

POLYTECHNIQUE MONTRÉAL

affiliée à l'Université de Montréal

**Large field of view Raman imaging clinical prototype to guide brain cancer
resection surgery**

FRANÇOIS DAOUST

Institut de génie biomédical

Thèse présentée en vue de l'obtention du diplôme de *Philosophiæ Doctor*

Génie biomédical

Avril 2024

POLYTECHNIQUE MONTRÉAL

affiliée à l'Université de Montréal

Cette thèse intitulée :

Large field of view Raman imaging clinical prototype to guide brain cancer resection surgery

présentée par **François DAOUST**

en vue de l'obtention du diplôme de *Philosophiæ Doctor*

a été dûment acceptée par le jury d'examen constitué de :

Frédéric LESAGE, président

Frédéric LEBLOND, membre et directeur de recherche

Kevin PETRECCA, membre et codirecteur de recherche

Michel MEUNIER, membre

Daniel ELSON, membre externe

DEDICATION

To my parents: Michèle and Daniel, who've always encouraged me in my pursuit of crazy projects.

ACKNOWLEDGEMENTS

This roller coaster ride of a Ph.D. has been a life-changing adventure. I've had the chance to work with so many amazing people throughout the last six years which helped shape me as a person as well and shape the work that is presented in this dissertation. Although it won't do justice to the level of appreciation that I have for everyone who has helped me, I will still acknowledge below these individuals and institutions as best I can.

First and foremost, my deepest appreciation goes to my thesis supervisor, Frédéric Leblond, for his exceptional mentorship, his generosity in time and his belief in me. From the moment I started an internship in his lab, I felt how privileged I was to work so close to patients with groundbreaking technology that had a clear impact. Ever since my internship, I've had the privilege of having Frédéric guide me in my research (including a Master's and PhD) and mentor me on my path to accomplish my dream of founding a technology-based business. Frédéric trusted me to represent the lab during the large Amaranta project's and to lead our new innovative startup *Exclaro*. Not only this, but he also always provided all the resources I needed to accomplish my ambitions. His rigor in work and leadership have always inspired me and are models that I always try to emulate. Our collaboration continues with *Exclaro* which has already reached many significant milestones thanks to his guidance and efforts. I could not have asked for a better supervisor, mentor, and co-founder. Thank you Fred!

I extend my sincere thanks to my thesis co-supervisor, Dr. Kevin Petrecca, a renowned neurosurgeon, and researcher, who is passionate about helping his patients and developing new technologies to improve the standard of care. By enabling me to view firsthand a glioma surgery, on my second week of internship in 2016, he allowed a roaring fire to be born within which has since driven my desire to build technologies to help patients. This fire drives me even now in my entrepreneurship efforts to develop new technologies to detect diseases early and prevent them. With his input, I was able to understand and appreciate the importance of my research. Bringing and testing my investigational device in a hospital setting was an absolute privilege. He offered valuable insights from the clinical perspective which significantly help guide my work. His collaboration has been instrumental in shaping the clinical relevance and success of my research.

A special thanks goes to Joannie Desroches and Karl St-Arnaud for being the two individuals who've been my inspiration for pursuing my graduate studies. Joannie brought me to

view the first glioma surgery which sparked my passion to develop technologies to help patients. She also inspired me to pursue and complete the most insane physical challenge I've ever tried, the Canada Man triathlon. As for Karl, he gave me the mantel to continue his work in Raman imaging and helped me get started with my research project. The documentation that he left for me, and the quality of his work was exemplary which, I must admit was intimidating at first, but significantly propelled the speed and quality of my own research.

I express my gratitude to the Optech team, including Patrick, Marie-Maude, Jacques, Alexandre, Philippe, Isabelle, Alexandre, Keven and Maroun. Working with professionals like you during the Amaranta project was exhilarating. The knowledge and experience you contributed into this project was invaluable and I appreciate the opportunity to have worked with you in the laboratories of Optech.

I further express my gratitude to Dr. Marie-Christine Guiot, a devoted and hardworking neuropathologist, for her tremendous help in setting up specimen experiments, providing histology analyses, and enhancing my understanding of neuropathology. She was generous with her time, curious about our technology and always encouraged me in my work.

My appreciation goes to Jean-Paul Lévesques, the most helpful technician of Polytechnique Montreal, for his invaluable assistance in setting up experiments and finding the resources I needed. His expertise and creativity have been indispensable on numerous occasions.

A heartfelt acknowledgement is reserved for my dedicated lab colleagues: Andrée-Anne, Amélie, Joannie, Karl, Audrey, Guillaume, Katie, Fabien, Nassim, Sandryne, Trang, Myriam, Esmat, Frédérick, Jean-Philippe D., Hugo, Alireza, Alice, Tien, Mame-Kany, Émile L., Émile B., Jean-Philippe T., Michael, Arthur and Samaneh. Their camaraderie and collaborative spirit are what made our lab so special and such an enjoyable environment to work in.

My thanks also go to my friends from other laboratories: Jean-Sébastien, Frédéric and Sébastien for the emotional support, often given on the premises of the Pub McCarold and for helping me locate key equipment for my experiments.

Thank you to the interns which assisted me in the realization of my project: Hugo, Olivier, Théophile, Jérémie, Julie, Antoine, Solal, Emily, Vanessa.

A sincere thanks goes to ODS Medical, the Montreal Neurological Institute and Hospital, Optech, EmVision and the CHUM Research Center for their contribution (funds, expertise and/or space) for this project. It is thanks to this multidisciplinary environment that this project came to fruition.

I extend my thanks to Eric and EmVision for the enjoyable collaboration aimed at improving the spectrometer of the imaging system. Eric was always generous with his time and expertise and my numerous conversations with him were extremely insightful.

A big thank you to Marie Jorgault for her ability to solve any administrative challenge at the CHUM Research Centre. She always helped things run smoothly and I could always depend on her. Not only this, but she also always found a way to brighten my day with her great sense of humour.

To my family: Catherine, Patrick, Michèle and Daniel, a special thanks goes for their continuous support throughout this academic journey. My brother Patrick, was an inspiration to pursue studies in engineering physics and with his own doctoral experience, was able to lend an ear, understand the challenges I was going through and make important suggestions.

A very special thank you goes to Jessica, my girlfriend and partner, for her unwavering support and encouragement throughout this adventure. She has been my anchor throughout the challenges faced during my doctoral work. I feel privileged to have her by my side and I feel like we can accomplish anything together.

An immense thank you to the different organizations which helped make this research possible through funding: NSERC, TransMedTech, FRQNT, MESI, CIHR and ODS Medical.

Finally, I would like to express my gratitude to Audrey and Simon for their time and effort in reading through my thesis, providing valuable comments, and contributing to its overall improvement. Your insights have been immensely valuable!

THANK YOU ALL!

RÉSUMÉ

Avec 43 % des Canadiens prévus de développer un cancer au cours de leur vie, on s'attend à ce que les systèmes de santé soient surchargés sans interventions novatrices et plus efficaces pour les patients. Les tumeurs cancéreuses sont souvent enlevées chirurgicalement et lorsque ces interventions chirurgicales échouent à éliminer toutes les cellules cancéreuses, le risque de récurrence menace de ramener les patients au bloc opératoire ou même pire, de mettre fin prématurément à leur vie. Sans aucun outil pour visualiser le cancer résiduel, les chirurgiens ont le choix entre soit retirer aveuglément des tissus sains supplémentaires autour de la tumeur pour s'assurer que tout le cancer résiduel est réséqué, soit laisser le cancer résiduel chez le patient pour épargner les tissus sains. Dans le cas des chirurgies de tumeurs cérébrales, épargner les tissus sains est souvent préférable car la résection peut affecter les fonctions cognitives et motrices du patient. Cependant, cela entraîne une probabilité très élevée de récurrence dans certains types de tumeurs cérébrales. Si les chirurgiens pouvaient visualiser le cancer résiduel chez le patient ou sur les spécimens fraîchement réséqués pendant la chirurgie, cela leur permettrait de localiser et d'éliminer plus de cancer tout en épargnant les tissus sains et donc, d'améliorer le pronostic des patients.

Cette thèse vise au développement d'un système d'imagerie Raman à grand champ de vision pour fournir aux chirurgiens les moyens de visualiser le cancer résiduel pendant la chirurgie. Le premier objectif de la thèse consiste à développer et à caractériser le système d'imagerie Raman pour la caractérisation tissulaire moléculaire basée sur l'apprentissage automatique. Le système a été conçu pour imager la signature Raman du tissu sur un champ de vision de 1 cm². Il a été démontré qu'il acquiert de manière fiable la signature du tissu en moins de 5 minutes et que cette signature, associée à l'apprentissage automatique, pourrait être utilisée pour localiser les frontières tissulaires. Le deuxième objectif est d'améliorer le système Raman pour créer un prototype clinique portable destiné à être utilisé dans une salle d'opération. Le système a été monté sur un chariot et équipé d'un bras de support pour effectuer des mesures *in vivo*. Il a également été démontré que le système pouvait être déployé en toute sécurité pour être utilisé chez des patients humains et que le flux de travail d'apprentissage automatique était généralisable à un jeu de données de tissus factice. Le troisième objectif consiste à tester le système dans un contexte réel en capturant la signature de spécimens cérébraux humains provenant de tumeurs cérébrales excisées lors de chirurgies de glioblastome. Le système a été utilisé pour imager 19 spécimens provenant de 8 patients à l'Institut et Hôpital neurologiques de Montréal. Au cours de cette étude, un modèle d'apprentissage

automatique a été importé dans le dispositif d'imagerie pour prédire la probabilité de présence de cancer dans chaque pixel. Il a été démontré que le système d'imagerie capturait de manière fiable la signature Raman du cerveau normal et du glioblastome par rapport à un prototype commercial. Il a également été démontré que le système, associé au modèle d'apprentissage automatique importé, pouvait prédire l'emplacement et la distribution des cellules cancéreuses à la surface d'un spécimen. Les prédictions concordaient avec l'évaluation des annotations des spécimens réalisée par un neuropathologiste.

Les résultats présentés dans cette thèse illustrent l'évolution d'un dispositif de visualisation du cancer novateur basé sur l'imagerie Raman sur un grand champ de vision. Ce système a été développé et testé pour un contexte clinique et s'est avéré être une première étape vers la mise en œuvre de l'imagerie Raman pour la caractérisation tissulaire pendant la chirurgie. Le système est déjà prévu pour plusieurs autres études impliquant la détection du cancer du sein chez l'homme et la détection de la maladie d'Alzheimer chez les cerveaux de rats.

ABSTRACT

With 43% of Canadians predicted to develop cancer within their lifespan, it is expected that healthcare systems will be overburdened without novel and more effective patient interventions. Cancerous tumors are often removed surgically and when these surgeries are unsuccessful at removing all cancer cells, the risk of recurrence threatens to bring patients back to the operating room or even worse, to end life prematurely. Without any tools to visualize residual cancer, surgeons are left with the options to either blindly remove additional healthy tissue around the tumor to ensure all residual cancer is resected or leave residual cancer in the patient to spare the healthy tissue. In the case of brain tumor surgeries, sparing healthy tissue is often preferred since resection may affect the patient's cognitive and motor functions. However, this leads to a very high likelihood of recurrence in some brain tumors. If surgeons could visualize residual cancer in the patient or on freshly resected specimens during surgery, it would allow them to locate and remove more cancer while sparing healthy tissue and thus, would ameliorate the prognosis of patients.

This thesis aims at the development of a large field of view Raman imaging system to provide surgeons with the means to visualize residual cancer during the surgery. The first thesis objective consists of developing and characterizing the Raman imaging system for machine-learning-based molecular tissue characterization. The system was designed to image the Raman signature of tissue over a field of view of 1 cm². It was shown to reliably acquire the tissue's signature within 5 minutes and that this signature coupled with machine learning could be used to locate tissue borders. The second objective is to enhance the Raman system to create a portable clinical prototype destined for use in an operating room. The system was mounted on a cart and equipped with a support arm to conduct *in vivo* measurements. It was also shown that the system could be safely deployed to be used in human patients and that the machine learning workflow was generalizable in a toy dataset of tissues. The third objective consists of testing the system in a real setting by capturing the signature of human brain specimens from excised brain tumors during glioblastoma surgery. The system was used to image 19 specimens from 8 patients at the Montreal Neurological Institute and Hospital. During this study, a machine learning model was imported into the imaging device to predict the likelihood of cancer presence in each pixel. It was shown that the imaging system reliably captured the Raman signature of normal brain and glioblastoma when compared to a commercial prototype. It was also demonstrated that the system, coupled with the imported machine learning model, could predict the location and distribution of cancer cells on

a specimen's surface. The predictions agreed with the assessment of the specimens' annotations made by a neuropathologist.

The results presented in this dissertation showcase the evolution of a novel cancer visualizing device based on Raman imaging over a large field of view. This system was developed and tested for a clinical setting and proved to be a first step towards implementing Raman imaging for tissue characterization during surgery. The system is already planned for several other studies involving human breast cancer detection and the detection of Alzheimer's disease in rat brains.

TABLE OF CONTENTS

DEDICATION	III
ACKNOWLEDGEMENTS	IV
RÉSUMÉ.....	VII
ABSTRACT	IX
TABLE OF CONTENTS	XI
LIST OF TABLES	XVI
LIST OF FIGURES.....	XVIII
LIST OF SYMBOLS AND ABBREVIATIONS.....	XXVI
LIST OF APPENDICES	XXVIII
CHAPTER 1 INTRODUCTION.....	1
1.1 Motivation	1
1.2 Research objectives	4
1.3 Dissertation overview.....	6
CHAPTER 2 LITERATURE REVIEW	7
2.1 Raman spectroscopy.....	7
2.1.1 Scattering.....	7
2.1.2 Absorption	9
2.1.3 Fluorescence.....	10
2.1.4 Biological Tissue Raman spectra	12
2.2 System design for Raman spectroscopy in biological tissues	13
2.2.1 Equipment selection	14
2.2.2 Raman macroscopic imaging system configuration.....	17
2.3 Machine learning in Raman spectroscopy	19

2.4	Molecular and structural characteristics of high-grade gliomas	21
2.4.1	General characteristics of high-grade gliomas	21
2.4.2	Histology of high-grade gliomas	22
2.5	Raman spectroscopy in glioma surgery	24
2.6	Raman intraoperative imaging probe systems	29
CHAPTER 3	GENERAL METHODOLOGY	31
3.1	Development of a novel Raman imaging system	31
3.2	Development of a clinical prototype Raman imaging system for intraoperative use	34
3.3	Evaluation of the Raman imaging system in a clinical setting on human tissue	36
CHAPTER 4	ARTICLE 1: HANDHELD MACROSCOPIC RAMAN SPECTROSCOPY IMAGING INSTRUMENT FOR MACHINE-LEARNING-BASED MOLECULAR TISSUE MARGINS CHARACTERIZATION	38
4.1	Background	38
4.2	Abstract	39
4.3	Introduction	40
4.4	Methods	44
4.4.1	Imaging system	44
4.4.2	Optical design	47
4.4.3	Raman imaging of biological samples	48
4.4.4	Spectral data pre-processing	49
4.4.5	Classification	50
4.5	Results	51
4.5.1	Optical design	51
4.5.2	Raman Imaging of Biological Samples	54
4.5.3	Margin identification	57

4.6	Discussion	58
4.6.1	Optical design.....	58
4.6.2	Raman imaging of biological samples	60
4.6.3	Margin identification.....	60
4.6.4	Photonic budget limitation of the system.....	63
4.7	Conclusion.....	64
4.7.1	Acknowledgements	65
CHAPTER 5 ARTICLE 2: A CLINICAL RAMAN SPECTROSCOPY IMAGING SYSTEM AND SAFETY REQUIREMENTS FOR IN SITU INTRAOPERATIVE TISSUE CHARACTERIZATION		66
5.1	Background	66
5.2	Abstract	67
5.3	Introduction	67
5.4	Experimental	70
5.4.1	Clinical prototype Raman imaging system	70
5.4.2	Spectral data pre-processing.....	74
5.4.3	Imaging of biological tissue	74
5.4.4	Classification.....	76
5.4.5	Clinical safety assessment.....	77
5.5	Results	80
5.5.1	Raman tissue imaging	80
5.5.2	Classification & tissue border detection.....	83
5.5.3	Clinical safety assessment.....	85
5.6	Discussion	88
5.6.1	Raman imaging of biological tissue	88

