approximately 180mW. As this is significantly reduced from the >450mW typically delivered to a 5mm radius spot in the widefield imaging studies, the fiber was moved so as to illuminate only a small fraction of the FOV and maintain a high power density for SERS generation.

As shown in Figures (6.6, 6.7) widefield SERS imaging proved to be possible through a NIR-modified CFE endoscope. While good SNR images were possible using concentrated SERS nanoparticles (40pM) alone, the signal was markedly decreased in cells stained with the targeted nanoparticles where the total number of particles in the imaging volume is much lower. Based on the intensities recorded using fixed integration times for imaging pure tag solution alone, the system is approximately 10-20x less sensitive when imaging in CFE mode as compared to using the standard widefield objective lens.
Figure 6.6. White light (top row) and SERS (bottom row) images acquired using CFE widefield SERS imaging system from: (a,b) S481 reporter molecule at 40pM, (c,d) A549 cells stained with S481-EGFR nanoparticles, (e,f) A549 cells stained with S481-EGFR nanoparticles after pre-incubation with excess EGFR antibody for competitive inhibition. Integration time was 10s, averaged 3 times.
A significant portion of this reduction in sensitivity is likely due to the reduced illumination power delivered to the sample by the fiber in the accessory channel. It was necessary to move the fiber quite close to the sample in order to ensure sufficient power density to get a reasonable number of SERS photons in any given image pixel, which was done at the expensive of exciting only a small fraction of the endoscope’s FOV with the laser light. Additionally, it was noted that extending the fiber this far from the endoscope caused some shadowing/blocking in some images as the fiber itself became visible on the screen, necessitating a large amount of adjustment to get the sample ‘just right’ for imaging. Improving the light delivery to the sample, preferably by making use of the illumination channels already built into the endoscope, would be a significant step towards better SERS imaging provided the power density could be maintained. This will
likely necessitate the use of a significantly more powerful light source. However, care must be taken to ensure that the linewidth of the laser does not increase at higher power – since the SERS peak width is in part determined by the excitation linewidth, the ability to carry out multiplexed imaging depends on the maintenance of a narrow excitation line.

6.4.2 Scanning fiber endoscope SERS imaging

6.4.2.1 SFE point spectroscopy

In the most simplistic application of the SFE for SERS detection, the dead time between fiber scan cycles can be used for point spectroscopy from the tissue which is at the center of the endoscope’s FOV. As shown in Figure 6.8, the spectra from a pure SERS tag solution can be clearly resolved using the NIR SFE and, by applying basic peak integration, significant differences can be shown between labelled and unlabeled cells.

Figure 6.8. (centre) Scanning fiber endoscope image of cell suspensions stained with SERS AuNP probes. (left) Point spectrum acquired through SFE of pure EGFR-S481 AuNPs at a concentration of 15nM. (right) Integrated signal intensity from unstained A549 cells and cells stained/washed with 40pM EGFR-S481 nanoparticles, acquired with a bandpass of (863-866)nm.

The spectrum shown in Figure 6.8 was acquired using a significantly longer acquisition time (15s) than would be available in typical endoscopic imaging uses, which tend to be on the order of 1s or less. This long integration time was necessary primarily because of the inexpensive
spectrometer used (HyperFlux785 Beta, Tornado Medical Systems Inc.) which uses an uncooled, board-level CCD for signal detection and contains no Rayleigh rejection optics. This necessitated post-SFE beam collimation, filtering, and re-launching into a fiber to the spectrometer, the end result of which was a relatively weak and extremely noisy spectrum. Given the SNR that was achieved using this rudimentary arrangement, further testing with a dedicated Raman spectrometer coupled to the SFE should allow for nanoparticle detection within the unused portion of a single imaging frame (approximately 450ms).

