Quantitative and depth-resolved fluorescence guidance for the resection of glioma

By

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

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Doctor of Philosophy, 2010
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Abstract

The clinical management of glioma remains a challenge. The prognosis is poor— for glioblastoma multiforme, the most virulent of these brain cancers, survival is only ~1 year. Surgical resection of the tumor is the first line of defense. Several studies demonstrate a survival advantage in patients who undergo near-complete tumor resection; however, achieving complete resection is limited by the difficulty of visualizing residual tumor after de-bulking. Intraoperative fluorescence guidance is a promising candidate to better visualize residual tumor. The most clinically developed form uses protoporphyrin IX fluorescence, the precursor to heme in its biosynthesis which preferentially accumulates in tumor cells after the administration of 5-aminolevulinic acid. Challenges remain in quantitatively assessing the fluorescence to reduce variability of outcome and improve tumor detection specificity, and in observing sub-surface tumor fluorescence. To these ends, this work outlines the development of intraoperative techniques to 1) quantify tissue fluorescence using a handheld fiberoptic probe and 2) improve detection by reconstructing the depth-resolved fluorescence topography of sub-surface tumor.

As a critical component to achieve these objectives, a technique to measure the tissue optical properties was developed. This technique used diffuse reflectance measurements mediated by a handheld fiberoptic probe to derive the tissue optical properties. The handheld fiberoptic probe was further developed to include fluorescence spectroscopy. A novel algorithm to combine the fluorescence measurement and the tissue optical properties was derived in order to extract the quantitative fluorescence spectrum, i.e. fluorescence without confounding effects of tissue
optical properties. The concentration of fluorescent tumor biomarker can then be extracted. The quantitative fluorescence work culminated in deployment of the fiberoptic probe in clinical trials for the resection of intracranial tumors. The quantitative fluorescence probe out-performed a state-of-the-art fluorescence surgical microscope for a broad range of brain tumor pathologies.

A novel technique for depth-resolved fluorescence detection was developed utilizing multi-excitation fluorescence imaging. An algorithm to extract depth information from the multi-excitation images was derived, with validation in phantoms and a rat brain tumor model. This demonstrates the potential for depth-resolved fluorescence imaging, which there is a clear need for in tumor resection guidance.
Publications appearing in this thesis:


Patents pending:


Additional publications arising from this thesis:


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## Abbreviations

<table>
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<tr>
<td>AF</td>
<td>Autofluorescence</td>
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<tr>
<td>ALA</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td>a.u.c.</td>
<td>Area-under-the-curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>dF</td>
<td>Depth-resolved fluorescence</td>
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<tr>
<td>FGR</td>
<td>Fluorescence-guided resection</td>
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<td>FWHM</td>
<td>Full-width, half maximum</td>
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<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Karnofsky Performance Scale</td>
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<td>LDA</td>
<td>Linear discriminant analysis</td>
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<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>LGG</td>
<td>Low-grade glioma</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PpIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>qF</td>
<td>Quantitative fluorescence</td>
</tr>
<tr>
<td>RMS</td>
<td>Root-mean-square</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver-operating characteristic</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
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## Symbols for various physical, physiological and mathematical properties

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>( \lambda )</td>
<td>Wavelength of electromagnetic radiation</td>
</tr>
<tr>
<td>( \mu_a )</td>
<td>Absorption coefficient</td>
</tr>
<tr>
<td>( \mu_s )</td>
<td>Transport scattering coefficient</td>
</tr>
<tr>
<td>( g )</td>
<td>(Scattering phase function) anisotropy</td>
</tr>
<tr>
<td>( \mu'_s )</td>
<td>Reduced scattering coefficient</td>
</tr>
<tr>
<td>( \sigma' )</td>
<td>Reduced albedo</td>
</tr>
<tr>
<td>( R )</td>
<td>Reflectance</td>
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<tr>
<td>( T )</td>
<td>Transmissivity</td>
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<tr>
<td>( \mu_{af} )</td>
<td>Fluorophore absorption coefficient</td>
</tr>
<tr>
<td>( \mu_{eff} )</td>
<td>Effective attenuation coefficient</td>
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<tr>
<td>( Q )</td>
<td>Fluorescence quantum yield</td>
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<tr>
<td>( F )</td>
<td>Detected fluorescence</td>
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<tr>
<td>( qF )</td>
<td>Quantitative fluorescence</td>
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<tr>
<td>( f )</td>
<td>Quantitative fluorescence</td>
</tr>
<tr>
<td>( \square_x )</td>
<td>Subscript denotes excitation ( \lambda )</td>
</tr>
<tr>
<td>( \square_m )</td>
<td>Subscript denotes emission ( \lambda )</td>
</tr>
<tr>
<td>( f_{HB} )</td>
<td>Hemoglobin concentration</td>
</tr>
<tr>
<td>( StO_2 )</td>
<td>Oxygen saturation fraction</td>
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1 The problem of glioma and the need for quantitative and depth-resolved fluorescence guidance during brain tumor resection surgery

1.1 Chapter overview
The clinical management of glioma continues to be a significant problem. Even with all available treatment options (surgical resection, radiotherapy and chemotherapy) the prognosis is grim. For glioblastoma multiforme (GBM), the most virulent form of this disease, the median survival is still only ~1 y, with little chance for long-term survival [1]. This introductory chapter reviews what is known about glioma and discusses the clinical diagnostic and therapeutic challenges of combating this deadly cancer. The array of conventional imaging techniques to aid in intraoperative guidance during resection surgery is reviewed. A particular emphasis is placed on fluorescence image guidance—it is the elaboration of this technique that is the focus of this thesis. The clinical rationale behind development of quantitative and depth-resolved measurements during resection surgery is discussed in light of the current limitations of state-of-the-art intraoperative surgical guidance. To cap off this introductory chapter, fluorescence in the context of tissue optics is introduced since modeling of light-tissue interaction is necessary for solving the quantitative and depth-resolved fluorescence problems.

1.2 Glioma
Gliomas are so named because they are thought to arise from glial cells—non-neuron brain cells that provide nutrition, protection and support to cells of the central nervous system (CNS). Gliomas make up the majority of primary CNS tumors (in general, neurons do not form tumors
because they do not divide). These tumors are named by the glial cell type that they most resemble, namely oligodendromas, ependymomas and astrocytomas. Since gliomas attack the brain—the seat of consciousness and regulator of critical life functions—the demands on diagnostics and therapeutics are unique compared with the rest of the human body.

In Canada, it is estimated that 2,600 people were diagnosed with brain cancer in 2009, with 1,750 people succumbing to the disease [2]. Unfortunately, the worst form of brain cancer, glioblastoma multiforme (GBM), is also one of the most common, making up 23% of all primary brain tumors [3]. Age is the most significant risk factor for gliomas, with a small peak in the pediatric population and a steady increase with age. As well, being male increases risk slightly. Aside from prior irradiation of the cranium [4], the environmental risk factors of primary brain cancer remain largely unknown. Predisposition to glioma is to date not related to cell phones, alcohol consumption or even smoking [3].

Gliomas occur along a continuum of malignancy. The World Health Organization (WHO) of tumor grading is the system that is most widely adopted in classifying central nervous system tumors [5]. The grading is a scale from I-IV, with Grades I and II denoted as low-grade gliomas (LGGs), and Grades III and IV being high-grade. In the WHO grading system, the I-IV grade for the entire tumor applies to the highest grade cell that is found under histological examination. Generally, high-grade gliomas (HGGs) are considered incurable. HGGs exhibit anaplasia, a histological hallmark for malignant cancer, where the cells appear undifferentiated. Tumor grading is based on pleomorphism, anaplastic hallmarks and percent necrosis. “Tumor staging” in the conventional sense generally does not apply here since CNS primary tumors rarely metastasize to other parts of the body prior to the tumor killing the patient. Even in the rare situation where glioma metastasizes to other sites in the body (in one study, the most common
metastasis sites are in the lungs, pleura, lymph nodes and bone, with extracranial metastasis occurring in <0.5% of patients [6]), the intracranial tumor is generally the most life-threatening.

The manner of glioma proliferation compared with other solid tumors exacerbates their destructive capability. Gliomas grow at the expense of healthy tissue, since growth of the tumor is physically limited by the skull. Though some space may be taken up by mechanical compression of the ventricles, only about 15% of the brain is taken up by the cerebrospinal fluid cavities, and thus space must be made by tissue destruction. There is evidence that neuron cell kill is caused by gliomas producing an excess of the neurotransmitter glutamate [7,8]. Unscavenged, excess glutamate near the tumoral space causes an excitatory toxic effect by opening wide calcium ion channels resulting in a flood of Ca\(^{2+}\) ions that triggers neuron cell death (this catastrophic event chain is also to blame for much of the brain damage caused by a stroke).

Cell migration is another mechanism that enhances the destructiveness of glioma. Satellite gliomas are often found distant from the main tumor bulk. Glioma cells migrate through the parenchyma via the white matter tracks (intrafascicular spread) and may travel along vessels or neurons (perivascular and perineuronal satellitosis) [9]. An example of this dispersal takes the form of a “butterfly” glioma, where the tumor cells migrate across the corpus collosum. It has been known since the 1960’s from autopsy studies that as many as 52% of high-grade gliomas have crossed into the contralateral hemisphere [10], and approximately 25% of patients with GBM have multifocal disease at death [11]. Moreover, there is evidence that this migration is active (rather than solely passive migration through the bloodstream, cerebrospinal fluid circulation or the white matter tracks). There are several molecular mechanisms that are suspected of enhancing glioma migration. Growth factors, adhesion molecules and extracellular
matrix components are all implicated in glioma cell motility [12]. One hope for treatment is to target these cell motility factors to prevent or arrest the spread of glioma. Microglia, the “macrophages” of the central nervous system, are implicated in aiding and abetting the active migration of glioma cells throughout the brain; it at least appears that microglia congregate towards the tumor as a non-specific immune response to the presence of the lesion, similar to their response to other types of brain damage [13]. Due to these and many other unknown migration mechanisms, gliomas proliferate in a diffuse manner throughout the brain, which is a major factor in the failure of conventional treatments.

The brain is the seat of consciousness and the physical embodiment of personality, emotion and memory. The therapeutic defense of the brain from glioma is paramount, yet the mechanisms of tumor growth, infiltration and motility make the disease extremely difficult to treat effectively. In light of this background, we now turn to the diagnostics and treatment of glioma in the next two sections. This general review of the standard-of-care for glioma provides context for the discussion on the use of fluorescence to intraoperatively guide neurosurgery.

1.3 Diagnosis
A brain tumor may present as any of a large number of neurological symptoms. Headaches, seizures, nausea, stroke-like symptoms and progressive memory deficits may indicate an intracranial tumor. Visual loss due to pressure or invasion of the optic nerve or optic chiasm may also be symptoms. If a tumor is suspected, a physician will order an imaging scan of the brain.

Magnetic resonance imaging (MRI) is the best imaging modality for diagnosing glioma. Both gadolinium contrast-enhanced and non-contrast imaging may be necessary to localize the tumor and potentially identify the pathology. Typically, high-grade gliomas appear as contrast-
enhancing lesions that display a mass effect, such as shown in Figure 1.1. Large tumors may display a ring-enhancing lesion given a contrast agent, with an inner region of necrosis. Contrast enhancement indicates a breakdown of the blood-brain barrier, a hallmark of high-grade gliomas. High-grade tumors arise in the white matter as a general rule.

Low-grade gliomas are typically non-contrast-enhancing infiltrative lesions that show enhancement under T2-weighted MR images. Distortion of the normal anatomy can indicate tumor infiltration, for example, a midline shift or ventricle compression. In the situation where a patient cannot undergo an MRI, for example, due to the presence of ferromagnetic surgical implants, a computed tomography (CT) image may be acquired in its stead. CT has some benefits over MRI, such as the ability to visualize tumor calcifications with high contrast.

Figure 1.1: A pre-operative magnetic resonance image of a contrast-enhancing lesion later diagnosed as an oligoastrocytoma. Image courtesy of Pablo A. Valdes and Dr. David Roberts, Dartmouth-Hitchcock Medical Center.
In general, MRI and CT are favored over positron emission tomography (PET), since there is a limited clinical benefit to nuclear imaging that cannot be obtained with the other two higher-resolution modalities. However, tumor grade is correlated to uptake of the radiopharmaceutical fluorodeoxyglucose (FDG, as imaged by PET), which is a glucose analog that has preferential uptake in glioma cells [14]. This takes advantage of the “Warburg effect”, a phenomenon whereby malignant tumor cells have a high rate of glycolysis.

MRI is reasonably capable of classifying tumors as high-grade or low-grade, with sensitivity and specificity in one typical study being 72.5% and 65%, respectively [15]; however, tissue biopsy remains the only method to determine pathology with confidence. The tumor grade (for example, as defined by the WHO grading scheme [5]) is important to determine the patient’s prognosis and may possibly inform and impact the treatment plan. Biopsies are always taken during surgical resection, since the risk of intraoperative biopsy is not greater than the concurrent resection procedure. Stereotactic biopsy prior to surgery (or in the situation where surgery is not an option) may also be performed in a standalone procedure; however, stereotactic biopsies are always dangerous due to the risk of tumor cell seeding and the inherent risk of inserting invasive instruments into the brain. Other important histological information may include the (apparent) glial precursor of the tumor cells. The extent of necrosis as determined via radiological imaging and biopsy may potentially have ramifications concerning radiotherapy, since extensive necrotic, hypoxic regions are less affected by radiation than normoxic tissue.

1.4 Treatment

Gliomas are so lethal because they stubbornly resist conventional cancer treatments. As a rule, it is currently not possible to cure malignant glioma. This is in part due to glioma cells diffusely
infiltrating throughout the brain parenchyma, making treatment, especially surgery, extremely challenging. However, with treatment, life may be extended. Often, treatment is a combination of all available alternatives. Treatment planning is also highly dependent on where the tumor is located. With the availability of anatomical and functional mapping tools such as MRI, CT, electroencephalography (EEG) and functional magnetic resonance imaging (fMRI), treatment should be tailored to avoid critical structures of the brain. The following is an overview of the battery of treatments that are used to combat glioma. In particular, the focus is on surgical intervention and the role of intraoperative image guidance, which leads into the discussion on the need for fluorescence guidance in neurosurgery.

Radiation therapy

Radiation therapy plays an important role in the treatment of glioma, and is typically administered following surgery. It may be the only localized therapy available if the tumor is in an area where surgery is impossible, such as the brain stem. Standard treatment is limited irradiation of the contrast-enhancing mass for high-grade gliomas, typically 2 cm beyond the radiologically-determined tumor margin [16]. Low-grade gliomas have been shown in separate studies to respond equally well to low radiation doses compared with high doses (Karim et al. is one representative study, where they used 45.0 Gy for the low dose and 59.4 Gy for the high dose [17]). High-grade gliomas require a high dose range between 50-60 Gy for optimal effectiveness [16]. Interestingly, standalone brachytherapy or stereotactic radiosurgery has not shown any added survival benefit compared with external beam radiation [3,19,20]; however, the potential for increased spatial accuracy of the dose delivery using these two techniques may be of benefit for sparing of normal tissue.
Regardless of the modality of radiation dose delivery, radiation necrosis is a potentially difficult complication that may arise. This is a problem because necrotic tissue may produce a mass effect requiring removal of the dead tissue in a separate procedure than the primary surgical resection. As well, radiation necrosis is often indistinguishable from high-grade glioma lesions, which may be a confounding factor when monitoring a low-grade tumor for increasing malignancy [3].

**Chemotherapy**

Chemotherapy in the brain is often ineffective due to the heterogeneity of gliomas and especially due to the blood-brain barrier (BBB) preventing drug delivery to targeted tissues. Nonetheless, chemotherapy is often included in treatment plans despite the limited benefit. Efforts to improve chemotherapy have focused on getting through or around the BBB. One strategy to bypass the BBB is to implant drug-eluting wafers into the surgical cavity after a resection surgery to be left to dissolve over time. A recent multi-centre trial demonstrated that patients who had intracavitary Gliadel™ wafers containing carmustine (Eisai Inc., Woodcliff Lake, NJ) placed in the resection bed during surgery have a 2.2 month median survival advantage compared with patients receiving a placebo [21]. Another strategy currently at the preclinical level involves the use of MRI-guided focused ultrasound in conjunction with exogenous preformed microbubbles to locally disrupt the BBB in order to deliver doxorubicin to brain tissue in mice at cytotoxic levels [22].

Although the benefits of chemotherapy for treating glioblastoma is somewhat limited [3], chemotherapy has shown to work effectively against anaplastic oligodendromas with deficiencies in chromosomes 1 and 19 [23,24]. This difference in therapeutic response further underlines the importance of histological confirmation of the tumor type when obtaining surgical
or stereotactic biopsies. Temozolomide is a recently developed alkylating agent that is now routinely used in glioma chemotherapy in large part because it readily penetrates the BBB. Temozolomide has been shown to impart 2.5 months additional median survival for patients with glioblastoma multiforme when it is administered concomitantly and as an adjuvant to radiotherapy [25].

**Pharmaceutical supportive therapy**

Pharmaceutical treatment may be used to support the primary forms of treatment (*i.e.* surgery, radiotherapy and chemotherapy). Dexamethasone, a corticosteroid, is routinely used to relieve cerebral edema and mass effect. Dexamethasone is particularly vital in controlling inflammation during and post-surgery. Drugs to control seizures, such as phenobarbital, carbamazepine and primidone may be used to relieve symptoms. Such enzyme-inducing anticonvulsants (EIACs) were long thought to be antagonistic to chemotherapy drugs; however, recent research has shown an unexpected and paradoxical finding: EIACs actually extend the median progression-free survival of glioblastoma patients by 0.8 months and median overall survival by 2.6 months [26].

**Surgery**

Of all available treatments, surgical resection of the tumor mass is the first line of defense. The objectives are 1) to reduce the mass effect of the tumor, 2) to obtain an accurate histological diagnosis to aid in determining the patient’s prognosis and potentially guide treatment and 3) to attempt a “cure” by gross total resection. Of these, 3) is the most difficult to accomplish, and indeed is impossible for high-grade glioma. Despite this difficulty, efforts to achieve complete tumor resection are likely to have life-extending benefit.

Although it is a reasonable supposition that surgical cytoreduction results in at least a partial “cure”, this claim is actually quite controversial. There is a dearth of prospectively randomized
clinical trials attempting to answer the question of whether or not surgical resection of the tumor imparts a survival benefit to the patient [27-29]. Although having matched, randomized controls would be ideal for a definitive, prospective study to answer this question, to defer surgery for research purposes is inherently unethical: aside from the curative goal, surgical resection alleviates many neurological symptoms, provides histological diagnosis that may guide the treatment plan and may allow adjuvant and concomitant therapy to work more effectively due to the tumor burden being reduced by several orders of magnitude.

One solution to get around this ethical dilemma is to perform retrospective studies comparing glioma patients who underwent surgical resection and those who only had stereotactic biopsy for confirmation of diagnosis. Examples of two such studies were reported by Devaux et al. from the Mayo Clinic [31] and Kreth et al. from the University of Freiburg [32], both published in 1993. The results were a mixed bag, with the Devaux study claiming that resection patients had a median survival of 50.6 weeks versus 33.0 weeks for non-resection patients; on the other hand, the Kreth study found no statistical significance between the two study arms. These two studies highlight the problem with retrospective analysis: there is an inherent, unknown bias in the results since those patients who underwent surgical resection likely had different clinical conditions and pre-surgical prognoses than those patients who did not.

Due to these confounding biases, many studies attempt to determine the effect of the degree of tumor resection completeness on patient survival, with a positive correlation indicating that surgery is a beneficial component in treatment. An oft-cited study by Lacroix et al. has demonstrated a survival advantage for patients with more complete tumor resection. Patients with >98% tumor mass removal were imparted with a relative survival advantage of 4.2 months
compared with patients with a resection <98% [29]. While not large in absolute terms, this does represent clinically significant improvement.

Even though this type of study is more “retrospectively randomized”, there is still possible bias in the Lacroix study concerning the neurosurgeon’s resection decision-making. There may be an interrelation between younger, healthier patients and the degree of resection; surgeons may decide this subset of patients can withstand more aggressive surgical intervention (this decision-making may even be subconscious to the neurosurgeon). At the same time, age and clinical condition is a very strong predictor for a good prognosis and long overall survival regardless of treatment plan, thus introducing the bias [3,27,28,33]. In perhaps the best effort to date at accounting for bias in a tumor resection completeness study, Stummer et al. retrospectively analyzed data from their own 243 GBM patient study where approximately half the patients underwent fluorescence-guided resection surgery (a topic to be discussed in more detail in the next two sections), resulting in a large fraction (50.2%) of patients receiving radiologically-determined\(^1\) 100% tumor resection completeness [33]. The Stummer study identified a survival bias in age and clinical condition as quantified by the Karnofsky Performance Scale score (KPS), and created subgroups in their survival analysis according to these parameters, as well as a subgroup for tumor location. In every subgroup, patients who underwent complete tumor resection survived significantly longer than those with incomplete resections (among all patients, 16.7 months versus 11.8 months, respectively, \(p<0.0001\)). Notwithstanding the confusion and controversy as to the survival benefit of surgical resection, it appears that there is a burgeoning consensus that all other factors being equal, near total tumor resection does indeed extend life.

\(^1\) As will be discussed near the end of this section, radiologically-determined tumor resection completeness (i.e. via MRI or CT) is not equivalent to true tumor resection completeness when it comes to glioma, since tumor cells are always found beyond the detected tumor margin.
The fact that gliomas nearly always recur at the surgical margins [3,27,28,29] underlines the need for maximal tumor resection. With total resection the goal, intraoperative technology has focused on image-based guidance to aid the neurosurgeon in identifying and delineating tumor margins with increasingly exquisite accuracy.

Intraoperative image-guidance systems have been developed to more effectively and safely identify tumor extent and effect a complete resection. Surgical tools and the resection bed can be registered to pre-operative MRI or CT images via optical or magnetic tracking technology, creating an environment referred to as frameless stereotaxy. This has afforded surgeons the ability to intraoperatively localize the three-dimensional tumor margin and important brain structures (as determined from pre-operative imaging) within the context of the two-dimensional resection bed. Even fMRI may be used to map out the eloquent areas of the brain such that the surgeon may take these areas into consideration during resection decision-making.

Though frameless stereotaxy has become standard technology in most modern neurosurgery theatres, there is an inherent problem with increasingly larger spatial errors between the pre-operative tomography and the intraoperative situation as the surgery progresses, since the brain deforms due to a change in pressure brought about by the craniotomy, cerebrospinal fluid (CSF) drainage and mechanical displacement due to the surgeon’s intervention. In an attempt to address the problem of degrading navigational accuracy, intraoperative MRI and CT have become a clinical reality; however, there are still issues with the practicality and cost-effectiveness of performing the surgery in an intraoperative MRI or CT suite. There may be long interruptions during the surgery for the purpose of producing a tomogram. The patient may have to be moved into and out of, say, an MRI bore during the surgery to acquire the images, an undesirable and
potentially risky situation. MRI compatibility of all surgical instruments and radiation safety concerning intraoperative CT are critical issues.

Finally, there is the major issue (and possibly critical flaw) that MRI and CT is inadequate to identify the last residual tumor cells that remain in the resection bed. There are many studies where tumor cells are found as far as 3-4 cm outside the tumor margins as indicated by contrast-enhanced MRI and CT [34,35,36]. As well, in the case of intraoperative MRI and CT, there is the problem of minimum detectable tumor signal near the end of the resection procedure since the surgeon is essentially removing tumor voxels from the tomogram. A representative threshold for minimum detectable tumor volume via MRI is $175 \text{ mm}^3$ [37]. For a 2 cm diameter tumor, this lower detection limit represents 4.2% of the total tumor volume, which may lead to only 95.8% tumor resection, less than the >98% tumor resection required for a significant increase in patient survival according to the Lacroix study [30]. Though MRI- and CT-enabled neurosurgical navigation is invaluable in the modern neurosurgical suite, a microscopic procedure able to detect functional and molecular tumor cell characteristics is required to complete the final stage of a resection procedure.

Though there are a variety of techniques available to combat gliomas, surgery is the primary means of attempting cytoreduction of the tumor. However, complete resection is difficult using standard intra-operative approaches, because of the difficulty of directly visualizing the residual tumor tissue. Aggressive resection at the tumor margin must be balanced by the increased risk of causing neurological deficit, underlining the need for better residual tumor detection. The problem of intraoperative detection of residual tumor cells is challenging, and it is the objective of this thesis to address this. The next section focuses on how fluorescence guidance is a
promising candidate for improving visualization and detection of residual tumor for the purpose of achieving more complete resections.

1.5 Fluorescence-guided resection surgery

Within the past decade, fluorescence-guided resection (FGR) surgery has emerged as a strong candidate to highlight residual brain tumor during resection surgery without the aforementioned issues using radiographic methods (i.e. navigational errors in the pre-operative imaging due to intraoperative brain distortions, costly and unwieldy intraoperative MRI and CT). Furthermore, the fluorescence modality has the potential for functional, molecular imaging and detection, allowing for unprecedented in vivo identification of microscopic tumor cell nests. Recent evidence has come to light where fluorescence guidance enables greater tumor resection completeness and even clinically demonstrating extension of progression-free survival [37,38].

The following discussion on using fluorescence as a tumor detection modality involves various endogenous fluorescence sources and exogenous contrast agents. Since aminolevulinic acid-induced protoporphyrin IX contrast is a key component of this thesis, it is only briefly introduced in this section on FGR surgery and treated in greater detail in the next section.

Autofluorescence

Endogenous fluorescence can be attributed to intracellular nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and porphyrins, as well as a modicum of collagen in the blood vessels and neurons. Lipofuscin, “junk material” in the cytosol that accumulates in the brain with age, is also fluorescent. Due to the weak quantum yield and scarcity of endogenous fluorophores in the brain, long acquisition times (in the order of several seconds to tens of seconds) and near-total elimination of ambient light are necessary for in vivo detection. Also,
variations in the tissue optical properties may obscure diagnostic ability. This is especially a problem with autofluorescence, since most endogenous fluorophores excite in the range of 300-470 nm, where variation in the absorption coefficient is high due to differences in tissue blood perfusion.

The major benefit of using autofluorescence contrast is that the patient does not require additional drugs or contrast agents, avoiding complications such as toxic accumulation in the kidneys and liver, skin phototoxicity and allergic reactions. Driven by this main advantage, researchers have developed techniques to explore the possibility of detecting cancer using autofluorescence contrast. The most promising and comprehensive findings using autofluorescence to intraoperatively detect glioma were produced by Vanderbilt University researchers, who used a fiberoptic contact probe to interrogate the brain tissue to measure the autofluorescence (emission from 350-750 nm) and diffuse reflectance (400-800 nm) spectra for diagnostic purposes [39,40]. Measuring the diffuse reflectance as well as the fluorescence at least partially compensates for the variation in the tissue optical properties. A study performed in 24 patients with glioma found that the spectral measurements were able to discriminate between normal tissue and infiltrative tumor margins with a sensitivity of 94% and specificity of 93%; however, the trade-off was a long acquisition time (30 seconds).

There are other modalities to use autofluorescence contrast for tumor detection. Using a time-resolved fluorescence detection system, Marcu et al. demonstrated that gliomas have different fluorescence lifetimes compared with normal brain tissue [41]. An interesting concept from Duke University is to use hyperspectral fluorescence emission imaging for wide-field spectral analysis [42].
Exogenous fluorescence contrast in clinical use

The most clinically advanced exogenous fluorescence agents that are used in humans are fluorescein, indocyanine green (ICG) and aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX). The main advantage of these contrast agents is that the fluorescence signal is much higher than the autofluorescence background, such that real-time imaging and detection is facilitated.