5.6.2	Classification and tissue border detection	89
5.6.3	Clinical safety assessment	90
5.7	Conclusions	92
5.7.1	Author Contributions	92
5.7.2	Conflicts of interest	93
5.7.3	Acknowledgements	93
5.8	Supplementary Material	93
5.8.1	Installation of a surgical drape on the clinical prototype for intraoperative imaging	93
5.8.2	Imaging system parameters for laser safety assessment	94
5.8.3	Maximal permissible exposure to skin calculation	97
5.8.4	Maximal permissible exposure to eyes calculation	98
5.8.5	Prominent Raman features in porcine muscle and adipose tissue	100
CHAPTER 6	ARTICLE 3: INTRAOPERATIVE WHOLE-SPECIMEN RAMAN SPECTROSCOPY IMAGING DETECTS HIGH CANCER CELL DENSITY CLUSTERS IN GLIOBLASTOMA	102
6.1	Background	102
6.2	Abstract	103
6.3	Introduction	103
6.4	Results	105
6.4.1	Hyperspectral Raman imaging instrument	105
6.4.2	Cancer detection machine learning model	106
6.4.3	Patient specimens and histology	107
6.4.4	Biomolecular information content and inter-system spectral discrepancies	111
6.4.5	Consistency between machine learning predictions and histology	113

6.4.6	Biomolecular alterations between different anatomical regions	115
6.5	Discussion	117
6.6	Methods	120
6.6.1	Raman imaging system	120
6.6.2	Patient selection, specimen preparation and histology analyses	121
6.6.3	Spectral pre-processing	123
6.6.4	Cancer detection machine learning model	124
6.6.5	Cancer likelihood maps of Raman images	125
6.7	Data availability	126
6.8	Code availability	126
6.9	Contributions	126
6.10	Ethics declaration	127
CHAPTER 7	GENERAL DISCUSSION.....	128
7.1	Raman line scanning system design and fabrication.....	128
7.2	From proof of concept to clinical prototype Raman imaging system	130
7.3	Raman imaging system tested in a clinical setting.....	132
7.4	The COVID-19 Pandemic and a novel Raman-based saliva test.....	133
7.5	Thesis work limitations	134
7.6	Future scope of work with the Raman imaging system	135
CHAPTER 8	CONCLUSION AND RECOMMENDATIONS.....	138
REFERENCES.....		140
APPENDICES.....		169

LIST OF TABLES

Table 2.1: Prominent Raman bands in glioma and normal brain, their associated molecular bond, molecular assignment, molecular family, and their association with tissue phenotype. Highlighted Raman bands indicate common markers of glioma found in the literature [38], [88], [92], [97], [134], [135], [141], [142], [143].	28
Table 3-1: Technical specifications and their respective characterization method	32
Table 4-1: Raman imaging system technical parameters selected for the <i>ex vivo</i> experiments.	49
Table 4-2: Prominent Raman features for porcine muscle and adipose tissue. Bands highlighted in boldface correspond to peaks most relevant for tissue classification.	56
Table 4-3: Parameter differences between Raman line scanning and Raman point systems comparing the overall photon collection budget for an identical interrogated area and acquisition time.	64
Table 5-1 : Raman imaging system parameters selected for the <i>ex vivo</i> tissue experiments.	75
Table 5-2 : Helium leak test results validating muzzle hermiticity	85
Table 5-3 : MPE for eyes calculated and measured according to distance from the probe nose tip for an exposure time >10 s.	86
Table 5-4 : Excitation line laser intensity at the tissue surface for different exposure times and a power of 0.9 W compared to the MPE of skin of 3.27 W/cm^2	87
Table 5-5 : Imaging system parameters for MPE calculations	96
Table 5-6 : Laser MPE for skin using the Raman imaging system.	98
Table 5-7 : Intensity of laser beam evaluated for several distances from the tip of the imaging probe muzzle.	99
Table 5-8 : Prominent Raman features for porcine muscle and adipose tissue.	100
Table 6-1. Information from retained <i>ex vivo</i> specimens, histological diagnosis, number of pixels retained in the Raman image and machine learning (ML) predictions. PX refers to a patient where X is the patient's identifying number and SPY refers to a specimen where Y is the sample's identifying letter.	109

Table 7-1 : Technical specification changes between each iteration of the Raman imaging system, from the proof of concept to the finalized clinical prototype.....	129
---	-----

LIST OF FIGURES

Figure 1-1: MRI images of glioblastoma in a 64-year-old female patient where (A) is a contrast-enhanced T1 weighted image showing a large tumor mass with a necrotic core in the left frontal lobe and (B) is a T2 weighted image showing extensive edema surrounding the tumoral mass [8] (CC BY 4.0).....	1
Figure 1-2: Intraoperative view of fluorescence-guided surgery using 5-Aminolevulinic acid for glioblastoma resection. In (a), the surgical cavity is shown under a white-light microscope and in (b), emitted red fluorescence from protoporphyrin IX under a blue light source highlights the location of the glioblastoma [35] (CC BY 4.0).....	3
Figure 2-1 : Jablonski diagram illustrating the light-matter interaction of Rayleigh scattering (elastic scattering), Stokes Raman scattering (inelastic scattering energy loss) and anti-Stokes Raman scattering (inelastic scattering with energy gain).....	9
Figure 2-2 : Absorption spectrum of principal absorbent molecules found in biological tissues in normal concentrations. These molecules are oxy-hemoglobin (HbO_2), deoxy-hemoglobin (HbR), lipids, water, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), melanin, reduced myoglobin (MbR), oxy-myoglobin (MbO_2) and bilirubin [49] (CC BY NC SA). ..	10
Figure 2-3 : Jablonski diagram illustrating the light-matter interactions leading to fluorescence emission.....	11
Figure 2-4 : Fingerprint Raman spectroscopy measurement of adipose tissue where the green spectrum corresponds to the captured signal from a spectroscopy measurement, the blue plot is the fluorescence-dominated baseline and the red plot consists of Raman scattering obtained from subtracting the baseline to the luminescence. In this dissertation, Raman scattering is the signal of interest for characterizing tissues.	13
Figure 2-5 : Example Raman spectroscopy system showing the key components to capture Raman scattering.	16
Figure 2-6 : The three most common configurations for Raman imaging: A) raster scanning, B) line scanning and C) wide field imaging.....	18

Figure 2-7 : Histology of human brain from glioma patients showcasing A) normal brain with differentiated structures and cells, B) tumor infiltrations in the tumor periphery, C) increased cellularity and mitotic activity from a grade III astrocytoma, D) high cellularity, mitotic activity, pleomorphic cells and loss of brain structures (e.g. neurons) in a high-grade glioma, E) pseudopalisading necrosis (black arrow) and microvascular proliferations (red arrows), both indicators of grade IV glioma (glioblastoma) and F) large area of necrosis from the tumor core following cell death, also a marker of glioblastoma. 23

Figure 2-8 : Mean fingerprint Raman spectra of frozen human brain specimens for normal, white matter, gray matter, glioblastoma, infiltrating tumor, and necrosis [86] (CC BY 4.0). 25

Figure 2-9 : A) Handheld Raman point probe equipped with a neuronavigational apparatus used to interrogate tissue *in vivo* during glioma resection surgery. B) Intraoperative view of the probe *in situ* showcasing in the location of the probe tip overlayed with the preoperative MR images [107] (CC BY). 27

Figure 2-10: A) Fiber-delivered, Handheld SRS microscope [40] (reprinted with permission). B) Handheld wide-field Raman spectroscopy imaging probe with large FOV. [60], [150]. C) Acquisition head of a large FOV Raman imaging probe system used for skin cancer detection [152] (CC BY 4.0). 30

Figure 3-1: Raman line scanning imaging system consisting of (A) an excitation branch, (B) an imaging probe and (C) a collection branch. 32

Figure 4-1: (a) Handheld Raman imaging probe and schematic of the imaging system consisting of: (b) the imaging probe with its excitation fiber bundle (EFB), a series of optics (lase line filter (LLF), mirror (M) and long pass filter (LPF)) to propagate white light and laser light onto the sample to interrogate the tissue, a series of optics (LPF, optical window (OW) and notch filter (NF)) to collect the generated Raman signal and reflected white light, and a collection fiber bundle (CFB); (c) the excitation branch generating a line-shaped laser excitation at the entrance of the EFB mounted on a translation stage (TS) while also shining white light into the EFB; (d) the collection branch capturing white light and Raman signal from the collection fiber bundle (CFB), separating the two optical components with a scanning high pass filter (SHP), obtaining a white light image on an RGB camera while extracting spectral features with an imaging spectrometer. All illustrated lenses are spherical unless

labelled LH for cylindrical lenses with their curvature in the horizontal direction or LV for cylindrical lenses with their curvature in the vertical direction. 46

Figure 4-2: (a) Laser line profile relayed through the excitation fiber bundle and imaged distal face of the excitation fiber bundle. Simulated laser line profile generated by the excitation branch at the distal end of the 4 x 4 mm fiber bundle on the x-axis (b) and on the y-axis (c). Measured laser line profile on the x-axis (d) and on the y-axis (e). 52

Figure 4-3: (a) Schematic of the imaging probe and collection branch illustrating components marking the start and end of the optical path selected for optical transfer function (OTF) simulation. (b) OTF of the imaging probe from 1-2 for a spatial resolution of 2.00 cycles/mm and for an illumination wavelength of 785 nm. (c)-(d) OTF from 1-3 for (c) IR light (800-950 nm) at a spatial resolution of 2 cycles/mm and (d) visible light (400-650 nm) at a spatial resolution of 10 cycles/mm. (e) OTF from 4-5 for visible light (400-650 nm) at a spatial resolution of 10 cycles/mm (black) and (f) from 4-6 for IR light (800-950 nm) at a spatial resolution of 2 cycles/mm. 53

Figure 4-4: Study of the imaging system using a pork chop. (a) Photograph of the full pork chop, (b) bright field image of the FOV, (c) uncorrected line scan luminescence (Raman and background fluorescence resulting from 785 nm laser line illumination) intensity image (d) line scan background intensity image and (e) line scan Raman intensity image. Each intensity value within the intensity images corresponds to the integral over the entire spectral range of the system. Black column on the right of (b)-(d) correspond to pixels with relative illumination intensity <40%.....54

Figure 4-5: Training set imaged samples. (a) Bright field image of porcine adipose tissue, (b) bright field image of porcine muscle and (c) corresponding averaged spectrum and standard deviation (shading) of each tissue obtained following 10 repeat measurements. Blue triangles designate Raman bands associated with protein content while red circles designate Raman bands mostly associated with lipid content. An arbitrary vertical offset was added to the adipose tissue mean spectrum and standard deviation to facilitate the visualization the spectra from both tissues. Variations across adipose tissue spectra are too small to observe the standard deviation (red shading) in the figure.....55

Figure 4-6: (a) Bright field image of the sample tissue margin and (b) corresponding mean and standard deviation (shading) of 126 Raman spectra for pork adipose tissue (red) and muscle (blue). Rectangle area corresponds to the selected areas from which the spectra were selected. Black triangles correspond to the two most important features for tissue discrimination identified by the L1-SVM feature selection tool.....57

Figure 4-7: (a) Bright field image of the sample tissue margin, (b) classification map with red indicating predicted adipose tissue and blue indicating predicted muscle tissue and (c) classification confidence map ranking from 0.5 (lowest confidence) to 1.0 (highest confidence). The green line shows the ground truth for the tissue margin location. 58

Figure 5-1: (A) Cart-mounted clinical prototype of a Raman imaging system designed for intraoperative clinical measurements with (B) the computer aided design of the imaging system illustrating the location of several parts: (1) the collection branch covered by a light tight enclosure, (2) the excitation branch covered by its light tight enclosure, (3) the medical isolation transformer, (4) the articulated arm and (5) the imaging probe with its contact measurement muzzle. 71

Figure 5-2: Imaging probe with its attached probe muzzle for contact measurements, mounted on an articulated support arm allowing the user to move the probe with ease. A manual translation stage with 8 cm travel distance lets the user position the probe ensuring contact with the tissue area targeted for imaging.....73

Figure 5-3 : (A) Contact measurements probe muzzle mounted on a hermetic silicon base for a helium leak test and (B) leak test setup using a helium leak detector.....78

Figure 5-4 : Study non-contact dataset consisting of (A) the training set (7 measurements from pig #1), (B) the validation set (7 measurements from pig #2) and (C) the testing set (7 measurements from pig #3). For each subdivision of the data set, bright field images of porcine adipose and muscle tissue and their corresponding visually selected labels are shown. Black labels correspond to muscle tissue while white labels correspond to adipose tissue. Each square delimits a field of view of roughly $10 \times 10 \text{ mm}^2$. In some bright field images, false colours including green tint were caused by an automatic white balance error introduced by the system's software. 81

Figure 5-5 : Mean Raman spectra (solid line) and its 1x standard deviation (shaded area) for adipose (blue) and muscle (red) tissue of (A) the training dataset and (B) the contact muzzle testing set. For (A) and (B), the upper left spectra correspond to the mean raw luminescence counts and its 1x standard deviation for the same tissue types. Inverted black triangles correspond to the Raman features manually selected from the training data set for the classification. 82

Figure 5-6 : (A-G) Raman images from the non-contact testing set (pig #3) with, from left to right respectively, the bright field image of the interrogated tissue, its corresponding labels (white for adipose tissue and black for muscle tissue), the classification predictions from the best performing SVM model and the assigned prediction probability. A prediction probability of 0.9 (black) indicates near certain prediction while a probability of 0.5 (white) corresponds to random prediction. 84

Figure 5-7 : Temperature difference following maximum laser exposure to blood (black), muscle (red) and adipose (blue) tissue for a total exposure time of 60 seconds. Each dotted line represents from left to right, 1x, 10x and 100x the skin MPE respectively. Only laser exposure to blood would exceed a safe limit of $CEM_{43^{\circ}C} = 5$ min after an exposure time of 10.5 s if adding the recorded temperature difference to a basal body temperature of $36.9^{\circ}C$ 87

Figure 5-8 : Raman imaging probe in a clinical setting with a sterile drape covering the probe body and the articulated arm. The surgical drape is secured using sterile rubber bands and sterile tape. The probe muzzle is sterilized, ready to be inserted in a patient surgical cavity and it presents a minimal infection risk to the patient.....94

Figure 5-9 : Imaging probe drape covering procedure consisting of 6 steps: (A) recover the sterilized probe nose and remove the sterile drape from its packaging, (B) secure the probe nose to the sterile drape, (C) cover the probe body and articulated arm with the sterile drape, (D) secure the probe nose to the probe body, (E) secure the sterile drape to the probe body using sterile tape or rubber bands and (F) secure the sterile drape to the articulating arm and fiber bundles. 95

Figure 5-10 : Laser line profile dimensions of the imaging system compared with the exposure area (i.e. limiting aperture) evaluated in maximum permissible exposure calculations. 97

Figure 6-1: Graphical depiction of the workflow leading to cancer prediction maps of specimens resected from glioblastoma patients that were imaged using the Raman spectroscopy imaging

system. A) The experimental workflow showcases how glioblastoma and normal brain Raman spectra are collected. Spectral fingerprint measurements are acquired using a single point probe during neurosurgery. B) Biopsy workflow showing how histopathological data is linked with each spectral measurement. A biopsy is recovered immediately following the Raman measurement, the specimen is prepared and then annotated by a neuropathologist. C) Shows the combination of Raman single point spectra, with prevalent Raman peaks, and the corresponding histological labels which allow the creation of machine learning classifiers based on only a select number of differentiating Raman features. In this context, the phenylalanine band (1004 cm^{-1}), the tryptophan band (1340 cm^{-1}) and the protein/lipid band ($1430\text{--}1460\text{ cm}^{-1}$) are used to create a cancer (i.e. glioblastoma) prediction model. In D), the imaging system uses line scanning to image a freshly resected brain specimen. Finally, E) the cancer prediction model is applied to each pixel on the Raman image to recover a cancer likelihood map which depicts areas of dense tumor (red), areas of normal tissue (blue) and diffuse areas in between (white) associated with tumor infiltration. 108