6.4.2.2 SFE NIR imaging

As an initial test of the SFE NIR detection module, an aliquot of a fluorophore which is efficiently excited at 785nm (IRDye800CW, Li-Cor Biosciences) at a concentration of 10µM was imaged using the SFE. The diversion line which bypasses the AOTF was used, so that all light passing the laser rejection filter and RGB pickoff dichroic was passed directly to the NIR PMT. As shown in Figure 6.9, there was significant noise present in the image acquired using maximal PMT gain. As the per-point dwell time is quite low (<50ns), a frame-averaging approach was used to increase the SNR, whereby 30 frames constituting 1s of data collection were stored in a memory buffer, then averaged and displayed. The resultant average frame is also shown in Figure 6.9, and demonstrates significantly less noise.
It was observed that there was very little dynamic range available in the fluorescence channel images, which appears may be related to a mismatch in input voltage ranges between the offset-adjusted PMT amplifier and the ADC. Further investigations are ongoing to determine if rescaling the PMT output to fill the ADC’s digitization range will improve the resultant images. Since this fluorescence signal is at least two orders of magnitude larger than the expected SERS peak intensity, SERS imaging with the SFE will not proceed until the NIR fluorescence images are optimized.

6.5 Discussion
6.5.1 Raman CFE detection
The coherent fiber endoscopic SERS images generated in this work have shown that CFE SERS imaging is possible, but it has also highlighted a number of issues which must be addressed prior
to further device development. In large part the hurdles faced by CFE SERS are the same as those facing CFE fluorescence (and even RGB) imaging, namely the poor optical efficiency and limited spatial resolution of CFEs. The fiber bundle in a CFE contains a significant amount of non-imaging cladding area which blocks signal from being transmitted from the tissue to the detector, in addition to the intrinsic losses in the fibers as well as reflections at either end of the bundle. Since the image of the fiber bundle is generated by an eyepiece lens that is fixed in place, an additional relay optical system must be incorporated to carry the signal through the filter module to the CCD, which incurs additional losses.

Given the size of even a miniaturized version of the tunable filter module and CCD used here, a CFE-based SERS imaging system would no longer be hand held by a user and would have to be mounted to a weight-supporting cable system similar to early Xillix autofluorescence systems. It is noteworthy that the Xillix approach was eventually abandoned as it was clinically unpalatable to use the suspended camera system. Discussions with Olympus have covered the possibility of using a flexible light guide to relay the signal from the endoscope eyepiece to a filter and CCD mounted on a nearby cart but, as this is essentially another fiber bundle added in continuation to the endoscope, they have estimated throughput as low as 20%. Considering the weak signal levels generated with direct attachment of the system to the endoscope, further losses from an additional fiber bundle would render the system unusable.

Conventional endoscopes have in large part shifted format away from fiber bundles to videoscopes in which a miniaturized CCD is located at the distal tip of the endoscope, eliminating all the losses associated with additional optical elements and fibers. Fluorescence versions of these endoscopes do exist, though most implementations consist of a fixed longpass filter and second monochrome CCD which is located beside the RGB imaging CCD. This design obviously increases the overall size and cost of the endoscope and is not directly amenable to SERS imaging as there would be no room for a tunable filter, only a fixed filter to select one or two distinct SERS peaks.
A compromise ‘hybrid’ endoscope design does exist which would be more suited to SERS imaging, in which a fiber bundle relays the image to a detector located within the endoscope handle, eliminating the need for an eyepiece and relay optical system to reach the detector. The small footprint of the endoscope handle would likely preclude the use of a rotational filter, but may accept a miniature solid state filter like an LCTF or AOTF. Given the considerable expense of such filters and the significant manufacturer R&D required to design and build such a system, it seems unlikely that one would be available for research without more compelling bench test data and clinical impetus.

The SERS CFE will likely retain its utility as a purely research tool, to be used for proof-of-principle studies where the endoscope may be rigidly fixed to allow for long exposure imaging to compensate for its poor optical transmission. Because of the standard optical interface used on most Olympus devices, the same system may be interfaced with clinical endoscopes used for oropharyngeal, gastric, colonic, and even neurosurgical endoscopy to explore other potential application areas relatively easily. Once feasibility has been demonstrated using the CFE system, additional resources could be devoted towards the development of a more clinically palatable and optically sensitive endoscope design.