For intravenous administration of organic fluorescent molecules such as fluorescein and ICG, the tumor-to-normal contrast is primarily driven by the enhanced permeability and retention effect (EPR) within solid tumors [43]. The EPR effect results from the abnormal anatomical and pathophysiological properties of tumor tissue, especially vasculature. When tumors grow past a few millimetres, angiogenesis is induced to support the growth of the tumor. Tumor vasculature is characterized by a chaotic, tortuous three-dimensional pattern. The vessels themselves are leaky due to pathophysiological factors such as vascular endothelial growth factor, allowing enhanced extravasation of macromolecules into the interstitium. Poor lymphatic drainage from tumor tissues retains macromolecules in the tissue, further increasing tumor-to-normal tissue uptake of exogenous organic molecules.

Researchers have investigated the use of fluorescein for delineating brain tumor margins as early as the 1940’s [44]. A major benefit of fluorescein is that it may be used as a simple absorption dye that may be visualized under white light, with no additional fluorescence detection equipment necessary. A recent Turkish study demonstrated use of fluorescein in delineating GBM tumors in 47 patients, compared with 33 control patients [45]. In this study, there was no statistically significant increase in median survival, possibly due to the fact that the fluorescein was not molecularly targeted.
Indocyanine green is a near-IR fluorescence dye that has a fluorescence emission peak at 815 nm in distilled water. This makes it a useful tool for deep tissue imaging, and has been used extensively in blood vessel and lymph angiography [46,47]. Deep-tissue imaging is achieved with ICG at the detriment of poor surface imaging resolution. Using ICG at long excitation and emission wavelengths (i.e. in the region of low tissue absorption), surface fluorescence appears more diffuse than, say, imaging fluorescein, which excites and emits $\text{<}600$ nm in the region of high hemoglobin absorption. Despite its extensive clinical use in angiography, there has been limited use of ICG specifically for brain tumor demarcation outside of pre-clinical animal studies.

Aminolevulinic acid-induced protoporphyrin IX has been extensively studied for application in photodynamic therapy (PDT) and for surgical image-guidance, which is sometimes referred to as the blanket term photodynamic diagnosis (PDD). PpIX fluorescence is endogenously synthesized by systemic administration of ALA, a precursor in heme biosynthesis, which exists in all mammalian cells (Figure 1.2). PpIX production is the penultimate step before forming heme, and fluoresces in the deep red, with a fluorescence peak at 635 nm and a secondary “shoulder” at 705 nm (Figure 1.3). Administration of ALA overloads this pathway, causing fluorescent PpIX to accumulate at varying levels in different tissue types.

The ALA-PpIX system is a metabolic marker that is highly specific to malignant cells, particularly in the brain. An animal model example is shown in Figure 1.4. A CNS-1 tumor was grown in rat brain, with the tumor cells labeled with green fluorescent protein (GFP). ALA was administered intraperitoneally 3 hours prior to imaging. The GFP fluorescence and PpIX fluorescence are co-localized, demonstrating good tumor specificity. ALA-PpIX produces
diagnostically useful tumor-to-normal contrast in tumors of the breast [48], brain [38], esophagus [49], lung [50] and many other sites [51].

Figure 1.2: The heme biosynthesis pathway, in the context of exogenous application of 5-aminolevulinic acid to produce excess protoporphyrin IX for fluorescence contrast. Chevrons indicate enzymatic reactions.

Figure 1.3: Fluorescence excitation and emission spectra of protoporphyrin IX, with a color bar showing the wavelength-dependent color perception of the human eye.
Figure 1.4: A CNS-1 tumor grown in the left hemisphere of a rat brain, with the tumor cells labelled with green fluorescent protein (GFP). A 1 cm craniotomy was performed and dura removed, exposing both hemispheres. With intraperitoneal administration of ALA, the tumor also fluoresces in the red, indicating PpIX contrast. (a) GFP image (470 nm excitation, 510 nm bandpass emission); (b) PpIX image (546 nm excitation, 600 nm longpass emission); (c) In vitro spectrofluorometry of CNS-1<sup>*pp</sup> cells after being incubated for 1 hour in 1 mM of ALA, demonstrating both GFP and PpIX spectral features. In the images, LH and RH denote the left and right hemispheres, with the measurement bars being 2 mm.

Of the many fluorescence contrast mechanisms, the ALA-PpIX system is by far the most clinically advanced for glioma detection. As discussed in the next section, the state-of-the-art in ALA-PpIX mediated image-guidance has demonstrated improvement in tumor resection completeness in clinical use. At the same time, recent studies have demonstrated that there are areas for improvement and unanswered questions that need to be addressed for the state-of-the-art to advance.
1.6 State-of-the-art clinical use of ALA-PpIX in fluorescence-guided resection

The neurosurgeon’s chief imaging tool is the surgical microscope (frameless stereotaxy and intraoperative neuronavigation notwithstanding). All of the tumor resection procedure is performed while viewing the tissue through the scope; hence, it is natural that modified surgical microscopes retrofitted to visualize fluorescence represent the current state-of-the-art modality for ALA-PpIX-mediated FGR. Through-microscope systems are available from Leica Microsystems GmbH (Wetzlar, Germany) and Carl Zeiss Meditec (Jena, Germany). Endoscopic implementation is also possible, such as instrumentation from Karl Storz (Tuttlingen, Germany).

Our group’s collaborative project with researchers at Dartmouth College (Hanover, NH) uses the Zeiss OPMI® Pentero™ microscope retrofitted with a fluorescence kit (Figure 1.5a). Fluorescence visualization is achieved with an added violet-blue light source and fluorescence emission filter. The excitation light from the microscope has a full-width half-maximum (FWHM) pass-band of 400-420 nm, with a long-pass 460 nm emission filter placed in front of the detection light path for fluorescence visualization. A small amount of remitted light from 430-460 nm resulting from a slight overlap between the excitation and emission bands was allowed to leak into the emission path in order to provide anatomical detail relative to the functional fluorescence image. Standard white light visualization can be switched back-and-forth with the fluorescence mode. A separate emission light path leads to a CCD camera capable of acquiring still images and video.

An example of the imaging clarity that is possible with a through-microscope system is shown in Figure 1.5. Panel (b) shows a white light image of a meningioma tumor, and panel (c) displays the PpIX fluorescence of the tumor, with the purposefully-leaked blue light providing anatomical context. The meningioma example is excellent for demonstrating image quality; however,
glioma imaging is more difficult due to the more diffuse nature of that pathology. Indeed, the current prevailing opinion is that fluorescence guidance is only practical for high-grade gliomas; low-grade gliomas are considered to have no contrast [37,39,40], a claim that is refuted by work presented in Chapter 4 of this thesis. In addition, ALA-PpIX contrast in meningioma and metastatic tumors has not been fully explored: this thesis demonstrates some of the first clinical fluorescence data showing that these tumors have excellent ALA-PpIX contrast.

Figure 1.5: (a) Photograph of a Zeiss OPMI® Pentero™ surgical microscope modified to visualize fluorescence (www.zeiss.de). (b) White light image of a meningioma tumor; (c) corresponding fluorescence image displaying fluorescing tumor due to PpIX over-production in the tumor cells. Blue excitation light was allowed to leak into the fluorescence image to provide anatomical detail. Courtesy of D. Roberts, F. LeBlond, P. Valdes, Dartmouth-Hitchcock Medical Centre.
ALA is usually mixed in water and administered to patients orally, though it may also be injected intravenously. Injection is the preferred route for dose delivery to animals in pre-clinical trials, since ALA has an unpleasant taste, so that it is difficult to get animals to ingest the proper dose of ALA voluntarily. Cancer patients can tolerate the oral route with little to no side effects or discomfort. Clinically, ALA is typically administered at 20 mg/kg bodyweight, 3-4 hours prior to when fluorescence imaging is expected to be used to delineate the tumor margins [37,38]. Higher doses than 20 mg/kg of ALA are considered to be in the range where photodynamic therapy may be performed, rather than simply providing fluorescence contrast for diagnostic purposes. Interestingly, there has been no prospective study published on ALA dose dependence in humans (i.e. ALA dose concentration and time for peak tumor-to-normal contrast) for the purposes of intraoperative glioma tumor margin demarcation.

A 2006 study by Stummer et al. is by far the most comprehensive study to date to determine the effectiveness of FGR on tumor resection completeness and patient survival for high-grade glioma cases [37]. The Stummer study was a German multicentre effort that assigned approximately half of patients for fluorescence-guided resection and half for standard white light micro-surgery. This study demonstrated that complete resections (as determined using MRI) were achieved in 65% of the patients who had FGR surgery; on the other hand, only 36% of the white light control subjects had complete resections. Historically, ~20% of patients have complete resections performed [33]. As well, the 6-month progression-free survival for the FGR patients was 41.0%, versus 21.1% for patients who underwent white light surgery.

The promising Stummer study provides an encouraging impetus to further elaborate the use of fluorescence in neurosurgery; however, despite the increase in tumor resection completeness and progression-free survival imparted by FGR, all patients still succumb in a short duration of time.
There is a clear a need for improvement in the technique. The next section elucidates the clinical challenges that this thesis addresses, as well as the driving hypothesis for this work.

1.7 Clinical rationale for quantitative and depth-resolved fluorescence guidance

The previous sections have outlined the extreme difficulties in the clinical management of glioma. Fluorescence-guided resection surgery has emerged as a promising modality enabling near-complete resection of the tumor; however, further elaboration of FGR is necessary.

Challenges remain in observing the fluorescence quantitatively and objectively. To date the fluorescence guided resection technique has largely been qualitative. Tissue seen as ‘solidly’ fluorescing is generally indicative of coalescent tumor, and ‘faint’ fluorescence indicates infiltrative tumor [38]. The interpretation of fluorescence guides resection decision-making; however, the impression of fluorescence may be prone to subjectivity. Moreover, the intensity differences between strong, weak, and non-fluorescing tissue are as likely to be due to differences in tissue absorption and scattering as to PpIX concentration. Many studies on fluorescence image-guidance involve the use of fluorescence excitation in the high absorption band of heme (UV-violet-blue), where variation in the optical properties are large [37,39,41]. The fluorescence intensity is also highly dependent on the viewing distance and angle relative to the tissue surface, especially in a resection cavity with surface variations. As well, color perception varies from person-to-person, especially for individuals with color vision deficiencies such as anomalous trichromacy. There is a clear need to investigate how a more quantitative evaluation of the fluorescence signal may improve FGR. The balance between preserving normal brain tissue and removing tumor tissue means that there is little margin for error.
The benefit of probing a few millimetres into tissue to detect residual tumor nests at depth is also clinically valuable. Gliomas recur at the tumor margin, implying the presence of unresected residual tumor cells near the tumor margin. Gliomas are characterized by diffuse infiltrations along the vasculature, pseudopalisading tracts and migrations of cells from the main tumor mass [3,5,27]. The likelihood that there exists at the end of resection surgery tumor cell nests that are completely occluded from the neurosurgeon’s view gives impetus for a technique to detect and map tumor cell nests at depth within the tissue. The work of Lacroix et al. [30] and Stummer et al. [33,37] (that demonstrates that tumor resection completeness does extend overall patient survival) provides strong evidence that a major mode of recurrence is through residual tumor cells not removed during surgery. Therefore, there is good rationale for depth-resolved tumor detection in the brain.

It is worth underlining an important distinction concerning the motivation behind developing quantitative and depth-resolved fluorescence techniques for increased tumor resection completeness. The techniques in this thesis are not intended to be used for most of the brain tumor resection procedure. The bulk of tumor resection can be performed under standard white light resection alone, with fluorescence imaging through a fluorescence-capable neuromicroscope (such as the aforementioned Zeiss-built instrument) enabling a more refined removal of most of the residual tumor cells that remain in the resection bed. The quantitative and depth-resolved fluorescence techniques developed for this thesis are meant to provide exquisitely sensitive and specific detection of the last residual nests of tumor cells left in or just beneath the resection bed, perhaps as few as 1%, 0.1% or 0.01% of the original tumor volume. Recall the Lacroix study, where the overall survival difference between patients who received >98% and <98% of tumor resection was 4.2 months [30]. Since gliomas always reproliferate, likely due to
these last remaining surviving cells from surgery, it is critical that new technology is developed to detect and consequently enable destruction of as many tumor cells as possible.

The **hypothesis driving the work presented in this thesis** is that quantitative and depth-resolved fluorescence modalities can better identify tumor margins, leading to increased tumor resection completeness and consequent extension of patient survival. A corollary to this hypothesis is that the current state-of-the-art fluorescence imaging modalities (*i.e.* through-microscope implementations) do not take full advantage of the tumor-to-normal contrast afforded by the ALA-PpIX system.

As reviewed more thoroughly at the start of Chapters 3 and 5, there have been many and varied developments of both quantitative and depth-resolved measurement of fluorescence. The objective of this thesis is to develop quantitative and depth-resolved fluorescence techniques that are practical within the context of ALA-PpIX-mediated fluorescence guided resection of glioma. Since these techniques are based upon the physics of light-tissue interactions, it is useful to review fluorescence within the context of the field of tissue optics, a topic we turn to now.

### 1.8 Fluorescence from a tissue optics perspective
The field of tissue optics aims to describe how light propagates in biological tissue, which is an optically scattering and absorbing media. The apparent fluorescence intensity viewed from, say, a camera modified to detect fluorescence is affected by a large number of factors: detection geometry (*i.e.* detector-to-tissue distance and viewing angle), excitation irradiance, emission sensitivity of the detector, tissue refractive index, excitation optical properties, emission optical properties, fluorophore concentration and fluorophore distribution within the tissue. Without factoring in these many effects, the detected fluorescence is difficult to interpret. In the context
of clinical applications using fluorescence guidance, the issue of properly interpreting tissue fluorescence is paramount. The following is a brief overview of fluorescence, how it relates to tissue optics, the definitions of the quantitative and depth-resolved fluorescence problems, and how fluorescence can be modeled to solve these problems.

Fluorescence is a phenomenon that occurs in some substances where light is absorbed at one wavelength and re-emitted at a longer wavelength. This process can be illustrated by a Jablonski diagram, a depiction of radiative transitions and electronic states within a molecule (an example depicting a fluorescence process is shown in Figure 1.6). For a single photon fluorescence process, a photon with energy $h\nu_x$ is absorbed by a fluorophore (subscript $x$ denoting excitation), with the fluorophore going from the ground state to an excited electronic state ($A \rightarrow B$). Some vibrational, non-radiative transitions may occur through which energy is lost to the surroundings as heat ($B \rightarrow C$). The radiative transition to the ground state produces a longer wavelength, lower energy photon with energy $h\nu_m$ ($C \rightarrow D$), (subscript $m$ denoting emission).

![Figure 1.6: Jablonski diagram of fluorescence phenomenon. The squiggly lines represent photons, arrows electronic transitions and each sphere represents a step in a series of electronic transitions.](image-url)
This molecular-level fluorescence emission in tissue is generally difficult to quantify directly in vivo, primarily due to the fluorescence signal being distorted by the tissue optical absorption and scattering.

Figure 1.7(a) displays an example of a schematic for modeling fluorescence phenomena with a view to solving the quantitative fluorescence (qF) problem. The qF problem is to determine the average fluorophore concentration (or, alternatively, the quantitative fluorescence spectrum) within some interrogation volume. There are a number of implicit assumptions in this diagram. One assumption is of homogeneous tissue optical properties at the excitation and emission wavelengths. The local tissue optical properties are the absorption coefficient, $\mu_a$, the scattering coefficient, $\mu_s$, and the anisotropy, $g$, at both the excitation and emission wavelengths. As well, the fluorescence quantum yield, $Q$, and fluorophore absorption coefficient, $\mu_{af,x}$, are assumed to be homogeneous within the interrogation volume. In general, the objective in solving the qF problem is to determine the quantitative fluorescence, $f_{x,m}$, which in this work is defined as the fluorophore absorption coefficient multiplied by the quantum yield at a given excitation and emission wavelength, i.e. $f_{x,m} = \mu_{af,x} Q_{x,m}$, where $\int_{-\infty}^{\infty} Q_{x,m} d\lambda_m = Q$. In other words, $Q_{x,m}$ gives the fluorescence emission spectral shape with the area under the curve being the quantum yield. The quantitative fluorescence spectrum is then a combination of the individual fluorophores, from which fluorophore concentrations may be calculated. The challenge here is to accurately model the fluorescence phenomena taking into account the measurement geometry and tissue optical properties to calculate $f_{x,m}$.

Figure 1.7(b) shows a slightly modified diagram that may be used for modeling the depth-resolved fluorescence (dF) problem. The challenge in the dF problem is to determine the depth of buried fluorescence in optically turbid media such as tissue. In this situation, assuming
homogeneous tissue optical properties greatly simplifies the modeling problem. Also implicit in the modeling diagram is that the fluorescing object has a definite boundary, although in reality tissue fluorescence may be somewhat diffusely distributed throughout the tissue.

The clear difference between the qF and the dF problems is that, in the former, fluorophores are assumed to be uniformly distributed in tissue (or at least, the measurement device is averaging over some localized interrogation volume); in the latter problem, the fluorescing tissue is spatially localized within a bed of non-fluorescing tissue. These are actually not conflicting assumptions, when considering the spatial scales for each problem. The qF problem is to determine fluorophore extent in a small, localized volume of tissue (in the order of \(~1 \text{ mm}^3\) ), whereas the dF problem is to determine the location of buried fluorescence over a relatively larger field-of-view (in the order of \(~\text{cm}^2\) ). In addition, there are two main detection modalities that may be used to collect data to solve both qF and dF problems: non-contact and contact detection. A non-contact detector or camera may be employed to detect fluorescence. This has the advantage of detecting or imaging over a large field-of-view; however, there are difficulties in compensating for the variations in position and orientation of the detector relative to the tissue. Contact detection is generally mediated with optical fibers on the tissue and has the advantage of a strictly defined measurement geometry and high sensitivity due to the intimate contact with tissue; however, it is generally constrained to point detection and does not take into account the full field-of-view of, say, a surgical site. Appropriately modeling the detection modality is critical to solving these fluorescence problems.
In addition to this, there is the issue of solving the tissue optical properties (TOP) problem, since the optical properties are required as inputs to solve the qF and dF problems. The TOP problem may be resolved using non-contact or contact methods, and generally the assumption of homogeneous optical properties within the detection volume is required to constrain the solution. There are many and varied techniques to solve the TOP problem; a technique will be presented in this thesis to aid in solving the qF and dF problems.

In order to solve the TOP, qF and dF problems, an appropriate modeling framework is necessary. In tissue optics, there are two techniques that are often used for modeling—diffusion theory and Monte Carlo simulation.

It is easy to think of light transport through tissue as a diffusion process. Photons propagate through turbid media in a similar fashion as gas molecules diffuse throughout a room. The diffusion theory equation for photon transport is an approximation to the Boltzmann radiative transfer equation (RTE), which is very difficult to solve analytically. The diffusion theory equation for the steady-state situation is an inhomogeneous ordinary differential equation that can be solved given adequate boundary conditions:
\[ D \nabla^2 \phi(r) - \mu_a \phi(r) + S_0(r) = 0 \]  
(1.1)

where \( D \) is the diffusion constant, \( \phi \) is the fluence rate, \( r \) is the position vector and \( S_0 \) is the light source term.

As a consequence of the diffusion theory approximation, the scattering coefficient, \( \mu_s \), and the anisotropy, \( g \), are reduced to a single lumped parameter called the reduced scattering coefficient, \( \mu'_s = (1-g) \cdot \mu_s \). As a result, there must be sufficient randomization due to scattering for the diffusion approximation to hold true. There are many analytical solutions to Eq. (1.1) depending on the measurement geometry and boundary conditions, allowing for efficient computation of the photon transport in tissue.

Monte Carlo simulation of photon transport is a more accurate, but computationally intensive, solution than diffusion theory. Essentially, Monte Carlo simulates the path of a photon “packet” through an optically scattering and absorbing medium until it is detected or otherwise terminated. A simple computer program written in, say, the C computer language can provide the framework to perform the simulations. Typically, millions of photon packets need to be simulated to achieve a precise solution, since a Monte Carlo simulation of a single photon packet is inherently based on statistical probabilities. Since the Monte Carlo technique models photon transport very closely to what actually happens in turbid and absorbing media, Monte Carlo is often considered the standard in biomedical optics for simulating light-tissue interactions. In this thesis, the online C-code developed by Jacques was used as a framework for developing custom Monte Carlo simulations [52].

It is useful to present a simple example of how diffusion theory and Monte Carlo may be used to model tissue fluorescence as a preview to the more complex modeling in this thesis. Figure 1.8 displays a schematic of this example. An isotropic point source emits light through an infinite
tissue-like medium, which propagates according to the excitation tissue optical properties, \( \mu_{a,x} \) and \( \mu_{s,x}' \). A fraction of this light encounters a fluorescence inclusion located a distance \( r \) away. The fluorophores absorb the light according to its absorption coefficient, \( \mu_{af,x} \), and an average pathlength, \( L \), through the inclusion. For a point source, the fluorescence conversion is given by \( Q \mu_{af,x} L \), where \( Q \) is the fluorescence quantum yield. The fluorescence emission light propagates according to the emission tissue optical properties, \( \mu_{a,m} \) and \( \mu_{s,m}' \). A fraction of this emission is detected by an isotropic detector at an identical distance \( r \) from the fluorescence inclusion, which measures the fluorescence fluence rate, \( F_m \) [W cm\(^{-2}\)]. This example illustrates how \( F_m \) can be modeled for 3 sets of optical properties. The detected fluorescence can be modeled as

\[
F_m = E_x \phi_x \left( r \right) Q \mu_{af,x} L \phi_m \left( r \right)
\]

(1.2)

where \( \phi_x \) and \( \phi_m \) are the normalized fluence rates at the excitation and emission wavelengths for a given \( r \), and \( E_x \) = 1 W/cm\(^2\). This modeling example therefore reduces to solving for these normalized fluence rates. These may be calculated via the 1-D solution to the diffusion theory equation (Eq. (1.1)) \[53,54\]:

\[
\phi \left( r \right) = \frac{\mu_{\text{eff}}^2 \exp\left(-\mu_{\text{eff}} r\right)}{4\pi r \mu_a}
\]

(1.3)

where \( \mu_{\text{eff}} = \sqrt{3\mu_a \left( \mu_a + \mu_s' \right)} \). Alternatively, \( \phi_x \) and \( \phi_m \) may be solved using Monte Carlo simulations. In this example, the following properties are held constant: \( \mu_{a,m} = 0.01 \text{ cm}^{-1} \), \( \mu_{s,m}' = 5 \text{ cm}^{-1} \), \( \mu_{af,x} = 0.1 \text{ cm}^{-1} \), \( L=0.01 \text{ cm} \) and \( Q_{x,m} = 0.001 \text{ nm}^{-1} \). The distance, \( r \), is varied as 0.1 -5 mm. Table 1.1 lists the excitation optical properties for each of the 3 cases that are simulated.

Both diffusion theory and Monte Carlo simulations were employed to calculate the \( F_m \) versus \( r \) graphs for each of the 3 cases, with the results plotted in Figure 1.9. The results from both methods agree closely for \( r > 0.8 \text{ mm} \). For \( r < 0.8 \text{ mm} \), as \( r \) decreases, the diffusion
theory calculation increasingly deviates from the Monte Carlo data. This breakdown in accuracy at short $r$ is typical of diffusion theory. Although this example is very simple, it exemplifies how to solve the various photon transport modeling problems that are encountered in this thesis, i.e. the TOP, qF and dF problems.

Figure 1.8: Schematic of the fluorescence modeling example.

<table>
<thead>
<tr>
<th>Case</th>
<th>$\mu_{a,x}$ (cm$^{-1}$)</th>
<th>$\mu_{s,x}'$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 1.9: Wavelength-dependent fluence rate plotted against $r$, as per the fluorescence model example. The symbols are the Monte Carlo data; the solid lines are based on the diffusion theory model.


### 1.9 Thesis objectives and overview

The main thesis objectives are to develop (1) quantitative fluorescence and (2) depth-resolved fluorescence techniques to elaborate and improve upon intraoperative fluorescence-guided resection surgery of brain tumor. In this thesis, the objective of quantitative fluorescence is achieved by using a handheld fiberoptic probe to interrogate small volumes of tissue. The objective of depth-resolved fluorescence is accomplished using multi-spectral excitation imaging to reconstruct the sub-surface fluorescence topography of the underlying tumor. Essential to solving both these problems is the development of a technique to determine the tissue optical properties.

The thesis proper begins by outlining work on the development of a method to extract tissue optical properties, called spectrally-constrained diffuse reflectance, mediated with a contact fiberoptic probe (Chapter 2). Chapter 3 adds a fluorescence modality for quantitative fluorescence evaluation using the intraoperative handheld fiberoptic probe. Here, a fluorescence model is developed to extract the quantitative fluorescence, which is directly related to the fluorophore marker concentration. The work in these two chapters culminated in the development of a clinical quantitative fluorescence system. Figure 1.10(a) displays a schematic showing how fluorescence and diffuse reflectance spectroscopy may be measured by a fiberoptic probe. The diffuse reflectance spectrum is used to estimate the tissue optical properties, which are then used to correct the measured fluorescence spectrum such that the quantitative fluorescence spectrum may be calculated (i.e. the qF spectrum is undistorted by the tissue optical properties and is a function of fluorophore extent alone). Photographs of the handheld probe are shown in Figure 1.10(bc).
Figure 1.10: Quantitative fluorescence is achieved using a fiberoptic probe that contacts tissue to perform spectroscopic measurements. (a) Cross-section of a fiberoptic-mediated probe tip that can measure the tissue fluorescence and diffuse reflectance spectrum by sequentially exciting the tissue with fluorescence excitation light and white light via source fibers located a fixed distance away from a detector fiber. The fibers are potted in a black epoxy within a 1.1 mm diameter stainless steel sheath. (b) Photograph of the handheld fiberoptic probe; (c) probe acquiring a measurement during glioma resection surgery.

Figure 1.11: Depth-resolved fluorescence is achieved using multi-spectral excitation imaging. (a) Different wavelengths of light penetrate within tissue at different depths: for example, light at 405 nm penetrates very shallowly in brain tissue (~100 µm) due to high hemoglobin absorption whereas light at 505 nm penetrates deeper (~3 mm). In this way, multi-spectral excitation information is encoded with depth-resolved information of buried fluorescent objects. (b) Photograph of the pre-clinical imaging system (used in Chapter 5) built into an existing small-animal fluorescence microscope.
Since September 2009, the probe has been in active clinical use, with in vivo patient data and initial clinical experience discussed in Chapter 4 to close off the work on quantitative fluorescence. Although the focus of this thesis is on addressing the serious problems involving surgical treatment of glioma, the clinical data also consists of measurements in meningioma and metastatic tumors, with very promising results.

Chapter 5 reviews work done on a novel technique that we refer to as sub-surface fluorescence topography—essentially a technique to build depth-resolved maps of buried fluorescing tissue. Depth-resolved fluorescence is accomplished using multi-spectral excitation imaging, as shown in Figure 1.11(a). By imaging at different excitation wavelengths, depth-resolved information of buried fluorescence can be recovered. This concept was embodied in a pre-clinical imaging system built into an existing fluorescence microscope (Figure 1.11(b)).

Chapter 6 closes off the thesis describing where our research team is taking the work next.

1.10 References


2 Measurement of tissue optical properties using spectrally constrained diffuse reflectance

Measurement of the tissue optical properties is fundamental to solving both the quantitative and depth-resolved fluorescence problems. Thus, the following technique was developed where the tissue optical properties are determined using a handheld fiberoptic reflectance probe that takes a measurement as it lightly contacts tissue. A spectrally-constrained, diffuse reflectance model is then applied to the reflectance measurements to extract the absorption and reduced scattering coefficient spectra. This work is novel in that multiple fiber separations are used to measure the reflectance for the purpose of expanding the dynamic range of derived tissue optical properties.

The following chapter is based on material published in an Optics Express paper [1]. As well, a review on methods of tissue optical properties was produced based on this research, published as a book chapter in the 2nd edition of *Optical-Thermal Response of Laser-Irradiated Tissue* [2].