Figure 6-2: Glioblastoma specimen shown in decreasing order of quantity of predicted cancer pixels (cancer likelihood >0.8) with corresponding photograph, machine learning predictions from single point Raman spectroscopy (SPRS), the haematoxylin and eosin (H&E) stained digital scan and cellular density map. Cancer likelihood predictions gave a score from 0.0 (blue) to 1.0 (red) where 1.0 or red corresponds to a high probability of cancer cells and 0.0 corresponds to a low probability of cancer (normal tissue). Cellular density maps were produced by counting the number of nuclei per mm^2 with black areas labelled as necrosis. 110

Figure 6-3: Overlay of the average fingerprint spectra from the single-point (solid) and imaging (dashed) dataset. Blue bands correspond to regions showing similar signatures between the two systems, red regions represent bands that were used during the classification, and green regions represent bands showing differences between single-point and imaging systems.. 112

Figure 6-4: Differences between the intensity of inelastic scattering bands of spectra acquired with the single-point probe system and the Raman imaging system. Boxplots associated with the Raman band intensity of all spectra at 1004 , 1064 , 1090 , 1128 , 1300 , 1340 , 1442 , 1620 and 1660 cm^{-1} 113

Figure 6-5: A) Average Raman spectra (solid line) and standard deviation (shading) of specimens recovered from the tumor core, the tumor interphase and the tumor periphery shown beside the average Raman spectra and standard deviation of all normal-predicted spectra using the single point Raman machine learning model. B) Average difference and standard deviation between the Raman signature of specimens from the tumor core, tumor interphase, tumor periphery and the normal-predicted spectra. The dashed grey line corresponds to 0. The spectral features used in the cancer prediction model are highlighted in red and other spectral features which differ significantly (i.e. at least one standard deviation) between tumor and normal spectra are highlighted in blue. 116

Figure 6-6: Raman imaging workflow for brain specimen imaging and producing assessment. (A) Brain matter is resected during surgery and B) shaped into square specimens of 10 mm x 10 mm x 3 mm. C) Specimens are imaged for 20 to 40 min and D) a collocated brightfield image and a 40 x 42 hyperspectral Raman image are produced. E) Specimens then undergo a first 18 to 24 h fixation in paraformaldehyde 10% before undergoing F) border inking for orientation identification. Finally, specimens undergo regular standard tissue preparation G) consisting of formaldehyde fixation, paraffin-embedding (FFPE). In H), the paraffin block is sliced, the slices are hematoxylin and eosin stained and scanned digitally for pathological annotations. The digital scans are oriented with the Raman images using the ink visible in the digital scan. 122

Figure 7-1: Future scope of work expected with the Raman imaging system. 137

Figure 8-1 : Current imaging probe's optomechanical schematic showing all optical components and their labels (i.e. laser line filter (LL), lens (L1, L2... L7), beam splitter (BS), optical window (W), mirror (M), notch filter (NF)), excitation fiber bundle (EFB) and collection fiber bundle (CFB). 169

Figure 8-2: Bright field image of a brain tumor border acquired with A) angled bright light, B) angled and colinear bright light simultaneously and C) only colinear bright light. Collinear bright field illumination produces green dominated images with specular reflections. 171

Figure 8-3 : Proposed LED ring light location at the tip of the imaging probe for near vertical bright field illumination. 172

Figure 8-4: Normalized mean spectra acquired with imaging system in air showing the luminescence profile generated by the focusing achromatic lens of the imaging probe.....176

LIST OF SYMBOLS AND ABBREVIATIONS

AI	Artificial Intelligence
AR	Anti-Reflective
BF	Bright Field
CCD	Charge-Couple Device
CRCHUM	Centre de Recherche du Centre hospitalier de l'Université de Montréal
CNN	Convolutional Neural Network
DNA	Deoxyribonucleic Acid
ETO	Ethylene Oxide
FFPE	Formalin Fixation and Paraffin-Embedding
FGS	Fluorescence-Guided Surgery
FOV	Field Of View
H&E	Hematoxylin and Eosin
IMRI	Intraoperative Magnetic Resonance Imaging
LDA	Linear Discriminate Analysis
ML	Machine Learning
MNI	Montreal Neurological Institute and Hospital
MPE	Maximum Permissible Exposure
MRI	Magnetic Resonance Imaging
MSRS	Multi-Spectral Raman Spectroscopy
MUHC	Montreal University Health Centre
NA	Numerical Aperture
NIR	Near-InfraRed

NN	Neural Network
OR	Operating Room
OTF	Optical Transfer Function
PCA	Principal Component Analysis
QF	Quality Factor
REB	Research Ethics Board
RNA	Ribonucleic Acid
SORS	Spatial Offset Raman Spectroscopy
SVM	Support Vector Machine
WHO	World Health Organization

LIST OF APPENDICES

Appendix A – Proposed System Optimizations.....	169
Appendix B – List of Scientific Contributions & Awards	177

CHAPTER 1 INTRODUCTION

1.1 Motivation

Gliomas are malignant brain tumors which are among the deadliest forms of cancers. Their poor prognosis is due to their frequent diagnosis at an advanced stage combined with the limited effectiveness of treatment options [1]. They are the most diagnosed primary brain tumors representing around 20,000 new cancer diagnoses in the United States in 2023 [2]. These brain tumors originate from glial cells and are classified by the World Health Organization (WHO) from grade I to grade IV: from best prognosis to worst. The two most diagnosed forms of gliomas are glioblastomas (WHO grade IV) accounting for approximately 60 to 70% of malignant gliomas, followed by anaplastic gliomas (WHO grade III) accounting for 20 to 25% [3], [4], [5]. These high-grade gliomas are linked to especially high morbidity and mortality, despite extensive treatment, with an average median survival of 13.5 months and 3.5 years for patients with glioblastomas and anaplastic gliomas respectively. They can develop at any age but are more often diagnosed in patients over 50 years old [6]. Diagnosing gliomas is generally achieved with magnetic resonance imaging (MRI) which in positive cases, will show the presence of an enhancing mass with surrounding inflammation and fluid (i.e. edema) [7]. This is illustrated in Figure 1-1 where two contrast-enhanced MRI scans showcase a large mass in the left frontal lobe of a 64-year-old woman diagnosed with glioblastoma.

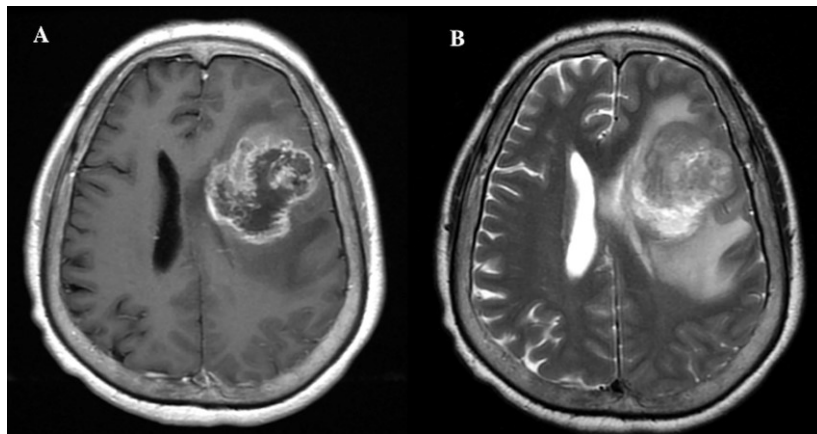


Figure 1-1: MRI images of glioblastoma in a 64-year-old female patient where (A) is a contrast-enhanced T1 weighted image showing a large tumor mass with a necrotic

core in the left frontal lobe and (B) is a T2 weighted image showing extensive edema surrounding the tumoral mass [8] (CC BY 4.0).

The standard treatment for newly diagnosed gliomas involves surgical resection and, in some cases, adjuvant radiotherapy and chemotherapy. One of the objectives of the surgery is to attenuate symptoms occurring from the pressure created by the mass and edema. Additionally, it is shown that the volume of resection of brain cancer is associated with a better patient prognosis [9]. Despite treatments, gliomas almost always return with an 80% recurrence rate [10], [11]. This is because gliomas are infiltrative and have diffuse tumor borders which make it impossible for current medical imaging modalities to capture their full extent [12]. Furthermore, these diffuse tumor borders make residual cancer cells invisible to surgeons which would be forced to remove healthy tissue to ensure an entire tumor resection. Because of the poor outcome on patient motor and cognitive functions associated with removing extensive healthy brain tissue[13], residual cancer cells are almost always left behind after surgery [14]. Some glioma surgeries require the patient to be awake (i.e. intraoperative mapping) to ensure motor and cognitive functions are not permanently impaired due to the surgery [15]. The inability to completely resect these diffuse tumors safely is linked with their high rate of recurrence and the poor prognosis of glioma patients [16], [17], [18], [19], [20].

To improve glioma patient prognosis, several surgical guidance modalities were developed to increase the safety and extent of glioma resection. Such modalities include Intraoperative MRI (iMRI) [21], [22], contrast-enhanced ultrasound [23], [24] and Fluorescence-Guided Surgery (FGS) [25], [26], [27]. iMRI is performed using a mobile or nearby MRI device which locates residual cancer during surgical procedures. This method is expensive [28], available in only a select number of hospitals [29] and it requires the surgery to be interrupted during iMRI. Intraoperative ultrasound aims to provide real-time assessment of tumor margins using the differentiating mechanical properties of tissues. Although this method can be performed in real-time, it has a lower resolution than MRI imaging and lacks accuracy specifically in diffuse tumor borders [30].

In FGS, patients ingest a fluorescent agent (e.g. the precursor 5-Aminolevulinic acid) which is metabolised by high-grade gliomas into a fluorophore (e.g. protoporphyrin IX). This fluorophore emits fluorescence when illuminated with a light source of the appropriate wavelength, enabling

pathological tissue visualization. In Figure 1-2, an intraoperative view of a glioblastoma is shown (Figure 1-2 (a)) under white light and under blue excitation light (Figure 1-2 (b)) for FGS with 5-Aminolevulinic acid. In this example, the bulk of glioblastoma emits a distinct red or pink color following blue light excitation. This color facilitates the localization and resection of diseased tissue throughout the surgery.

Although the above-mentioned methods can guide surgeons in removing additional diseased tissue safely, they are not well suited to locate areas with low cancer cell densities at the tumor periphery [31], [32] and therefore only delay the recurrence of malignant gliomas [33]. Another disadvantage of these methods is their requirement for a contrast agent (ingested or injected) which complicates regulatory approval pathways and can induce undesirable side effects (e.g. anemia, photosensitivity, leukocytosis, and temporary liver problems), rendering them inaccessible for some patients [34]. There is a current need for alternative surgical guidance modalities which are agent-free and more accurate for the complete resection of gliomas and specifically the resection of tumor infiltrations at the tumor periphery. In this dissertation, the work focuses on developing a novel intraoperative guidance technique for high-grade glioma surgery using a modality which does not require any contrasting agents.

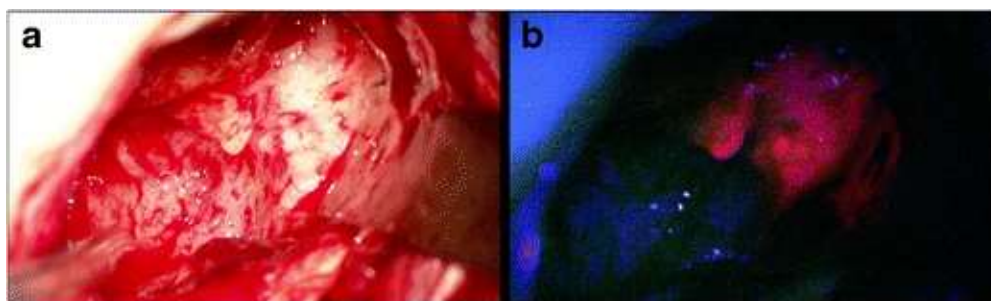


Figure 1-2: Intraoperative view of fluorescence-guided surgery using 5-Aminolevulinic acid for glioblastoma resection. In (a), the surgical cavity is shown under a white-light microscope and in (b), emitted red fluorescence from protoporphyrin IX under a blue light source highlights the location of the glioblastoma [35] (CC BY 4.0).

Among emerging technologies capable of discriminating cancer from normal tissue during surgery, Raman spectroscopy stands out as a promising candidate. This method uses a laser source to interrogate, non-invasively and non-destructively, molecular vibrations within a sample. Using

this molecular-specific information, normal tissue can be differentiated from diseased tissue to locate tumors. Raman spectroscopy has already shown significant potential to guide glioma surgery in several intraoperative studies [36], offering neurosurgeons the information necessary to remove additional cancer during the surgery. Despite its potential, the method has only been demonstrated intraoperatively using single-point acquisition [37], [38], [39] or microscopy imaging [40], [41]. Point measurements do not offer spatial context which can lead to the unsuccessful identification of real tumor margins or even to overlook localized cancer infiltrations. As for microscopy imaging, surgical cavities from glioma resections are too large (i.e. several cm^2) to be promptly interrogated by this method.

These limitations of single-point measurements and intraoperative microscopy motivate the development of macroscopic Raman imaging to interrogate large areas of tissue to identify tumor infiltrations and the extent of diffuse tumor borders during glioma surgery. Although this type of Raman imaging may show promise for detecting residual cancer surgery, there are no currently available imaging systems for this clinical need. Existing systems are cumbersome, have a small field of view (FOV) (i.e. $\text{FOV} < 1 \text{ cm}^2$) or do not acquire images in a clinically acceptable timeframe (i.e. $< 5 \text{ mins}$) [36], [42]. Additionally, none of the existing laser-based systems can be safely used in patients during surgery. Since these Raman imaging systems are not suitable for characterizing brain samples during glioma surgery, a novel Raman imaging system may be able to answer the clinical need.

1.2 Research objectives

The current challenges in surgical resection of brain tumors lead us to the following research question: how can large areas of tissue be characterized intraoperatively to guide surgeons towards improved surgical outcomes? The proposed means to answer the question is by utilizing a large field of view macroscopic Raman imaging system adapted for intraoperative tissue characterization. This goal is based on the following three hypotheses:

1. Intraoperative molecular characterization with Raman imaging over a large area ($\geq 1 \text{ cm}^2$) can assist surgeons in locating diseased tissue borders.
2. Molecular characterization with Raman imaging over a large area can be achieved in a practical timeframe for surgeries.

3. *In situ* molecular characterization with Raman imaging over a large area can be conducted without endangering patients and clinical staff.

To achieve this research goal, three specific objectives were identified and addressed.

Objective 1: Design and develop a large field of view ($\sim 1 \text{ cm}^2$) Raman imaging system proof of concept for machine-learning-based molecular tissue characterization and demonstrate its potential in animal tissue. This objective is further divided into 3 sub-objectives.

Sub-Objective 1.1: Determine the technical specifications required for a Raman imaging system to be tested in an operating room in human patients.

Sub-Objective 1.2: Design, construct and validate the Raman imaging system to the required technical specifications.

Sub-Objective 1.3: Characterize the Raman signature content of a sample animal tissue and develop an accurate ($>90\%$ accuracy) machine-learning classification model for tissue discrimination.

Objective 2: Enhance the Raman imaging system proof of concept to create a safe and portable clinical prototype suitable for *in situ* intraoperative tissue characterization.

Sub-Objective 2.1: Develop and validate the capability of the system to conduct *ex vivo* (i.e. outside of the patient) and *in vivo* (i.e. within the patient) measurements in humans within an operating room.

Sub-Objective 2.2: Optimize and validate the clinical prototype's imaging workflow (i.e. acquisition parameters, pre-processing and machine-learning) in a large dataset (>20 samples) from animal tissue.

Sub-Objective 2.3: Assess the safety of the system for users and patients in a clinical setting.

Objective 3: Evaluate the feasibility, practicality, and performance of the clinical prototype in a pilot study on resected tumoral tissue from patients affected by brain tumors.

To accomplish these different objectives, the research project will be conducted in collaboration with an Engineering School (Polytechnique Montreal), an applied research center specialized in optical technology (Optech), a Raman equipment manufacturer (EmVision), a

clinical research center (*Centre hospitalier de l'Université de Montréal* Research Center (CRCHUM)), a hospital with a neurosurgery department (Montreal Neurological Institute and Hospital (MNI)) and a medical device company specialized in neurosurgical guidance (Reveal Surgical).