6.5.2 Raman SFE

The scanning fiber endoscope represents a completely novel approach to endoscopic imaging, and is appealing for SERS imaging thanks to its flexible optical layout. Because the return light from the SFE is not spatially organized into an image, as with a CFE, the tasks of collimating and filtering the signal prior to the detector are greatly simplified and do not suffer from artifacts such as FOV center wavelength gradients encountered in Raman imagers using a CCD detector. Illumination of the sample is also straightforward, since all that is needed is to couple the source laser into a singlemode fiber and the VIS-NIR objective lens will project a nearly diffraction limited spot onto the sample.
The SFE’s optical simplicity is gained at the expense of collection efficiency, which varies based on the actual probe design. In the standard ultra-miniature arrangement, a ring of multimode collection fibers collects reflected/emitted light from the tissue, relying on their high NA to collect as much light as possible without any additional collection lensing. This approach is unsurprisingly inefficient and, as shown in this work, necessitates significant frame averaging to collect sufficient SERS signal to be detected above baseline. This collection efficiency can be improved by using larger core fibers, but care must be taken to select materials with sufficient transmission in the NIR and that do not increase the size of the SFE probe significantly. An alternative probe design being developed by the University of Washington employs a dual-clad scanning fiber which would collect the emitted light in a coaxial fashion using the objective lens for both excitation and emission, and would result in a probe with the possibility of confocal collection as well as a reduced outer diameter.

The SFE design is also appealing as it would allow for point spectroscopy to be integrated with widefield fluorescence or Raman imaging in addition to standard RGB reflectance. Areas of interest identified with any of the widefield modalities could be interrogated by utilizing the dead time of the scan cycle in which the beam points directly forwards and is not scanned to collect a point spectrum of any variety (reflectance, fluorescence, time-resolved fluorescence, Raman, etc.). This approach would combine the extremely high sensitivity/low throughput of a technique such as point Raman spectroscopy with the ‘screening’ level throughput of widefield SERS imaging. One could also imagine the use of the SFE’s scanning fiber to deliver therapeutic light either at high powers to achieve ablation or coagulation of tissue or at lower power to active photodynamic therapy (PDT) drugs. Such treatments could also be monitored in real time using either point spectroscopy or widefield imaging to detect signals such as singlet oxygen phosphorescence in the case of PDT. By modulating the therapeutic laser intensity it would also be possible to ‘paint’ the light dose onto a region of interest defined on a widefield image, allowing for sparing of dose to vital structures and normal tissue (which would be more clearly defined by the enhanced spectral imaging afforded by the SFE).
While the optical image quality afforded by the SFE is remarkable given its small size, it may be its potential for therapeutic delivery which results in the largest shift in the paradigm of modern endoscopy. It transforms the endoscope from a passive observational tool to an active interventional device, and has the ability to provide a multitude of simultaneous or interleaved imaging modes which cannot be achieved with any other endoscopic technology. Clearly there are an enormous number of possible research avenues based on the SFE, as we have only just begun to define all the potential clinical applications - hopefully the recent successes of ourselves and others using the SFE in-vivo will generate further interest and development on the commercial side to support swift clinical adoption.

6.6 Conclusion

In this section, two approaches to implementing widefield Raman imaging on available endoscopic platforms have been shown. SERS imaging using a clinical CFE is the most straightforward approach, but suffers from poor illumination and collection efficiency. Nevertheless, the WRI CFE was able to image and differentiate between cells stained with targeted SERS AuNPs and inhibited control cells. The SFE represents an appealing approach to WRI SERS endoscopy due to the flexibility in the optical design, however the technical challenges of integrating an additional NIR detector into the existing hardware interface have not been straightforward. The NIR detector module has successfully been used to carry out fluorescence imaging in a spectral region identical to that planned for SERS imaging, and frame averaging has been implemented to improve the SNR in low light conditions. With improvements to both designs it appears that endoscopic widefield SERS imaging of targeted nanoparticles in vivo is a viable possibility.
7 Conclusions and Future Directions

7.1 Summary of work to date

Surgical resection or ablation remains the primary curative treatment strategy for most early-stage solid cancers and as such an enormous amount of effort has been dedicated to technologies which augment the vision of the surgeon to better ‘see’ the margins of the tumor for removal. With our ever-expanding knowledge of the genetic changes which underpin malignancies, the idea of adding ‘biochemical vision’ to the surgeon’s decision making process should allow for clearer identification of margins, more complete resections with no residual tissue left in-situ, and consequent improvement in recurrence rates.