2.1 Introduction and Background

Accurate measurement of tissue optical properties, specifically the absorption ($\mu_a$) and transport (reduced) elastic scattering ($\mu_s'$) coefficient and their spectral dependence, is central to many diagnostic and therapeutic optical techniques. For example, the outcome of treatments such as photodynamic therapy [3] and laser interstitial thermal therapy [4] depends on the light dose distribution in the target tissue (such as tumor), which is governed by these optical properties at the treatment wavelength. Similarly, diagnostic procedures can exploit the changes in the optical properties due to disease, since these properties are sensitive to changes in tissue microstructure (collagen fibers, nuclei and other particulates) and chromophores (hemoglobin, water, etc.) [5,6].
There are a variety of methods to determine these optical properties. One broad class involves the measurement of the diffuse reflectance and transmittance of an excised tissue sample of known thickness in an integrating sphere setup [6]. This requires *ex vivo* tissue samples, adding uncertainty due to distorting factors such as deoxygenation, loss of blood and the effects of tissue handling (*e.g.* cryofreezing) [7]. Hence, there has been considerable effort to develop minimally-invasive techniques that can be used *in vivo*, especially for clinical applications. These are generally based on either fiberoptics in contact with, or a non-contact detector in close proximity to, the tissue. Frequency-domain [5,8] and time-resolved diffuse reflectance [9] are among these *in vivo* methods, where the dynamic migration of photons through tissue is measured. Steady-state fluence rate [1] or radiance measurements [4,10] have also been advanced for interstitial measurements. Spatially-resolved, steady-state diffuse reflectance measurements represent another technique, where in general a source fiber launching light into the tissue is set at varying distances from several collector fibers, such that the optical properties may be derived from the spatially-resolved reflectance measurements [11,12]. A spatial-frequency domain method has been explored as a corollary to this technique, where the tissue is imaged with spatially-modulated illumination [13].

The aforementioned techniques typically rely upon large sampling volumes and long acquisition times. One typical time-domain diffuse reflectance system has source-collector fiberoptic distances of 15-20 mm, with acquisition times from 20-30 s [9], while another frequency-domain system for non-invasive measurements of breast tumors has a fiberoptic separation of 25 mm [5]. The spatially-modulated reflectance technique of Cuccia *et al.* takes images with sinusoidal frequencies of up to 0.63 mm$^{-1}$ projected on the tissue, thus integrating over tissue surface areas ~1 cm$^2$ [12]. The analogous spatially-resolved diffuse reflectance
technique requires source-collector distances that vary over several mm’s [12,14]. While large volume and/or wide field measurement of the optical properties is desired in many applications (such as bulk measurements in breast) and where measurement time is not a major constraint, there are many clinical situations where rapid, localized measurements are desired, such as during interstitial procedures, operating in a surgical field, during endoscopy and performing measurements on small anatomical structures.

To address this need, spectrally-constrained diffuse reflectance methods have been developed that allow the use of a single fiberoptic source-collector pair (Figure 2.1). The source fiber delivers broadband white light and the diffuse reflectance spectrum is detected by the collector fiber located at a distance, \( r \). Since there is only one reflectance measurement per wavelength, \( \lambda \), solving for \( \mu_a \) and \( \mu_s' \) relies upon spectral constraints, \textit{i.e.} applying \textit{a priori} knowledge of the shapes of \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) in a forward model, which can then be used to solve for the absolute coefficient values. Recent efforts using this approach have explored the use of very small source-collector distances, \(<1 \) mm [15]. This results in tissue-sampling volumes \(~1 \) mm\(^3\) and fast acquisition times \(<1 \) sec to \(~\)seconds).

In addition, these small reflectance probes can be implemented in interstitial format. A major advantage over the fluence rate and radiance interstitial techniques is that the source-collector distance is fixed and known (within the housing of the reflectance probe); by contrast, fluence rate and radiance techniques generally have the source fiber and the collector fiber independently inserted into the tissue, which is a major source of uncertainty in determining the tissue optical properties and requires an independent imaging technique to determine the fiber positions.

In the present work we address one of the outstanding problems with the spectrally-constrained diffuse reflectance method, namely the limited dynamic range of \( \mu_a \) and \( \mu_s' \) that can
be measured with a single source-collector distance. Here, three source-collector distances (260, 520 and 780 µm) were used. Since each distance spans a unique range over which $\mu_a$ and $\mu_s'$ can be measured, overlap of the reflectance measurements at the three distances extends the dynamic range beyond that of each distance separately. This approach is distinct from the aforementioned spatially-resolved diffuse reflectance techniques in that the multiple source-collector distances are used to expand the dynamic range of optical properties measurement, not to constrain the solution. We believe that this approach has distinct advantages over other reported methods, in particular the extended range of optical properties over which it is valid and the ability to make rapid, highly localized measurements of these properties which is advantageous in many applications.

Figure 2.1: Source-collector pair for measuring the diffuse reflectance spectrum. A broadband white light source is used to illuminate the tissue. A collector fiber connected to a spectrometer records the diffuse reflectance spectrum.
2.2 Theory

2.2.1 Using diffusion theory and spectral constraints to extract optical properties

The absorption spectrum can be modeled as a linear combination of the separate chromophore contributions. In the visible spectrum, it is commonly expressed using hemoglobin concentration and an oxygen saturation term [11,16]:

\[
\mu_a(\lambda) = f_{Hb} \left[ SIO_2 \mu_{a^{oxyHb}}(\lambda) + (1 - SIO_2) \mu_{a^{deoxyHb}}(\lambda) \right]
\]  

(2.1)

where \( \mu_{a^{oxyHb}}(\lambda) \) and \( \mu_{a^{deoxyHb}}(\lambda) \) are the wavelength-dependent absorption coefficients of oxygenated hemoglobin and deoxygenated hemoglobin, respectively, in units of \([\text{cm}^{-1} \cdot \text{L/g}]\). \( f_{Hb} \) is the total hemoglobin concentration \([\text{g/L}]\) and \( SIO_2 \) is the oxygenation fraction. Only the significant chromophores should be included in order to have an accurate absorption model. In this work, optical properties in the visible light range are measured, with hemoglobin being dominant. Other absorbers, such as beta carotene in breast tissue [17], should be included if their concentration is measurable. Water absorption is neglected here, since the diffuse reflectance is measured in the range 450-850 nm where water is optically clear relative to hemoglobin.

The reduced scattering coefficient spectrum of bulk tissue has been shown to fit well to a power law dependence on wavelength [11,15,16] given by

\[
\mu_s'(\lambda) = A \lambda^k
\]  

(2.2)

where \( A \) and \( k \) are constants. \textit{A priori} knowledge of the shapes of these spectra can be combined with a forward model of the diffuse reflectance. Non-linear fitting can then be applied to extract the free parameters, \( f_{Hb}, SIO_2, A \) and \( k \), from which \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) can be computed using Eqs (2.1) and (2.2). Our approach was to use the well-known diffusion theory equation for steady-state diffuse reflectance, \( R_{DT} \), as the forward model, with the assumption of homogeneous optical
properties in the volume of light interrogation [12]. In this embodiment, \( r \) is then fixed and \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) are wavelength-dependent:

\[
R_p(\lambda) = \frac{a'}{4\pi} \left[ z_0 \left( \frac{\mu_{\text{eff}}}{r_1} + 1 \right) \frac{e^{-\mu_{\text{eff}} r_1}}{r_1^2} + \left( z_0 + 2z_b \right) \left( \frac{\mu_{\text{eff}}}{r_2} + 1 \right) \frac{e^{-\mu_{\text{eff}} r_2}}{r_2^2} \right]
\]

where \( z_0 = 1/\mu_s' \), and \( r_1^2 = z_0^2 + r^2 \) and \( r_2^2 = (z_0 + 2z_b)^2 + r^2 \). The parameters \( z_0, r_1, r_2, z_b \) and \( \mu_{\text{eff}} \) are then all wavelength-dependent. \( z_b \) depends on \( \mu_a, \mu_s' \) and the internal reflection parameter \( \kappa = (1 + r_{id})/(1 - r_{id}) \) due to index mismatch between tissue and the external medium. The extrapolated boundary distance is given by \( z_b = 2\kappa D \), where \( D \) is the diffusion constant given by \( D = (3\mu_s')^{-1} \). To quantify the index mismatch at the boundary, \( r_{id} \) has been empirically determined as \( r_{id} = -1.44n_{rel}^{-2} + 0.71n_{rel}^{-1} + 0.67 + 0.0636n_{rel} \) [18]. Refractive index matching indices at the tissue surface yields \( \kappa = 1 \), and we have used this condition since the external medium is that of ink-blackened epoxy surrounding the probe fibers, which has approximately the same refractive index as tissue. The effective attenuation coefficient is expressed as \( \mu_{\text{eff}} = (3\mu_a\mu_s')^{1/2} \), and \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) are given by Eqs. (2.1) and (2.2).

The diffusion constant, \( D \), has been variously cited as \((3\mu_s')^{-1}\) or \([3(\mu_s' + \mu_a)]^{-1}\) [12,14,19]. Additionally, the way in which the probe light source is modeled impacts the form of Eq. (3), as outlined by Farrell et al. [12]. Thus, if it is modeled as a buried point source, the diffusion equation takes the form in Eq. (2.3) but without the reduced albedo, \( a' = \mu_s'/(\mu_s' + \mu_a) \). If it is modeled as an exponentially decaying line source extending into the tissue, then Eq. (2.3) includes the \( a' \) term. This will be discussed further below, where we empirically show that \( D = (3\mu_s')^{-1} \) and the exponential line source model is most suitable for this fiberoptic geometry. Note that for \( \mu_a = 0 \) this issue does not matter, since then \( D = (3\mu_s')^{-1} \) and \( a' = 1 \) in all cases.
2.2.2 Upper and lower bounds of validity of the diffusion theory model

In practice, it is not a simple matter to apply the above method, since, for a given $r$ value, there is a range of $\mu_a$ and $\mu_s'$ values over which Eq. (2.3) can be accurately applied to solve the inverse problem to derive the optical properties. Hence, in order to increase the overall range, we have used three source-collector distances ($r = 260, 520$ and $780$ $\mu$m). For a fixed $r$, the diffuse reflectance does not monotonically increase with increasing $\mu_s'$, i.e. there is a peak reflectance (Figure 2.2). Hence, to avoid ambiguity correlating $\mu_s'$ to reflectance, the range of $\mu_s'$ must be restricted to either the monotonically increasing or monotonically decreasing part of the curve. The absorption coefficient does not have this problem, since increasing $\mu_a$ always reduces the reflectance signal. Here, we have used the monotonically increasing part since the reflectance is far more sensitive to changes in $\mu_s'$ over this region, as evidenced by the steeper slope in Figure 2.2. The peak reflectance then represents an upper bound for estimating $\mu_s'$. As shown in Table 2.1, this upper bound decreases with $\mu_a$, so it should be taken as the largest expected $\mu_a$ value (here, $10$ cm$^{-1}$). In practice, this was set as the value of $\mu_s'$ at $90\%$ peak reflectance, to provide an additional safety margin. From Table 2.1, the upper bounds for $r = 260, 520$ and $780$ $\mu$m were then $\mu_s' = 52.9, 26.1$ and $17.1$ cm$^{-1}$, respectively. Note that the existence of the upper bound has nothing to do with diffusion model accuracy in the monotonically decreasing part of the reflectance-$\mu_s'$ curve; rather, the upper bound is placed to ensure that only the monotonically increasing part of the curve is used (for the reasons stated above) to solve the inverse problem to extract $\mu_a$ and $\mu_s'$, as detailed later on in Section 2.3.3.
Figure 2.2: Diffuse reflectance varying with $\mu_s'$ as per the diffusion theory model equation (Eq. (2.3)), with $r=500 \, \mu m$ and $\mu_a = 0.01 \, cm^{-1}$.

Table 2.1. $\mu_s'$ values at the maximum of the diffuse reflectance curve and at 90% of the maximum, for each of the three source-collector separations

<table>
<thead>
<tr>
<th>Fiber separation</th>
<th>$\mu_a = 0.1 , cm^{-1}$</th>
<th>$\mu_a = 1 , cm^{-1}$</th>
<th>$\mu_a = 10 , cm^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_s'$ (cm$^{-1}$) at $R_{max}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>260</td>
<td>89.2</td>
<td>87.1</td>
<td>80.1</td>
</tr>
<tr>
<td>520</td>
<td>44.4</td>
<td>42.8</td>
<td>38.4</td>
</tr>
<tr>
<td>780</td>
<td>29.5</td>
<td>28.2</td>
<td>24.7</td>
</tr>
<tr>
<td>$\mu_s'$ (cm$^{-1}$) at 90% $R_{max}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>260</td>
<td>54.9</td>
<td>54.4</td>
<td>52.9</td>
</tr>
<tr>
<td>520</td>
<td>27.4</td>
<td>27.0</td>
<td>26.1</td>
</tr>
<tr>
<td>780</td>
<td>18.3</td>
<td>17.9</td>
<td>17.1</td>
</tr>
</tbody>
</table>

There is also a lower bound of validity for the diffusion model. At small $\mu_s'$, single scattering dominates, invalidating the Similarity Principle defining $\mu_s' = \mu_s(1-g)$, where $g$ is the scattering anisotropy, and thus also invalidating the diffusion approximation. This condition was investigated using Monte Carlo modeling for a pencil light beam incident on an optically semi-infinite turbid medium, using the on-line C-code implementation developed by S. Jacques and colleagues [20]. The roulette technique was employed to increase computational speed. The epoxy-packed space around the fibers was considered to be a perfect absorber and index-matched to the tissue. This Monte Carlo model was used to create 3-D look-up tables for each $r$.
value and for $\mu_s' = 2-20$ cm$^{-1}$, $\mu_a = 0-1$ cm$^{-1}$ and $g = 0.6-0.95$. This range of $g$ is typical for tissues as determined \textit{ex vivo} in the breast [21], brain [6] and bone [22], as well as the phantom material Intralipid [23]. A Matlab (MathWorks, Natick, MA, USA) 3-D interpolation algorithm was applied to the data in the $\mu_s'$, $\mu_a$ and $g$ dimensions. Examples of the resulting Monte Carlo reflectance \textit{versus} $\mu_s'$ curves are shown in Figure 2.3(a), together with the diffusion theory model graphs. The root-mean-square (RMS) error, $\varepsilon_{RMS}$, of the Monte Carlo data compared to the diffusion theory model was used as a measure of the goodness of fit, as illustrated in Figure 2.3(b). We applied an $\varepsilon_{RMS}$ cut-off of 10% to determine the ranges of validity of the diffusion theory model. As would be expected, as $r$ decreased the corresponding $\mu_s'$ value at $\varepsilon_{RMS}$ increased, since single scattering effects are amplified with smaller inter-fiber distances. An interesting artifact is evident in (b), where the $\varepsilon_{RMS}$ increases slightly with increasing $\mu_s'$ after reaching a minimum. This is likely due to loss of photons outside the finite (2 cm dia.) tissue volume used in the Monte Carlo simulations to reduce the computation time.

![Figure 2.3](image)

Figure 2.3: (a) Reflectance as a function of $\mu_s'$ for $r = 520$ µm, with $\mu_a = 0.1$ cm$^{-1}$ and a range of $g$. The line-connected symbols are Monte Carlo values; the thick solid line is the diffusion theory graph. (b) RMS errors of the Monte Carlo data compared to diffusion theory ($g = 0.6-0.95$) as a function of $\mu_s'$, for the three source-collector separations.
Table 2.2 shows the $\mu_s'$ values at the 10% $\varepsilon_{RMS}$ crossover points, for the three $r$ distances and for $\mu_a = 0.1, 0.5$ and 1 cm$^{-1}$. Based on the above, Eq. (2.3) has lower limits of $\mu_s' =16.4, 10.1$ and 5.8 cm$^{-1}$ for $r = 260, 520$ and 780 $\mu$m, respectively. Figure 2.4 then displays the corresponding ranges of validity of the diffusion theory model. Recall that the motivation for using three source-collector distances was to provide overlapping regions of validity to increase the overall dynamic range of the optical coefficient measurements. The resulting dynamic range then has $\mu_s'$ ranges of 5.8-52.9 cm$^{-1}$ for a $\mu_a$ range of 0-10 cm$^{-1}$. Note that if the cut-off is >10%, then the lower limit of $\mu_s'$ for all fiber distances will be decreased. Figure 2.4 also displays the calculated ranges for $r = 1$ and 2 mm to further demonstrate how increasingly larger fiberoptic separations have a desirable decrease in the lower limit, but also an undesirable corresponding decrease in the overall range. This trade-off is important to inform the design of fiberoptic reflectance probes using this technique.

Table 2.2. $\mu_s'$ values (cm$^{-1}$) below which there is >10% RMS error between Monte Carlo and diffusion theory ($g = 0.6$-0.95) for the three $r$ distances

<table>
<thead>
<tr>
<th>Fiber separation, $r$ (µm)</th>
<th>$\mu_a = 0.1$ cm$^{-1}$</th>
<th>$\mu_a = 0.5$ cm$^{-1}$</th>
<th>$\mu_a = 1$ cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>260</td>
<td>16.4</td>
<td>16.4</td>
<td>16.4</td>
</tr>
<tr>
<td>520</td>
<td>10.1</td>
<td>9.7</td>
<td>9.3</td>
</tr>
<tr>
<td>780</td>
<td>5.8</td>
<td>5.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Figure 2.4: Range of $\mu_s'$ within which the diffusion theory model can be used to solve for $\mu_a$ and $\mu_s'$ using spectral constraint, for each $r$. The bars shaded in gray are for the $r$ values that are used in the fiberoptic probe; the white bars are for larger $r$ distances than currently used in the probe to graphically display the trend with increasing $r$. 
2.3 Materials and methods

2.3.1 Instrumentation

A schematic and photographs of the fiberoptic probe and control system are shown in Figure 2.5. An optical multiplexer (Model MPM-2000, Ocean Optics, Dunedin, FL, USA) was used to control the flow of input and output optical signals. A tungsten-halogen white light source (Model LS-1, Ocean Optics) was connected to one of the ports of the optical multiplexer such that the light could be selected to enter the probe fibers as well as be fed back into the spectrometer (Model S2000, Ocean Optics). The four fiberoptic leads were connected to the multiplexer so that the detected light could be measured sequentially by the spectrometer. The optical multiplexer, light source and spectrometer were installed in an electronics enclosure. A measurement consists of the following steps: 1) a background measurement is taken, 2) a reference spectrum is measured of the white light source to compensate for variations in lamp output, 3) the white light is sequentially channeled to each of the 3 source fibers, with spectrometer measurements taken from a collector fiber, thereby generating diffuse reflectance spectra for each $r$. The total time for each measurement was approximately 3 sec.

The day-to-day standard deviation of the reflectance measurements was calculated from 6 measurements of a standard Intralipid phantom taken on different days spanning a period of 4 months. The instrument demonstrated excellent repeatability, with the reflectance measurements (over all $r$ distances) having a standard deviation of only 0.37 cm$^{-2}$, or 3.6%.
2.3.2 Calibration

Calibration of the measured reflectance spectrum to the diffusion theory model is critical to retrieve $\mu_a$ and $\mu'_s$. Fortuitously, the diffuse reflectance curve with respect to $\mu'_s$ has a characteristic peaked shape (Figure 2.2) that may be exploited. The approach was to measure the reflectance in a scattering fluid that was diluted over a range such that the reflectance *versus* $\mu'_s$ curve (of all dilutions) captures the peak reflectance over all wavelengths. The aliquot fraction of scattering fluid was a 3% concentration of Intralipid-20% (Fresenius Kabi, Uppsala, Sweden) in distilled water. The dilutions were then 3, 6, 9, ..., 48%. Figure 2.6(a) displays the uncalibrated reflectance at 600 nm, with both the x- and y-axis needing calibration.

The reflectance measurements were then polynomial-fitted to obtain a smooth curve as a function of dilution. The peak of the reflectance *versus* $\mu'_s$ curve was then used to fit the
reflectance measurements to the diffusion theory model with the appropriate \( x \)- and \( y \)-axis scaling. The \( y \)-axis scale at each wavelength and source-collector distance is calculated as

\[
Scale(\lambda, r) = \frac{\langle R_{\text{th}}(\mu'_{s} \in V) \rangle}{\langle R_{\text{raw}}(\mu'_{s} \in V) \rangle}
\]

(2.4)

where \( V \) is the subset of \( \mu'_{s} \) for which the diffusion theory model is valid (see Figure 2.6(b) for properly scaled, calibrated data). This scale factor (Eq. (2.4)) was used to scale the (relative) reflectance measurements with the probe in order to yield the reflectance in absolute units (\( i.e. \) \( \text{cm}^{-2} \)) so that the inverse problem may be solved to extract \( \mu_{a} \) and \( \mu'_{s} \).

Figure 2.6: (a) Experimental diffuse reflectance measurements (□’s) of diluted Intralipid concentrations measured at 600 nm and \( r=520\mu\text{m} \). Here, the absolute value of \( \mu'_{s} \) for each dilution is unknown; however, the relative scatterer concentration of each dilution is known, and the uncalibrated result is graphed with the diffusion theory curve. (b) The \( \mu'_{s} \) value at the peak reflectance is used to scale the data to match that of diffusion theory curve. Although the data shown are at 600 nm, the calibration was done at every wavelength and source-collector distance.

2.3.3 Inverse algorithm to recover optical properties

For each acquisition, three reflectance spectra are taken in sequence, one for each \( r \) (260, 520 and 780 \( \mu\text{m} \)). To recover \( \mu_{a}(\lambda) \) and \( \mu'_{s}(\lambda) \), a Levenberg-Marquardt non-linear least squares algorithm was applied to Eqs. (2.1)-(2.3) over the spectral range \( \lambda = 450-850 \) nm. This
minimized the variance between the diffusion theory reflectance equation, \( R_{DT}(\lambda) \), and the reflectance measurement, \( R_{meas}(\lambda) \), with \( f_{Hb}, StO_2, A \) and \( k \) as the free parameters. The optical properties spectra, \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \), can then be derived. Only one reflectance spectrum is required to estimate \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \). The selection of this reflectance spectrum is based on the optical properties range for each \( r \), as shown in Figure 2.4. The following inversion algorithm was found to be suitable, with values for the \( \mu_s' \) boundaries taken from Figure 2.4.

i) Perform inversion with \( r = 260 \mu m \). If \( \mu_s' > 16.4 \text{ cm}^{-1} \) for \( >50\% \) of the spectral range (\( i.e. 450-850 \text{ nm} \)), output \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) and end; else go to ii).

ii) Perform inversion with \( r = 520 \mu m \). If \( \mu_s' > 10.1 \text{ cm}^{-1} \) for \( >50\% \) of the spectral range, output \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \) and end; else go to iii).

iii) Perform inversion with \( r = 780 \mu m \). Output \( \mu_a(\lambda) \) and \( \mu_s'(\lambda) \).

2.3.4 Phantom measurements for diffusion theory model validation

Phantom measurements were used to validate and optimize the diffusion model for the probe geometry. Although this type of analysis has been reported by others [12,19], the fiber separation distances are shorter (\( i.e. <1 \text{ mm} \)) relative to these previous studies, necessitating confirmation of the model. As mentioned in the Theory section, there are different expressions for the diffusion coefficient, \( D \). The general form is \( D = [3(\mu_s'+\alpha \mu_a)]^{-1} \), with \( \alpha \) variously cited as 0, 1 or some function of the optical properties [12,19]. As also discussed above, there may be two different forms of Eq. (2.3), depending on how the light source in tissue is modeled (buried point or exponential line source). Hence, phantom measurements were used to determine the optimal form of \( D \) and light source model for our probe geometry.

Naphthol Green (NG) dye and Intralipid were used as the absorber and scattering medium, respectively. Reflectance measurements at 715 nm (the absorption peak of Naphthol Green) were used for the following analysis. Nine phantoms were formulated, with \( \mu_a=1, 5 \) or \( 10 \text{ cm}^{-1} \) and \( \mu_s'=7, 14 \) or \( 21 \text{ cm}^{-1} \), prepared in all combinations. Since there are three \( r \) distances, this results in...
27 total data points. This range of $\mu_a$ and $\mu'_a$ spans the majority of tabulated *in vivo* optical properties in the review by Kim and Wilson for $\lambda=450$-850 nm [1]. Note that this phantom experiment does not include cases where $\mu_a$ is close to zero, since both forms of $D$ and both forms of the light source model converge to the same model at $\mu_a=0$.

The reduced scattering coefficient was verified using the single integrating sphere technique (see next section). The absorption coefficient of NG was measured in a standard spectrophotometer. Probe measurements were taken of the nine phantom solutions. The probe reflectance measurements were then compared to the diffusion model with both forms of the diffusion constant and the light source model. Note that probe calibration (with the varying dilutions of Intralipid) is not affected by the different forms of Eq. (2.3), since $\mu_a$ of Intralipid alone is assumed to be negligible for $\lambda = 450$-850 nm.

### 2.3.5 Phantom measurements to determine probe accuracy

A set of phantom data was prepared to obtain accuracy statistics for the probe-derived optical property values, using the NG absorption curve instead of that of hemoglobin in Eq. (2.1). The probe estimates of optical properties were compared with measurements made using the single integrating sphere technique. The latter has been reported in detail previously [24]. Briefly, the samples were placed in a custom sample holder consisting of two quartz disks separated by a 1 mm thick ring spacer. A tungsten-halogen lamp (L.O.T. Oriel: Darmstadt, Germany) and collimating optics were used to illuminate the sample with a 5 mm diameter white-light beam. The total diffuse transmittance, $T_d(\lambda)$, and diffuse reflectance, $R_d(\lambda)$, spectra were measured with a 15 cm diameter integrating sphere (SphereOptics: Contoocook, NH, USA) coupled to a spectrometer (S2000, Ocean Optics). A Monte Carlo simulation was used to calculate the
expected $R_d$ and $T_d$ for $\mu_a$ and $\mu_s'$ values ranging from 0-100 cm$^{-1}$ and 0-100 cm$^{-1}$, respectively. The tissue optical properties were then calculated from $T_d(\lambda)$ and $R_d(\lambda)$ using an inverse interpolation algorithm. For this set of measurements, the phantoms were formulated first with $\mu_a = 21.7$ and $\mu_s' = 24.7$ cm$^{-1}$ (at 715 nm) and then diluted into serial fractions of 95 to 5%, such that $\mu_a$ and $\mu_s'$ scale linearly with concentration. Each of the 20 phantoms was measured by both the probe and the integrating sphere.

2.3.6 *In vivo measurements to demonstrate utility*

In order to test the fiberoptic probe *in vivo*, as well as to obtain brain optical properties for separate studies on optical diagnostics during brain resection surgery, female Lewis rats (Charles River, QC, Canada) were used, under institutional ethics approval (University Health Network, Toronto). The animals were brought under general anesthesia with 4% isofluorane (oxygen flow at 2 L/min) and sustained by an injection of ketamine/xylazine (80/13 mg/kg, *i.p.*), and the eyes lubricated with tear gel. The scalp was reflected and a 1 cm dia. craniotomy was performed using a 1 mm drill bit, exposing both hemispheres. The dura was cut, exposing the cortical surface. The fiberoptic probe was placed in gentle contact with the brain tissue and measurements taken. As well, measurements were obtained from exposed facial muscle adjacent to the craniotomy site. After measurements were taken, 120 mg/kg bodyweight of Euthanyl under heavy anesthesia (2.5% isofluorane with 1 L/min oxygen) was used for euthanasia. In this article, representative data from these measurements are presented to demonstrate the utility of the probe in an *in vivo* application, although 5 animals were used in total. All studies were carried out under institutional animal-care approval (University Health Network, Toronto, Canada).
2.4 Results

2.4.1 Phantom measurements for diffusion theory model validation

The errors between the phantom reflectance measurements and diffusion theory reflectance values were calculated for all $r$, $\mu_a$ and $\mu'_s$ (with the exception of the data with $\mu'_s$ values lower than the lower bounds on the diffusion theory model, as defined previously). The fit to the variations in the diffusion theory model was quantified using the coefficient of determination ($R^2$) and the normalized root-mean-square error (NRMSE). This was done for all combinations of the two diffusion constant variations, and the two light source models (buried point source and exponential line source). The statistics for these four cases are shown in Table 2.3. Based on this analysis, the exponential line source model and a diffusion constant of $D=(3\mu'_s)^{-1}$ were found to be the best fit for the probe geometry among the tested cases. The measured reflectance values (from the probe) were then plotted against the modeled reflectance values (based on $\mu_a$ and $\mu'_s$ measurements from the integrating sphere), again with the proviso that, for a given $\mu'_s$, the values were within the range of validity as discussed in the Theory section (Figure 2.7).