1.3 Dissertation overview

The dissertation is organized into 9 chapters. Chapter 1 includes the motivation of the work, the research objectives, and the dissertation organization. Chapter 2 presents a literature review covering the concepts required to understand the content of this dissertation. These concepts are tissue Raman spectroscopy, optical systems design, machine learning applied to spectroscopic data, histology and glioma molecular biology. Furthermore, a review on Raman spectroscopy applied to glioma characterization during surgery and on Raman imaging probe systems for tissue characterization is presented. Raman spectroscopy in gliomas and Raman macroscopic imaging in tissues. In Chapter 3, the general methodology is detailed. Chapter 4 showcases the first article of this work and introduces the proof-of-concept Raman large field of view imaging system. The article addresses Objective 1 and was published in the *Journal of Biomedical Optics* (impact factor: 3.47). Chapter 5 describes the second manuscript of this work and showcases the enhanced clinical prototype Raman imaging system designed for intraoperative use. The manuscript was published in *Analyst* (impact factor: 4.2), a scientific journal of the Royal Society of Chemistry, and its work is related to Objective 2. In Chapter 6, the third article produced in this work is presented. The article was submitted to *Scientific Reports* (impact factor: 4.6) and showcases the results of an intraoperative pilot study with the imaging system in *ex vivo* human brain at the MNI. The results of this study marked the accomplishment of the Objective 3 of this dissertation. In Chapter 7 is presented the general discussion, which discusses each of the thesis objectives and how they were addressed. The impact of the Covid-19 on the work presented is also discussed along with the limitations of this work and the future scopes of work. Chapter 8 marks the conclusion of the thesis along with the recommendations for future work. Appendix A details the proposed technical improvements to improve the system towards a practical solution. Finally, Appendix B lists my accomplished scientific contributions including patents, manuscripts, and conference presentations as well as the awards obtained for my doctoral work.

CHAPTER 2 LITERATURE REVIEW

2.1 Raman spectroscopy

To guide residual cancer resection during glioma surgery, it is necessary to characterize large areas of tissue intraoperatively using a modality capable of highlighting the intrinsic differences between cancerous and normal anatomical structures. This dissertation focuses on Raman spectroscopy to accomplish this task due in part to its ability to provide near real time specific information about the molecular content and structure of a target. Raman spectroscopy is a non-destructive method using non-ionizing radiation to illuminate a sample and recover its molecular fingerprint. The detected signal result from light-matter interactions with the molecular bonds of the sample. Raman spectroscopy is a challenging modality since the light signal of interest (inelastic scattering) is minute when compared to other light-matter interaction processes. In this section, the principal competing light-matter interactions (i.e., elastic scattering, absorption, and fluorescence) and their effects on the collected optical signal, are described. The section is concluded with the illustration of an example of a collected Raman spectroscopy spectrum with details regarding its principal characteristics and informational content.

2.1.1 Scattering

When light interacts with matter, photons may scatter, deviating from their original trajectory. This phenomenon is influenced by the wavelength of the incident light and the structures within the material, such as cellular structures including the lipids membranes and the organelles. Tissue scattering is often characterized by the reduced scattering coefficient μ_s' describe by the following equation:

$$\mu_s' = \mu_s(1 - g) [mm^{-1}] \quad (2-1)$$

where μ_s is the scattering coefficient and g the anisotropy factor. The scattering coefficient measures the likelihood of light being scattered per unit of distance, while the anisotropy factor quantifies the directionality of the scattered light. Together, these factors contribute to the overall light scattering behavior in tissue described by μ_s' .

As depicted in the Jablonski diagram of Figure 2-1, some scattering events can result when molecular bonds absorb photons and reach virtual excited states. Virtual states are unstable and quickly release a photon within picoseconds with a change of directionality and with either equal energy (i.e. elastic scattering) or a slightly shifted energy (i.e. inelastic scattering) relative to the incident light. Elastic scattering, which accounts for most scattering events, is referred to as Rayleigh scattering. Inelastic scattering, known as Raman scattering (named for its discoverer Sir Chandrashekhara Venkata Raman), occur when the incident photon has gained or lost energy from the interaction with molecular vibrational modes [43]. This process occurs rarely (i.e. approximately 1 in 10 million photons) and spontaneously which is why it is often referred to in the literature as *spontaneous* Raman scattering.

The shift of photon energy during Raman scattering is specific to the differences of energies between different vibrational modes of molecular bonds. When a material is exposed to monochromatic light, this scattering will manifest in the form of sharp peaks in a spectrum (see Figure 2-4). Under the right measurement conditions, Raman spectroscopy can be used to specifically identify which molecular bonds and vibrational modes were responsible for the observed peaks associated with a specimen. For Raman scattering to be observed associated with a specific molecular bond, it needs to be susceptible to a change in polarizability from an incoming electromagnetic field. However, few biomolecules are unsusceptible to a change in polarizability [44]. The cross section of Raman scattering is inversely proportional to λ^{-4} where λ is the excitation wavelength which means shorter wavelengths generate more effective Raman scattering in samples.

Inelastic scattering resulting in a loss of energy of incident light is known as Stokes Raman scattering. If the scattering event results in a gain of energy as the phenomenon is called anti-Stokes Raman scattering [45]. At room temperature, as well as body temperature (i.e. around 36.9 °C in humans), the majority of Raman scattering events are Stokes scattering since most vibrational states in materials and tissues are at a ground state at these temperatures [46], [47]. This dissertation is centered on using Raman scattering for *in situ* characterization of tissues, specifically in surgical oncology during the resection of a tumor. Throughout the remainder of the dissertation, the term 'Raman scattering' will specifically denote Raman Stokes scattering unless explicitly stated otherwise.

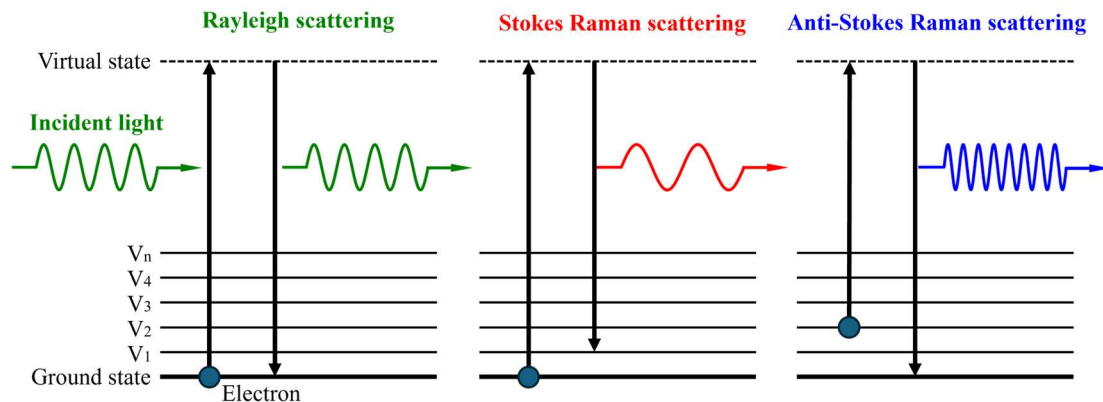


Figure 2-1 : Jablonski diagram illustrating the light-matter interaction of Rayleigh scattering (elastic scattering), Stokes Raman scattering (inelastic scattering energy loss) and anti-Stokes Raman scattering (inelastic scattering with energy gain).

2.1.2 Absorption

Absorption is the process by which tissue absorbs light resulting in the transition of electrons from lower energy states to higher energy states within molecules. This is also a wavelength dependant light-matter interaction and is driven by different biomolecules (e.g. hemoglobin and melanin) which have their own unique absorption spectrum (see Figure 2-2). Absorption in tissues is described by the absorption coefficient μ_a [mm^{-1}] which describes the rate at which light is absorbed in the media per unit of distance. When exposed to a light source, absorption may lead to radiative emission by fluorescence and phosphorescence or non-radiative absorption (i.e. photon energy converted into heat. Fluorescence emission is further described in subsection 2.1.3. As for non-radiative absorption, light may be converted into heat and increase the local temperature or, in some cases, may even result in an alteration in the chemical structure of the tissue through thermal effects [46], [47]. This makes absorption an important consideration when performing Raman spectroscopy in a clinical setting, since the wrong choice of excitation wavelength and light intensity may lead to permanent tissue alterations and patient injuries [48].

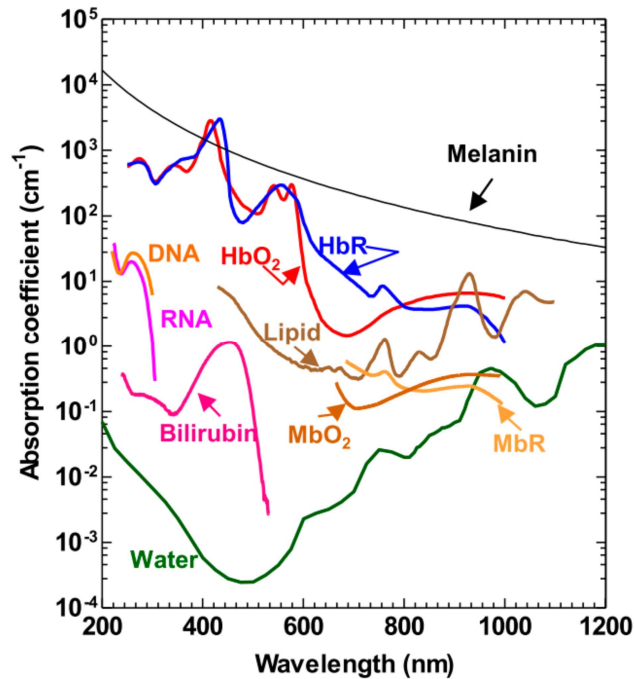


Figure 2-2 : Absorption spectrum of principal absorbent molecules found in biological tissues in normal concentrations. These molecules are oxy-hemoglobin (HbO_2), deoxy-hemoglobin (HbR), lipids, water, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), melanin, reduced myoglobin (MbR), oxy-myoglobin (MbO_2) and bilirubin [49] (CC BY NC SA).

2.1.3 Fluorescence

The interaction of light with matter can result in the absorption of photons into excited electron states which then relax radiatively after undergoing several non-radiative transitions [50]. This process, known as fluorescence, is shown in the Jablonski diagram in Figure 2-3, and occurs in fluorophores which have a specific molecular and structural configuration susceptible to absorption. In biological samples, biomolecules such as riboflavin, tryptophan, and tyrosine are fluorophores responsible for most of observed fluorescence emission [51]. Fluorescence emission upon excitation at a given wavelength is observed over a range of wavelengths, resulting in a spectrum with features characteristics of the tissue. This is due to the large number of possible radiative transitions enabled by the combination of close vibrational energies and the thermal

motion of molecules [52]. The timescale for fluorescence emission is in the order of nanoseconds to microseconds.

Since the fluorescence intensity and emission profile is molecule specific, this information can be exploited for tissue discrimination such as in the case of FGS [53]. However, fluorescence has been reported to be significantly quenched by tissue optical properties (i.e. scattering and absorption) which does not always make fluorescence tissue-specific [54], [55]. Fluorescence and Raman scattering are competing light-matter interactions and, as shown in Figure 2-4, they will both often manifest in a detector's overall photon count when performing a Stokes Raman measurement.

Absorption can also lead to a process known as phosphorescence emission. While this emission shares a similar electronic transition pathway with fluorescence, it differs significantly in both timescale and intensity. Phosphorescence emission typically occurs over a timescale ranging from milliseconds to seconds, allowing for only a few emission events during long-duration measurements [51]. Consequently, fluorescence remains the dominant competing radiative emission when capturing Raman scattering.

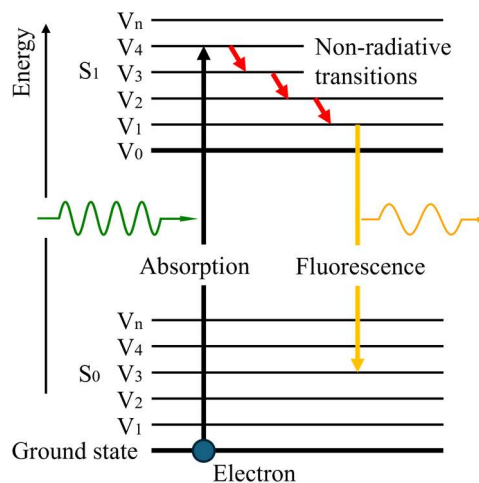


Figure 2-3 : Jablonski diagram illustrating the light-matter interactions leading to fluorescence emission.

2.1.4 Biological Tissue Raman spectra

When conducting a Raman spectroscopy measurement, a light spectrum (Figure 2-4) is acquired where the shape of the spectrum corresponds to a combination of Raman scattering (i.e. peaks) and the baseline. The latter consists of luminescence from fluorescence (dominant effect), and other relatively weak light-tissue interactions (e.g. phosphorescence). The total photon count and the spectrum's shape are also driven by the wavelength-dependent absorption and scattering of the interrogated sample. An example spectrum of adipose tissue following 785 nm excitation is depicted in Figure 2-4 where the total collected light is shown in green, the baseline in blue. The red plot showcases the Raman spectrum with the baseline subtracted. As observed in Figure 2-4, the x -axis of a Raman spectrum is presented using Raman shifts ν calculated using the following equation:

$$\nu \text{ (cm}^{-1}\text{)} = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda} \right) \quad (2-2)$$

where λ_0 [cm] is the excitation wavelength and λ [cm] is the wavelength of the scattered photon. Raman shift is used to visualize molecular vibrational energies in an axis that is independent of excitation wavelength. Each peak in the Raman spectrum corresponds to a specific vibrational mode of the molecules present in the sample, providing insight about the sample's molecular content. The height of each peak can be correlated with the concentration of the corresponding molecular species within the sample, enabling both qualitative and quantitative analysis. The literature discusses two regions where Raman peaks are observed in biological samples: the fingerprint region (200-2000 cm^{-1}) and the high wavenumber region ($>2000 \text{ cm}^{-1}$) [56], [57]. As the name suggests, the fingerprint region contains most of the molecular information that is required to identify a material. It is why the fingerprint region is the most preferred region when the combination of light sources and spectrometers only allow one of the two spectral ranges to be acquired [46], [47], [55].

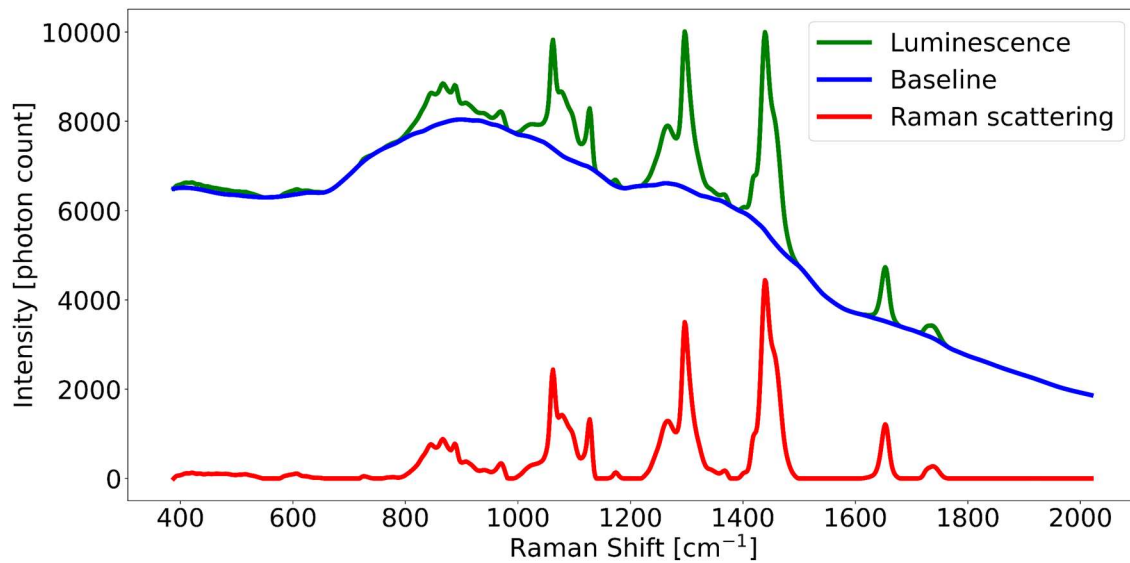


Figure 2-4 : Fingerprint Raman spectroscopy measurement of adipose tissue where the green spectrum corresponds to the captured signal from a spectroscopy measurement, the blue plot is the fluorescence-dominated baseline and the red plot consists of Raman scattering obtained from subtracting the baseline to the luminescence. In this dissertation, Raman scattering is the signal of interest for characterizing tissues.