Molecular imaging using SERS nanoparticles represents a platform which is well-suited for cancer detection in-vivo. It has already been established that SERS offers the ability to carry out multiplex assays far in excess of what is possible with fluorescence, that the SERS signal is detectable at concentrations far below what is resolvable with similar amounts of fluorophores, and that the SERS signal is temporally stable and does not photobleach over time. At the time this project was undertaken, encouraging results were already being reported showing tumor differentiation from normal tissue using molecularly-targeted SERS nanoparticles and point-based spectroscopy detection.

This body of work has developed a framework for detecting these SERS-labelled tumors, not using slow point-by-point screening, but rather fast large-area widefield imaging. While many early papers reported protocols for the generation of targeted SERS AuNPs, these protocols were often incomplete or unrepeatable in our lab and by others, such that a significant effort was invested in developing a reliable method for generating antibody-functionalized AuNPs. As shown in Chapter 2, the resulting synthetic procedure allows for efficient conjugation of numerous subtypes of antibodies to the surface of NIR-optimized AuNPs, and has been
successfully applied in our own lab as well as those of collaborators at The Hospital for Sick Children and SUNY Stony Brook.

Widefield Raman imaging had previously only been shown using a microscope as the optical base and studied semiconductor samples, which provide strong Raman signals with relatively little background or movement to contend with. By contrast, SERS sensing in biological samples generates significant amounts of fluorescence background signal which must be dealt with in order to extract the minute SERS signals of interest when detecting rare molecular markers of disease. Chapters 3 and 4 cover the development of the necessary hardware for widefield SERS detection as well as a data processing pipeline which applies the usual fluorescence subtraction scheme used in spectral analysis but has extended it to remove the fluorescence contribution from entire images. These works represent the first demonstration of widefield SERS imaging in-vitro and in-vivo, as well as containing the first demonstration of quantitative multiplex SERS widefield imaging in the same settings.

Building upon the refined probe design and novel imaging hardware previously described, Chapter 5 demonstrated for the first time multiplex widefield SERS imaging from tumor cells and tissue that have been stained with antibody-targeted nanoparticles. This work also showed that the proposed topical application/washing strategy could deliver sufficient AuNPs for imaging detection during an intervention by staining a tumor in-situ during surgical exposure, and generating quantitative multiplex SERS images from the operative field.

Finally, Chapter 6 presented the proof-of-principle results from efforts to translate the widefield imaging approach to two distinct endoscopic imaging platforms. It was shown that the previously developed widefield imaging hardware was directly compatible with clinical coherent fiber endoscopes and that, with sufficient illumination, good SNR images of cells labelled with targeted SERS AuNPs could be detected. The extension of the widefield SERS imaging
approach to scanning fiber endoscopy was also demonstrated in principle, and the possibility of combined widefield and point-Raman spectroscopy with the SFE was shown for the first time.

7.2 Future Directions

7.2.1 Widefield Raman imaging

The widefield Raman imaging (WRI) system as developed in this body of work has clearly demonstrated the enormous potential of SERS imaging in pre-clinical investigations in-vivo, but there is still significant room for improvement in terms of technological implementation. The WRI system, while designed for Raman imaging, is essentially equivalent in its optical layout to bioluminescence imaging systems which are commercially available (Xenogen/Maestro) and design changes to mimic these more refined products would likely bring significant improvements in Raman performance.