2.4.2 Phantom measurements to determine probe accuracy

For the 20 phantom measurements, the RMS deviation between the probe and integrating sphere measurements were 5.4% and 4.3% for $\mu_a$ and $\mu'_s$, respectively. Figure 2.8(a) shows corresponding optical spectra for one measurement from this data set, demonstrating good correlation between the probe and integrating sphere measurements. Figure 2.8(b) then also shows good agreement between the diffuse reflectance measurement and the fit to the diffusion theory model.
Table 2.3. $R^2$ and NRMS error quantifying the goodness-of-fit of the four different forms of the diffusion theory model compared with phantom measurements

<table>
<thead>
<tr>
<th>Model</th>
<th>$R^2$</th>
<th>NRMS Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential line source model</td>
<td>$R^2 = 0.812$, NRMS = 11.0%</td>
<td></td>
</tr>
<tr>
<td>Buried point source model</td>
<td>$R^2 = 0.256$, NRMS = 22.0%</td>
<td>$R^2 = 0.863$, NRMS = 9.4%</td>
</tr>
</tbody>
</table>

\[
D = \left[3(\mu_s' + \mu_a)\right]^{-1}, \quad D = (3\mu_s')^{-1}
\]

Figure 2.7: Diffuse reflectance derived from the diffusion theory model versus measured reflectance values using the probe in the nine Intralipid-NG phantoms for the three $r$ values (providing that the reflectances measured are within the range of validity). Here, the form of the diffusion theory model shown is with the exponential line source and $D=(3\mu_s')^{-1}$, with $R^2 = 0.984$. Representative error bars at the maximum value and in the mid-range are displayed, with $y$-axis error bars representing the uncertainty in the reflectance model due to uncertainty in the $\mu_s'$ value as measured by the integrating sphere; $x$-axis error bars represent standard deviation in the reflectance measurements.
2.4.3  *In vivo* measurements to demonstrate feasibility

Representative *in vivo* optical properties data measured at the rat brain cortical surface and facial muscle are shown in Figure 2.9(ac). The estimated free parameters from the brain measurement are $f_{Hb}=4.89$ g/L, $StO_2=40.5\%$, $A=125.46$ and $k=0.2576$. From the muscle data set, the values are $f_{Hb}=2.66$ g/L, $StO_2=68.5\%$, $A=12.49$ and $k=0.0934$. The diffusion theory model of reflectance fits very well to both data sets, with $R^2=0.963$ and 0.971 for brain and muscle, respectively (Figure 2.9(bd)).

It would be helpful to have a useful comparison between the *in vivo* probe data and the *in vitro* integrating sphere technique; however, this was not technically feasible due to optical properties distortions moving from the *in vivo* to the *in vitro* situation. For example, we took probe measurements in cortical brain tissue *in vivo* and also after the brain was extracted post-sacrifice. We found on average that $\mu_a$ dropped by 44% at 500 nm from the *in vivo* to the *ex vivo* situation due to loss of blood perfusion upon death and/or brain extraction ($n=5$ animals). Since tissue needs to be further prepared by slicing for integrating sphere measurements (and possibly...
frozen to preserve the tissue prior to these measurements) this is highly likely to introduce further handling artifacts, as demonstrated by Chan et al. [7]. For these reasons, the integrating sphere technique was applied with phantoms as in Section 2.4.2 for validation purposes.

Note that these single measurements in tissue are to demonstrate the utility of the probe in vivo as well as to demonstrate algorithm convergence in tissue—estimates of the inter-animal variation on the derived optical properties in larger sets of animals are planned and will be reported in future work, with the focus of this current article on instrument/algorithm development and validation.

Figure 2.9: Representative in vivo absorption and scattering spectra as measured by the probe in (a) brain cortical tissue and (c) muscle. The corresponding reflectance measurements and fits to the diffusion theory model are shown in (b) and (d), with $R^2$ values of 0.963 and 0.971, respectively.
2.5 Discussion

One of the main issues addressed with this technique is that there is a limited range of $\mu_a$ and $\mu_s'$ values that can be determined from the reflectance measurements for any given source-collector distance, $r$. Using multiple inter-fiber distances to overlap these ranges increases the overall dynamic range of optical properties that can be accurately measured. The lower limit of $\mu_s'$ is the major issue in utilizing the technique described here. One potential improvement is to add reflectance measurements at $r > 780 \mu m$ to reach $\mu_s' < 5.8 \text{ cm}^{-1}$. There is a tradeoff, however: with increasing $r$, the signal-to-noise decreases and the probe head necessarily has to be larger (limiting the versatility of the technique). As well, the overall $\mu_s'$ range decreases with increasing $r$, as shown in Figure 2.4. Restricting $r$ to small values also limits the effective tissue sampling depth of the measurements, which is advantageous for highly localized measurements and for application in small tissue structures. Figure 2.10 shows Monte Carlo modeling using the current probe geometry to determine the effective sampling depth (90% of the detected photons) for the different $\mu_s'$ and $\mu_a$ values, with the deepest penetration occurring at low $\mu_s'$ and low $\mu_a$. It is useful to compare our results with those in the literature concerning diffusion theory breakdown with fiberoptic separation distances <1 mm. Reif et al. implemented a probe with $r=250 \mu m$, and used Monte Carlo simulations of the reflectance signal to determine the effect of varying $g$ while holding $\mu_s'$ constant [15]. The variation was <15% for $g = 0.75-0.95$ and $\mu_s' = 5, 10$ and $20 \text{ cm}^{-1}$. We report very similar findings extrapolated from our Monte Carlo data, with the reflectance at $r = 260 \mu m$ reflectance having a variation <15% for $\mu_s' > 5.1 \text{ cm}^{-1}$ for $g = 0.6-0.95$. A similar diffusion theory analysis to that used here was performed by Sun et al. [25], using Monte Carlo simulations to determine how errors in the diffusion theory model vary with $r$: for $\mu_a = 2.5 \text{ cm}^{-1}$, $\mu_s' = 6.4 \text{ cm}^{-1}$ and $g = 0.84$, the diffusion theory errors were <20% for $r > 500 \mu m$. Our results are
comparable, with the $r = 520 \mu m$ fiber giving an error <10% for $\mu_s' > 9.3 \text{ cm}^{-1}$ ($\mu_a = 1 \text{ cm}^{-1}$, $g = 0.6-0.95$) (see Figure 2.3(b)).

We have used the form $D=(3\mu_s')^{-1}$ as the diffusion constant, so that $z_0=(\mu_s')^{-1}$ and $\mu_{\text{eff}}=(3\mu_a\mu_s')^{1/2}$. In practice, this simpler form of $D$ is not only more accurate, it also makes the inversion of Eq. (2.3) more robust than the alternative with $D=(3(\mu_a+\mu_s'))^{-1}$ (and, thereby, $z_0=(\mu_a+\mu_s')^{-1}$ and $\mu_{\text{eff}}=[3\mu_a(\mu_a+\mu_s')]^{1/2}$): it was found that the nonlinear least squares algorithm used for inversion often did not converge in this alternative formulation. We note also that the exponential line source model is more accurate than the buried point source model under these experimental conditions, possibly because of the close source-collector separations used.

![Figure 2.10: Effective sampling depth of the probe as a function of $\mu_a$ and $\mu_s'$. The $r$ distance used for these depth calculations depends on the range of validity for each $\mu_s'$, i.e. for the $\mu_s'=5$ and 10 cm$^{-1}$ data points, $r=780\mu m$; for $\mu_s'=15 \text{ cm}^{-1}$, $r=520 \mu m$; for $\mu_s'=20 \text{ cm}^{-1}$, $r=260 \mu m$. Representative error bars are shown at the highest and lowest data points. Error bars represent standard deviation in the Monte Carlo simulations with five repetitions of the simulations.](image)

For skin studies, the presence of melanin and the shape of the $\mu_s'$ spectrum may cause difficulties with this technique, as outlined in Tseng et al. [26]. The spectral shape of melanin is similar to the power law-dependence of $\mu_s'$, making it difficult to spectrally constrain the
solution. As well, it has been shown in skin that a piece-wise power law function fits better to $\mu_s'$, rather than a single power law function as used in this work. For these reasons, the technique presented in this work is likely not well-suited to skin in its present form, although it may be extended or modified to include skin. It is not obvious \textit{a priori} that an algorithm based on homogeneous tissue will translate to layered turbid media such as skin.

One potentially confounding issue with tissue optical properties measurement is the effect of probe contact pressure on optical spectroscopy measurements of \textit{in vivo} biological tissues. Reif \textit{et al.} demonstrated that oxygen saturation decreases and $\mu_s'$ increases with increasing probe pressure for a fiberoptic pair with 250 $\mu$m separation distance [27]. Likewise, Chan \textit{et al.} demonstrated using an \textit{in vitro} integrating sphere technique that tissue pressure increases the reduced scattering coefficient [28]. Physiologically, pressure may affect optical measurements by increasing the local density of optical scatterers in the pressurized volume, as well as pushing blood out of capillary beds thereby reducing the local hemoglobin concentration. Good contact is necessary to provide good index matching with the probe tip; however, it may be necessary to quantify force using a load cell in series with the probe tip if contact force is found to be a major issue with the present fiberoptic probe geometry. An intriguing possibility is to employ non-contact diffuse reflectance measurements [14,29] to solve the problem of pressure variations; however, non-contact approaches give rise to complications such as specular reflectance and signal loss due to detection at a distance.

An interesting consequence of the Similarity Principle breaking down at low $\mu_s'$ and low $r$ is that scattering phase function information is encoded in the reflectance signal. The Monte Carlo results in Figure 2.3 suggest that, for $\mu_s' < 16.4$ cm$^{-1}$ at low absorption ($\mu_a = 0.1$ cm$^{-1}$), the reflectance at $r = 260$ $\mu$m will be sensitive to both $\mu_s'$ and $g$, whereas at $r = 780$ $\mu$m it is sensitive
only to $\mu_s'$. The idea that anisotropy is encoded into the reflectance signal at very small $r$
separations may be useful in some applications. Since the scattering phase function depends only
on the ‘morphology’ of the scattering structures, while $\mu_s'$ depends on both morphology and
abundance, a measure of both may provide additional biological information.

In this study, we have chosen to apply diffusion theory to solve the inverse problem, rather
than say, using Monte Carlo-generated look-up tables for reflectance versus $\mu_a$ and $\mu_s'$ and then
applying a *post hoc* spectral constraint to determine the optical properties. The main advantage
of using the diffusion theory approach is that it allows use of a simple, closed-form analytic
equation that integrates the spectral constraint with the reflectance model, allowing the inverse
problem to be solved in a straightforward manner. At this time, it is not obvious which approach
(diffusion theory or Monte Carlo) is better for this probe geometry and under what conditions.
Relevant factors include the overall accuracy, ease of implementation, computational speed and
robustness against, for example, tissue inhomogeneity, out-of-range $\mu_a$ and $\mu_s'$ values and
measurement noise. This would be an interesting subject for future studies.

It is perhaps surprising that the diffusion modeling and the experimental data are in such good
agreement given the number of transport mean free paths ($1/\mu_s'$) travelled by the detected
photons is relatively small; Monte Carlo simulations show that this is in the range of roughly 2-5
transport mean free paths for $\mu_s'$ between 5 and 25 cm$^{-1}$. We speculate that this may be partially
due to the fact that we are applying a spectral constraint to the model, which differs from the
more typical situation for when diffusion theory is applied at single wavelengths, which might
require a larger number of transport mean free paths to be robust. It may also be that the
exponential line source model is more accurate than the point source model for close fiber
separations.
2.6 Conclusions

This chapter reviews a technique to recover accurate tissue optical absorption and reduced scattering coefficients using a diffusion theory model of the diffuse reflectance collected at the tissue surface. Multiple source-collector distances enabled spanning of a large range of \( \mu_a \) and \( \mu_s' \) values, beyond that of any single source-collector separation. The dynamic range is \( \mu_s' = 5.8-52.9 \text{ cm}^{-1} \) for \( \mu_a = 0-10 \text{ cm}^{-1} \) for this geometry. Optical phantoms experiments demonstrated that the derived \( \mu_a \) and \( \mu_s' \) values are accurate to 5.4% and 4.3%, respectively, when compared against integrating sphere estimates. In the next chapter, this technique is used in combination with a fluorescence model to measure the quantitative fluorescence spectrum in tissue.

2.7 Statement of contributions

The instrumentation and algorithm development presented in this chapter was developed by me. The phantom and animal experiments were designed and managed by me, with contributing help from Mathieu Roy (University of Toronto, Toronto, ON), Farhan Dadani (Ontario Cancer Institute, Toronto, ON) and Elizabeth Munro (University of Toronto, Toronto, ON).

2.8 References


3 Quantification of in vivo fluorescence decoupled from the effects of tissue optical properties using fiberoptic spectroscopy measurements

This chapter tackles the problem of tissue fluorescence quantification. Recall that the objective here is to improve the sensitivity and specificity of intracranial brain tumor identification during resection surgery, as mediated by fluorescent protoporphyrin IX. Here, fluorescence quantification is achieved using a handheld fiberoptic probe (with identical fiberoptic configuration to that used in Chapter 2) that measures the fluorescence and white light reflectance spectra of tissue in situ. A simplified method to decouple the fluorescence from distorting effects of the tissue optical absorption and scattering is applied to extract the absolute fluorescence content. This technique builds upon the work from Chapter 2 on tissue optical properties measurement, with updated hardware capable of fluorescence measurement and also implementation in a neurosurgical operating theatre.

The following was adapted from a paper submitted to the Journal of Biomedical Optics. As well, a provisional patent application was filed for this invention (Patent Pending # US 61,297,969).

3.1 Introduction and Background

Fluorescence measurements are of considerable interest in biomedical optics for applications such as photosensitizer dosimetry during photodynamic therapy [1], fluorescence image-guided surgery [2], detection of cancerous or dysplastic lesions [3] and uptake of exogenous contrast agents [4]. The shape and intensity of the fluorescence spectrum contain valuable information on the identity and extent of fluorophores in tissue. Accurate, quantitative fluorescence
measurement is complicated by the distorting effects of tissue absorption and scattering. Untangling the effects of these optical properties on the measured fluorescence spectrum is highly desirable for quantitative analysis.

Several methods have been reported for this purpose, many of which use the diffuse reflectance to correct the fluorescence signal. Thus, Wu and coworkers have developed a fluorescence photon migration model to derive a relationship with the diffuse reflectance that can be exploited to extract the quantitative fluorescence in tissue [3,5]. In a different approach, Diamond and coworkers have advanced the idea to use a single optical fiber for both source and collection [4]. The concept is that detectable fluorescence events occur so close to the fiberoptic tip that absorption and scattering effects are minimal, analogous to how these effects are minimal for very thin tissue sections. Empirical methods with similar underlying concepts have also been developed. Thus, Finlay et al. used the single fiber method in conjunction with an empirically-derived correction factor that depends on the tissue optical properties at the emission wavelength to compensate for high tissue attenuation in the prostate during PDT studies [1]. Weersink et al. used a fluorescence/reflectance ratio to quantify fluorophore concentration, but with the fluorescence and reflectance measured at different source-collector fiberoptic distances, with the distances optimized using Monte Carlo simulations [6]. Ex vivo extraction techniques have also been developed that are based on homogenizing the tissue and diluting the analyte to the point that effects due to optical scattering and absorption are small [7]. These ex vivo procedures are typically time-consuming and error-prone due to tissue handling and/or cryofreezing for later processing.

In this work, we present an in vivo method to extract the quantitative fluorescence spectrum (i.e. the full fluorescence emission spectrum corrected for optical properties effects, in absolute
units), and consequently fluorophore concentration, using a simple, closed-form equation. The tissue optical properties, diffuse reflectance spectrum and measured fluorescence spectrum (i.e. the raw, uncorrected fluorescence measurement that is distorted by the optical attenuation) are inputs to the model. Since the tissue optical absorption and scattering properties need to be known, a method to extract these is reviewed, using the spectrally-constrained diffuse reflectance technique outlined in Chapter 2.

Our specific interest here is to develop a technique to quantitatively measure the fluorescence in situ during surgical resection using 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX). Studies on the drug ALA to promote the over-production of the naturally-occurring fluorophore PpIX has shown strong selectivity to high-grade glioma tumor cells. Intra-operative fluorescence imaging, mediated by ALA-PpIX, has proven useful in increasing completeness of tumor tissue resection [2]. However, to date, resection decisions based on fluorescence are made using qualitative assessment of the fluorescence [2,9]. The interpretation of the fluorescence intensity guides resection decision-making; however, the procedure is highly subjective. Moreover, the differences between strong and weak tissue fluorescence intensities are as likely to be due to differences in tissue absorption and scattering as to varying fluorophore concentration. Many published studies on fluorescence image-guidance involve the use of fluorescence excitation in the high absorption band of heme around 400-420 nm [9,10,11], where variation in the optical properties is severe. The fluorescence intensity is also highly dependent on the viewing distance and angle relative to the tissue surface, especially in a resection cavity with large surface contour variations. There is a clear need to devise more quantitative instrumentation to evaluate the fluorescence signal to improve decision-making during
fluorescence image-guided surgical resection in order to achieve its full potential as an objective, reproducible and more operator-independent technique.

Most published work on extracting fluorophore concentrations in tissues involves excitation wavelengths where the tissue attenuation is low [4,6,12]. However, the challenge here is to decouple the quantitative fluorescence from the tissue optical properties under high attenuation conditions to take advantage of the 405 nm PpIX absorption peak. For this, we have derived a simple, closed-form analytical model to extract the quantitative fluorescence spectrum with excitation wavelengths in regions of high absorption. In order then to extract the true fluorescence signal, the tissue optical properties must be known at the excitation wavelength. For this we use the technique of spectrally-constrained diffuse reflectance. Fluorophore concentrations can then be calculated from the quantitative fluorescence spectrum through spectral decomposition using \textit{a priori} basis spectra. The fluorescence model is implemented into a handheld fiberoptic probe for investigations of ALA-PpIX tumor contrast in guided resection surgery of brain tumors. However, the approach is generic to any visible/near-infrared emitting fluorophores and any tissue.

3.2 Theory

3.2.1 Fluorescence model

The fiberoptic geometry for fluorescence collection is shown in Figure 3.1(a). The following fluorescence model is based on the assumption that the optical absorption at the excitation wavelength, $\lambda_x$, is high relative to that of the emission wavelength, $\lambda_m$.\footnote{Variables at the excitation and emission wavelengths are denoted with subscripts $x$ and $m$, respectively.} This is generally true in
tissue if the excitation wavelength is in the UV-blue end of the visible spectrum (~380-450 nm). As a result, most fluorophore absorption events occur close to the source fiber.

![Diagram](image)

Figure 3.1: (a) Fiberoptic geometry for fluorescence collection; (b) fiberoptic geometry for measuring the diffuse reflectance spectrum.

The migration paths of the fluorescence emission photons at \( \lambda_m \) are then approximated by those of the reflectance photons at \( \lambda_m \) emitted and collected using the same fiberoptic geometry. Given constant optical properties at \( \lambda_x \) and constant fluorophore concentration, it follows that the measured fluorescence, \( F_{x,m} \), is linearly proportional to the diffuse reflectance, \( R_m \), at the emission wavelength:

\[
F_{x,m} = S R_m \quad (3.1)
\]

where \( S \) is the fraction of excitation photons launched into the tissue that are absorbed and produce fluorescence.

The term \( S \) can be modeled as the fraction, \( S_1 \), of the total excitation photons that are retained within the tissue at steady-state (i.e. those photons that are not diffusely re-emitted through the tissue surface, represented by \( R_{t,x} \)), multiplied by the fraction, \( S_2 \), of the total absorbed photons.
that are re-emitted as fluorescence. \( R_{tx}, \) i.e. the total diffuse reflectance, depends on the internal reflection parameter, \( \kappa = (1 + r_{id})/(1 - r_{id}) \) that arises from refractive index mismatch between the tissue and the external medium, and the reduced albedo at \( \lambda_x \), \( a'_x = \mu'_x/(\mu_{a,x} + \mu'_s,x) \). It is given by diffusion theory as [13]:

\[
R_{tx} = \frac{a'_x}{1 + 2\kappa(1 - a'_x) + (1 + \frac{2\kappa}{3}\sqrt{3(1 - a'_x)})}.
\]

(3.2)

An empirical formulation of \( r_{id} \) for index-mismatched boundaries has widely been used, where \( r_{id} = -1.44n_{rel}^{-2} + 0.71n_{rel}^{-1} + 0.67 + 0.0636n_{rel} \), and \( n_{rel} = n_{tissue}/n_{external} \) [14]. For matching internal and external refractive indices, \( \kappa = 1 \), and this was used here since the ink-blackened epoxy surrounding the probe fibers acts as the external medium (the epoxy-tissue interface was approximated as index-matched). \( S_1 \) is the fraction of photons that are not diffusely reflected out of the tissue, so that \( S_1 = (1 - R_{tx}) \).

The quantitative fluorescence, \( f_{x,m} \), in units of nm\(^{-1}\) cm\(^{-1}\) is defined here as the product of the wavelength-dependent fluorescence quantum yield, \( Q_{x,m} \), and the fluorescence absorption coefficient at the excitation wavelength, \( \mu_{af,x} \). It is, therefore, an intrinsic property of the tissue fluorophores, rather than depending on the collection geometry or tissue optical properties. The fraction of total absorbed photons that undergo fluorescence, \( S_2 \), is simply the quantitative fluorescence divided by the total absorption. If the fluorophore absorption contribution is negligible compared to the tissue absorption, i.e. \( \mu_{af,x} \ll \mu_{a,x} \), then the total absorption can be approximated by \( \mu_{a,x} \):
\[ S_2 = \frac{Q_{x,m} \mu_{af,x}}{\mu_{a,x}} \]  

(3.3)

The measured (uncorrected) fluorescence can now be expressed as:

\[ F_{x,m} = \left( 1 - R_{t,x} \right) \left( \frac{Q_{x,m} \mu_{af,x}}{\mu_{a,x}} \right) R_m \]  

(3.4)

A closed form equation for the quantitative fluorescence is then:

\[ f_{x,m} = Q_{x,m} \mu_{af,x} \left( \frac{\mu_{a,x}}{1 - R_{t,x}} \right) \left( \frac{F_{x,m}}{R_m} \right) \]  

(3.5)

Clearly, if \( \mu_{a,x} \) goes to zero, then \( f_{x,m} \) should not go to zero. Recall, however, the underlying assumption that \( \mu_{a,x} \) is high, so that Eq. (3.5) would be invalid at low excitation absorption and the model is not applicable.

Some caution is necessary in using the diffusion theory model in Eq. (3.2) for the total diffuse reflectance. For high reduced albedo, i.e. \( a' > 0.5 \), the relation has been shown to be accurate; however, in this work the reduced albedo is often <0.5 due to the strong absorption in the excitation band. Despite this, Eq. (3.2) can still be used to accurately calculate the quantitative fluorescence in spite of the fact that it is technically invalid due to low albedo. Figure 3.2(a) shows \( R_t \) plotted against \( a' \) using both diffusion theory and Monte Carlo calculations. At low \( a' \) the diffusion theory curves deviates from the Monte Carlo values. Since Eq. (3.5) uses the factor \( 1/(1-R_{t,x}) \), this factor was plotted against \( a' \) (Figure 3.2(b)) to determine if a diffusion theory equation is suitable for calculating this factor. This figure shows that even with very high absorption (\( \mu_a > 1 \)) and at low albedo, diffusion theory is adept at producing accurate values for the corrective factor \( 1/(1-R_t) \), justifying Eq. (3.2)’s use in the fluorescence model.
The corrected emission spectrum, $f(\lambda)$, can be used to quantify fluorophore concentration, $c$, given an \textit{a priori} fluorescence basis spectrum, $b(\lambda)$, for unit concentration. The relation is:

$$f = b c$$  \hspace{1cm} (3.6) \\

where $f$ and $b$ are $f(\lambda)$ and $b(\lambda)$ in column vector form. Solving for $c$:

$$c = (b^T b)^{-1} b^T f$$  \hspace{1cm} (3.7) \\

where $T$ indicates the matrix transpose. Generalizing to $N$ fluorophores with distinct spectra, a basis matrix, $B = [b_1 \ b_2 \ldots \ b_N]$, can be built with the individual fluorophore basis spectra as its columns, with a corresponding fluorophore concentration vector, $c = [c_1 \ c_2 \ldots c_N]^T$. Solving for $c$:

$$c = (B^T B)^{-1} B^T f$$  \hspace{1cm} (3.8) \\

To recapitulate the assumptions in the fluorescence model:
1. Reflectance photons and fluorescence photons traverse similar path lengths given the same fiberoptic distance, given that $\mu_{a,x} >> \mu_{a,m}$, which is generally true in tissue if the excitation wavelength is in the UV-blue range.

2. $\mu_{a,x} >> \mu_{af,x}$. In many cases, the fluorophore contribution to $\mu_{a,x}$ may be small compared to the high absorption of hemoglobin in the range 380-450 nm, but this should be considered based on the expected maximum concentration of the fluorophore of interest. In the situation where fluorophore absorption at $\lambda_x$ is significant compared with tissue absorption, the albedo becomes $a'_x = \mu'_s x / \left( \mu_{a,x} + \mu_{af,x} + \mu'_s x \right)$, and Eq. (3.3) becomes $S_2 = (Q_{s,m} \mu_{af,x}) / \left( \mu_{a,x} + \mu_{af,x} \right)$, and a priori knowledge of the quantum yield is required to solve for the quantitative fluorescence in Eq. (3.4).

3.2.2 Tissue optical properties extraction using spectrally-constrained diffuse reflectance

The fluorescence model of Eq. (3.5) requires the excitation tissue optical properties, $\mu_{a,x}$ and $\mu_{s,x}'$. The method described in Chapter 2 was used to measure the visible light range of optical properties by employing fiberoptic source-collector pairs to measure the steady-state diffuse reflectance spectrum, as shown in Figure 3.1b. The objective here is to determine $\mu_a(\lambda)$ and $\mu'_s(\lambda)$ over a wavelength range (450-720 nm) to provide a good model fit, then extract $\mu_a(\lambda_x)$ and $\mu'_s(\lambda_x)$ by extrapolating to the excitation wavelength, which in this work is $\lambda_x = 405$ nm.

As outlined in Chapter 2, deriving the optical properties is not as simple as applying the inverse algorithm to any $r$; for each $r$, there is a range of validity that is constrained by the peak of the reflectance-$\mu_s'$ curve, and the diffusion theory model breakdowns at low $\mu_s'$. By using reflectances measured at several $r$, the ranges of validity overlap, thus increasing the total dynamic range. For the quantitative fluorescence work, fiber distances of $r = 260$ and 520 µm
were selected. In Chapter 2, it was determined that for these values of $r$, the range for the spectral constraint technique was found to be $\mu_s'=10.1-52.9 \text{ cm}^{-1}$ and $\mu_a=0-10 \text{ cm}^{-1}$. The $r = 260$ and $520 \mu$m source-collector distances were used because it was known that the brain is the target site of interest and the brain optical properties have been measured as within this range of validity in previous laboratory experiments on murine tissues.