2.2 System design for Raman spectroscopy in biological tissues

This section covers key considerations for building a Raman imaging system for biological tissue interrogation. Since Raman scattering is a rare occurring event and tissues are rich in fluorophores, careful system design considerations are required to ensure sufficient Raman signal can be collected despite fluorescence emission from tissues and despite potential noise sources [58], [59]. Furthermore, an emphasis on overall measurement efficiency wherever possible will be considered to minimize image acquisition times to ensure rapid measurements can be achieved in a clinical setting. A brief overview of equipment selection is described for performing a Raman spectroscopy measurement. Following this, a summary of Raman imaging configurations and considerations is presented.

2.2.1 Equipment selection

To perform a Raman spectroscopy measurement, several key components are required: 1) an excitation light source to generate Raman scattering in the sample, 2) a wavelength isolating component to discern the intensities and positions of Raman spectral features, 3) a photosensitive sensor to collect Raman-scattered photons, 4) a low-pass filter to remove any remnant Rayleigh scattering from the excitation source in the collected signal, and 5) a series of collection and excitation optics to deliver the laser intensity to the material and to collect Raman scattering [60]. The three most important components (i.e. the excitation light source, the wavelength isolating component and the photodetector) and their implications are further described below.

Light sources for acquiring Raman spectra in biological samples often consist of monochromatic lasers with a narrow linewidth (i.e. < 0.5 nm) to obtain a high spectral resolution (i.e. ≥ 8 cm^{-1}) and narrow Raman peaks [36]. Narrow peaks facilitate the detection of different vibrational modes which may be used to identify molecules and perform tissue identification tasks. As for the wavelength excitation laser, the choice of wavelength needs to account for the proportionality of the Raman cross section to λ^{-4} , for the absorption and emission spectral profiles of tissue fluorophores, for the quantum efficiency and cost of available photodetectors, and for the wavelength-dependent optical properties of tissue. The most common wavelengths for Raman tissue interrogation are 670, 785, 830 and 1064 nm [61]. Multiple competing considerations are associated with the choice of excitation wavelength: 1) shorter wavelengths (i.e. visible blue light) generate too much tissue fluorescence [51], 2) biomolecules such as hemoglobin are much more absorbent in the visible part of the electromagnetic spectrum (see Figure 2-2), inducing potentially problematic thermal effects [49], 3) tissue absorption at longer wavelengths is reduced, resulting in larger light penetration depths through diffusion (> 1 mm) [62] and 4) longer wavelengths are much less effective at generating Raman scattering.

Once the excitation wavelength is selected, the wavelength isolating component (e.g. tunable filter or passive spectrographic element) has the role of separating Raman scattering into its constituent wavelengths before reaching the photosensitive sensor. Considerations when selecting this component include its spectral resolution, spectral range, and light detection efficiency.

Systems used for tissue interrogation are most often reported to have a spectral resolution $\leq 8 \text{ cm}^{-1}$ while a spectral range covering the fingerprint region.

Considerations in selecting the detector include sensitivity (i.e. quantum efficiency), dynamic range, readout noise, thermal noise, pixel size and readout speed. Detectors for sensitive spectroscopy applications call upon deep cooled sensors ($< -50 \text{ }^{\circ}\text{C}$) to reduce the impact of thermal noise [63]. In system configurations using a spectrometer, the detector's pixel and sensor sizes must be matched with the spectrometer as they may influence the spectral resolution and spectral range, respectively [60].

Coupled with the corresponding filters and optical components, the right excitation and detection setup will enable the collection of spontaneous Raman scattering in tissue (example configuration shown in Figure 2-5). This equipment will also be susceptible to environmental light sources which, if not controlled, can drown the optical signal of interest. For example, in an operating room environment, light pollution can be associated with a surgical microscope, a neuronavigational unit (infrared light), operation room neon lights, and computer screens [63], [64]. Light enclosures for *ex vivo* measurements, shutting main lighting in the room, signal preprocessing, and installing filters on external light sources (e.g. the surgical microscope) help prevent this light pollution [64], [65].

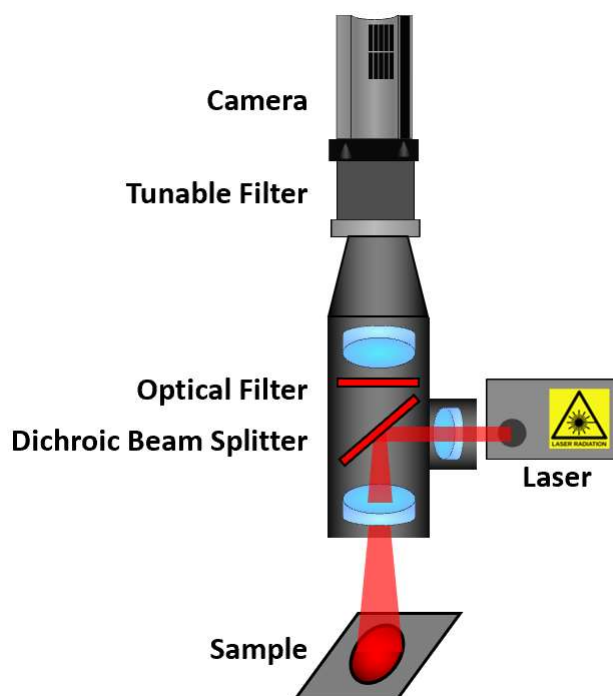


Figure 2-5 : Example Raman spectroscopy system showing the key components to capture Raman scattering.

Some specialized system configurations can enable complex Raman signal collection methods using non-linear optical techniques (e.g. Coherent Anti-Stokes Raman Spectroscopy (CARS) [66], [67], Surface-Enhanced Raman Spectroscopy (SERS) [68], [69] and Stimulated Raman Spectroscopy (SRS)) [70], [71], [72]. CARS needs up to three lasers (i.e. a pump, stokes and probe) to stimulate anti-Stokes Raman scattering, thus allowing the use of shorter wavelengths while also avoiding fluorescence in the captured spectrum. SRS uses a pump laser to bring electrons to virtual states followed by sweeping a probe laser to stimulate Stokes Raman scattering and increase the Raman signal to noise ratio. SRS can be used to probe specific Raman peaks and reduce the acquisition time to observe a molecular contrast in tissues at microscopic scales [73]. CARS and SRS require tissue to be exposed to significant laser intensities to produce the desired non-linear optical effects. These high intensities drastically increase the risk of damaging tissues through thermal or photochemical damage. Additionally, the high laser intensity requirement constrains the measurement to a microscopic field of view which makes large surgical cavities

challenging to interrogate rapidly with these techniques [36]. As for SERS, the method relies on nanoparticles or nanostructured surfaces to enhance the electric field surrounding adsorbed molecular bonds and significantly increase the occurrence of Raman scattering (i.e., plasmonic effect) [74]. Although this method may -in principle- enhance Raman signal up to a factor of $\sim 10^{10}$ [75], the reproducibility of SERS signal for diagnostics is known to be quite challenging due to the variability in biological samples and to the highly sensitive and specific surface signal enhancement from the surface plasmonic effects [76], [77], [78]. Moreover, external agents such as nanoparticles, require extensive clinical testing before being approved by regulatory bodies for clinical intervention in patients. As an example of the challenge of adopting new contrast agents for surgical guidance, it took close to 20 years after its first use in humans in 1998, to have the fluorescence-guided surgery label Gleolan® approved by the FDA for high-grade glioma surgery (approved in 2017) [79].

2.2.2 Raman macroscopic imaging system configuration

Raman imaging requires Raman spectra to be collected over a two-dimensional space which results in a data cube (i.e. two spatial axes and one spectral axis). The three common configurations employed for Raman imaging (shown Figure 2-6) are raster scanning, line scanning and wide field imaging [80], [81]. Raster scanning consists of acquiring one spectrum per point area and sweeping the measurements over the desired 2D area. Each point measurement consists of a pixel within the final hyperspectral image. Line-scanning consists of interrogating one line at a time and scanning the line across the sample to cover the desired image area. Each interrogated line is projected on a 2D sensor where one of the sensor dimensions captures the spectra and the other sensor dimension captures the line's spatial dimension. Wide field imaging consists of illuminating the entire field of view (FOV) of the system and acquiring intensity images over that FOV. Hyperspectral images are reconstructed by acquiring intensity images at different wavelengths using, for example a tunable filter or a filter wheel that is placed in front of the photodetector [82].

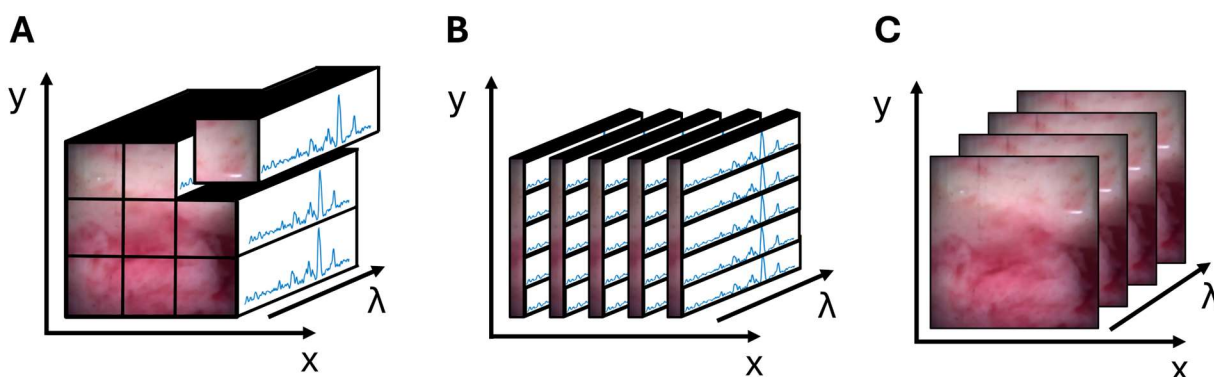


Figure 2-6 : The three most common configurations for Raman imaging: A) raster scanning, B) line scanning and C) wide field imaging.

Among the three configurations, raster scanning is the simplest, is the best for high laser intensity requirements such as for nonlinear Raman measurements (e.g. CARS and SRS) and is used with large numerical aperture objective lenses in the case of microscopy. However, the method is known to be the slowest at imaging large areas [80]. Line scanning is faster than raster scanning due to its ability to recover spatial and spectral information simultaneously during each line measurement. Reconstructing a hyperspectral image over a large surface area can be achieved quickly by scanning the line over the target area [80], [83]. However, line scanning is more complicated to implement due to the requirement of synchronizing the scanning mechanism with the image capture. Wide field imaging is much faster for acquiring intensity images over a large area $>1 \text{ cm}^2$. However, to acquire spectra it is required to cycle through multiple wavelengths. for the latter is challenging for Raman spectroscopy [84]. Current tunable filters (e.g. liquid crystal filters) for visible and NIR wavelengths have a spectral linewidth that is large ($> 7 \text{ nm}$) which significantly hampers the ability to observe well-defined Raman peaks in tissues. An alternative setup involves the use of narrow linewidth filters ($\leq 0.5 \text{ nm}$) but, their use would require mechanical switching of over 250 filters just to cover the fingerprint spectral range (800 - 950 nm for 785 nm excitation). With a cost per filter exceeding \$999, it is not an economical nor practical solution to image the fingerprint range.

2.3 Machine learning in Raman spectroscopy

Machine learning (ML), a subset of artificial intelligence, encompasses algorithms capable of autonomously performing tasks by discerning patterns and making inferences from data without explicit instructions. ML is highly relevant for Raman spectroscopy as it efficiently recognizes complex spectral patterns (e.g. many different peak intensities between tissues) and enables the automation of tissue discrimination, thereby accelerating decision-making and potentially reducing human error [85]. Additionally, ML extracts valuable insights from multivariate Raman spectra, enabling applications such as disease diagnosis and tissue characterization with improved sensitivity and efficiency, ultimately making Raman spectroscopy suitable for clinical use [86]. ML predictive models are constructed by training them with data (here Raman spectra) and tissue labels i.e. whether a given sample contains cancer cells or not. Through this training process, the models learn to recognize patterns that minimize prediction errors. Implementation of ML algorithms in Raman spectroscopy applied to brain is often reported using two principal steps: feature engineering followed by supervised learning [36].

Feature engineering in Raman spectroscopy involves selecting, transforming, or creating features (i.e. variables) to enhance the performance of machine learning (ML) models and improve their biochemical interpretability. The primary goal of this process is to extract only relevant information necessary for the ML task, thereby mitigating the impact of irrelevant features, confounding factors, and noise. Techniques such as spectral preprocessing, peak detection, baseline subtraction, normalization, and dimensionality reduction are employed to extract meaningful insights from Raman spectra, ensuring the quality and accuracy of ML predictions [87]. Many groups report using principal component analysis (PCA) to reduce the dimensionality of their Raman spectra [56], [88], [89], [90], [91], [92], [93]. PCA works by converting the initial Raman spectra into a new ensemble of orthogonal variables termed principal components (PCs). These PCs represent linear combinations of the original spectral variables and are ranked based their ability to describe the data's variance. Typically, the initial PCs encapsulate the bulk of the variance within the spectral data, facilitating the reduction of dimensions while preserving most of the spectral information [87]. Some groups describe using feature extraction such as band fitting or peak ratios to create new variables [94], [95], [96]. This process requires a much deeper

understanding of the features of the spectrum and what they represent in the classification task. Feature selection (manual or automated) was employed by some groups as a much simpler way to recover the data points responsible for the most interclass variations [97], [98], [99]. This method requires little to no modifications of the original data but can introduce a bias in the ML task.

Following feature engineering, supervised ML algorithms are trained with the retained features to discriminate tissue, e.g. diseased vs healthy tissue. Although many algorithms exist for supervised ML, algorithms used in the Raman spectroscopy literature mostly consisted of linear discriminate analysis (LDA) [100], [101], support vector machines (SVM) [39], [102], [103], [104], [105], decision trees [38], [106], [107], [108], [109], and neural networks (NN) [65], [110], [111], [112], [113]. LDA is a classification technique that finds the linear combinations of features that best separate different classes. LDA works well for linearly separable classes, but it also requires the features to be normally distributed which is not the case in biological Raman spectra [114]. The SVM algorithm finds the hyperplane that best separates different classes in a high-dimensional space. It can handle high-dimensional data and is effective in cases where the data is not linearly separable by using kernels to map data into higher dimensions [115]. SVM can be computationally expensive, especially with large datasets. It also requires careful selection of hyperparameters and kernel functions [116]. Decision trees are tree-like structures where each internal node represents a feature, each branch represents a decision based on that feature, and each leaf node represents the decision outcome. Decision trees are easy to understand and interpret and automatically select important features. However, they are prone to overfitting, especially with complex datasets. They can also be sensitive to small variations in the data [116]. Neural networks are computational models inspired by the structure and function of the human brain. They consist of interconnected nodes organized into layers, including an input layer, hidden layers, and an output layer. Neural networks can learn complex patterns from large amounts of data and are highly flexible in modeling nonlinear relationships such as observed in biological samples [117]. Neural networks require large amounts of data for training and are computationally expensive. They are also black-box models, making them less interpretable compared to the previously mentioned algorithms.