The first area identified for future work would be the detector itself – at present the system uses a high-speed, high-noise EMCCD which is designed for video rate imaging in the visible spectrum, not long exposure NIR spectroscopy. A back-thinned, large pixel, water-cooled EMCCD with deep-capacity wells in both the array and gain registers would allow for significantly longer exposure images without saturation and smearing as presently is common in heterogeneous samples. The present EMCCD also does not possess a linearized EM gain scale that is locked in hardware, meaning that one cannot calculate even relative differences in intensities in images acquired with different gain settings or on different days – this problem is solved and is addressed in modern scientific EMCCDs, and would be a welcome addition to a future WRI system to allow day-to-day experimental comparisons.

A second interesting area for development is the implementation of the tunable interference filter module. At present the filter offset is fixed, which sets the bandwidth of the composite filter, and is rotated on a stage to select the center wavelength for imaging. To maximize multiplexing
efficacy the filters are presently quite offset, which produces an extremely narrow filter FWHM at the expensive of relatively poor transmission (<60%). This is in stark contrast to the filters’ baseline transmission when they are used individually, typically >90%. It is straightforward to imagine uncoupling the two filters onto individual rotation stages, such that the offset could be adjusted to be small (broad FWHM, high %T) for single peak imaging, then the offset could be increased (small FWHM, reduced %T) for multiplex detection. While this would necessitate some re-engineering of the mechanical layout to avoid lengthening the optical path, it would significantly improve the ultimate limit of detection of the system, as well as opening the door to alternate methods of background signal acquisition; it may then be possible to acquire the fluorescence image for subtraction using a single wide-spectrum frame instead of the present stokes/anti-stokes narrow-image approach.

There is also development potential in the illumination system for the WRI, which at present consists of a simple post-fiber collimated beam with a radially decaying beam profile (the launch fiber is multimode to support diode powers above 100mW). The spatial non-uniformity of illumination makes it impossible to discern whether intensity variations in signal are due to biological or laser intensity differences, and presently restricts SERS imaging to only the centermost portion of the illuminated field. Considering the size mismatch between the illumination spot (5-12mm) and the system FOV (25mm), a significantly more powerful laser source, along with suitable optics to generate a top-hat beam profile over the entire FOV, would allow for imaging of much larger areas without the necessity for intensity compensation.

The value of these system revisions, which involve elements of considerable expense, must be weighed against the cost of commercial widefield Raman imaging systems which are emerging on the market. Discussions with a manufacturer of microscopic SERS imaging systems (RIMA, Photonetc, Montreal QC) has indicated that such systems are now available on a research basis. As an example; a RIMA system, based on a large format volume Bragg grating, would provide a filter transmission >90%, FWHM of 3.5cm⁻¹, and a tuning range of 0-4000cm⁻¹ when operating at 785nm. These systems have been in use in astronomical applications for over a decade and as
such issues like center wavelength FOV gradients have largely been solved much more quantitatively than is presently done in the prototype WRI system described here.

Looking retrospectively, it is remarkable to see how much of the work contained within this thesis was enabled by technologies which were developed only very recently. The high power 785nm diode laser source used for widefield illumination is a relatively inexpensive spectrum-stabilized unit which is no larger than a shoebox and weighs ~1kg; by comparison a similarly specified unit would have occupied an entire optical bench with components totaling an excess of 100kg only 25 years ago. The angle-tunable filters used in the widefield imaging system were only introduced in 2011 (for applications far removed from Raman imaging), and there exists significant potential for the development of narrower transmission, broader-coverage filters for SERS imaging in the near future. Similarly, the present approach to SERS imaging on the SFE makes use of a standard AOTF which is a free-space unit, necessitating dispersion and collimation of the fiber-optic SFE return light along with associated losses. All-fiber AOTFs are now becoming commonplace in telecommunications applications, and adaptation of such a probe to the working wavelength for NIR SERS (800-900nm vs. 1310/1550nm) could lead to a greatly simplified SERS SFE system with a far reduced cost and increased mechanical stability. Based on the pace of innovation in spectral imaging technology to date, and assuming continued reductions in the cost of NIR CCDs and large-format filters, it is easy to imagine that a widefield Raman imaging system in the next 10 years may have both a higher optical throughput and significantly reduced cost.