### 3.3 Materials and methods

#### 3.3.1 Measurement system

The instrument was designed for clinical use to quantify fluorescence signal during resection surgery of brain tumors. Photographs of the sterilizable handheld probe are in Figure 3.3(abc), with a schematic of the probe tip geometry shown in Figure 3.3(d). A linear array of 4 optical fibers (FG200LCC: ThorLabs, Newton, NJ), spaced apart every 260 $\mu$m, were epoxied into 18 Ga hypodermic needle tubing. The silica core of the fiber was 200 $\mu$m, with a numerical aperture of 0.22. The hypodermic needle part was affixed to a stainless steel handle, with the four fibers extending 3 m away to SMA 905 leads.

The control system (see Figure 3.3(e)) directs the flow of optical signals into and out of the probe handle. This control system is an updated, clinically-applicable and fluorescence-capable version of the “bench top” system developed in Chapter 2. The white light sources for the diffuse reflectance measurements and the 405 nm source for fluorescence excitation are LEDs (LEDengin, Santa Clara, CA, USA), controlled by computer via an analog data output card (Measurement Computing, Norton, MA, USA). The fluorescence LED is filtered with a 550 nm shortpass filter (Edmund Optics, Barrington, NJ, USA). The spectrometer is a USB2000+ model (Ocean Optics, Dunedin, FL, USA). The control system unit consisted of these elements in an
electronics enclosure that was custom-built in our lab. The instrument met electrical requirements for the Canadian Standards Association.

A custom Labview (National Instruments, Austin, TX, USA) application on a laptop computer was used to control the LED signals and spectrometer acquisition. The program acquired the following sequence of measurements:

1. White light reflectance spectrum at \( r = 260 \) \( \mu \)m
2. White light reflectance spectrum at \( r = 520 \) \( \mu \)m
3. Fluorescence spectrum (405 nm excitation) at \( r = 260 \) \( \mu \)m
4. Background signal (no light through probe)

For \textit{in vivo} PpIX measurements (as in the \textit{in vivo} rabbit work to be described later on in Section 3.3.4), data acquisition times adequate for good signal-to-noise were determined to be 125 msec for the fluorescence spectrum, 25 msec for each of the two reflectance spectra, and 500 msec for the background spectrum (\textit{i.e.} ambient lighting), with the total acquisition time per measurement being \( \sim 0.675 \) sec. In this work, the reflectance and fluorescence spectra obtained at \( r = 260 \) \( \mu \)m were used for the quantitative fluorescence and spectral fitting calculations (Eqs. (3.5) and (3.7)). The reflectance spectra at \( r = 260 \) and 520 \( \mu \)m was used for the extraction of optical properties using the spectrally-constrained diffuse reflectance method outlined previously.

The reflectance measurements were calibrated according to a technique we previously published using fractionated Intralipid samples, such that the absolute reflectance is determined [cm\(^{-2}\)] [8]. The fluorescence measurements were calibrated according to a Intralipid liquid phantom with known \( \mu_{a,x}, \mu_{s,x}', \) and [PpIX]. A previously published fluorescence quantum yield of 0.5\% for PpIX [9] was used to scale the quantitative fluorescence to units of nm\(^{-1}\)cm\(^{-1}\).
Repeatability performance tests were carried out as follows. Silicone-based solid phantoms with titanium oxide, TiO$_2$, for optical scattering were formulated according to a protocol developed at the University of California, Irvine (www.bil.uci.edu/ntroi/pubs/pdf/si_recipe.pdf). The probe tip was placed in contact with the silicone-TiO$_2$ phantom and a measurement taken. The entire system, including the computer, was then shut down, the probe leads were disconnected and then reattached. The system was re-booted and the phantom measurements repeated. This was done 5 times. The standard deviations of the phantom measurements of the fluorescence and diffuse reflectance fibers (at $r = 260$ and 520 $\mu$m), normalized to the mean values, were 1.2, 2.1 and 2.0%, respectively.
### 3.3.2 Phantom experiments to test the accuracy of the fluorescence model

Phantom experiments were carried out to validate the fluorescence model. Intralipid fluid (Fresenius Kabi, Uppsala, Sweden) was used to provide the tissue-like background scattering. Yellow food coloring (McCormick Canada, London, ON, Canada) was used to control the absorption coefficients. Protoporphyrin IX (Sigma-Aldrich) was used as the target fluorophore.

The absorption coefficients of the yellow dye were measured using a spectrophotometer (Cary 5000, Varian Inc., Palo Alto, CA, USA). The reduced scattering coefficient spectrum of Intralipid has been quantified for varying concentrations previously [8]. A set of nine phantoms were mixed, giving the optical properties shown in Table 3.1.

<table>
<thead>
<tr>
<th>Phantom A</th>
<th>Phantom B</th>
<th>Phantom C</th>
<th>Phantom D</th>
<th>Phantom E</th>
<th>Phantom F</th>
<th>Phantom G</th>
<th>Phantom H</th>
<th>Phantom I</th>
</tr>
</thead>
<tbody>
<tr>
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<td>40</td>
<td>40</td>
<td>60</td>
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<td>60</td>
</tr>
<tr>
<td>( \mu_{s,x}' ) (cm(^{-1}))</td>
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<td>20</td>
<td>25</td>
<td>15</td>
<td>20</td>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>( \mu_{a,m} ) (cm(^{-1}))</td>
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<td>0.04</td>
<td>0.06</td>
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<td>8.7</td>
<td>11.6</td>
<td>8.7</td>
<td>11.6</td>
<td>14.5</td>
</tr>
</tbody>
</table>

PpIX was mixed in six concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.15625 µg/mL) for each set of 9 optical properties, for a total of 54 phantoms. Probe measurements were taken in each phantom and Eq. (3.5) was applied to extract the quantitative fluorescence spectra and PpIX concentrations. Images of the phantom surfaces (at \([\text{PpIX}] = 5 \, \mu\text{g/mL}\)) were also taken using a fluorescence stereomicroscope (MZ FLIII: Leica, Wetzlar, Germany) in order to compare the observed fluorescence intensities with the quantitative measurements using the fiberoptic probe technique.
3.3.3 Ex vivo tissue validation studies

All animal experiments were carried out with institutional animal-care approvals (University Health Network, Toronto). A mouse tumor model was used to validate the accuracy of the probe and model in measuring photosensitizer concentration in various tissue types, again with PpIX as the target fluorophore, and compared with measurements of diluted, solubilized tissue in a cuvette-based fluorometer using an established protocol [7].

Tumor induction: Five male mice (20 g) were anesthetized with 2% isoflurane (oxygen flow rate at 2.5 L/min) and placed on a warming blanket. The skin at the injection site was swabbed with 70% ethanol, and $10^6$ B16 melanoma cells in 20 µL of phosphate buffered saline were injected subcutaneously into the left flank.

PpIX measurement in different organs: After tumors had grown for 7 days to approximately 4-7 mm diameter, each mouse was injected via the tail vein with 100 mg/kg ALA (Sigma-Aldrich) at 0.5, 1, 2, 3 or 4 h before sacrifice, which ensured a large range of PpIX concentration across all tissue samples. The mice were sacrificed by cervical dislocation under isoflurane general anesthesia (2% isoflurane, 2.5 L/min oxygen flow rate). Blocks (~ 5-10 mm across) of each tissue (brain, heart, kidney, liver, muscle, skin and tumor) were rapidly excised under subdued lighting and probe measurements were taken on each sample at 3 different positions. The samples were weighed, placed into cryotubes, snap frozen in liquid nitrogen and stored at -70°C in a light-tight container until the tissue solubilization procedure was carried out in batches.

Tissue solubilization: The tissue solubilization protocol for measurement of absolute fluorophore concentration was developed by Lilge et al [7]. Each tissue sample was combined with 2 mL Solvable (Perkin Elmer, Waltham, MA, USA) and placed in an undulating water bath at 50°C for 1h. The resulting material was homogenized (Tissue Tearor, Biospec Products,
Bartlesville, OK, USA) in the original vial. 200 µL of this homogenate was combined with 3 mL of distilled water and 1 mL of Solvable. This solution was incubated in the water bath at 50°C for 1h. The optical density was measured and diluted to <0.1 if required. The resulting solution was transferred to a quartz cuvette and placed in a fluorometer (Fluorolog: Jobin Yvon, Edison, NJ, USA), using an excitation wavelength of 401 nm. A look-up curve was constructed by measuring known concentrations of PpIX in 75%/25% distilled water/Solvable solution, with the detector nonlinearity taken into account. The estimated accuracy of the solubilization technique for measuring [PpIX] is ±13.5%.

3.3.4 In vivo rabbit brain tumor model

An intracranial brain tumor model (VX2) in rabbits was used to test the instrument sensitivity and to get baseline measurements in the in vivo surgical setting for the intended fluorescence-guided resection application. Female New Zealand white rabbits ~4 kg (Charles River, Montreal, QC, Canada) were used.

Tumor induction: The VX2 tumor cells were propagated in the flank muscle and harvested at ~1 cm diameter. VX2 cells were extracted with a strainer and implanted into the brain within 2 h. For this, the antibiotic Baytril (Bayer, Toronto, ON, Canada) at 7.5 mg/kg was administered subcutaneously (SQ) 1 day prior and for 3-5 days post cell implantation. Immediately prior to induction 10 mg/kg of the sedative acepromazine was administered SQ, and the animals were then anaesthetized and maintained with 1-2.5% inhaled isoflurane (oxygen flow rate at 2.5 L/min) as required. The site was shaved and swabbed with betadine. The head was affixed to a rabbit-adapted stereotactic frame. A 2 cm midline incision was made, the scalp reflected and the cranium exposed. Using a handheld drill with a 1 mm diameter drill bit, a burr hole was
performed over the right hemisphere, anterior to the coronal suture and 5 mm to the right of the bregma, leaving the dura intact. A 100 mL Hamilton syringe was introduced to a depth of 2 mm beneath the dura and $2 \times 10^5$ VX2 cells in 50 µL Hank’s media were inoculated intracerebrally, over a period of 2-3 min under low manual pressure to avoid mechanical damage. Bone wax was used to close the burr hole and the incision was closed with sutures. After surgery the animals were monitored continually until fully recovered and Buprenorphine (0.05 mg/kg SQ) was administered for analgesia every 8 h for the first 24 h.

**Craniotomy and fluorescence measurements:** At 7-9 days after VX2 implantation, animals underwent craniotomy surgery to expose the tumor. For this, the rabbit was injected intravenously (*i.v.*) with 20 mg/kg ALA (Sigma-Aldrich) as a solution in PBS buffered to pH 4-5. Prior to anesthesia, acepromazine (10 mg/kg SQ) was given. For surgery, the animals were brought under general anesthesia and maintained with inhaled isoflurane (1-2.5%, with oxygen at 2.5 L/min). The eyes were lubricated with tear gel. The heart rate and oxygen saturation were monitored with a pulse oximeter and blood pressure monitor. The scalp was shaved and betadine was applied to the surgical site. A 2-4 cm incision was made in the scalp along the mid-line. A craniotomy was performed using the handheld drill with a 1 mm drill bit. Gentle suction was used to remove irrigating fluid and bone dust. The bone flap was gently elevated, aiming to keep the entire dura intact. Once the bone flap was removed, both left and right hemispheres were exposed and the dura was cut using microsurgery scissors and forceps. Under dim room lighting, 10 probe measurements were taken near the tumor cell injection site, with an additional 10 measurements in normal brain in the contralateral hemisphere to the injection site. The animals were sacrificed post-surgery (*i.v.* Euthanyl, 1 mL/kg).
3.4 Results

3.4.1 Phantom experiments to test the accuracy of the fluorescence model

This set of experiments were used to validate the fluorescence model in Eq. (3.5) and (3.7), with a priori knowledge of the excitation optical properties. The measured fluorescence spectra, \( F_{x,m}(\lambda_m) \) for the set of 9 phantoms A-I, all with a PpIX concentration of 5 \( \mu \)g/mL, is shown in Figure 3.4(a). Applying Eq. (3.5) to the data produces the quantitative fluorescence spectra, \( f(\lambda) \), shown in Figure 3.4(b). The relative standard deviation (normalized to the mean) at the 635 nm peak is 53.1% for the measured fluorescence and 10.1% for the quantitative fluorescence estimate.

The quantitative fluorescence model was applied to the entire data set of 54 phantoms, with the results plotted against PpIX concentration. Figure 3.6(a) shows the measured, raw fluorescence intensities at 635 nm, compared to the estimated [PpIX] concentration shown in Figure 3.6(b), calculated from the raw data using Eqs. (3.5) and (3.7). Fluorescence microscope images of the phantoms were taken in order determine the improvement in fluorescence quantification using the fiberoptic probe as compared to fluorescence imaging, as well as to get a visual conception of the fluorescence intensity variation due to changes in optical properties. Phantom surface images are shown in Figure 3.5 for [PpIX] = 5 \( \mu \)g/mL, the intensity differences between phantoms easily distinguishable by eye. The highest intensity difference in this set of images is between phantom C (highest \( \mu_{s,x}' \), lowest \( \mu_{a,x} \)) and G (lowest \( \mu_{s,x}' \), highest \( \mu_{a,x} \)), where the fluorescence intensity of C is 4.0 times that of G.
Figure 3.4: a) Measured fluorescence, $F_{x,m}(\lambda_m)$, and b) model-derived absolute quantitative fluorescence spectra, $f(\lambda)$, for Phantoms A-I at [PpIX] = 5 µg/mL.

Figure 3.5: Images of PpIX phantoms A-I, all with a PpIX concentration of 5 µg/mL.
Figure 3.6: (a) Measured fluorescence, $F_{x,m}$, at 635 nm and (b) estimated fluorophore concentration using the fluorescence model and spectral fitting using a basis spectrum for PpIX for phantoms A-I, indicated by the symbol legend below the graphs. The excitation optical properties are shown in the symbol legend for reference. The dashed line in (a) represents the best straight line fit through the origin to the data; the dashed line in (b) is the unity line.

Fluorescence images of the phantoms are shown in Figure 3.5 at a fixed PpIX concentration (5 µg/mL) to give an idea of the visual appearance of the fluorescence intensity variations due to changes in the optical properties. The most marked difference, corresponding to a factor of 4:1 in intensity, is between phantoms C (highest $\mu''_{r,x}$, lowest $\mu_{a,x}$) and G (lowest $\mu''_{r,x}$, highest $\mu_{a,x}$).

The measured fluorescence intensity at the 635 nm PpIX peak from each of the 54 phantoms is shown as a function of the known PpIX concentration in Figure 3.6(a), while panel (b) shows a similar graph for the PpIX concentration derived from the model applying Eqs. (3.5)-(3.7) to the measured data. Linear fits to these data show a marked increase in the goodness-of-fit, from $R^2 = 0.639$ to 0.976. The root-mean-square (RMS) deviation from the mean and the maximum
deviation from the mean, each normalized to the mean value, were calculated for the 5 µg/mL data (Table 3.2). This shows a 5- to 7-fold reduction in these metrics in applying the model to the raw data, though there is still a small dependence of quantitative fluorescence on the optical properties (10.1% RMS deviation for [PpIX] estimation).

Table 3.2: RMS deviation from the mean and maximum deviation from the mean, comparing the raw fluorescent image intensity, the uncorrected probe measurement at 635 nm, and the model-derived fluorophore concentration for phantoms A-I at [PpIX] = 5 µg/mL.

<table>
<thead>
<tr>
<th></th>
<th>RMS deviation from mean</th>
<th>Maximum deviation from mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence image intensity</td>
<td>56.5%</td>
<td>150.2%</td>
</tr>
<tr>
<td>Measured fluorescence $F_{x,m}(\lambda_{m}=635 \text{ nm})$</td>
<td>52.3%</td>
<td>94.9%</td>
</tr>
<tr>
<td>Estimated [PpIX]</td>
<td>10.1%</td>
<td>14.2%</td>
</tr>
</tbody>
</table>

3.4.2 *Ex vivo mouse model for quantitative fluorescence validation in tissue*

Figure 3.7(a) shows the measured fluorescence signal exemplified by the PpIX peak at 635 nm plotted against the [PpIX] measurement from the tissue solubilisation technique for 34 tissue samples.\(^3\) The data shows large scatter, both between tissues as would be expected, but also for individual tissues. The coefficient of determination was $R^2=0.255$ for the best line fit through the uncorrected fluorescence data in Figure 3.7(a). Applying the quantitative fluorescence model gives marked improvement in the correlation. Thus, the coefficient of determination for all tissues lumped together is $R^2=0.709$ fitting the data to the unity line, demonstrating that the technique compensates substantially, although not completely, for the variations in tissue optical properties. The best line fit through the origin, which has a slope of 0.85, is also included in Figure 3.7(b) with $R^2=0.871$. Note the best line fit is not the unity line: this may be due to a difference in the quantum yield of PpIX in tissue and in the Intralipid calibration phantom.

\(^3\) There was no data for liver at 4 hours due to a tissue handling error during the solubilisation stage.
Figure 3.7: Fluorescence measurements in 7 types of *ex vivo* murine tissues. 5 mice were used, each sacrificed at different time points after ALA injection (0.5, 1, 2, 3 and 4 hours). a) Uncorrected fluorescence intensity at 635 nm versus PpIX concentration from the tissue solubilization data: the y-axis scale is in 2 parts to display better the range of data. b) Model-derived [PpIX] values versus tissue solubilization values. Y-axis error bars are ± 1 s.d. from 3 measurements on each tissue sample. X-axis error bars were calculated from the RMS errors estimated by Lilge *et al.* [7]

It is worth noting also the large range of absorption and scattering coefficients between tissues at the excitation wavelength: 7.0 - 68.3 cm\(^{-1}\) and 5.3 – 26.3 cm\(^{-1}\), respectively (including data between the 1\(^{st}\) and 9\(^{th}\) deciles). It is also useful to check the optical property ranges compared to the validated dynamic range of the spectrally-constrained diffuse reflectance method. The absorption spectrum as calculated from the diffuse reflectance spectrum (450-720 nm) ranged from 0.091 – 6.64 cm\(^{-1}\) and the reduced scattering spectrum ranged from 4.9 – 22.9 cm\(^{-1}\) across all tissue measurements. Note that lower limit of the \(\mu'\) range is out of the dynamic range of the spectrally-constrained diffuse reflectance method (recall that the \(\mu'\) dynamic range is from 10-50 cm\(^{-1}\)). Both the non-unity slope and the large scatter in the [PpIX] data may be due to the tissue optical properties lying somewhat out of range of the validated dynamic range of the model.
Resolving this would require an additional fiber separation to decrease the lower limit of the dynamic range for $\mu'_s$; however, this necessitates a larger probe diameter.

### 3.4.3 In vivo rabbit brain tumor model

Table 3.3 shows data in 4 rabbits for the maximum and minimum [PpIX] estimates around the tumor injection site and in the contralateral normal brain, making 10 different measurements in each case. Figure 3.8 shows representative spectra, with a strongly positive signal in the tumor site and low signal in the contralateral normal brain. The objectives were to demonstrate that the probe can detect fluorescence \textit{in vivo}, in both tumor and normal tissue, as well as to obtain baseline measurements of the PpIX concentration to inform the clinical studies in glioma patients during fluorescence image-guided surgery. The ratio of the average PpIX concentration between tumor and normal measurements was approximately 35:1, which is the same order of magnitude as reported in other brain tumor models using the \textit{ex vivo} tissue solubilization technique with similar dose parameters [18]. It is also seen that there is considerable point-to-point variation in the measured values, showing the heterogeneity of ALA uptake and/or PpIX synthesis throughout the tumor: bear in mind, however, that the tumors are only a few mm’s in diameter, so that there may be a contribution from the exact probe positioning.

<table>
<thead>
<tr>
<th></th>
<th>Contralateral normal brain</th>
<th>Near tumor cell injection site</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PpIX] in µg/mL (max/min for $N=10$)</td>
<td>[PpIX] in µg/mL (max/min for $N=10$)</td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>0.097 / 0.001</td>
<td>1.081 / 0.004</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0.013 / 0.001</td>
<td>0.224 / 0.003</td>
</tr>
<tr>
<td>Rabbit 3</td>
<td>0.027 / 0.001</td>
<td>1.951 / 0.017</td>
</tr>
<tr>
<td>Rabbit 4</td>
<td>0.010 / 0.001</td>
<td>2.451 / 0.028</td>
</tr>
</tbody>
</table>
Figure 3.8: Representative fluorescence spectra corrected for optical properties for one animal (Rabbit #3), with the spectrum taken near the tumor injection site, showing enhanced PpIX levels compared with the spectrum taken from normal brain tissue; the corresponding model-derived PpIX concentrations were 1.951 and 0.016 µg/mL for the tumor and normal sites, respectively.

3.5 Discussion and Conclusion

One of the main challenges of this work was to produce a simple analytic equation that models fluorescence measurements in tissue, and that can be rapidly computed to enable *in situ* application. The model presented here achieves this goal and, from initial clinical studies in progress [19], has been found to be highly practical in the real intra-operative environment. However, there are inherent limitations that are due to the assumptions made in the model.

Recapitulating, the assumptions used are (1) that reflectance and fluorescence photons traverse similar paths providing that the tissue absorption at the excitation wavelength is much larger than at the emission (detection) wavelengths (*i.e.* \( \mu_{a,x} \gg \mu_{a,m} \)), (2) that the intrinsic tissue absorption at the excitation wavelength is much larger than the added absorption due to the fluorophore (*i.e.* \( \mu_{a,x} \gg \mu_{af,x} \)), and (3) that hemoglobin is the dominant absorber in tissue. Assumptions (1) and (2) are used primarily in the quantitative fluorescence model (Eq. 5) and were validated by the phantom experiments. Thus, applying the quantitative fluorescence model
with known optical properties resulted in a ~5-fold decrease in the RMS deviation compared with the measured, uncorrected fluorescence signal for a given fluorophore concentration. The improvement in the coefficient of determination ($R^2 = 0.639$ for the uncorrected data and 0.976 after applying the model) further supports the validity of the model, given accurate values of the tissue optical properties as inputs.

The *ex vivo* organ study was carried out mainly to test assumption (3) across different tissue types, *i.e.* to test if the technique is ‘universal’. From Figure 3.7, the estimates of [PpIX] derived using the model indicate that absolute fluorophore measurements are feasible, notwithstanding residual errors for some tissue types. For example, liver and the B16 melanoma tumor are highly pigmented tissues, causing $\mu_a$ to be underestimated, which in turn leads to underestimation of the quantitative fluorescence—indeed there seems to be a systematic bias for these two tissues as shown in Figure 3.7(b). From Figure 3.2(b), the factor $1/(1-R_t)$ is nearly constant at low albedo ($i.e. a' <0.5$) so that, given that the reflectance spectrum at the emission wavelengths is measured correctly, the major factor that affects the fluorescence correction model, Eq. (5), is the $\mu_{a,x}$ estimate. We surmise, therefore, that this technique is more suitable for tissues where hemoglobin dominates the absorption in the excitation band, such that $\mu_{a,x}$ is estimated accurately, rather than in highly pigmented tissues with additional chromophores. It may be possible to incorporate other chromophores into a more sophisticated spectrally-constrained diffuse reflectance model. Another potential source of error in the murine tissue experiment is the heterogeneity in PpIX localization. Solubilizing the tissue has the effect of averaging this, whereas the fiberoptic probe interrogates only a superficial region of the tissue of each organ.

Although the mouse organ experiment was not designed as a pharmacokinetics study, it is useful to compare the results with other data on ALA-PpIX, as summarized in Table 3.4. Thus,
Sroka et al. injected mice with 50 mg/kg i.v. via the tail vein [20], while Moan et al. used intraperitoneal injection at 1.5 mM/kg [21], and the results seem to be consistent (even though our data has only $N=1$ per time point). It is encouraging that there is no apparent systematic bias introduced by the fiberoptic probe technique, so that it should be reliable for time-dependent or longitudinal studies.

Table 3.4: Comparison between PpIX kinetics measured in the mouse study and other published data.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Our data</th>
<th>Sroka et al. [20]</th>
<th>Moan et al. [21]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>3</td>
<td>3.1</td>
<td>4</td>
</tr>
</tbody>
</table>

The rabbit tumor experiment also demonstrated that this technique works well in vivo for use in fluorescence-guided resection of brain tumors, our initial clinical application. Since the fluorescence signals had high signal-to-noise (in the rabbit study the average SNR was 43 dB) at this clinically-relevant ALA dose (20 mg/kg) [2], we believe that the technique will perform as expected in the operating room environment—indeed this is borne out by recent preliminary clinical work, as detailed in Chapter 4. It is of interest to ask what the fractional tumor cell density in the tissue that can be detected. This can be estimated from the rabbit brain tumor data in which the average tumor-to-normal contrast ratio was 35:1. If the detectability threshold is considered conservatively to be 5:1, then the handheld probe technique should be able to detect ~14% ($\frac{5}{35} \times 100\%$) tumor “infiltrate”.

As mentioned in Section 2.5, the tissue optical properties may be affected by the probe contact pressure, possibly as a result of squeezing the local microvasculature and increasing the local density of optical scattering particles. Note that changes in the tissue optical properties due
to pressure should be largely corrected for by Eq. (3.5). For example, if $\mu_{a,x}$ increases, this is offset by a corresponding decrease in the measured fluorescence, $F_{x,m}$. However, if there is a change in the local fluorophore concentration due to pressure, this will affect the quantitative fluorescence measurement. Since PpIX is thought to be intracellularly retained and possibly bound to membrane lipids, it is not likely that pressure would cause PpIX molecules to, say, be squeezed out into the interstitium, lymphatic network or microvasculature; however, further study is necessary to determine whether or not local [PpIX] is affected by probe pressure.

In conclusion, absolute quantification of fluorophore concentration in tissues in vivo is a challenging problem, complicated primarily by the (unknown) variations in tissue optical properties. In this work we have focused on absolute quantification of the fluorescence spectrum in order to determine the true concentration of a target fluorescent species. The model is successful in reducing the distortion of the fluorescence spectrum and signal intensity due to tissue optical properties, when used under conditions of high tissue attenuation (380-450 nm). We expect this method is capable of quantifying many fluorophores other than PpIX, potentially including those comprising the tissue autofluorescence. This technique is adept at measuring the concentration of PpIX in situ for a variety of murine tissues, as well as in the case of an in vivo tumor. This instrument is currently deployed in clinical trials in glioma patients undergoing fluorescence guided resection surgery mediated with ALA-PpIX. The next chapter reviews the data from the first clinical trial using the quantitative fluorescence probe.

3.6 Statement of contributions

The quantitative fluorescence probe system and algorithms were designed and developed by me. The phantom and animal experiments were also done by me, with help from my colleagues Mamta Khurana (University of Toronto) and Yumi Moriyama (Ontario Cancer Institute).
3.7 References


4 Clinical trial to quantitatively measure ALA-PpIX fluorescence in normal brain and intracranial tumors

4.1 Introduction and Background

The previous two chapters outlined the physical modeling, design, development and validation of a handheld fiberoptic probe that is capable of measuring the tissue optical properties and quantitative fluorescence. The objective of developing these techniques was to provide neurosurgeons with a tool to quantify ALA-PpIX tumor contrast during fluorescence-guided resection surgery.

One of the exciting developments of the quantitative fluorescence project is the deployment of this device into clinical trials for the fluorescence guided resection of glioma. Our research group is in collaboration with Dartmouth College (Hanover, NH) and their associated medical centre, the Dartmouth-Hitchcock Medical Center (DHMC, Lebanon, NH) to elucidate how quantitative fluorescence may be able to provide improved performance for tumor margin demarcation.

Chapter 4 is divided into two parts. Part 4A presents clinical work-to-date on the in vivo qF probe data collected during surgery from 14 patients with intracranial tumors between September 2009 and March 2010. Probe measurements were taken in tumor and normal intracranial tissues during open craniotomy procedures; as well, at each probe measurement site a score for fluorescence intensity interpretation according to the neurosurgeon’s view through a fluorescence-capable surgical microscope was recorded for comparison. The data demonstrate that the quantitative fluorescence probe and correction algorithms impart an increase in the sensitivity and specificity of tumor detection compared with the neurosurgeon’s evaluation through the fluorescence surgical microscope. In addition, the autofluorescence and other optical
parameters such as oxygen saturation also have diagnostic value, and were used to improve the sensitivity and specificity of the probe data using a multivariable technique called linear discriminant analysis (LDA). It is worth noting that the data presented here are the first truly quantitative measurements of PpIX concentration in human intracranial tumors and normal brain tissue \textit{in vivo}. The raw clinical data in this chapter were used to produce a manuscript that will be submitted mid-2010 in collaboration with Dartmouth College; however, the entirety of the data analysis, interpretation and conclusions in this chapter are original to this thesis.