2.4 Molecular and structural characteristics of high-grade gliomas

Developing an AI-based surgical guidance tool requires an understanding of the characteristics of the tissues interrogated in relation to the signal acquired. Understanding the hallmarks of the tissues of interest and their physicochemical environment has four main purposes: 1) the recovered signals (i.e. Raman spectra) need to be adequately labelled (e.g. cancer or normal) to train effective classifiers, 2) recovered Raman spectra need to be linked to actual molecular characteristics in the tissues of interest and not external effects (e.g. human errors in measurements or system noise), 3) classifier predictions need to be reviewed for their accuracy to ensure the Raman imaging is effective at locating residual cancer cells and 4) the behaviour of the classifiers needs to be explainable to facilitate clinical adoption of the method. In this section, the characteristics of malignant gliomas are described, with an emphasis on differences with normal brain, from the perspective of molecular biology and histology (i.e. the study of the microscopic structure of tissues). This will help understand what are the expected molecular and structural differences which will manifest when interrogating normal and cancerous tissues in brain using Raman imaging.

2.4.1 General characteristics of high-grade gliomas

Gliomas are renowned for their invasiveness, angiogenesis, and heterogeneity. High-grade gliomas exhibit aggressive infiltrations into neighboring healthy brain tissue [12]. Their diffuse tumor borders cannot be observed with medical imaging (MRI and CT), contributing to the challenge of complete tumor resection [13], [14]. Peritumoral edema, characterized by fluid accumulation in the brain's interstitial spaces, is a common feature of high-grade gliomas and is often associated with neurological symptoms such as headaches, seizures, and neurological deficits [118]. Moreover, high-grade gliomas are reported to be among the most vascularized tumors in humans [119], with microvascular proliferation sustaining rapid tumor growth and contributing to metastasis [120]. These tumors are also heterogeneous, with various cell types—including endothelial, stromal, cancer stem, and tumor cells—contributing to tumor growth and forming different tissue structures (e.g. blood vessels, stromal tissue and pseudopalisades) within the tumor [121]. Spatial heterogeneity is another hallmark of gliomas, with distinct tumor regions displaying variations in histological features, genetic profiles, and metabolic activity. Notably, the necrotic

core observed in glioblastoma, detectable in MRI and CT scans, reflects rapid cell death in response to a hostile microenvironment with limited oxygen and nutrient availability [122]. This necrotic core is associated with lower metabolic activity and reduced intrinsic fluorescence [123]. Proton magnetic resonance spectroscopy has revealed metabolic alterations in gliomas, including increased choline levels indicative of membrane turnover and decreased N-acetyl aspartate levels reflecting decreased neuronal cellularity compared to healthy or necrotic brain tissue [124].

2.4.2 Histology of high-grade gliomas

To diagnose diseases, assess patient prognosis and help guide therapies, pathologists study the microscopic structures and the biomolecular characteristics of tissues in a discipline known as histopathology. Tissue microstructures are a direct manifestation of cellular behaviour and their physicochemical environment. When cells start growing uncontrollably such as in the case of cancer, these alterations, invisible during the surgery, are visible under microscope and can be specifically characterized with the use of different staining agents. Histopathology is the current gold standard for glioma diagnosis and grading [5]. It is commonly used as a label for developing AI-based classifiers in Raman spectroscopy [125], [126].

Histopathological analyses of specimens necessitate biopsies, wherein tissue extracted from the patient is meticulously prepared through fixing and embedding to preserve cellular structures. Specimen preparation methods either involve freezing the specimen or fixing the specimen in formaldehyde and embedding in paraffin. The specimens are then sectioned into thin slices (3-5 μm) and stained to increase the contrast in the observed microstructures. Frozen sections enable rapid specimen preparation (~30 min) for margin assessment during surgery but are less common in glioma surgery due to errors in intraoperative diagnoses introduced by the method [127], [128]. It is for this reason that formalin-fixed paraffin embedding (FFPE) is preferred specimen preparation method for diagnosing gliomas. For assessing high-grade gliomas, hematoxylin, and eosin (H&E) staining is the primary method employed [5]. Hematoxylin delineates nuclear morphology (e.g., shape, size, and mitotic activity) by staining acidic structures (e.g., nucleic acids) blue, while eosin highlights extracellular matrix and cytoplasm by staining basic structures pink. Additionally, immunohistochemical staining agents such as Ki-67 [129], p53 [130] and isocitrate

dehydrogenase 1 [99], [131] are used by neuropathologists to provide molecular characteristics by identifying specific mutations or protein.

The histological hallmarks observed in high grade gliomas using H&E staining are shown in Figure 2-7 and include pleomorphic cells (cells with varying sizes and shapes), visible mitotic activity (increased mitotic figures indicating a high rate of cell division) and nuclear atypia (deformation of cellular nuclei and deviation from regular cell shape) [132], [133]. High-grade gliomas and glioblastomas show a deteriorated cell structure, an increase in cellularity and a loss of differentiation of cells. In the case of glioblastomas (grade IV glioma) particularly, additional histological features include necrosis (dead cells resulting from tissue hypoxia and nutrient deprivation), pseudopalisading necrosis (generation of palisades of cells surrounding a necrotic area) and vascular and microvascular proliferation (generation of new blood vessels to enable tumor growth). Generally, high nuclear to cytoplasmic ratios and the presence of multinucleated cells are observed. Nuclei in malignant gliomas are usually oversized while also showing deformation known as nuclear atypia.

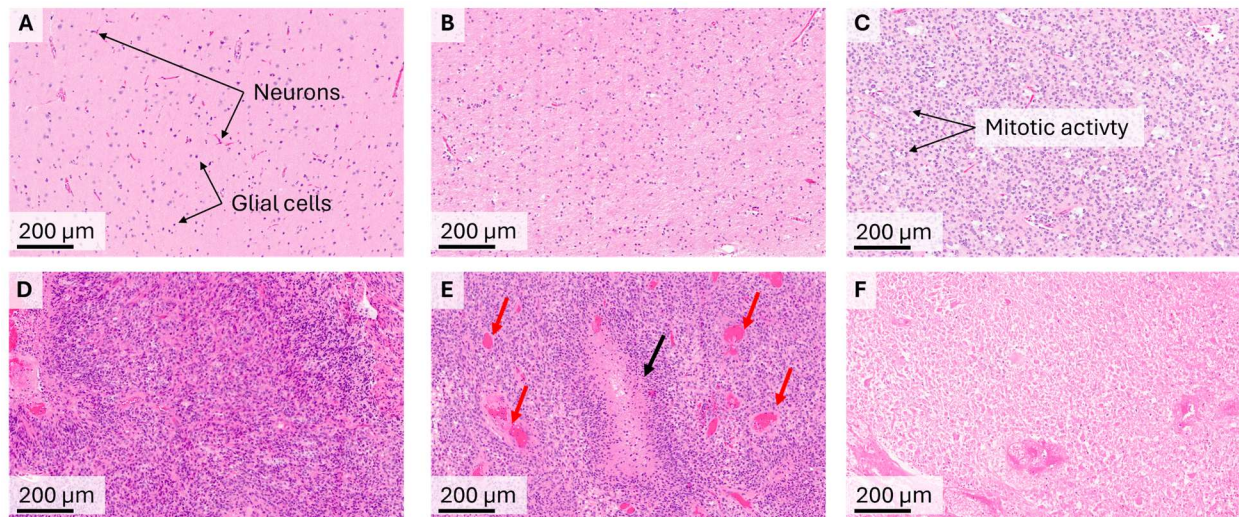


Figure 2-7 : Histology of human brain from glioma patients showcasing A) normal brain with differentiated structures and cells, B) tumor infiltrations in the tumor periphery, C) increased cellularity and mitotic activity from a grade III astrocytoma, D) high cellularity, mitotic activity, pleomorphic cells and loss of brain structures (e.g. neurons) in a high-grade glioma, E) pseudopalisading necrosis (black arrow) and

microvascular proliferations (red arrows), both indicators of grade IV glioma (glioblastoma) and F) large area of necrosis from the tumor core following cell death, also a marker of glioblastoma.

2.5 Raman spectroscopy in glioma surgery

Raman spectroscopy has emerged as a promising tool for intraoperative tissue characterization and surgical guidance. By capturing the molecular composition and employing trained ML models to interpret Raman spectra, this approach may offer specific molecular insights to aid surgeons in real-time decision-making. This section reviews the studies aimed at characterizing brain and glioma lesions during surgery using Raman spectroscopy.

Many Raman spectroscopy studies in glioma have been conducted *ex vivo* in either fresh or frozen specimens using microscopy systems with the aim of characterizing molecular composition and provide intraoperative margin assessment. The most common experimental setup for Raman spectroscopy was used for frozen tissue section analyses. The system of choice was an InVia Microscope (Renishaw, Gloucestershire, UK) equipped with a 785 nm excitation laser, a spectral range of 600 – 1800 cm^{-1} and a spectral resolution of $<4 \text{ cm}^{-1}$ [97], [98], [103], [134], [135], [136]. Although some groups reported collecting Raman signal over areas $> 1 \text{ mm}^2$ through raster scanning, their acquisition times ($>1 \text{ h}$) exceeded practical clinical timeframes for intraoperative assessment. As an example of reported Raman spectra in brain, Figure 2-8 shows the mean fingerprint spectra acquired by Auger et al. in frozen brain specimens for normal, white matter, gray matter, tumor (GBM), infiltrating tumor, and necrosis [86]. This example showcases the subtle differences captured by Raman spectroscopy between the different tissue types.

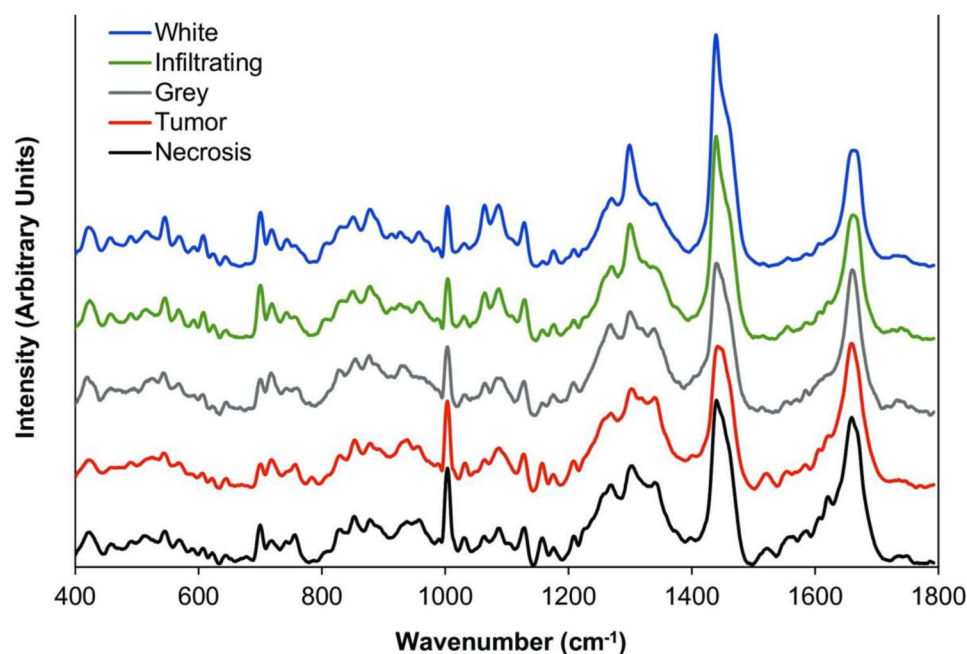


Figure 2-8 : Mean fingerprint Raman spectra of frozen human brain specimens for normal, white matter, gray matter, glioblastoma, infiltrating tumor, and necrosis [86] (CC BY 4.0).

More recently, SRS has been suggested as a faster and highly resolved imaging technique which can provide intraoperative histology assessment of brain specimens. SRS imaging of the CH_2 Raman shift ($2,845\text{ cm}^{-1}$) and the CH_3 Raman shift ($2,930\text{ cm}^{-1}$) can enable digital reconstruction of images reproducing the information content of H&E-stained sections, from frozen or fresh specimens. The first reported use of SRS histology in human brain was in 2015 by Ji et al., where tumor-infiltrated tissue sections were automatically and accurately assessed to reveal the presence of cancer cells in frozen sections [137]. Following this, SRS histology was tested in fresh brain specimens by Lu et al. (2016). The study revealed that SRS histology retains morphological information that is otherwise lost during tissue preparation and H&E staining [138]. Orringer et al. then tested, in 2017, a mobile SRS system that was deployed in a hospital setting and was used to digitally replicate H&E staining (2.5 min per area of $400 \times 400\text{ }\mu\text{m}$) [125]. They were able to automatically classify the imaged tissues and proved that the classification was reliable when compared with histopathology. In 2018, Hollon et al. reported the successful use of a random forest model to classify tissue *in situ* with 100% accuracy [126]. That study used fresh pediatric brain tumors analysed with SRS. These works demonstrate the potential of SRS histology as a

viable method for assessing surgical margins. However, a major limitation of this approach is that it necessitates the resection of tissue for analysis, leading to the need to resect healthy tissue to observe negative margins.

With the aim of keeping healthy tissue in the patient and resecting only cancerous tissue, two *in vivo* animal studies were published in which a Raman spectroscopy system was used to characterize gliomas *in vivo* in animal models. Beljebbar et al. (2010) reported using a single point Raman spectroscopy system with a fiber optics probe (400 μm central excitation fiber surrounded by nine 200 μm collection fibers) to interrogate gliomas in a rat model [88]. This system acquired fingerprint Raman spectra (900 – 1800 cm^{-1}) with a 830 nm excitation laser, within 5 seconds and with a spectral resolution of 4 cm^{-1} . The study from Minbiao et al. (2013) reported using a microscopy system to capture SRS images in human glioblastoma xenografts, in mice [70]. They captured high-wavenumber Raman signal (spectral range: 2800 – 3100 cm^{-1}) using a pump laser of 720 - 990 nm and a Stokes laser at 1064 nm. They reported that the ratio of Raman signals at 2930 and 2845 cm^{-1} reflects the different lipid and protein contents which allowed the visualization of tumors in mouse models ($n = 6$). By combining several patches, they were able to cover an area $\sim 4 \text{ mm}^2$ with an acquisition time $>8 \text{ s/mm}^2$.

The pioneers in testing Raman spectroscopy in humans *in vivo* is the Leblond group which published their first intraoperative glioma study in humans in 2015 [108]. This study was conducted using a sterilizable single point probe [107] (shown in Figure 2-9) at the Montreal Neurological Institute & Hospital. The single point system acquired fingerprint Raman spectra during glioma surgery using a 785 nm excitation laser, over a diameter of 500 μm and within a few seconds. They report testing supervised ML models (boosted trees and SVMs) detecting brain cancer with a sensitivity of 93% and a specificity of 91%. In a subsequent study in 2017, using a combination of Raman, fluorescence and diffuse reflectance spectroscopy, the group reported a supervised ML model with a prediction specificity, sensitivity and accuracy of 93%, 100% and 97%, respectively, for differentiating normal brain from grade 2-4 gliomas *in vivo* [139].

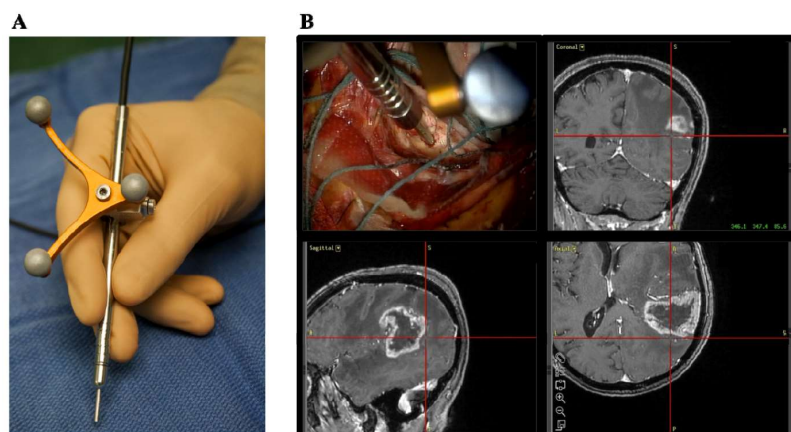


Figure 2-9 : A) Handheld Raman point probe equipped with a neuronavigational apparatus used to interrogate tissue *in vivo* during glioma resection surgery. B) Intraoperative view of the probe *in situ* showcasing in the location of the probe tip overlaid with the preoperative MR images [107] (CC BY).