7.2.2 Translation to first-in-human studies

At a minimum there remain two important barriers to the translation of the aforementioned SERS nanoparticles to in-human testing; pharmaceutical-grade manufacturing and safety/toxicity studies. In Canada at the present time, to satisfy the first requirement necessitates production under good manufacturing practices (GMP) for active pharmaceutical ingredients (APIs). To synthesize the SERS nanoparticles under such conditions is straightforward (in principle) as
GMP regulations do not restrict the actual chemical agents used in the product but rather outline a system of record keeping, chemical sourcing, and an analytical framework which should be used in the creation of any agent to enter a human body. By and large the manufacture of SERS nanoparticles under GMP conditions is restricted only by the investment required, which can be significant considering the cost of USP-grade reagents, dedicated analytical hardware, dedicated cleanroom handling/bottling, and the human costs associated with record keeping and regulatory agency interaction. The current construction of the “NanoMedFab” GMP core facility in Toronto will eliminate a number of the infrastructure-based costs associated with GMP production such as a bottling cleanroom and dedicated analytical hardware, but will still necessitate a large operating investment to cover the chemical and administrative fees.

With regards to toxicity, the route of administration must be carefully considered. Gambhir et al. have aggressively pursued toxicology studies of the same commercially available AuNPs for topical application in the colon. These works have shown that the large outer diameter (~120nm) particles cannot efficiently cross the epithelial lining of the mouse colon and are largely excreted with the first bowel movement after administration. This is in contrast to the situation where the particles are given IV, in which case they are rapidly cleared by the reticuloendothelial organs where they are sequestered long term and produce a limited inflammatory and apoptotic reaction. Importantly, there were no adverse neurological, cardiovascular, or biochemical abnormalities noted in animals in which the AuNPs were given through either route. However, the long term sequela of AuNP retention in the body following IV administration would require significantly more study to determine any side effects which may present beyond the 2 week period studied to date. As the topically administered particles were unable to penetrate into tissue in the gut, it follows that they would only be useful for labelling tumour tissue which is on the epithelial surface and protruding into the lumen. Fortunately this growth pattern is the most commonly encountered scenario in colorectal cancer, and is also true of bronchogenic carcinomas in the lung.
The topical application route, while appealing from a toxicity point of view, requires some critical assessment in the case of lung cancer. Unlike the colon, the lung does not tolerate high volume fluid flushing and as such the administration of AuNPs would need to be more directed towards a sub-region of clinical suspicion identified with standard bronchoscopy. Alternatively, it may be possible to formulate an aerosol suspension of AuNPs which could be inhaled into the lung and allowed to be cleared by mucociliary action except where they are retained by their targeting moieties onto tumour tissue. This approach would require considerable additional research into factors including probe stability, homogeneity of distribution, and maximum deliverable probe concentration prior to attempting any in-vivo tests but would likely be much more palatable clinically than pulmonary lavage. With either approach one must also consider the relevance of the preclinical colon data in light of the preponderance of macrophages located within the lung; these cells function to clear the lung of foreign particulate matter by phagocytosis and it is not unreasonable to expect that they would take up any delivered nanoparticles with great avidity through the same mechanism, likely increasing systemic uptake and nonspecific background on imaging. A further concern arises from the silica encapsulant which is used in the current AuNP formulation – inhalation of particulate silica is known to produce a robust inflammatory and fibrotic reaction (silicosis) in the lung which can cause permanent disability, and so the total delivered amount must be carefully considered especially in the setting of chronic exposure through screening. Given these considerations, it would be prudent to carry out a complete biodistribution and toxicity study of the targeted particles delivered topically to the lungs rather than attempting to rely on the previously published colonic data.