**Part 4B** outlines work done on \textit{ex vivo} fluorometry of tumor biopsies obtained during resection surgery. Prior to and separate from the \textit{in vivo} qF probe measurements, our group has been measuring the \textit{ex vivo} fluorescence of tumor biopsies received from DHMC during their FGR trials. The \textit{ex vivo} fluorometry on the biopsies was performed because up until the deployment of the qF probe device (in September 2009), there was no better way to measure [PpIX] quantitatively. The protocol for the \textit{ex vivo} fluorometry assay for these brain biopsies is a modified version of the tissue solubilisation assay that was used in Chapter 3 (refer to Section 3.3.3). Currently, we are measuring the initial batch of 237 biopsies from 22 patients; as of this writing, 45 biopsies from 5 patients with high-grade gliomas have been measured and the data processed. Although there is currently no data set where both \textit{in vivo} probe data and \textit{ex vivo} fluorometry data overlap, the current \textit{ex vivo} data set can provide supporting evidence that the \textit{in vivo} probe is measuring [PpIX] values within an expected physiological range.

Parts 4A and 4B are treated separately in terms of the “Materials and Methods” and “Results” sections. The “Discussion and Conclusions” section includes both Parts 4A and 4B in the discussion.
Part 4A: Clinical trial to quantitatively measure ALA-PpIX fluorescence in normal brain and intracranial tumors in vivo

4.2 Materials and methods (Part 4A)

4.2.1 Instrumentation and data processing

The quantitative fluorescence probe system (as detailed in Chapter 3) was used to take the measurements in normal and tumorous brain tissue. The tissue optical properties and quantitative fluorescence algorithms (see Chapters 2 and 3) were calculated post-procedure. For quality assurance purposes, the TiO$_2$ silicone phantom outlined in Section 3.3.1 was measured by the probe every 2-3 surgical procedures to ensure that the probe measurements were repeatable.

The fluorescence spectral fitting for these qF probe measurements require some additional basis spectra to fit to the real clinical situation. It is known that flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide, (NADH) and lipofuscin are fluorophores found in brain tissue [1-4]. A linear combination of these three endogenous fluorophores was used to model the background autofluorescence.

Neurosurgical imaging was performed using a Zeiss OPMI® Pentero™ surgical microscope (Carl Zeiss Meditec: Jena, Germany) with the added fluorescence kit, as described in Section 1.6. Figure 4.1 shows images from the Zeiss microscope before (white light image, panel (a)) and during (fluorescence image, panel (b)) a probe measurement. A large advantage over our qF probe system versus others (e.g. Toms et al. [1,2]) is that ambient lighting can often be tolerated. All probe measurements were taken during fluorescence illumination from the microscope, allowing the neurosurgeon to view the surgical field while taking a measurement. It is interesting that probe measurements can be taken under the Zeiss microscope’s fluorescence lighting as well.
as low-level incandescent lighting with no complications; however, probe measurements happen to be completely compromised by the sharp spectral lines and time-variance of standard cool-white fluorescent light bulbs used ubiquitously for room lighting.\(^4\)

Figure 4.1: Images taken from the Zeiss surgical microscope of the handheld probe taking a measurement on a meningioma during open craniotomy surgery. (a) White light image; (b) fluorescence image. The point of measurement is in the centre of the field-of-view, in the cross-hairs shown in panel (a). Note that the fluorescence image was taken at the moment of a probe measurement, with a flash of white light noticeable at the probe tip. For a video of this procedure, please refer to the T-Space link.

4.2.2 Clinical protocol for in vivo probe measurements

Measurements were performed at DHMC with the handheld fiberoptic probe instrumentation developed in Toronto (see Chapter 3). The clinical protocol was developed by both of our groups and approved by the DHMC Institutional Review Board in mid-2009. Patients received 20 mg/kg ALA in water to drink 3 h prior to anesthesia. During the open cranial procedure, fiberoptic probe measurements were taken at varying positions on the brain tumor, at different stages of the resection. Normal tissue data were also acquired as control data, typically on an area of brain tissue that is far away from the known location of the tumor as determined by intraoperative

\(^4\) Cool-white fluorescence room lights are particularly devastating for taking probe measurements for the FGR application due to the unfortunate location of the spectral lines of mercury at 546.4 nm and europium phosphor at 611.6 nm. These time-varying, high-intensity peaks in fluorescence room lighting completely interfere with one of the absorbance peaks of oxy-hemoglobin at 546 nm and the PpIX peak at 635 nm.
navigation tools (i.e. frameless stereotaxy) and the neurosurgeon’s judgment. In the case of meningioma, the dura mater was considered to be the control tissue, since these tumors arise from the meninges. The neurosurgeon for all cases was Dr. David W. Roberts at DHMC, who was also the principal investigator for this study.

The protocol for each probe measurement was as follows. The probe was positioned onto the tissue site of interest. The Zeiss surgical microscope acquired a white light image and a fluorescence image of the field-of-view with the probe in place. Under fluorescence illumination, 3 probe measurements were taken in sequence. The neurosurgeon scored the fluorescence intensity of the qF probe measurement site as visualized through the operating microscope (scores are from 0 to 4, with ‘0’ being no visible fluorescence and ‘4’ being high fluorescence intensity). Even though measurement results were available almost immediately, the neurosurgeon was blinded to all results from the probe, since it was approved for research purposes only and not yet cleared as a resection decision-making aid.

If neurosurgeon decided to resect the tissue, a biopsy was obtained from the probe measurement site and immediately frozen in dry ice. The biopsy was later divided for several purposes: hemotoxylin and eosin (H&E) histological examination, immunohistochemistry (IHC), and other analyses (such as mass spectrometry to measure Gadolinium contrast administered for MRI contrast). Biopsies were not taken for sites deemed to be normal controls. Figure 4.2 shows representative fluorescence and H&E brightfield images of thin sections of these biopsies. Panels (a,b) show a strongly fluorescing glioma sample with correspondingly high [PpIX].

Fourteen patients were enrolled in this study and gave informed consent. The median age of the patients was 56 (range: 27-74). Table 4.1 displays the pathologies and number of
measurement sites in this study. It should be noted that this is an ongoing study, with designs to continue until 2012.

Figure 4.2: (a,c) Fluorescence microscopy of thin (10 µm) frozen sections of brain tumor biopsies taken using a Zeiss Axiovert 200M inverted microscope and a PpIX filter block kit (excitation 405 nm, emission 610 nm longpass); (b,d) corresponding H&E sections. Panels (a,b) display a highly fluorescing glioma with [PpIX]=2.08 µg/mL. Scale bars are 50 µm. This sample was assessed to have a tumor grade of IV and a tumor burden of III.

Table 4.1: Baseline characteristics for the 14 patients in the in vivo quantitative fluorescence probe study

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Patients</th>
<th># of control sites</th>
<th># of biopsy sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG-II</td>
<td>2</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>HGG-IV</td>
<td>3</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Meningioma</td>
<td>6</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

4.2.3 Histopathology

H&E sections were prepared for each of the biopsies extracted during resection surgery. Dr. Brent T. Harris was the neuropathologist handling all of the cases in this study. Each tissue sample was evaluated for cell type, tumor burden, histological grade and percent necrosis. Cell typing is the classification of the tumor cells as the cell type that they most closely resemble (e.g. astrocytoma, oligodendroma, etc.). Tumor burden scores range from 0-III. A score of (0) indicates normal or fully necrotic tissue; (I), (II) and (III) indicate viable tumor burdens of >1-
33%, 33-67% and 67%-100%, respectively. Histological grade per biopsy sample are assigned independently of the overall WHO grade of the patient, and range from 0-IV. Table 4.2 shows the characteristics that the neuropathologist looks for to assign histological grades. Similar to tumor burden, percent necrosis has scores of (0), (I), (II), (III) and (IV), indicating 0%, >1-33%, 33-67% and 67-100% necrosis, respectively. All of the in vivo probe measurements and ex vivo tissue solubilisation fluorometry data were correlated to histopathological analysis.

Also, all of the samples taken from each patient were used to grade the patient according to the WHO primary CNS tumor grading scheme, with the highest grade biopsy determining the patient tumor grade.

Table 4.2: Characteristics of each histological grade.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Characteristics in H&amp;E section</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal or fully necrotic tissue</td>
</tr>
<tr>
<td>I</td>
<td>Diffusely infiltrating tumor cells</td>
</tr>
<tr>
<td>II</td>
<td>Greater number of infiltrating tumor cells with pleomorphism present, and no necrosis, mitotic figures and endothelial proliferation</td>
</tr>
<tr>
<td>III</td>
<td>Large degree of pleomorphism and mitotic figures, with no observable necrosis and no endothelial proliferation</td>
</tr>
<tr>
<td>IV</td>
<td>Large degree of pleomorphism, mitotic figures, as well as observable necrosis and endothelial proliferation</td>
</tr>
</tbody>
</table>

4.2.4 Statistical analysis of in vivo probe measurements taken during surgery

Statistical significance tests, linear discriminant analysis and receiver-operator characteristic analysis were performed on the in vivo data set acquired by the quantitative fluorescence probe during resection surgery.

Statistical significance tests: Since the data were expected to be non-parametric, a Wilcoxon rank-sum test was selected in order to determine how statistically-significant were the several
optical parameters derived from the *in vivo* probe data at distinguishing between normal and tumor tissues. PpIX concentration was the most relevant parameter to be tested. In addition, the following optical parameters were tested for statistical significance in differentiating normal from tumor tissue: the autofluorescence (AF) at 600, 635, 650 and 700 nm, diffuse reflectance (for both $r = 260$ and $520 \, \mu m$ fiber distance) at 575 and 600 nm, oxygen saturation ($StO_2$), total hemoglobin concentration, $f_{\text{ih}}$, and $\mu_a$ and $\mu_s'$ at 575 and 600 nm.

*Linear discriminant analysis:* Statistically-significant optical parameters that disprove the null hypothesis according to Wilcoxon rank-sum tests were evaluated as to their physiological and photochemical relevance to brain cancer. The selected parameters were used in a linear discriminant analysis (*i.e.* Fisher’s linear discriminant) in order to find the vector in this feature space such that the normal and tumor classes were separated to a maximal extent (at least, in a linear fashion).

*Receiver-operator characteristic analysis:* The receiver-operator characteristic (ROC) curves were generated using the PpIX concentrations as the parameter comparing normal tissue to these tumor populations: all tumors, all gliomas, LGGs, HGGs, meningiomas and metastases. Optimal sensitivity and specificity values were extracted to determine performance of [PpIX] as a tumor-specific marker. Its performance was compared with a metric for the uncorrected, raw fluorescence spectrum (*i.e.* not corrected for optical properties), and the neurosurgeon’s scoring of the visible fluorescence through the Zeiss microscope (scores are from 0 to 4). The uncorrected fluorescence metric was the magnitude of the PpIX fluorescence peak from the raw fluorescence spectrum, *i.e.* $F_{x,m}$ at 635 nm (refer to Eq. (3.4)). Note that the three qF probe measurements at each site were used separately to calculate the ROC curve (rather than averaging the triplicate measurements at each site).
As well, ROC analysis was performed for the above pathologies using linear discriminant analysis. In other words, multiple variables were used to attempt to separate the normal and tumor classes to the maximum extent. Since the “training” data set used to train the LDA cannot be the same as the “validation” data set used to evaluate the LDA using ROC analysis, a cross-validation algorithm was set up to assess the performance statistics. The cross-validation scheme that was used was repeated random sub-sampling validation [6]. Essentially, half of the data set is randomly sampled and assigned as the training data set to train the LDA. The remaining half is used for validation using ROC analysis. The process was repeated several times and the performance statistics were averaged. In this work, the random sampling process was run 50 times. Note that the measurement sites were randomized, not the measurements themselves (recall that there are 3x more measurements than measurement sites due to triplicate measurements taken at each site). The low number of patients in this study (see Table 4.1) does hamper the interpretation of the LDA results—ideally, the patients would be randomized, rather than the measurement sites given a larger patient cohort. However, given that this clinical trial represents merely the initial “pilot-sized” portion of an ongoing effort, it is worth evaluating the performance of LDA at this point to forecast its use for future, much larger data sets.

All of the above analysis techniques were implemented in Matlab (MathWorks: Natick, MA).

4.3 Results (Part 4A)

4.3.1 In vivo probe measurements

The data fits to the reflectance and fluorescence measurements from the handheld probe during resection surgery were generally good across all tissues. Figure 4.3 and Figure 4.4 display data from a patient with an HGG and a meningioma patient. Panels (a) and (b) display normal tissue
data; (c) and (d) show tumor data. The reflectance model fits very closely to the reflectance measurements. Figure 4.5 shows quantitative fluorescence data from a meningioma patient. The tumor data show a strong PpIX signal; however, the normal tissue data show AF in the same range as the PpIX signal. These demonstrate the usefulness of accurate AF modeling. It is worth noting that the “AF signal” likely has some component of excitation light leaking into the emission band. This may be a partially confounding factor in using the autofluorescence for diagnostic purposes; however, if the goal is AF/excitation leakage subtraction from the desired PpIX signal, this is less critical.

![Figure 4.3](image)

Figure 4.3: (a,c) In vivo tissue optical properties spectra and (b,d) reflectance data with model fit. Panels (a) and (b) are from measurements in normal brain parenchyma; panels (c) and (d) are from measurements in a high-grade glioma.
Figure 4.4: (a,c) *In vivo* tissue optical properties spectra and (b,d) reflectance data with model fit. Panels (a) and (b) are from measurements in normal dura mater; panels (c) and (d) are from measurements in a meningioma.

- Panel (a): $\text{StO}_2 = 66.3\%$, $f\text{Hb} = 0.5 \text{ g/L}$
- Panel (b): $R^2 = 0.910$
- Panel (c): $\text{StO}_2 = 35.7\%$, $f\text{Hb} = 2.7 \text{ g/L}$
- Panel (d): $R^2 = 0.987$
Figure 4.5: *In vivo* fluorescence data as measured by the handheld probe from a patient with a meningioma. Panels (a) and (b) show data from the meningioma; panels (b) and (d) show data from normal dura. (a) and (c) show the quantitative fluorescence measurement and model fit; (b) and (d) display the spectrally unmixed signals.\(^5\)

Figure 4.6 exemplifies the additional sensitivity to fluorescence that is afforded by the probe as compared to the fluorescence microscope during tumor resection surgery. Panels (a,b) display fluorescence images from two sites in the same patient that were both histologically confirmed as tumor. The first site shows visible fluorescence, with the surgeon scoring that site as a ‘2’. This was confirmed by the probe measurement with a signal of 12.9 µg/mL PpIX. However, the

\(^5\) Note that the scale units for quantitative fluorescence is in \(\text{nm}^{-1}\text{cm}^{-1}\); recall from Chapter 3 that these units arise from the multiplication between fluorophore absorption coefficient (\(\mu_{af,x}\) in \text{cm}^{-1}\)) and the quantum yield (when represented as a wavelength distribution, the units are \(\text{nm}^{-1}\) since the integration over all wavelengths is the dimensionless quantum yield).
second site (panels (b,d)), measured after the visible tumor from the first site was removed, has no visible fluorescence (surgeon’s score of ‘0’) yet there is a measureable PpIX concentration of 0.36 µg/mL.

![Image](image_url)

**Figure 4.6:** Fluorescence images (a,b) and corresponding quantitative fluorescence spectra (c,d) from a low-grade glioma patient during tumor resection surgery. The first site (a,c) expressed visible fluorescence via the operating microscope. The second site (which was examined after the tumorous areas of the first site were removed) (b,d) had no visible fluorescence. Probe measurement sites are indicated by the white arrows; also, the probe shaft is visible at the point of contact. Both sites had measureable PpIX concentrations via the fiberoptic probe, with the first site having [PpIX]=12.9 µg/mL and the second site with [PpIX]=0.36 µg/mL. Both sites were histologically confirmed as tumor.

Table 4.3 displays PpIX concentrations measured *in vivo* by the probe in normal and tumor tissues for the various pathologies. The tumor-to-normal (T/N) ratios for all pathologies are of the same order of magnitude and average out to T/N = 200. It seems that the [PpIX] in normal tissue from patients with LGGs, HGGs and metastases are approximately the same, although the
[PpIX] in normal tissue from HGG patients is slightly elevated. Recall that the control measurements for LGG, HGG and metastases patients were all in normal brain tissue; however, the control sites for meningioma patients were on the dura mater. The [PpIX] levels in dura mater were higher than in normal brain tissue.

Table 4.3: Comparison of in vivo [PpIX] levels found in normal and tumor tissue for each of the pathologies under study. The ± appendages denote standard deviations.

<table>
<thead>
<tr>
<th></th>
<th>[PpIX] (µg/mL)</th>
<th>[PpIX] (µg/mL)</th>
<th>[PpIX] T/N ratio (based on averages)6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal tissue</td>
<td>Tumor tissue</td>
<td></td>
</tr>
<tr>
<td>All tumors</td>
<td>0.008 ± 0.018</td>
<td>1.27 ± 3.78</td>
<td>155</td>
</tr>
<tr>
<td>LGGs</td>
<td>0.004 ± 0.002</td>
<td>0.98 ± 3.22</td>
<td>266</td>
</tr>
<tr>
<td>HGGs</td>
<td>0.007 ± 0.007</td>
<td>1.48 ± 5.56</td>
<td>225</td>
</tr>
<tr>
<td>Meningioma</td>
<td>0.014 ± 0.028</td>
<td>1.59 ± 2.89</td>
<td>116</td>
</tr>
<tr>
<td>Metastasis</td>
<td>0.003 ± 0.005</td>
<td>0.66 ± 0.96</td>
<td>222</td>
</tr>
</tbody>
</table>

Fifteen (15) optical parameters were tested as to their statistical significance in differentiating between normal and tumor tissue in vivo (Table 4.4). The range of significance levels used to reject the null hypothesis was \( p<0.05 \). This analysis was performed for the glioma data set and the all-tumors data set. Partly based on this, [PpIX], AF at 600 nm, the reflectance at both fiber separations at 600 nm, \( StO_2 \) and \( f_{Hb} \) were used for the multi-variable linear discriminant analysis.

There are physiological and photochemical reasons for these selections as well. [PpIX] is of course the chief tumor biomarker. Autofluorescence has been shown to be tumor-specific [1,2], and the autofluorescence at 600 nm (i.e. shortest wavelength available to us in our fluorescence data collection range) is likely to have the strongest AF signal, since AF peaks in the green region of the spectrum. Figure 4.7 shows cluster plots (for the all-tumors data set and the HGG

6 Standard deviations for the T/N ratio were difficult to calculate, since a large number of normal tissue data had [PpIX]=0. Also, the [PpIX] distributions were non-Gaussian and non-uniform, so it was not feasible to use, say, a Cauchy distribution to calculate the standard deviations. Because stating T/N standard deviations would be ambiguous at best, here they are not presented.
data set) with AF on the y-axis and [PpIX] on the x-axis, demonstrating (at least qualitatively, by eye) that autofluorescence can aid in separating the normal and tumor data clusters. Reflectance values at 600 nm are likely to be dominated by scattering, which in turn are affected by cell organelle size and morphology. Using reflectance measurements at the two fiber separations potentially contain encoded information on not only the reduced scattering coefficient, but also the scattering phase function, as per the argument in Section 2.6. Finally, $f_{\text{Hb}}$ and $S\text{tO}_2$ have been shown to be diagnostically useful in distinguishing between glioma and normal tissues [7].

Figure 4.7: Cluster plots of the in vivo quantitative autofluorescence (AF) on the ordinate and [PpIX] on the abscissa. (a) All tumors; (b) high-grade gliomas.
Table 4.4: List of diagnostic variables that were tested for significance in differentiating between normal and tumor tissue. \( h \) denotes if the null hypothesis has been disproved (‘1’ indicating null hypothesis is not true, ‘0’ indicating it is true).

<table>
<thead>
<tr>
<th>Diagnostic variable</th>
<th>Using \textit{in vivo} glioma data</th>
<th>Using \textit{in vivo} all-tumors data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( p )</td>
<td>( h ) ((h=1 \text{ if } p&lt;0.05))</td>
</tr>
<tr>
<td>[PpIX] (( \mu g/mL ))</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>AF (( \lambda=600 \text{ nm} ) (( \text{nm}^{-1} \text{ cm}^{-1} )))</td>
<td>0.346</td>
<td>0</td>
</tr>
<tr>
<td>AF (( \lambda=635 \text{ nm} ) (( \text{nm}^{-1} \text{ cm}^{-1} )))</td>
<td>0.974</td>
<td>0</td>
</tr>
<tr>
<td>AF (( \lambda=650 \text{ nm} ) (( \text{nm}^{-1} \text{ cm}^{-1} )))</td>
<td>0.792</td>
<td>0</td>
</tr>
<tr>
<td>AF (( \lambda=700 \text{ nm} ) (( \text{nm}^{-1} \text{ cm}^{-1} )))</td>
<td>0.445</td>
<td>0</td>
</tr>
<tr>
<td>( R (r=260 \mu \text{m}, \lambda=575 \text{ nm}) (\text{cm}^{-2}) ))</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>( R (r=260 \mu \text{m}, \lambda=600 \text{ nm}) (\text{cm}^{-2}) ))</td>
<td>0.039</td>
<td>1</td>
</tr>
<tr>
<td>( R (r=520 \mu \text{m}, \lambda=575 \text{ nm}) (\text{cm}^{-2}) ))</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>( R (r=520 \mu \text{m}, \lambda=600 \text{ nm}) (\text{cm}^{-2}) ))</td>
<td>0.003</td>
<td>1</td>
</tr>
<tr>
<td>( \mu_s (\lambda=575 \text{ nm}) (\text{cm}^{-1}) ))</td>
<td>0.002</td>
<td>1</td>
</tr>
<tr>
<td>( \mu_s (\lambda=600 \text{ nm}) (\text{cm}^{-1}) ))</td>
<td>0.045</td>
<td>1</td>
</tr>
<tr>
<td>( \mu_r (\lambda=575 \text{ nm}) (\text{cm}^{-1}) ))</td>
<td>0.251</td>
<td>0</td>
</tr>
<tr>
<td>( \mu_r (\lambda=600 \text{ nm}) (\text{cm}^{-1}) ))</td>
<td>0.245</td>
<td>0</td>
</tr>
<tr>
<td>\textit{StO2} )</td>
<td>0.002</td>
<td>1</td>
</tr>
<tr>
<td>( f_{\text{Hb}} ) (g/L) )</td>
<td>0.002</td>
<td>1</td>
</tr>
</tbody>
</table>

4.3.2 \textit{ROC analysis of the in vivo probe data}

Figure 4.8 displays the ROC curves for (a) the \textit{in vivo} all-tumors data set and (b) the \textit{in vivo} HGG data set. Interestingly, the raw uncorrected fluorescence measurement at the 635 nm PpIX peak performed the worst. The surgeon’s subjective fluorescence scoring was better than the raw fluorescence metric. Note that the surgeon, Dr. Roberts, has much clinical experience with ALA-PpIX FGR, so that this result may be unrepresentative. However, the quantitative [PpIX] metric performed better than the surgeon’s scoring, with the LDA model improving the tumor detection accuracy. Tables 4.5-4.9 displays the ROC area under the curve (a.u.c.) data, sensitivity and specificity values for the \textit{in vivo} all-tumors, HGG, LGG, metastasis and meningioma data sets, respectively. For all tissue types with the exception of metastatic tumors, the general trend holds that the order of increasing performance is: raw fluorescence at 635 nm, surgeon’s score, [PpIX] and then LDA. For the lung metastatic tumor data, the ROC area-under-curve is slightly higher.
for [PpIX] than the LDA classifier, though both these have better performance than the surgeon’s score and raw fluorescence metric. The baseline characteristics (i.e. number of normal and tumor sites and the number of patients for each pathology) for the following ROC figures and tables are listed in Table 4.1.

![ROC curves for tumors and high-grade gliomas.](image)

**Figure 4.8:** ROC curves for (a) all tumors; (b) high-grade gliomas. The diagnostic variables considered were: the surgeon’s visible fluorescence score as determined through the Zeiss microscope; the PpIX peak (635 nm) fluorescence magnitude from the raw, uncorrected fluorescence spectrum; PpIX concentration; metric derived from linear discriminant analysis.

**Table 4.5:** ROC data from *in vivo* all-tumors and normal tissue measurements. (s.e.) is the standard error and a.u.c. is the area under the curve.

<table>
<thead>
<tr>
<th></th>
<th>ROC a.u.c. (s.e.)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon’s visible fluorescence score</td>
<td>0.734 (0.029)</td>
<td>58.6</td>
<td>78.0</td>
</tr>
<tr>
<td>Raw fluorescence spectroscopy @ 635 nm</td>
<td>0.571 (0.035)</td>
<td>51.6</td>
<td>84.8</td>
</tr>
<tr>
<td>[PpIX] (µg/mL)</td>
<td>0.880 (0.019)</td>
<td>79.7</td>
<td>86.9</td>
</tr>
<tr>
<td>Linear discriminant analysis</td>
<td>0.922 (0.022)</td>
<td>87.1</td>
<td>89.1</td>
</tr>
</tbody>
</table>
Table 4.6: ROC in vivo data from high-grade gliomas.

<table>
<thead>
<tr>
<th>Method</th>
<th>ROC a.u.c. (s.e.)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon’s visible fluorescence score</td>
<td>0.775 (0.051)</td>
<td>62.6</td>
<td>83.1</td>
</tr>
<tr>
<td>Raw fluorescence spectroscopy @ 635 nm</td>
<td>0.577 (0.052)</td>
<td>55.0</td>
<td>98.3</td>
</tr>
<tr>
<td>[PpIX] (µg/mL)</td>
<td>0.945 (0.022)</td>
<td>88.3</td>
<td>93.3</td>
</tr>
<tr>
<td>Linear discriminant analysis</td>
<td>0.967 (0.016)</td>
<td>92.8</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Table 4.7: ROC in vivo data from low-grade gliomas.

<table>
<thead>
<tr>
<th>Method</th>
<th>ROC a.u.c. (s.e.)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon’s visible fluorescence score</td>
<td>0.542 (0.095)</td>
<td>50.2</td>
<td>54.3</td>
</tr>
<tr>
<td>Raw fluorescence spectroscopy @ 635 nm</td>
<td>0.247 (0.049)</td>
<td>22.2</td>
<td>73.3</td>
</tr>
<tr>
<td>[PpIX] (µg/mL)</td>
<td>0.651 (0.059)</td>
<td>47.2</td>
<td>93.3</td>
</tr>
<tr>
<td>Linear discriminant analysis</td>
<td>0.800 (0.068)</td>
<td>77.4</td>
<td>89.0</td>
</tr>
</tbody>
</table>

Table 4.8: ROC in vivo data from intracranial lung metastasis tumors.

<table>
<thead>
<tr>
<th>Method</th>
<th>ROC a.u.c. (s.e.)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon’s visible fluorescence score</td>
<td>0.750 (0.062)</td>
<td>60.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Raw fluorescence spectroscopy @ 635 nm</td>
<td>0.664 (0.070)</td>
<td>66.7</td>
<td>95.8</td>
</tr>
<tr>
<td>[PpIX] (µg/mL)</td>
<td>0.984 (0.016)</td>
<td>91.7</td>
<td>95.8</td>
</tr>
<tr>
<td>Linear discriminant analysis</td>
<td>0.978 (0.050)</td>
<td>95.9</td>
<td>97.3</td>
</tr>
</tbody>
</table>

Table 4.9: ROC in vivo data from meningiomas.