Desroches et al. (2018-2019), reported two studies where a Raman spectroscopy probe was deployed to capture high wavenumber [39] and both fingerprint and high wavenumber [140] spectra through a biopsy needle. Using 671 and 785 nm excitation for high wavenumber and fingerprint collection respectively, they recorded measurements between 1 and 7 seconds with a reported spectral resolution of 1.8 cm^{-1} . Using an SVM model, they obtained sensitivity and specificity of 80% and 90% respectively. A most recent publication from the same group has reported Raman feature engineering involving peak fitting and quality control of data can increase glioma vs normal classification performance over 95% [94]. This publication presented high-quality Raman spectra of *in vivo* glioma grade 2-4 high cancer density vs normal brain and low-density cancer. From these spectra, the groups reported a comprehensive Raman peak assignment methodology which links molecular families (e.g. DNA, lipids, and proteins) and their significance for disease discrimination. No other group has reported *in vivo* use of Raman spectroscopy in human brain.

Raman spectra of normal brain and glioma tissues have been extensively analyzed in the literature. Table 2.1 provides a condensed collection of Raman shifts associated with molecular families and their association with brain tissue phenotypes. Although Raman studies differ in their findings of which Raman peaks are markers of gliomas, there is a consensus that 1004

(phenylalanine), 1339 (nucleic and amino acids) and 1159 and 1523 (collagen and carotenoid) are markers of gliomas [38], [88], [92], [97], [134], [135], [141], [142], [143].

Table 2.1: Prominent Raman bands in glioma and normal brain, their associated molecular bond, molecular assignment, molecular family, and their association with tissue phenotype. Highlighted Raman bands indicate common markers of glioma found in the literature [38], [88], [92], [97], [134], [135], [141], [142], [143].

Raman band (cm ⁻¹)	Associated bond	Assigned molecule	Molecular family
782	Uracil ring breathing	Nucleic acids	DNA
829	O–P–O stretch PO ₂ -stretch	Nucleic acids, tyrosine and proline	DNA proteins
852	Ring breathing	Tyrosine and proline	Proteins
877	C–C–N symmetric stretching C–O–C ring	Choline, tyrosine, hydroxyproline and myelin	Lipids proteins
1004	Symmetric ring breathing νC–C	Phenylalanine, collagen, heme and carotenoid	Proteins
1032	C–H CH ₂ CH ₃ bending	Phenylalanine and collagen	Proteins
1064	C–O stretch C–O–C symmetric stretch C–C stretch	Proline, phospholipids and cholesterol	Proteins lipids
1086	C–C stretch PO ₂ -symmetric stretch C=O vibration	Phospholipids and nucleic acids	Lipids DNA
1130	C–C stretch C–N stretch CH ₂ vibration	Phospholipids, cholesterol and glucose	Lipids glucose
1159	C–C C–N	Collagen and carotenoid	Proteins
1268	Delta = CH amide III	Fatty acids and collagen	Lipids proteins
1300	CH ₂ twist and wag amide III	Phospholipids, palmitic acid, cholesterol and collagen	Lipids proteins
1339	CH₂/CH₃ wag Amide III	Nucleic acids, aliphatic amino acids and tryptophan	DNA proteins glycogen
1441	CH ₂ /CH ₃ deformation	Lipid side chains, amino acids, cholesterol, cholesterol ester and collagen	Lipids proteins
1523	C=C stretch	Carotenoid	Proteins
1553	νC=C	Porphyrin and tryptophan	Proteins
1659	Amide I ν C=C	Nucleic acids, collagen, and unsaturated fatty acids	DNA proteins lipids
1670	Amide I C=C stretch	Cholesterol, cholesterol ester and collagen	Proteins
2845	CH ₃ stretch	Fatty acids	Lipids
2885	CH ₃ stretch CH ₂ assym. stretch	Lipids and Proteins	Lipids
2930	CH ₃ stretch CH ₂ assym. stretch	Fatty acids Proteins	Proteins lipids

2.6 Raman intraoperative imaging probe systems

The progress of tumor characterization with Raman spectroscopy has led to a growing interest in using Raman imaging probe systems to provide molecular insights over broader surface areas in the operating room [36], [144]. In the case of glioma surgery, these imaging systems could help surgeons identify and visualize diffuse glioma tumor borders during the surgery [145], [146]. In this section we cover the progress in Raman endoscopes and imaging probes systems designed for intraoperative characterization of tissues.

To conduct Raman imaging intraoperatively, some groups have proposed and tested compact CARS endoscopes, however none have yet been used *in vivo* or on brain tissue [147], [148], [149]. The reported CARS endoscopes had FOVs up to $350 \times 350 \mu\text{m}$, had spatial resolutions as low as $1 \mu\text{m}$ and acquired images in as little as 0.8 seconds using raster scanning [148]. Although the endoscopes are ergonomic and can be handheld, concerns were noted regarding their fragility for clinical settings [36]. Additionally, CARS systems rely on large free-space lasers and an optical table for alignment, necessitating further engineering to transition these systems towards commercial clinical deployment. As an alternative to CARS, Liao et al. (2018) have proposed a handheld SRS probe for producing highwavenumber ($2800\text{--}3100 \text{ cm}^{-1}$) images with a 15 cm^{-1} resolution over a FOV of $380 \times 380 \mu\text{m}$ (shown in Figure 2-10A) [40]. Using this system, the team imaged frozen sections of healthy and cancerous canine brain and they report acquisition times as low as 3 seconds per image. Although CARS and SRS probes have shown promise to rapidly image tissues intraoperatively, they remain limited to very small FOVs and thus remain impractical for large surgical cavities.

Some larger FOV systems were proposed to interrogate brain specimens in the operating room. Among these systems we find a Raman imaging system designed for *in situ* tissue characterization, consisting of a wide-field excitation, line scanning system, which can discriminate white matter from grey matter in veal brains using linear discriminant analysis. This system acquired images in 120 seconds over a FOV of $3.5 \times 4 \text{ mm}$ with a spatial resolution of $100 \mu\text{m}$ and spectral resolution of 6 cm^{-1} [150]. This system employed a handheld imaging probe with a coherent fiber bundle for fingerprint Raman signal collection (shown in Figure 2-10B). An alternative wide-field system was proposed by Papour et al. (2015) to interrogate bone *ex vivo*

using multispectral Raman imaging over $1 \times 1 \text{ cm}^2$ [82]. This system used a 785 nm excitation laser combined with a narrow band tunable filter to capture two monochromatic images with a spatial resolution $\sim 10 \text{ }\mu\text{m}$. By tilting the narrow band filter 10 degrees, the group could capture images at 849 or 852 nm (equivalent to 960 or 1002 cm^{-1}) and successfully capture with seconds the location of bone fragments in specimens. Schmälzlin et al. (2014 & 2018) reported on the development and testing of a Raman imaging system designed for human *in vivo* applications, specifically targeting skin cancer, with a notable measurement area of 1 cm^2 (shown in Figure 2-10C) [151], [152]. The group captured fingerprint spectra in both fresh skin tumor specimens and *in vivo* skin however, they did not demonstrate cancer margin detection or cancer detection with machine learning. Similar to other Raman imaging systems, this apparatus lacks sterilizability and is cumbersome, featuring a 20 mm head diameter mounted on a larger square head mount, rendering it impractical for surgical guidance purposes. Other imaging probe systems have called on using SERS nanoparticles to enhance the signal in tissues [153], however the use of nanoparticles to exploit SERS is out of the scope of this dissertation.

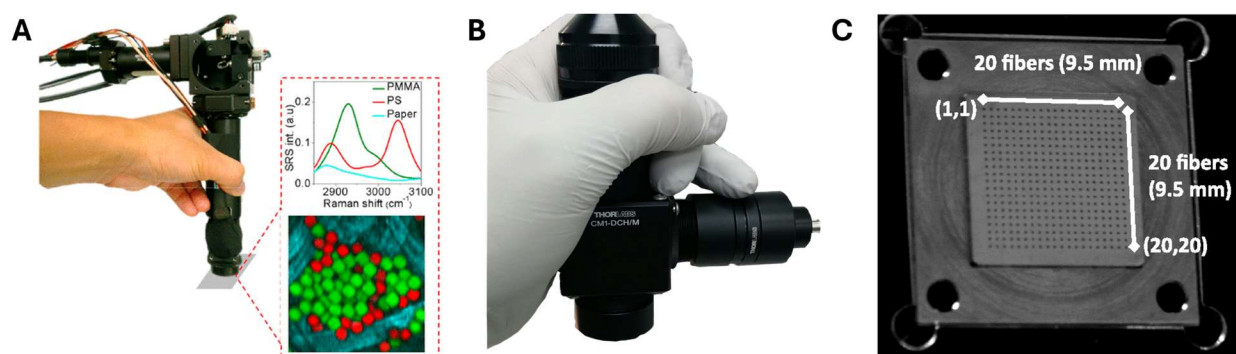


Figure 2-10: A) Fiber-delivered, Handheld SRS microscope [40] (reprinted with permission). B) Handheld wide-field Raman spectroscopy imaging probe with large FOV. [60], [150]. C) Acquisition head of a large FOV Raman imaging probe system used for skin cancer detection [152] (CC BY 4.0).

Despite these advancements, no Raman imaging system has yet been deployed to characterize *in situ* surgical margins in open surgery in humans [154]. Challenges such as sterilizability, lengthy acquisition times, and limited FOVs remain significant barriers to the clinical translation of Raman imaging for intraoperative guidance in human glioma surgeries.

CHAPTER 3 GENERAL METHODOLOGY

This chapter describes the general methodology employed for the work presented in this dissertation. The chapter is divided into three sections where each section presents one objective, the methodology to address that objective and all collaborators that were involved. For each section, all related published articles, submitted articles and patent applications are listed. Moreover, my contributions to the work accomplished are detailed.

3.1 Development of a novel Raman imaging system

Objective 1: Design and develop a large field of view ($\sim 1 \text{ cm}^2$) Raman imaging system proof-of-concept for machine-learning-based molecular tissue characterization and discrimination.

The interdisciplinary work presented in this thesis involved many collaborators. The industrial collaborator for this proof-of-concept system was Optech (Montreal, Canada) which specializes in optomechanical, industrial and optical systems design. Optech contributed laboratory space and technical consultation services while also handling the optomechanical design, optical simulations, and a portion of the system fabrication. Dr. Kevin Petrecca, (neurosurgeon and Chief of the Department of Neurology and Neurosurgery at the Montreal Neurological Institute and Hospital (MNI)), provided consulting services to identify relevant technical specifications for an imaging system destined for intraoperative use and provided user feedback. Development and testing of the imaging system were conducted at the LumedLab located at both the CRCHUM and Polytechnique Montreal. A previously designed benchtop Raman imaging system ([60], [150]) was used to guide some of the design choices of the novel imaging system.

Sub-Objective 1.1: Design and fabricate a Raman line scan imaging system for molecular tissue characterization.

The first objective required the creation of a novel Raman imaging system capable of tissue characterization. The choice of Raman imaging system configuration was a fingerprint (spectral range from 400 cm^{-1} to 2000 cm^{-1}) line scanning imaging modality combined with bright field imaging. Key technical specifications included an excitation wavelength of 785 nm, a FOV of 1

cm^2 , a Raman pixel size and spatial resolution $\sim 250 \mu\text{m}$, a Raman spectral resolution $\leq 8 \text{ cm}^{-1}$, a bright field resolution of $50 \mu\text{m}$ and a flexible imaging probe. The optical design of the system was generated using an optical design software and prioritized the use of off-the-shelf components. The optomechanical design of the system was developed using computer modeling software. A custom designed graphical user interface and software was created to control the system. The resulting system is shown in Figure 3-1 and consists of an excitation branch (Figure 3-1(A)), an imaging probe (Figure 3-1(B)) and a collection branch (Figure 3-1(C)). An imaging fiber bundles relays the excitation laser line between the excitation branch and the imaging probe while another imaging fiber bundle relays collected images from the probe to the collection branch.

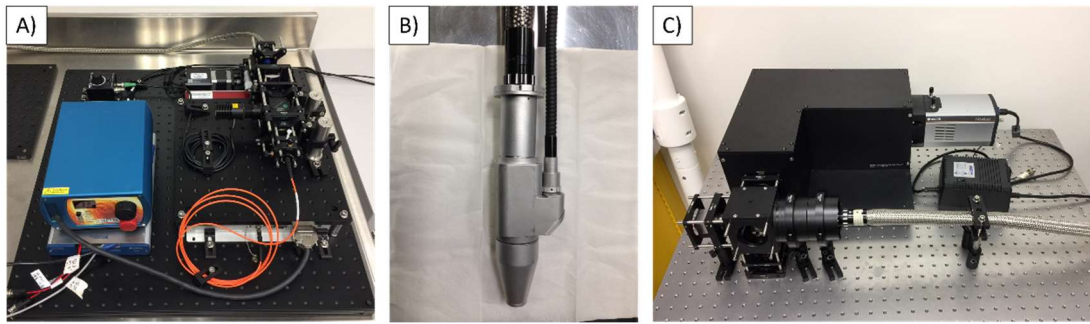


Figure 3-1: Raman line scanning imaging system consisting of (A) an excitation branch, (B) an imaging probe and (C) a collection branch.

Key system technical specifications were verified using the methods listed in Table 3-1.

Table 3-1: Technical specifications and their respective characterization method

Technical Specification	Characterization Method
Field of view	Ruler at probe focus
Spectral Resolution	CaCO_2 FWHM Raman Band at 1035 cm^{-1}
Spatial Resolution	USAF 1951 target
Line Profile	Camera placed at probe focus
Exposure Power	Power meter at probe focus
Laser Divergence	Optical simulation (Zemax OpticStudio)
Probe NA	Optical simulation (Zemax OpticStudio)

Spectral Range	Acetaminophen measurement
Exposure Time	Time required to fill camera dynamic range
Practicality	User feedback (Dr. Petrecca, MNI)
System Response	Raman NIST Standard (SRM 2241)

Sub-Objective 1.2: Characterize the molecular content of animal tissue using the Raman line scan system.

Sub-Objective 1.3: Evaluate the system's ability to locate tissue borders with machine-learning-based classifiers.

The system was used to image and characterize *ex vivo* porcine adipose and muscle tissue. A pre-processing workflow was established to extract only Raman spectral content from all optical signals collected. This workflow consisted of the following steps: 1) cosmic ray removal, 2) wavelength axis correction, 3) system response correction, 4) background subtraction and 5) spectra normalization. Spatially collocated bright field images were used to visually assign labels (muscle or adipose tissue) to the Raman images. Raman spectral band assignments were then conducted and compared with the literature to ensure biological relevance observed Raman spectra. SVM and RF machine learning models were derived from spectra of muscle and adipose tissue to perform pixel-wise discrimination of these tissue types. Machine learning models were trained and validated on three porcine samples and 4,446 spectra. The prediction maps were shown and pixel-wise prediction probabilities were used to highlight tissue borders.

The development of the Raman imaging system resulted in the publication of an article presented in Chapter 4 and two granted patents [155], [156]. My contributions to this work first involved identifying the technical requirements of the Raman imaging system destined for intraoperative testing. With the requirement of having a flexible probe, I conceived and demonstrated the feasibility of using coherent fiber bundles as a flexible medium to relay 2D laser excitation patterns (e.g. a laser line) for Raman imaging. I participated in a 3-year collaboration with industrial partner Optech where I partook in the optomechanical design of the system to meet its technical and clinical requirements. I assisted Optech in fabricating the imaging system. I

significantly upgraded an existing graphical user interface and software code (designed for a previous Raman imaging system [60]) to support the novel imaging system's functionalities (i.e. bright field imaging, translation stage control and line scanning synchronization). I performed and interpreted all the characterization methods listed in Table 3-1 except for optical simulations which were conducted by Optech. I acquired all Raman and bright field images with the system and pre-processed them. I participated in the writing of a granted patent and a patent pending patent application, both of which I have equal authorships (33.33%) with two additional authors. Finally, I authored >80 % of the published Article 1 presented in Chapter 4.

3.2 Development of a clinical prototype Raman imaging system for intraoperative use

Objective 2: Enhance the proof-of-concept Raman imaging system to create a safe and portable clinical prototype suitable for *in situ* intraoperative tissue characterization.