7.2.3 Future clinical applications of SERS endoscopy

Even if the aforementioned technical and regulatory challenges were overcome, the likelihood of clinical adoption of SERS bronchoscopy beyond first-in-man studies is also uncertain at this point in time. Autofluorescence bronchoscopy, which has been shown to have improved sensitivity to malignancy over white light imaging alone, has generated very little traction in North America despite its relative simplicity, safety, and straightforward integration with clinical
bronchoscopy. Considering one proposed workflow for SERS bronchoscopy (sedation and anesthesia, bronchoscopic navigation to region of interest, mucosal lavage, contrast agent administration and incubation, rinsing and aspiration, multiple SERS band image acquisition) would require at least an order of magnitude more time to complete, as well as further physician technical training and possible drug/contrast agent reactions requiring management, clearly this would not be palatable without sufficient clinical impetus showing improved detection or resection rates. While the results demonstrated in this thesis have shown that such improvements may be achievable, they have all been undertaken in the somewhat artificial setting of a monoclonal human tumour growing within the background of a mouse – this likely represents a best-case scenario in terms of achievable signal to background. A more realistic study, using an orthotopically implanted primary human xenograft to recapitulate the heterogeneity of actual cancers, or examination of surgical resection specimens would absolutely be necessary to generate sufficient data to warrant any pursuit of a clinical trial.

It may be that the first clinical applications of multiplex SERS imaging may lie outside the lung, or even outside the realm of flexible endoscopy. Given the optical complications described herein regarding the collection efficiency of both CFE and SFE endoscopes, a system based on a rigid endoscope or operative microscope would provide a far more suitable optical platform for initial testing. Operative microscopes are used daily for the fluorescence-guided resection of malignant neoplasms in the brain, and it would be straightforward to adapt the SERS tunable filter module to such a system, which would be free of the size/weight constraints imposed by handheld endoscopes. In such resections SERS contrast agents could be applied directly to the surgical field to identify residual tumour tissue after initial debulking, and irrigation and suction would be easily applied to remove nonspecific probe as compared to what is possible in bronchoscopy. Brain tumour pathogenesis and biology is somewhat more defined than the heterogeneity found in NSCLC, and as such a panel of markers may be more readily available to differentiate it from surrounding normal tissue. Similar arguments could be made in support of SERS-guided resection of dermatologic malignancies, though these studies would likely serve mainly as a proof-of-concept test given the relative simplicity of complete resection of most skin cancers using the pathology-guided Mohs surgery or similar approaches.
In a broad sense it is worth considering that SERS-based contrast agents have enjoyed much interest in recent years due to their apparent ability to overcome the limitations of fluorescence agents. Much press is made in the introductions of SERS papers about the multiplexing limits and photobleaching of conventional fluorophores, and yet every day flow cytometry experiments using ten or more conventional fluorophores are carried out without any particular difficulty or even necessitating the use of quantum dots. In this thesis the potential advantages of SERS bronchoscopy have been shown in comparison with fluorescence, and yet there has not been a clinical use demonstrated to date for even a single molecular marker which could be detected with NIR fluorescence. One could argue that fluorescence bronchoscopy has not taken hold due to the necessity for multiplex detection beyond what is possible with conventional fluorophores, but this has not been conclusively demonstrated anywhere in the literature to date. SERS imaging represents in some sense a refinement to multiplex fluorescence detection (with requisite additional complexities), and until such time as fluorescence imaging has been attempted and found insufficient it may be premature to pursue SERS development clinically.

What is certainly needed at the present time to truly test the utility of the proposed imaging scheme is a better ‘target’ definition from a biological point of view. While there has been an explosion of information recently regarding the genetic makeup of lung cancer, this information must now be integrated to determine a precise clinical target; be it 1, 3, 5, or 12 molecules of interest. Only then will it be possible to intelligently select an imaging modality suitable for the task at hand, which may indeed be SERS imaging, but Occam’s razor will ultimately prevail:

"Frustra fit per plura quod potest fieri per pauciora" (It is futile to do with more things that which can be done with fewer)
References


List of Publications


Publications in Progress


†Authors contributed equally to the work