<table>
<thead>
<tr>
<th>Method</th>
<th>ROC a.u.c. (s.e.)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon’s visible fluorescence score</td>
<td>0.800 (0.043)</td>
<td>65.5</td>
<td>86.2</td>
</tr>
<tr>
<td>Raw fluorescence spectroscopy @ 635 nm</td>
<td>0.694 (0.053)</td>
<td>58.3</td>
<td>100.0</td>
</tr>
<tr>
<td>[PpIX] (µg/mL)</td>
<td>0.898 (0.031)</td>
<td>78.3</td>
<td>87.2</td>
</tr>
<tr>
<td>Linear discriminant analysis</td>
<td>0.947 (0.057)</td>
<td>91.9</td>
<td>92.2</td>
</tr>
</tbody>
</table>
Part 4B: *Ex vivo* tissue solubilisation fluorometry of tumor biopsies obtained during brain tumor resection surgery

4.4 Materials and methods (Part 4B)

4.4.1 Acquisition of *ex vivo* biopsy specimens

Prior to the deployment of the quantitative fluorescence probe in neurosurgical trials, the best way to obtain qF data was via tissue solubilisation fluorometry of excised biopsies. As in the *in vivo* study, ALA at 20 mg/kg of bodyweight was given to the patients approximately 3 hours prior to anesthesia. Biopsies were extracted for H&E histology, IHC and *ex vivo* tissue solubilisation fluorometry. This *ex vivo* fluorometry was a modified protocol based on the tissue solubilisation protocol used in Chapter 3. Twenty-two (22) patients were enrolled for this part of the study. Baseline characteristics for these patients are shown in Table 4.10. *Ex vivo* fluorometry data that are correlated with *in vivo* probe measurements (14 patients, see Table 4.1) are also tabled for analysis in the near future, though as of yet these have not been processed.

4.4.2 Tissue solubilisation fluorometry

The tissue solubilisation protocol for measurement of absolute fluorophore concentration was developed by Lilge *et al.* [5]. The protocol was modified such that the overall dilution factor of the solubilised tissue was less than in the original, since the tissue biopsy weights were approximately an order of magnitude smaller than the original prescribed weight range of 100-400 mg. Each tissue sample was combined with 1 mL of Solvable and placed in a water bath at 50°C for 1 hour. The tissue/Solvable solution was homogenized with a Tissue Tearor tool (Biospec Products, Bartlesville, OK, USA). 500 μL of the tissue homogenate was combined with 1.125 mL of distilled water and 0.375 mL of Solvable. This solution was incubated in the water
bath at 50°C for 1 h. The optical density was measured and diluted down to <0.1 if necessary. The resulting solution was transferred to a quartz cuvette. The cuvette was analyzed by a fluorometer (Fluorolog: Jobin Yvon, Edison, NJ), using an excitation wavelength of 401 nm, and the PpIX concentration was calculated based on a known standard solution.

As of this writing, ex vivo fluorometry data has been collected for 45 biopsies from 5 high-grade glioma patients. This ex vivo data supplements the observations and conclusions from the in vivo data acquired by the quantitative fluorescence probe during surgery. Specifically, the [PpIX] distribution of the ex vivo data set was compared with the in vivo data set to determine if the quantitative fluorescence probe is measuring [PpIX] within an expected physiological range.

Table 4.10: Baseline characteristics for the 22 patients in the ex vivo fluorometry study. As of this writing, measurements and data processing of 5 HGG patients were completed.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Patients</th>
<th># of biopsy sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG-I</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>HGG-III</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>HGG-IV</td>
<td>15</td>
<td>173</td>
</tr>
</tbody>
</table>

4.5 Results (Part 4B)

4.5.1 Comparison between in vivo probe measurements and ex vivo tissue solubilization fluorometry

As mentioned before, ex vivo tissue solubilisation data currently exist for 5 high-grade glioma patients. The distribution of [PpIX] from the ex vivo data is comparable to the in vivo probe measurements in high-grade glioma, as shown in Figure 4.9. As well, the difference between ex vivo and in vivo [PpIX] distributions is not statistically significant ($p = 0.145$). Although the ex vivo and in vivo data sets have no patients in common (at least, not to date), it is encouraging that the [PpIX] distributions are similar, indicating that the in vivo qF probe is measuring [PpIX] in
the typical physiological range of high-grade glioma tissue. An interesting relationship is depicted in Figure 4.10, showing a positive trend between [PpIX] and tumor burden (i.e. percent of viable tumor cells seen in the histological section) in both in vivo and ex vivo data.

Figure 4.9: Distribution of PpIX concentrations from the ex vivo tissue solubilisation measurements for 5 patients (45 biopsies) and from the in vivo probe measurements taken from 3 patients (20 measurement sites), with all patients having high-grade glioma. The median and upper-quartile range are also shown.

Figure 4.10: [PpIX] vs. tumor burden for (a) in vivo probe measurements (all-tumors) and (b) ex vivo tissue solubilisation fluorometry. Data is represented as mean (□) and standard deviation (error bars).
4.6 Discussion and Conclusions (Parts 4A and 4B combined)

Although this chapter reports only a pilot study-sized data set, the sample size is large enough to derive several conclusions that support the overarching hypothesis of this body of work. It is very clear from these results that quantitative fluorescence measurement of PpIX concentration is superior to both non-quantitative fluorescence spectroscopy and the subjective assessment of the neurosurgeon (via the surgical microscope) for demarcating tumor margins during resection surgery. Linear discriminant analysis improves the performance of the probe’s diagnostic accuracy even further. The improvement in diagnostic accuracy that the probe provides is most marked in the high-grade glioma data set (Figure 4.8(b) and Table 4.6).

In addition to the PpIX contrast, autofluorescence has some value as a diagnostic feature. The AF signal in the range of 600 nm is slightly higher in normal tissue than in tumor, as shown in Figure 4.7 (1.5× higher in the glioma data set)—this trend is consistent with the autofluorescence studies of Toms et al. in glioma [2]. If this bears out in the future with larger sample populations, autofluorescence would seem to be a confounding factor for fluorescence imaging using, for example, the Zeiss surgical microscope. If tumor-positive PpIX is high and AF is low in neoplastic tissue (and vice-versa in normal tissue), the summation of both signals (as in fluorescence imaging) will be less tumor-specific than if the AF and PpIX signals are separated.

Table 4.3 demonstrates some interesting trends concerning PpIX accumulation in normal tissue that may have a physiological explanation. [PpIX] in the normal tissue of LGG patients is slightly lower than in HGG patients. This may be explained by the fact that the blood-brain barrier in LGG patients is relatively intact compared with HGG patients, where the BBB is often breached due to unchecked malignant tumor growth. ALA, as many drugs, has difficulty penetrating the BBB [10], resulting in lower PpIX synthesis in the brain tissue (both normal and
tumor tissue) of LGG patients. Among normal tissue populations, [PpIX] is highest in normal
dura mater. This may be because there is no blood-brain barrier in the dura mater proper and the
dura is richly vascularized [11], allowing ALA delivery and hence PpIX build-up in the dural
tissues. This may also explain why meningioma has the largest average PpIX uptake in this
study. The fact that [PpIX] in normal tissue is dependent on the patient’s disease state may be
diagnostically relevant. The optimal [PpIX] threshold for separating normal and tumor states
may be different for different tumor pathologies. It is encouraging to see that a universal
threshold is practical across all-tumors pathologies in this study, as exemplified by Figure 4.8(a)
and Table 4.5. A universal [PpIX] threshold is clinically desirable because, as any
neuropathologist will dictate, the pathology cannot be determined with certainty unless it is
histologically (i.e. post-operatively) confirmed. On the other hand, prior to surgery the
neurosurgeon generally has a very good idea as to the grade and type of tumor, based on
information from pre-operative imaging and clinical history, so tailoring the diagnostic algorithm
to pre-operative information may be feasible.

One interesting finding from both the in vivo and ex vivo data sets was that PpIX may be
photobleached by the surgical microscope illumination. Dysart et al. identified and measured the
spectral profiles of three photoproducts of PpIX: photoprotoporphyrin (Ppp, 675 nm peak),
Product II (658 nm peak) and Product III (620 nm peak) –these last two are to date putative,
molecularly unidentified, photoproducts [12]. The spectral peaks of Product III and Ppp were
noted in a small fraction of the ex vivo and in vivo measurements, as exemplified in Figure 4.11.
Typically, when photoproducts were detected, the samples had low concentrations of PpIX
(<0.01 µg/mL). This opens up the potential for using the handheld probe as a monitoring device
for PpIX-mediated photodynamic therapy, \textit{i.e.} increasing relative contributions of PpIX photoproducts may be used as a surrogate metric for explicit PDT dosimetry.

Figure 4.11: Photoproducts of PpIX found in (a) \textit{ex vivo} tissue solubilisation fluorometry and (b) \textit{in vivo} probe measurements. Both data were taken in glioma (different patients). Panel (c) displays PpIX and its photoproducts (all spectra peak-normalized).

As mentioned in the chapter introduction, a direct comparison between \textit{in vivo} probe measurements and \textit{ex vivo} tissue solubilisation fluorometry will be executed in the second half of
2010. It bodes well for this future study that, in the present data set, *in vivo* and *ex vivo* [PpIX] distributions (1.48 ± 5.56 and 1.66 ± 3.40 µg/mL, respectively) are similar for high-grade glioma. As well, both *in vivo* and *ex vivo* data sets demonstrate increasing PpIX expression with increased tumor infiltration. This is perfectly logical and expected, though it is encouraging to see the data bear out that a higher density of tumor cells is correlated to elevated tumor biomarker.

ALA-PpIX brain tumor time kinetics are not known for humans. Anecdotally, the standard protocol is 3 hours prior to induction of anesthesia [8]; however, this may not be optimal. Moreover, in the course of a procedure that is several hours long, there is no doubt that the PpIX fluorescence and tumor specificity fade over time due to completion of heme formation, passive transport, bleeding, edema and photobleaching. The time kinetics of the PpIX signal is definitely relevant to this work: for the 14 patients in the *in vivo* study, the span of time between the first probe measurement and the last during a given procedure spanned from 0.5 – 3.4 hours. The long duration of some of these surgeries definitely impacted diagnostic performance in this study: when taking into account only the first hour of probe measurements in high-grade glioma, for example, [PpIX] has an ROC a.u.c. of 0.96, sensitivity of 90.3% and specificity of 100%, a large improvement over using the data set throughout the entire procedure (see Table 4.6). It may be that tightly controlling the delivery time of ALA is the key to increasing the diagnostic performance of PpIX even more, perhaps through the intravenous route. For the ongoing Toronto-Dartmouth study, and given a large enough data set, there is the possibility of calculating the receiver-operating characteristic for specific time intervals after ALA administration (perhaps in bins of 0.5 or 1 hour). This would yield time-correlated (*i.e.*
longitudinal) tumor sensitivity and specificity plots and, at least partially, answer the question of the optimal time-after-dose for maximum tumor-to-normal contrast.

This data show that there are in fact measurable PpIX levels in low-grade glioma, contrary to other published findings and opinions [1,2,8,9]. PpIX expression, tumor sensitivity and specificity are lower in low-grade gliomas than in high-grade disease (Tables 4.3, 4.6 and 4.7); however, there is definitely diagnostic value in [PpIX]. Using [PpIX] in addition to other diagnostic variables (e.g. StO₂, fHb and AF) brings the tumor sensitivity and specificity for LGGs up to a practical level. The issue of the unknown ALA time kinetics in glioma may actually have had a large impact on the quality of the LGG data: for one of the two LGG patients in this study, probe data were acquired over a time span of 3.4 h, a long enough time anecdotally to suspect PpIX fluorescence drop-off.

The feedback from the neurosurgeon (D.W.R.) on the practicality and ease-of-use of the fiberoptic probe was very positive. The ability to use the probe under fluorescence lighting (rather than in complete darkness) was a large advantage. The integration of the handheld probe in the overall workflow was seamless, as the probe simply lay on the sterile tool area when not in use, did not interfere with the surgical microscope, and took only seconds to position, with the measurements themselves being essentially instantaneous. The probe was easily washable and sterilizable via the STERRAD process with minimal visible damage.

This chapter demonstrates the culmination of the device development of the handheld fiberoptic probe outlined in the previous two chapters. The clinical deployment of the probe device has been successful, showing a marked improvement in utilizing the PpIX biomarker compared with state-of-the-art neurosurgical fluorescence microscopy. The data shown in this
chapter represent a “pilot study”, the start of a larger, ongoing effort. These initial results are, however, encouraging for continuing systematic studies in quantitative fluorescence.

4.7 Statement of contributions

The development of the clinical protocol concerning the qF probe system and the clinical certification of the equipment was accomplished by myself, Pablo Valdes, Frederic LeBlond and Keith D. Paulsen (all of Dartmouth College, Hanover, NH). Neurosurgery was performed by Dr. David W. Roberts and histopathological analysis was done by Dr. Brent T. Harris. All data analysis and algorithms in this chapter are original to this thesis, and were done by me.

4.8 References


5 Depth-resolved topographic mapping of sub-surface fluorescent structures in tissue using multi-wavelength excitation

We now switch gears and turn to the other problem that this thesis tackles, the depth-resolved fluorescence problem. The brain is a unique surgical site for tumor resection in that healthy tissue must be spared as much as possible while maximizing tumor cytoreduction. There is a clear need for a non-invasive, intraoperative method to localize tumor cell nests buried at depth. This chapter demonstrates the development-to-date of a technique called sub-surface fluorescence topography, aimed at depth-resolved mapping of PpIX-expressing tumor cells.

Different colors of visible light penetrate to varying depths in tissue due to the wavelength-dependence of tissue optical absorption and elastic scattering. We exploit this to map the contour of the closest surface of a buried fluorescent object. This uses a novel algorithm based on the diffusion theory description of light propagation in tissue at each excitation wavelength to derive metrics that define the depth of the top surface of the object. The algorithm was validated using a tissue-simulating phantom. It was then demonstrated in vivo by sub-surface brain tumor topography in a rodent model, using the fluorescence signal from protoporphyrin IX (PpIX) that is preferentially synthesized within malignant cells following systemic application of aminolevulinic acid (ALA). Comparisons with histomorphometry in the brain post mortem show the spatial accuracy of the technique. This method has potential for fluorescence image-guided tumor surgery, as well as other biomedical and non-biological applications in sub-surface sensing. The following is adapted from a journal article accepted by the Journal of Biomedical Optics, currently undergoing final revisions. Since this method is novel in the field of depth-
resolved fluorescence, our group has also filed a provisional patent application (Patent pending # US 61,287,997) for the sub-surface fluorescence topography method.

5.1 Introduction and Background

The clinical management of malignant glioma continues to be a challenge. Even with the best available treatment options (surgical resection, radiotherapy and chemotherapy), the prognosis is grim. For glioblastoma multiforme, the most virulent form of this disease, the median survival is still only ~1 year, with little chance for long-term survival [1]. Surgical resection of the tumor mass is the first line of defense. Recent work has demonstrated a survival advantage for patients with more complete tumor resection, with >98% tumor mass removal giving a relative survival advantage of 4.2 months compared with patients with a resection <98% [2]. While not large in absolute terms, this does represent clinically significant improvement. However, such complete resection is usually not achieved by standard operative approaches, because of the difficulty of directly visualizing the residual tumor tissue. Aggressive resection at the tumor margin must be balanced by the increased risk of causing neurological deficit, underlining the need for better residual tumor detection.

Intraoperative fluorescence imaging is a potential solution to enhance localization of residual tumor in the resection cavity that is occult under white light. This could be done using tissue autofluorescence (steady-state or time-resolved) [3] or by use of an exogenous contrast agent. The latter, using protoporphyrin IX (PpIX) fluorescence that is endogenously synthesized by systemic administration of 5-aminolevulinic acid (ALA), a precursor in heme biosynthesis, is the most clinically advanced. Intraoperative imaging of ALA-PpIX fluorescence has been shown recently by Stummer and colleagues in multicenter clinical trials to provide survival advantage
when incorporated into an intraoperative microscope [4]. However, challenges remain in assessing the fluorescence quantitatively and objectively, and in detecting and assessing tumor fluorescence that is lying below the resection surface. The latter is the focus of the present work.

Various methods have been proposed to solve the general depth-resolved fluorescence problem in optically-turbid media such as tissue. Point detection methods have been developed, such as work by Swartling et al. using the depth-dependent distortion of the fluorescence emission spectrum by tissue absorption [5] or the work of Hyde et al. using spatially-resolved diffuse fluorescence to determine depth [6]. Since there are obvious limitations to point detection methods in a surgical field, wide-field methods have also been pursued. A method forwarded by Comsa et al. has the diffuse fluorescence imaged using broad-beam illumination, with the modeling assumption that the fluorescence source is point-like [7]. Laminar optical tomography has also been developed for full 3D reconstruction, where a laser is raster-scanned over the tissue surface and the fluorescence pattern imaged at each position [8,9].

A major challenge in optical tomography is that it is generally an ill-posed problem [10]. Concerning fluorescence tomography, there are many innovative approaches to constrain the inverse image reconstruction problem in both small animal and clinical imaging. These constraints are largely based on multiple projections of the fluorescence [11], with additional constraints provided by multiple excitation wavelengths and/or multi-spectral emission imaging wavelengths [12,13]. These techniques may require additional information external to the optical modalities, such as anatomical reference information from CT to aid in the optical modeling [12]. Tomographic techniques such as the multiple projection technique by Ntziachristos et al. are highly applicable in small animal imaging [14]; however, these techniques are not generally suitable for the restricted access that is typically available during, for example, neurosurgery.
Here, we introduce an alternative to point detection and tomographic approaches. We aim to recover topographic information of sub-surface fluorescent objects, based on imaging the tissue surface with broad-beam excitation at several wavelengths. A diffusion theory model is used to extract the depth of the upper surface of buried fluorescing tissue. The model is based on the wavelength dependence of the optical depth penetration due to the wavelength dependence of the tissue optical absorption and elastic scattering. In the example presented here, *a priori* values for the normal brain optical properties and the PpIX excitation spectrum are used as inputs.

It is important to note how this work differs from other approaches using multiple excitation wavelengths, for example, in autofluorescence-based diagnostics as in the work of Ramanujam and colleagues [15]. In those studies the objective was to extract more information from the different fluorescence excitation characteristics of the endogenous fluorophores. By comparison, the use of multiple excitation wavelengths here is to spectrally encode the fluorophore depth. We note also that we are working in the red-NIR spectral range where tissue autofluorescence is weak. As well, it is important to distinguish that this technique is not intended to produce a full fluorescence tomographic solution in, say, a mouse; rather, this is a depth-detection and topographic mapping problem with the target application being surgical guidance in patients.

5.2 Theory

5.2.1 Modeling of buried fluorescence

The imaging geometry is shown in Figure 5.1. The fluorescence re-emission, $F_{x,m}$ ($x$ denotes excitation; $m$ denotes emission), is a function of the fluorophore depth $z$. Here, $F_{x,m}$ can be calculated using the normalized excitation fluence rate $\Phi_x$ (*i.e.* normalized to the incident
irradiance) and the emission escape function (dimensionless) leaving the tissue surface, $R_m$. $F_{x,m}$ is given by

$$F_{x,m}(r,z) = E_x \eta_m S_f \mu_{af,x} \phi_x(z) R_m(r,z)$$

(5.1)

where $\eta_m$ is a constant that incorporates the optical efficiency of the collection chain (camera + optics) and the emission filter transmissivity, $E_x$ is the excitation irradiance, $\mu_{af,x}$ is the fluorophore absorption coefficient at the excitation wavelength, $Q_x$ is the fluorescence quantum yield at a given excitation wavelength, and $S_f$ comprises a fluorescence source factor which is a function of the shape of the fluorescing object. The independent variable $r$ denotes spatial distribution in the $xy$ (tissue surface) plane.

![Figure 5.1: Measurement and modeling geometry for buried fluorescence](image)

Using the signal at one of the excitation wavelengths as a reference, many of the terms in Eq. (5.1) cancel out (i.e. $\eta_m$, $S_f$ and $R_m$), leaving a depth-dependent metric, which is directly proportional to the ratio between excitation fluence rates at different wavelengths. In this work, the fluorescence from the 405, 546 and 495 nm excitations (in order of decreasing effective

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Note that Kasha’s Rule states that the fluorescence quantum yield is the same regardless of the excitation wavelength; however, there are exceptions to this rule, hence the general form of $Q_x$.  

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attenuation coefficient, $\mu_{\text{eff}} = \sqrt{3\mu_a (\mu_a + \mu_s')}$, of the tissue were used as the signal and the fluorescence from the 625 nm excitation (corresponding to the lowest $\mu_{\text{eff}}$) was used as the reference, producing three fluorescence ratio metrics, $M_1 = \alpha_1 F_{405\text{nm},700\text{nm}}/F_{625\text{nm},700\text{nm}}$, $M_2 = \alpha_2 F_{546\text{nm},700\text{nm}}/F_{625\text{nm},700\text{nm}}$ and $M_3 = \alpha_3 F_{495\text{nm},700\text{nm}}/F_{625\text{nm},700\text{nm}}$, where
\begin{align*}
\alpha_1 &= \frac{(E_{625\text{nm}} \mu_{\text{af},625\text{nm}} Q_{625\text{nm}})}{(E_{405\text{nm}} \mu_{\text{af},405\text{nm}} Q_{405\text{nm}})} \\
\alpha_2 &= \frac{(E_{625\text{nm}} \mu_{\text{af},625\text{nm}} Q_{625\text{nm}})}{(E_{546\text{nm}} \mu_{\text{af},546\text{nm}} Q_{546\text{nm}})} \\
\alpha_3 &= \frac{(E_{625\text{nm}} \mu_{\text{af},625\text{nm}} Q_{625\text{nm}})}{(E_{495\text{nm}} \mu_{\text{af},495\text{nm}} Q_{495\text{nm}})}
\end{align*}
(5.2)
The normalization coefficients $\alpha_1, \alpha_2$ and $\alpha_3$ may be obtained by imaging the target fluorophore in free space ($z = 0$) at each excitation wavelength, such that $\alpha_1 = (F_{625\text{nm},700\text{nm}}/F_{405\text{nm},700\text{nm}})|_{z=0}$, etc. Note that the excitation irradiance and quantum yield terms are already lumped into the normalization coefficients, and do not need to be determined explicitly.

Analytic expressions for $\phi_x(z)$ based on diffusion theory were used as the light-transport model [16]. A general solution to the diffusion theory differential equation is:
\[ \phi_x(z) = A \exp(-\mu_{\text{eff},x} z) + B \exp(-\mu_{t,x}' z) \]
(5.3)
where $\mu_{\text{eff},x} = \sqrt{3\mu_a (\mu_a + \mu_s')}$ is the effective attenuation coefficient and $\mu_{t,x}' = \mu_{a,x} + \mu_{s,x}'$ is the total attenuation coefficient, with everything in terms of the excitation wavelength.

At the surface boundary the photon current in the $z$ direction, $J_z$, leaving the tissue is
\[ J_z = -D_x \frac{\partial \phi_x(z)}{\partial z} \]
(5.4)
The energy balance at the tissue surface is expressed as
\[ \phi_x(z=0) = -2KJ_z = 2KD_x \frac{\partial \phi_x(z=0)}{\partial z} \]
(5.5)
where

$$K = \left( \frac{1 + R_j}{1 - R_q} \right)$$  \hspace{1cm} (5.6)

and

$$R_q = \frac{1}{\pi} \int_{2\pi} \int_{2\pi} R_{\text{Fresnel}}(\theta) \cos(\theta) \, d\Omega$$ \hspace{1cm} (5.7)

$$R_j = \frac{3}{\pi} \int_{2\pi} \int_{2\pi} R_{\text{Fresnel}}(\theta) \cos^2(\theta) \, d\Omega$$

For tissue, a common value used in biomedical optics is $n_{\text{tissue}} = 1.4$ \cite{19}, resulting in $R_q = 0.529$ and $R_j = 0.389$ \cite{16}.

The boundary condition from Eq. (5.5) combined with the assumed solution Eq. (5.3) yields

$$0 = \left(1 + 2KD_{s,\mu_{\text{eff},x}} \right) A + \left(1 + 2KD_{s,\mu_{\text{eff},x}} \right) B$$  \hspace{1cm} (5.8)

Substituting the assumed solution Eq. (5.3) into the diffusion theory differential equation, we get

$$\mu_{s,x}^{'} = \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right) A + \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right) B$$ \hspace{1cm} (5.9)

The terms $A$ and $B$ result from solving the diffusion theory equation using boundary conditions at the target surface that is created due to the index mismatch between air and the tissue.

$$A = \frac{-\mu_{s,x}^{'} \left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right)}{\left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right) \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right) - \left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right) \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right)}$$ \hspace{1cm} (5.10)

$$B = \frac{\mu_{s,x}^{'} \left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right)}{\left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right) \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right) - \left( 1 + 2KD_{s,\mu_{\text{eff},x}} \right) \left( \mu_{a,x} - D_{s,\mu_{\text{eff},x}}^2 \right)}$$ \hspace{1cm} (5.11)
The diffusion theory model solution for the fluence rate at depth was compared with Monte Carlo simulations from a modified online code from Jacques [17], as shown in Figure 5.2. The absorption coefficient was varied as $\mu_a = 1, 5$ and $10 \text{ cm}^{-1}$ and the scattering coefficient held at $\mu_s' = 30 \text{ cm}^{-1}$. The phase function was modeled as a Henyey-Greenstein function with $g = 0.8$. The fluence rate as determined by diffusion theory closely matches the Monte Carlo simulations, except for a slight overestimation.

![Figure 5.2: Excitation fluence rate with respect to depth for $\mu_a = 1, 5$ and $10 \text{ cm}^{-1}$ and $\mu_s' = 30 \text{ cm}^{-1}$. Symbols are Monte Carlo simulation data and the solid lines result from the diffusion theory model.](image)

These diffusion theory-derived equations may be used to calculate the fluence rate at depth, although there are also other methods, such as Monte Carlo simulation, that may be used for this calculation. An impetus to use Monte Carlo simulations is that Monte Carlo is known to provide more accurate results closer to the tissue surface than the diffusion theory solution.

In principle, only two wavelengths are required for a depth calculation. The purpose of using multiple wavelengths is that better $xy$ spatial resolution is expected near the surface for excitation wavelengths with high tissue attenuation; however, this is at the cost of poorer depth penetration.
The multiple wavelengths were intended to span a large range of both spatial resolution and depth penetration. The algorithm applied to retrieve the depth estimate of the fluorophore (tumor) surface was as follows:

1. Use the depth estimate from $M_1$ if $M_1 > T$, else go to 2.
2. Use the depth estimate from $M_2$ if $M_2 > T$, else go to 3.
3. Use the depth estimate from $M_3$ if $M_3 > T$, else no depth estimate available.

The threshold, $T$, was arbitrarily selected as 0.05 as the cut-off point. The absorption and reduced scattering coefficient spectra of rat cortical brain tissue, $\mu_a$ and $\mu'_s$ respectively, were measured \textit{ex vivo} using a diffuse reflectance fiberoptic probe, the details of which have been reported previously [18,19] and was the subject of Chapter 2 of this thesis. Figure 5.3 displays these tissue spectra, together with the absorption and fluorescence emission spectrum of PpIX (at 405 nm excitation), as well as the excitation and emission bands used for imaging.

5.3 Materials and methods

5.3.1 Imaging system

The experimental setup consists of an epifluorescence microscope (MZ FLIII: Leica, Richmond Hill, ON, Canada) custom retrofitted with a 12-channel filter wheel (AB304T: Spectral Products, Putnam, CT, USA) that filters a mercury arc lamp white light source (X-Cite 120: Exfo, Quebec, QC, Canada) for the fluorescence excitation. Excitation bandpass filters (Chroma, Rockingham, VT, USA) were mounted in the filter wheel, with central wavelengths at 405, 495, 546 and 625 nm (full-width at half-maximum (FWHM) of 20, 32, 28 and 47 nm, respectively), corresponding approximately to PpIX absorption peaks, as shown in Figure 5.3. The excitation power ranged from 3-11 mW, over a 1 cm Gaussian spot (FWHM) at the tissue surface (variations are due to
changes in the output source as well as varying excitation filter transmissivities). A cooled CCD camera (CoolSnap K4: Photometrics, Tucson, AZ, USA) mounted on the microscope eyepiece served to image the fluorescence emission. A corresponding white light image was also taken for anatomical reference. A 700 nm bandpass (50 nm bandwidth FWHM) filter was used in front of the camera to block the excitation light and pass the PpIX fluorescence. A custom software module (Labview v.7.1: National Instruments, Austin, TX, USA) was developed to select the excitation filters and acquire the corresponding images.

![Figure 5.3: Average \( N_{\text{animals}} = 5, N_{\text{measurements}} = 25 \) \( \mu_a \) and \( \mu_s' \) spectra of ex vivo rat brain tissue, measured at the cerebral cortex. The absorption spectra and normalized emission spectra of PpIX and the 4 excitation and single emission filter bands are overlaid.](image)

5.3.2 Phantom experiment

The validity of the above model was tested in a tissue-simulating phantom. A 1.15 mm inner diameter (0.20 mm wall thickness) glass capillary tube was filled with PpIX (Sigma-Aldrich, Oakville, ON, Canada) in dimethyl sulfoxide at a concentration of 35 \( \mu \text{M} \). A liquid phantom was formulated with Intralipid (Fresenius Kabi, Germany) to model tissue scattering, fresh rodent
blood to simulate tissue absorption, and distilled water. The optical properties of this phantom, listed in Table 5.1, were measured using the diffuse reflectance probe of Chapter 1[18], and adjusted to match approximately the rat brain tissue data. The capillary tube was placed in a large container (4× the diameter of the excitation beam spot) and liquid phantom material was added to vary the depth overlying the capillary tube in 0.25 mm increments. An in-house swept-source optical coherence tomography (OCT) system [20] was used to obtain a reference depth of the capillary tube at the start of the experiment. The OCT system operated with axial and transverse resolutions of 7 and 18 µm, respectively, and a field of view set to 3 mm in the depth (axial) direction and 5 mm in the transverse direction. Multispectral excitation images were taken at each depth increment (measurements below ~1mm were difficult to obtain due to surface tension effects around the thin capillary tube).

Table 5.1: Optical properties of the Intralipid phantom

<table>
<thead>
<tr>
<th></th>
<th>405 nm</th>
<th>495 nm</th>
<th>546 nm</th>
<th>625 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu_{a,x}) (cm(^{-1}))</td>
<td>37.7</td>
<td>2.2</td>
<td>5.8</td>
<td>0.12</td>
</tr>
<tr>
<td>(\mu_{s,x}') (cm(^{-1}))</td>
<td>20.1</td>
<td>16.5</td>
<td>15.5</td>
<td>13.5</td>
</tr>
</tbody>
</table>

5.3.3  *In vivo experiment using a rat brain tumor model*

In order to test the technique and algorithm *in vivo*, female Lewis rats (Charles River, QC, Canada) were used, under institutional ethics approval (University Health Network, Toronto). For tumor induction, the animal was placed under 4% isoflurane anesthesia (oxygen flow at 2 L/min), induced in a chamber and sustained by an injection of ketamine/xyazine (80/13 mg/kg, intraperitoneal (*i.p.*)). The eyes were lubricated with tear gel and the animal was placed on a warming blanket. The scalp was be shaved and disinfected with betadene and isoproponol prior to performing a 1.5 cm incision along the midline. The skull was carefully exposed and a 1 mm
burr hole was made in the left hemisphere, 3 mm posterior to the bregma and 2 mm to the left of the sagittal suture, exposing the dura but leaving it intact. Sub-surface intracranial brain tumors were induced by injection of $1.5 \times 10^5$ CNS-1-GFP cells (transfected with the green fluorescent protein gene) in 5 µL of RPMI 1640 media (Sigma-Aldrich) through the burr hole, at a depth of 2 mm below the dura using a 26G Hamilton syringe. Tumors were allowed to grow for 7-10 days.

For fluorescence topographic imaging, on the day of surgery, ALA (Sigma-Aldrich) was injected i.p. at 100 mg/kg, with imaging scheduled 3.5-4 hours later. The animal was brought under general anesthesia with 4% isoflurane (oxygen flow at 2 L/min) and sustained by an injection of ketamine/xylazine (80/13 mg/kg, i.p.), and the eyes lubricated with tear gel. The scalp was reflected and a 1 cm craniotomy was performed, exposing both hemispheres, including the original tumor cell injection site. The dura was cut and removed, exposing the cortical surface. Multispectral excitation images were taken, with each image integrated over typically 10 sec. Subsequently, after sacrifice by anesthetic overdose, the brain was removed intact, fixed in formalin and coronal hematoxylin and eosin (H&E)-stained histology sections were taken. GFP cells were used with the aim to confirm the depth of the tumor upon histological sectioning, but this was not done here for technical reasons. GFP interference into the emission band was negligible: the PpIX signal at 700 nm was ~50× higher than that of GFP in in vitro tests.

5.4 Results

5.4.1 Phantom experiment

The fluorescence intensity measured at the phantom surface is plotted in Figure 5.4(a) as a function of capillary tube depth (as measured from the top of the capillary tube). The metrics $M_1$, $M_2$,
$M_2$ and $M_3$ are plotted in Figure 5.4(b) versus capillary tube depth, together with the diffusion theory model predictions. The depth of the capillary tube estimated using the above algorithm, with the phantom optical properties at each wavelength used as input, is shown in Figure 5.4(c). There was excellent agreement ($R^2 = 0.918$ for linear regression) up to a depth of 3 mm. The model fails at depths > 3 mm, in part due to loss of fluorescence signal, but also due to the flattening of the $M_1$, $M_2$ and $M_3$ curves with increasing depth: in Figure 5.4(a), the poor depth estimate recovered from $M_3 < 0.05$ is shown (rather than discarded) in order to demonstrate how the depth estimate behaves at a very low signal. The performance may be enhanced by use of stronger light sources such as high-power LEDs and/or a more sensitive camera, such as an electron multiplying CCD. Both options are currently being pursued. Note that the effective penetration depth, $1/\mu_{\text{eff}}$, ranged from 0.12 to 4.47 mm for this phantom in the excitation range 405-625 nm. It is also worth noting that fluorescence was detectable at the 625 nm wavelength down to at least ~6 mm depth as shown in Figure 3a, even though the depth estimate can only be resolved < 3 mm.
Figure 5.4: (a) Fluorescence intensity as a function of capillary tube depth (measured from the top surface of the tube) in the tissue-simulating phantom, for the different excitation wavelengths; (b) \( M_1, M_2 \) and \( M_3 \) as a function of capillary tube depth, with the solid lines being the diffusion model predictions; (c) Depth estimate of the capillary tube compared with the true depth: the dashed line is the line of equality: \( R^2 = 0.918 \) for depths up to 3 mm.
5.4.2  *In vivo experiment using a rat brain tumor model*

Figures 4 and 5 show results from two animals, in the first of which the tumor was fully sub-surface, while in the second it was located close to the cortical surface. These individual images show the increasing light penetration with wavelength (panels (a-d)), as the tissue optical attenuation decreases. Using the above model, depth-resolved topographic images were derived and are shown in Figures 5(f) and 6(f), with the corresponding white light images in panel (e). The corresponding H&E-stained histology sections in panel (g) illustrate the location of the tumors relative to the tissue surface.

It is useful to approximate tumor depth in the histology images so as to compare them to the depth-resolved fluorescence maps. In Figure 5.5, the tumor is fully buried in the left hemisphere according to the depth-resolved fluorescence topography, ranging in depth from approximately 0.65-1.8 mm, with a central tumor mass closest to the surface at the 0.65 mm depth. The H&E-stained section indicates the tumor depth ranges from 0.95-2 mm in the left hemisphere, with the central tumor mass closest to the surface at 0.95 mm. In Figure 5.6, the depth-resolved topography indicates a large surface tumor located in the central region of the exposed left hemisphere and sub-surface tumor ranging from 0-0.6 mm deep surrounding this central tumor mass. The H&E stain also indicates a tumor mass in the central region of the left hemisphere, with small, dispersed tumor inclusions through the left hemisphere ranging in depth from 0-1 mm. These discrepancies between the fluorescence depth estimations and tumor depth in H&E-stained slides may be partly explained by the difficulties in quantitative spatial analysis of histology slides, *i.e.* thin tissue 5 µm thick may stretch or tear, and formalin-fixed tissue is spatially distorted; however, the depth approximations from H&E stains still demonstrate a reasonable accuracy of the depth-resolved fluorescence technique *in vivo.*
Figure 5.5: Topographic images of a sub-surface brain tumor in vivo: (a)-(d) individual fluorescence images at each excitation wavelength, in order of decreasing optical attenuation of the tissue (i.e. 405, 546, 495 and 625 nm). The color bars indicate the relative fluorescence intensity (arbitrary units); (e) white light image; (f) sub-surface fluorescence topographic data rendered as a 3-D surface; (g) coronal H&E histology with the arrows indicating the top edge of the tumor mass. The scale bars are 3 mm for (a)-(e), and 1 mm for (g).
Figure 5.6: As in Figure 5.5, for an animal in which the tumor was closer to the cortical surface.
5.5 Discussion and Conclusion

These first studies illustrate the feasibility of this technique, albeit in a small-rodent brain tumor model and show that one could expect to detect localized fluorescence up to ~3 mm below normal brain surface using PpIX. This in itself may be very useful in guiding the final resection steps in patients to ascertain the presence and location of any non-contiguous tumor nests lying below the surface of the surgical resection bed. For this and other applications, it would also clearly be of value to map the tumor topography to greater depths in tissue. The restricted depth of sub-surface imaging is a fundamental limitation of any fluorescence-based technique. One solution is to use fluorophores (including nanoparticle-based agents) with high quantum yield and/or excitation and emission in the red-to-NIR wavelength range (such as indocyanine green, though this dye has poor quantum yield). Such experiments are in progress.

Note that this technique works well with PpIX because it has a large Stokes shift and a wide absorption band that encompasses a spectral band where the tissue absorption has large variation. For fluorophores with small Stokes shifts the available part of the fluorescence emission band is reduced due to the absorption-emission overlap, and thus limits the signal to noise of the images. In the case of a narrow excitation spectrum, the effect will be to reduce the depth dynamic range over which imaging can be done.

With the modeling geometry in this work, diffusion theory typically has difficulty modeling the fluence rate near the surface, and at high albedo. This is generally not that much of a problem in this work. For example, using averaged brain tissue optical properties, 0.5 mm beneath the surface diffusion theory is within ~20% agreement with Monte Carlo simulations for $M_1$ (i.e. lowest albedo, poorest fit), 15% for $M_2$ (2nd lowest albedo) and 10% for $M_3$. The good accuracy at such a low albedo and shallow depth can partly be explained by the fact that diffusion theory
overestimates the fluence rate, especially at shallow depths. By taking a ratio, the overestimation of both the numerator and the denominator is reduced, resulting in a fortuitous reduction of the error in calculating $M_1, M_2$ and $M_3$.

It is of interest to know how buried fluorescing objects with complex shapes may be resolved. If there are multiple sources of fluorescence (e.g. several separate nests of tumor at different depths) and lateral separations, then the topographic appearance will depend on whether or not the objects are fully separate or overlapping in the lateral directions. Thus, if there are two laterally overlapping objects at different depths, then the reconstructed surface will show the top surface layer of the nearest object continuous with the top surface of the non-overlapping part of the deeper object. Two non-laterally overlapping objects, such as the separate tumors in the LH and the RH in Figure 5.6(e), would appear as two distinct objects at different depths; however, there is a clearly complex interplay between the lateral and depth resolutions that needs further investigation. Further studies using Monte Carlo models are planned to answer this question.

The concept of topographic imaging of sub-surface fluorescent structures could be useful for a range of different applications including, for example, determining subsurface blood vessels during surgery (using a vascular fluorophore), sentinel lymph node and lymph flow mapping with indocyanine green [21,22], determining the depth of dermatological or other vascular lesions to help target laser therapies, non-destructive testing of non-biological materials, and possibly for encryption and biosecurity.

### 5.6 Statement of contributions

The general concept and algorithm development for the depth-resolved work were accomplished by me, as well as phantom and animal experiments. Mathieu Roy spearheaded the hardware and
software development of the multi-spectral excitation system. Farhan Dadani assisted in the system development and especially the animal experiments.

5.7 References


6 Conclusions and Future Work

6.1 Accomplishments of this work

The treatment of glioma is confounded by the nature of the disease and the resistance of glioma to conventional therapies. Surgery is the primary means of combating glioma. The clinical and technological challenge is to identify the tumor margins during resection surgery with exquisite accuracy. Image guidance technologies such as frameless stereotaxy environments and intraoperative MRI and CT are sharpening the cutting edge of neuro-navigation during resection surgery; however, these technologies do not truly address the need for functional and molecular detection to maximize brain tumor cell removal. Fluorescence guidance has proven to be a valuable tool to visualize residual tumor cells during resection surgery, particularly using the ALA-PpIX biosynthesis system as tumor contrast. Even with the clear benefits of fluorescence guidance to both tumor resection completeness and patient survival, the technique requires further technological elaboration in order to achieve its full potential.

Recall that the hypothesis driving this work is that quantitative and depth-resolved fluorescence modalities can better identify tumor margins, leading to increased complete tumor resection completeness and consequent extension of patient survival. The work in this thesis has addressed this hypothesis through the development of technologies capable of quantitative and depth-resolved fluorescence measurements/imaging.

Quantitative fluorescence spectroscopy mediated with a handheld fiberoptic probe was developed in order to provide the neurosurgeon with true quantitation of positive tumor fluorescence signal. Intrinsic to achieving this was the development of a method to extract the tissue optical properties, since it is the variation of local optical absorption and scattering that is a
major factor in fluorescence distortion. The technique developed was the first to address and find a solution to the limitations in the dynamic range of optical properties measurement using spectrally-constrained diffuse reflectance. Research into tissue optical properties measurement also culminated in a book chapter in the 2nd edition of a standard text in biomedical optics [1].

Much of this work involved the validation of the quantitative fluorescence technique. Phantom experiments validated the fluorescence model. A mouse model was used to ensure that the probe technique worked well in different tissue types. The PpIX concentration in several mouse tissue types was extracted via ex vivo tissue solubilization and compared with the in situ probe measurements, with good correlation between the two. The quantitative fluorescence probe system was engineered such that it is safe and compatible with a surgical environment.

One of the exciting developments outlined in this thesis was the deployment of the quantitative fluorescence probe in clinical trials. The probe technique was compared to state-of-the-art imaging to determine its effectiveness in detecting several brain tumor pathologies in vivo (low- and high-grade gliomas, meningioma and metastases). Recall that the corollary to the hypothesis of this work is that the current state-of-the-art fluorescence imaging modalities (i.e. through-microscope implementations) do not take full advantage of the tumor-to-normal contrast afforded by the ALA-PpIX system. This research has generated evidence to prove this corollary. This work presented a clinical demonstration that quantitative fluorescence greatly improves sensitivity and specificity compared with either uncorrected fluorescence intensity or qualitative fluorescence imaging. Using multiple variables (namely PpIX, autofluorescence, reflectance and oxygen saturation), the diagnostic capability of the probe proved to be excellent in discriminating between normal and tumor tissue in vivo (sensitivity and specificity for the high-grade glioma population were 92.8% and 96.2%, and were 87.1% and 89.1% for the all-tumors population).
Moreover, the technique can be generalized over several different brain tumor pathologies and over an inter-patient (N=14 patients) data set. There is evidence that the diagnostic accuracy of ALA-PpIX can be improved even further if the time kinetics are known and the critical resection of residual tumor is performed at the time of peak tumor-to-normal contrast.

A novel technique to produce depth-resolved fluorescence maps was developed up to the proof-of-concept phase. Since the technique maps the topographic surface of the fluorescent tumor closest to the surface, we have named this technique sub-surface fluorescence topography. This technique was validated in phantom studies as well as in a rat brain tumor model. The potential benefit of this technique is to allow the neurosurgeon to identify tumor cell nests buried beneath the resection bed surface in order to guide the surgery.

6.2 Future work: Quantitative and depth-resolved fluorescence guidance of intracranial tumor resection

6.2.1 Continuation of current clinical trial on quantitative fluorescence and future publications

Our group’s collaboration with Dartmouth College and DHMC has been fruitful, and will continue to the end of the current National Institutes of Health (NIH) grant in 2012 and beyond. With the promising clinical results produced so far with the quantitative fluorescence probe, clinical trials will continue using the same protocol in intracranial resection surgery, with the aim to collect enough data for a far more comprehensive study on quantitative PpIX fluorescence than the current data set. Plans to renew the NIH grant are beginning to take shape, with the intention to continue the Toronto-Dartmouth partnership.

At least two future publications led by the Toronto group are planned for late 2010 based on the work-to-date. The first publication involves the ex vivo tissue solubilisation fluorometry for
biopsies from the first 22 patients in the Dartmouth clinical trials \((i.e.\) without correlating \textit{in vivo} probe measurements). The objective here is to attempt to correlate PpIX concentration and potentially also the autofluorescence to tumor pathology. Since biopsying normal tissue was impossible, this study will test the statistical significance and the relationships between the fluorescence markers and tumor grade, percent necrosis, WHO grade and histological grade, rather than determine the sensitivity and specificity of PpIX as a tumor marker. Time kinetics data of ALA-PpIX may also be gleaned from this sample population.

The second publication will be a comparison between intraoperative, \textit{in vivo} fiberoptic probe measurements during resection surgery and \textit{ex vivo} tissue solubilisation fluorometry of excised biopsies. Currently, there is a 14 patient overlap where both \textit{in vivo} and \textit{ex vivo} measurements/tissue samples are available. This publication may be approached in one of two ways. This may be considered a validation study of the fiberoptic probe modality and to determine its accuracy at deriving fluorophore concentrations. On the other hand, if the fiberoptic probe measurements are considered to be accurate, this may be approached as a study of how fluorophore concentration accuracy degrades with tissue extraction, tissue handling, cryofreezing and the solubilsation assay.

\subsection{6.2.2 ALA dose parameters and time kinetics clinical study}

Our group has been in talks with Carl Zeiss Meditec (Jena, Germany) and University Hospitals Case Medical Center in Cleveland, OH to initiate a dose parameter study for ALA-PpIX-mediated fluorescence-guided resection surgery. There have been no published clinical studies to determine the best ALA dose (in mg/kg bodyweight) and post-ALA administration time for optimal tumor-to-normal contrast. In fact, as mentioned in the concluding discussions of Chapter
4, the current clinical data suggests that ALA time kinetics is a critical issue. The aim for this study is to determine ALA dose parameters in a clinical study using the quantitative fluorescence fiberoptic probe in order to provide absolute quantitation of PpIX concentration.

6.2.3 Development of an intraoperative depth-resolved imaging system

Sub-surface fluorescence topography as a depth-resolved fluorescence modality is currently at the proof-of-concept phase. Our group is seeking to push the concept into the operating theatre by developing a stand-alone imaging system capable of acquiring multi-excitation fluorescence images. In addition to the technique in this thesis, our collaborators at Dartmouth have achieved proof-of-principle for a depth-resolved modality using multi-emission fluorescence images [2].

Table 6.1 lists the images required to satisfy requirements for both the multi-excitation (i.e. for sub-surface fluorescence topography) and multi-emission (i.e. for the Dartmouth depth-resolved fluorescence solution) techniques. We have identified two main routes to achieve these imaging objectives: (1) implementation through the surgical microscope and (2) a stand-alone imaging system. From a surgical workflow perspective, Option (1) is ideal, since the neurosurgeon performs the entire resection surgery while viewing through the microscope; however, this is fraught with logistical and physical difficulties. The current Zeiss microscope used to perform the clinical trials is not only prohibitively expensive (~US$600,000), but it is also on loan. A partnership with Zeiss at the engineering and development level is vital to open up and modify the surgical microscope, a partnership which to date has yet to mature. As well, there is a large amount of light loss from the long camera-to-tissue working distance (10-20 cm) for the Zeiss scope. There are additional light losses due to splitting the light paths several times (through the binocular viewer, to the video imaging system and finally to the detector for depth-
resolved imaging). There also needs to be excellent rejection of stray excitation light in the fluorescence collection path, which is an unknown variable since this likely was not as critical an issue when Zeiss designed their microscope. Option (2), on the other hand, allows us to develop and optimize the system independent of the Zeiss surgical microscope. Hence, we are pursuing the development of a stand-alone system, akin to the concept sketch shown in Figure 6.1, where the fluorescence imaging is achieved through a rigid-bore endoscope. One of the difficulties in implementing a stand-alone system is that the solution must work around the Zeiss surgical microscope, which is positioned approximately 10-20 cm above the surgical field—an endoscope or other such imaging end effector would solve this problem.

<table>
<thead>
<tr>
<th>Image #</th>
<th>Excitation wavelength</th>
<th>Emission wavelength</th>
<th>Multi-excitation algorithm (Toronto)</th>
<th>Multi-emission algorithm (Dartmouth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>405 nm</td>
<td>705 nm</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>505 nm</td>
<td>705 nm</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>3</td>
<td>546 nm</td>
<td>705 nm</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>4</td>
<td>635 nm</td>
<td>705 nm</td>
<td>❌</td>
<td>❌</td>
</tr>
<tr>
<td>5</td>
<td>635 nm</td>
<td>680 nm</td>
<td>❌</td>
<td>❌</td>
</tr>
<tr>
<td>6</td>
<td>White light</td>
<td>705 nm</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>White light</td>
<td>680 nm</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>8</td>
<td>No light</td>
<td>705 nm</td>
<td>❌</td>
<td>❌</td>
</tr>
<tr>
<td>9</td>
<td>No light</td>
<td>680 nm</td>
<td>❌</td>
<td>❌</td>
</tr>
</tbody>
</table>
6.2.4 Depth-resolved fluorescence using the fiberoptic probe modality

The difficulty with non-contact imaging is the massive loss of signal compared to a contact fiberoptic probe modality. There is an inverse relationship between fluorescence signal and camera distance to the tissue. As well, the air-tissue index mismatch further reduces the signal. An interesting possibility is to use the fiberoptic probe in depth-resolved mode for increased sensitivity to buried fluorescence. The concept would be similar to that presented in Chapter 5, except the multiple colors would be emitted through the fiberoptic tip. In addition, the multi-emission concept developed by Dartmouth may be concurrently implemented to provide an adjunct measurement.
A fiberoptic probe system tailored to the Dartmouth multi-emission spectral measurement was actually built recently for the purpose of trying out their depth-resolved algorithms in clinical trials. The new system, shown in Figure 6.2, is equipped with an additional red LED that emits light at 625 nm for deep tissue penetration. Since everything of import in the operating theatre is co-registered via the frameless stereotaxy environment, including the surgical microscope and the resection bed, there is a practical way to correlate the depth-resolved algorithms to knowledge of the tumor location, via pre-operative MRI. These trials are planned to start in mid-2010. This data set will also serve as a feasibility study to determine if the multi-excitation technique as mediated by the fiberoptic probe will work in a clinical setting.

![Fiberoptic probe system](image)

Figure 6.2: Fiberoptic probe system outfitted with a 625 nm red LED, 405 nm blue LED and 2 white LEDs. Here, the probe is disconnected to show the multi-color lights projecting through the optical ports.

6.2.5 Current and future commercialization initiatives

The quantitative fluorescence probe system and the sub-surface fluorescence topography concept are currently protected by provisional patents filed in partnership with the University Health Network (UHN) [3,4]. In light of the promising clinical and pre-clinical results-to-date, plans are
underway to commercialize both properties. INO (Quebec, QC) is one company that has shown interest in partnering with our lab to commercialize these two inventions.

6.3 Future work: Applications

6.3.1 Clinical and pre-clinical trials for photodynamic therapy drug biodistribution measurement

The uses for the quantitative fluorescence probe may be expanded to be used for fluorescent drug delivery applications, in particular, photodynamic therapy (PDT) drugs, many of which are fluorescent. Knowledge of the drug concentration in tissue is necessary to determine efficacy and safety in pre-clinical and clinical trials.

Currently, there are two applications for using the quantitative fluorescence probe for drug fluorometry. A pre-clinical trial was performed in 2009 to determine the time kinetics of a two-photon drug developed at Oxford University (Oxford, U.K.), a porphyrin dimer derivative [5], in brain, heart, liver, kidney, skin, muscle and a melanoma tumor model. A publication reporting results from this pre-clinical study has been submitted recently [6].

As well, a prostate clinical trial is planned at UHN to determine the drug uptake in prostate for a PDT drug, denoted as SL052 (Quest Pharmatech: Edmonton, AL), when injected directly into the prostatic artery.

6.3.2 Monitoring of heat coagulation fronts during laser interstitial thermal therapy

Focal therapy of prostate cancer can be achieved using laser interstitial thermal therapy [7,8]. Essentially, a fiberoptic-based light source is interstitially catheterized within the prostate treatment volume. Laser light is then delivered to the tissue, achieving tumor cell kill by
thermally coagulating the tissue. It is critical to avoid damaging structures local to the prostate—urethra, rectal wall—to avoid morbidity. Monitoring is therefore very important. One technique that will be implemented in an upcoming prostate clinical trial at UHN is to use the fiberoptic reflectance probe outlined in Chapter 2 to interstitially monitor the tissue optical properties at a point near critical structures. Thermal coagulation should effect substantial changes in the tissue optical properties [7], providing a potential stop-trigger to the procedure (see Figure 6.3(a)).

Initial experiments demonstrate that the reduced scattering coefficient increases with heat coagulation. Figure 6.3(b-d) shows data from a heat coagulation experiment in porcine muscle ex vivo. The infrared thermal laser probe and reflectance probe were placed 1 cm apart. A Luxtron (Lumasense: Santa Clara, CA) optical temperature probe was placed adjacent to the reflectance probe’s tip. The laser was turned on and data were acquired over approximately 2.5 minutes. Panels (b) and (d) demonstrate how the reduced scattering coefficient at 600 nm increases with increased temperature and thermal dose. Physiologically, what is likely occurring is that the heat-driven denaturation of proteins leads to cross-linking of protein super-structures that are large enough to become effective optical scatterers, increasing the optical scattering power, similar to what happens when cooking an egg white.

The clinical trial (~5 patients, depending on recruitment) will begin early June 2010 to determine the change in optical properties with heat coagulation of the prostate. The fiberoptic probe will be interstitially placed in the prostate transperitoneally at a known distance from the thermal fiber. Reflectance measurements will be taken every 5 sec during the thermal coagulation procedure. After the procedure, a radical prostatectomy will be performed and the gross anatomy and thin-section histology of the prostate will be examined to evaluate the extent of thermal damage surrounding the fiberoptic probe.
Figure 6.3: (a) Concept schematic of laser interstitial thermal therapy with the heat coagulation boundary monitored using a fiberoptic reflectance probe and temperature sensor located at distance $r$. (b) Temperature and thermal dose graph versus time in the porcine muscle experiment, showing the laser on-off states during the experiment. (c) Photograph of the coagulated tissue relative to the thermal and reflectance probe placements. (d) Reduced scattering coefficient versus time graph, showing the laser on-off states.

### 6.4 Conclusion

This thesis was written at a time when a consensus is emerging that near-complete tumor resection does extend the survival of patients with glioma. Fluorescence guidance is becoming increasingly accepted in surgical applications; however, there is a need for improved detection to fully exploit the diagnostic capability of fluorescence contrast. The work in this thesis has clearly demonstrated that the tumor-to-normal contrast provided by the endogenous ALA-PpIX system can be much better utilized by quantitative and depth-resolved fluorescence guidance compared
to current state-of-the-art imaging/detection. It is my hope that this work will drive the development of increasingly sophisticated instrumentation that will further elaborate the method of fluorescence guided resection. With the improved and more accurate tumor detection afforded by these techniques, this work will contribute to advances in extending the survival of patients with glioma and other intracranial tumors.

6.5 References