Involved industrial collaborators for this objective were Optech (Montreal, Canada) and EmVision LLC (Florida, USA) which specializes in Raman spectrometers, probes, and accessories for clinical settings. Clinical collaborator Dr. Kevin Petrecca provided consulting services regarding constraints found in an operating room and guided the development of the Raman imaging system towards a clinical prototype. Development and testing of the system were conducted at the LumedLab within the CRCHUM, and clinical safety assessments were conducted in an operating theatre of the MNI.

Sub-Objective 2.1: Develop and validate the capability of the system to conduct *ex vivo* (i.e. outside of the patient) and *in vivo* (i.e. within the patient) measurements in humans within an operating room.

For the system to be used within a clinical setting, the system first needed to be adapted for use in an operating theatre. Effectiveness, practicality, and safety of the system were prioritized in this adaptation. Effectiveness of the system was first improved by replacing the excitation source with a more powerful 5 W, 785 nm laser. It was then improved by replacing the system's spectrometer to obtain 100% more photonic collection. Guided by optical simulations, Raman and

bright field photon collection were improved by replacing key optical components of the collection branch. Practicality was addressed by mounting the system on a mobile cart capable of navigating a hospital. Custom securing mechanisms were manufactured for quick assembly and disassembly of the system with minimal disruption to optical alignment. Practicality was further improved by developing an extendable passive mechanical support arm to easily handle the imaging probe and to keep the probe in place during measurements. For system safety, sterilizable and biocompatible probe muzzles were designed and manufactured while a robust sterilization procedure was devised. Light tight enclosures for the system were fabricated to protect users from accidental laser exposures and to mitigate light pollution from exterior lighting. A custom light enclosure was also fabricated for *ex vivo* measurements.

Sub-Objective 2.2: Optimize and validate the clinical prototype's imaging workflow (i.e. acquisition parameters, pre-processing, and machine-learning) in a large dataset (>20 samples) from animal tissue.

Spectral and imaging pre-processing methods were improved based on the results and findings from Sub-Objective 1.3. A noise eliminating step and a novel baseline removal technique were introduced into the workflow. All pre-processing of Raman images and bright field images were conducted using a custom developed code. Imaging of biological tissue was conducted in 24 porcine muscle and adipose tissue samples from 3 pigs. Imaging of tissue was conducted with and without a contact probe muzzle. Raman features for classification were selected manually. Classification was achieved using RF and SVM models trained on the first pig, validated on the second pig, and tested on the third pig.

Sub-Objective 2.3: Assess the safety of the system for users and patients in a clinical setting.

Clinical safety assessments were conducted for biocompatibility, sterilisation, laser safety, electrical safety, and laser exposure safety. Materials for the probe muzzle were carefully selected to be biocompatible and sterilizable using multiple standard methods (e.g. steam sterilization). A sterilisation procedure was elaborated and tested using a combination of a surgical drape and sterilisation cycle. Optech performed leak tests in 3 manufactured probe muzzles to ensure no leaks occurred in the probe muzzles. Electrical safety was in part considered with the use of a medical

grade isolation transformer. Laser safety was assessed for accidental laser exposure in skin and eyes using ANSI laser safety standards. Laser exposure risk to interrogated tissue (i.e. porcine adipose and muscle tissue and rat blood) was assessed using the thermal time history known as CEM43°C. Transportability of the system and sterilization procedure was testing within an operating theatre at the MNI.

The development of the clinical Raman imaging prototype resulted in the publication of an article (presented in Chapter 5). My contributions to this second publication involved the identification, installation and testing of new system components (i.e. spectrometer and laser). I converted the system towards a portable cart-mounted prototype with a support arm with the help of colleagues. I coordinated the development with Optech of the customized probe mounts and probe muzzles for the imaging probe. I implemented the suggested optical component ameliorations in the system following Optech's optical simulations and tested the ameliorated photon collection of the system. I developed the methodology of the study in porcine tissue. I performed data acquisition in tissue, data processing and data analyses. I implemented a robust machine learning protocol for SVM and RF classifications and assessed all classification model performances with the assistance of data scientist, Frédérick Dallaire. I undertook all safety assessments and safety tests of the system except for leak tests which were conducted by Optech. Finally, I authored >80 % of the published Article 2 presented in Chapter 5.

3.3 Evaluation of the Raman imaging system in a clinical setting on human tissue

Objective 3: Evaluate the feasibility, practicality, and performance of the clinical prototype in a pilot study on resected tumoral tissue from patients affected by brain tumours.

A pilot study in eight patients with glioblastoma was conducted under principal investigator and neurosurgeon Dr. Kevin Petrecca at the MNI where craniotomies are performed weekly to surgically remove brain tumors. Dr. Petrecca obtained consent from patients, conducted surgery for glioma resection and provided the resected tissue specimens for the study. Recovered brain specimens originated the tumor core, the tumor interphase, or the tumor periphery. Dr Marie-Christine Guiot (neuropathologist at the MNI) was consulted for developing a measurement

protocol which was compatible with current hospital specimen examination procedures. She supervised several specimen preparation steps (i.e. paraffin embedding, shaving, sectioning, and staining) and provided histological analyses and labels for all human brain specimens. Raman imaging of brain specimens was conducted in the operating room within one hour of resection.

The medical device company Reveal Surgical, provided a large dataset of Raman spectra acquired in several glioma patients to assist in interpreting the Raman image data. A machine learning model was created from the dataset and integrated into the imaging system to predict the likelihood for each pixel containing cancer. The glioblastoma and normal brain spectra were compared with the Raman spectra provided by Reveal Surgical to ensure reproducibility. Pre-processing methods for Raman and bright field images as well as classification methods were inspired and improved from Objective 2. These improvements include removing an additive system response by subtracting a blank measurement and introducing a novel quality factor (QF) metric to assess and compare the quality of spectra within Raman images.

The *ex vivo* pilot study in the human brain resulted in the article (submitted for publication) presented in Chapter 6. My contributions to this third manuscript involved the creation of the imaging system for the study, the elaboration of a significant portion of the study methodology and coordination of the study with involved clinicians and interns. I operated the Raman imaging system in the operating room and performed tissue manipulations, and acquisition of Raman images with the assistance of the intern Hugo Tavera. I performed the initial pre-processing steps of Raman images and submitted them for pre-processing and machine learning classification with the data scientist of the team, Frédérick Dallaire. I partook in the interpretation of the results. Finally, I authored >60 % of the published Article 3 presented in Chapter 6.

CHAPTER 4 ARTICLE 1: HANDHELD MACROSCOPIC RAMAN SPECTROSCOPY IMAGING INSTRUMENT FOR MACHINE- LEARNING-BASED MOLECULAR TISSUE MARGINS CHARACTERIZATION

JOURNAL OF BIOMEDICAL OPTICS – SPIE

Published on February 12th, 2021

François Daoust,^{1,2} Tien Nguyen,^{1,2} Patrick Orsini,³ Jacques Bismuth,³ Marie-Maude De Denus-Baillargeon,³ Israel Veilleux,^{1,2} Alexandre Wetter,³ Philippe Mckoy,³ Isabelle Dicaire,³ Maroun Massabki,³ Kevin Petrecca,⁴ and Frederic Leblond,^{1,2}

¹ Polytechnique Montreal, Department of Engineering Physics, Montreal, QC H3T 1J4, Canada

² Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montreal, QC H2X 0A9, Canada

³ Optech, LaSalle, QC H8N 2J4, Canada

⁴ Department of Neurology and Neurosurgery, Montreal Neurological Institute-Hospital, McGill University, Montreal, QC H3A 2B4, Canada

4.1 Background

In previous chapters, we defined the scope of this dissertation, its objectives and what methods were employed to complete these objectives. We identified that Raman imaging is a potential solution to characterize large areas of tissues and locate cancer margins during surgery. To test this, it was determined that a novel imaging system first needed to be designed and fabricated to image a field of view of at least 1 cm². In this chapter, we present the work leading to completing our first Raman imaging prototype. This work was accomplished to answer this dissertation's Objective 1: *Design and develop a large field of view (~1 cm²) Raman imaging system for machine-learning-based molecular tissue characterization.* To assist in completing this objective, we consulted the Centre for Applied Research: Optech which significantly contributed to designing and fabricating this new device. We first established the technical specifications required for this novel imaging

system destined for use in an operational theatre. We then designed and fabricated the system in a line scanning configuration using a flexible imaging probe with a 785 nm excitation wavelength and bright field. We verified the technical specifications of the device and confirmed it had a field of view $\sim 1 \text{ cm}^2$. We tested its ability to recover Raman images by characterizing the Raman signature of porcine adipose and muscle tissue. From these characterizations, machine learning was successfully employed to locate tissue borders and discriminate adipose from muscle tissue with a 99% accuracy.

4.2 Abstract

Significance: Raman spectroscopy has been developed for surgical-guidance applications interrogating live tissue during tumor resection procedures to detect molecular contrast consistent with cancer pathophysiological changes. To date, the vibrational spectroscopy systems developed for medical applications include single-point measurement probes and intraoperative microscopes. There is a need to develop systems with larger fields of view for rapid intraoperative cancer margin detection during surgery.

Aim: To design a handheld macroscopic Raman imaging system for in vivo tissue margin characterization and test its performance in a model system.

Approach: The system is made of a sterilizable line scanner employing a coherent fiber bundle for relaying excitation light from a 785 nm laser to the tissue. A second coherent fiber bundle is used for hyperspectral detection of the fingerprint Raman signal over an area of 1 cm^2 . Machine learning classifiers were trained and validated on porcine adipose and muscle tissue.

Results: Porcine adipose versus muscle margin detection was validated ex vivo with an accuracy of 99% over the field of view of 95 mm^2 in ~ 3 minutes using a support vector machine.

Conclusion: This system is the first large field of view Raman imaging system designed to be integrated in the workflow of surgical cancer resection. It will be further improved with the aim of discriminating brain cancer in a clinically acceptable timeframe during glioma surgery.

Keywords: Raman spectroscopy, imaging systems, macroscopic imaging, machine learning, surgical guidance.

4.3 Introduction

The current standard treatment for malignant tumors involves the surgical resection of the compromised tissue. Tumor resection can alleviate patient suffering related to tumors pressing on healthy organs [157], [158], [159] and is shown to improve patient prognosis in breast [160] and pancreatic [161] cancer. Improved prognosis is especially true when maximizing the extent of cancer resected since residual cancer cells can lead to tumor recurrence [162], [163], [164], [165]. Prior to surgery, diagnosis of the tumor is usually achieved through means of palpation, structural imaging (e.g. magnetic resonance imaging (MRI), computer tomography (CT) and ultrasound), molecular imaging (e.g. positron emission tomography (PET)) or histology (e.g. biopsy). These methods serve to evaluate the location, severity and extent of disease, to determine surgical success and assess postoperative disease progression. Surgeons then establish their surgical plan and proceed to the tumor site through open or minimally invasive surgery where the bulk of the tumor is often identified by visual inspection and palpation. Additional tools such as frozen section histology and intraoperative MRI are also used in some tumor sites to further help in assessing disease margins. Tumor grade and location may warrant the resection of complete organs, as can be the case for prostate and breast cancer, while other sites (e.g. brain cancer), require meticulous maximal resection of malignant tissue while preserving the neighboring healthy tissue [13]. Histopathological analysis of the resected tissue during (i.e. Mohs micrographic surgery) or after surgery is used to assess the presence of positive margins, defined as cancer cells located at the periphery of the resected tissue. It is estimated that 15% to 60% of surgeries for head, neck, liver, oral, breast and prostate cancers will result in positive margins following the principal surgery [53]. To improve patient prognosis, positive margins can require an additional surgery from which ensues further patient trauma and complication risks [166], [167], [168]. On the other hand, it has been suggested that reducing healthy tissue resection can lead to fewer surgical complications, lower hospital length of stay and decreased patient discomfort motivating tissue conservation surgery in rectal cancer as an alternative to radical surgeries[169]. It is apparent that extending cancer resection during the principal surgery while preserving healthy tissue is a clinical need for surgical oncology and such a need can be addressed by real time and *in situ* surgical guidance technology.

Methods showing potential for real-time surgical guidance involve molecular imaging modalities which recover, non-invasively, distinct molecular characteristics of tissue and provide surgeons with tissue-specific information to visualize *in situ* tumor extent. Such methods currently employed for medical treatment include fluorescence-guided surgery (FGS), where precursors to endogenous fluorescent molecules or fluorescent molecular probes (e.g. 5-aminolevulinic acid (5-ALA) or indocyanine green (ICG)) are administered orally or injected preoperatively into the blood stream. These probes accumulate in organs in different concentrations and emit fluorescence (e.g. green light for ICG) upon illumination with an excitation light source. In glioma surgery, 5-ALA is metabolized preferentially in tumors into the fluorescent protoporphyrin IX, identifying residual dense cancer within the surgical cavity. Such approaches have demonstrated their effectiveness by increasing the occurrence of complete resection of the tumor (i.e. no visible contrast in postoperative MRI) in glioma surgery [123], [170]. However, FGS does not always show surgeons the full extent of cancer in part due to luminescence quenching, fluorescence probe breakdown or a lack in specificity of probe distribution in healthy and diseased tissue. This translates to absent or hard to detect fluorescence contrast in tissue where only subtle oncological, morphological and/or molecular changes occurred [171]. Studies have shown that postoperative imaging such as MRI, even when employed with contrast agents, is also unable to detect areas associated with low density of cancer cells associated with tumor infiltrations. When used as a gold standard for evaluating complete tumor resection in glioblastomas, tumor volume measured with MRI may confirm the resection of the tumor, but it does not guarantee the absence of residual cancer cells at the surgical site [31], [172]. This clinical reality is associated with the high tumor recurrence rate (85%) at the resection margin in glioblastoma patients and its very poor prognostic even when complete tumor resection is achieved [123], [173]. Efforts are currently being employed to develop new contrast-enhancing molecular probes (e.g. OTL38, LUM015 and SGM-101) with increased specificity for various cancer sites including prostate, breast and colon [174].

Label-free molecular imaging (e.g. diffuse reflectance and endogenous fluorescence detection) has gained interest as an alternative surgical guidance solution due in part to its independence from labels and dyes which require risky, expensive and lengthy safety studies prior to use in patients. Among label-free molecular imaging technologies, Raman spectroscopy (RS) stands out with its ability to recover a molecular specific fingerprint from interrogated tissue. RS is capable of

discriminating in real-time cancer from normal tissue [37] and has been shown to be superior to diffuse reflectance and endogenous fluorescence combined in discriminating between breast tissue histopathologic categories [175]. This non-invasive and non-destructive method employs a laser source to interrogate the intrinsic molecular vibrations of tissue content, i.e. DNA, lipids and proteins, and exploits this tissue-specific information to map tissue heterogeneity accordingly. RS relies on recovering signal from inelastic scattering in interrogated samples, a relatively rare event (approximately 1 in 10^7 photonic events) often difficult to decouple from high intrinsic tissue fluorescence. Improvements in data preprocessing methods, spectrometer resolution, camera sensitivity and laser excitation sources have led to the development and implementation of RS systems for *in vivo* discrimination of tissue [65]. RS for surgical guidance has been demonstrated with handheld single point probes in humans and has already shown across several studies its potential to extend cancer resection effectively following the main tumor resection such as during glioma surgery [65], gastrointestinal inspections [176], early lesions detection in oral cancer [177] and breast cancer lumpectomy [178]. However, point measurements do not offer spatial context which can make difficult the identification of margins and localized cancer infiltrations linked to subtle molecular changes [179]. The need for spatial context motivates the development of Raman imaging to accomplish the task of identifying residual cancer and its margins. Surgical time constraints and user adoption impose the challenging requirement of making Raman imaging a rapid modality which can quickly interrogate large surgical cavities (i.e. several cm^2).

Many Raman microscopes have been developed with the intention of guiding surgery by assisting pathologists in the visualization of tissue margins in excised tissue (e.g. breast [180], skin [181] and oral [182] cancer). These systems can cover areas up to several mm^2 , but they are unsuitable for performing molecular imaging *in vivo*. Handheld stimulated Raman scattering microscopy systems have been developed and tested successfully to address precise tissue margin localization *in vivo* in mice for skin [183] and brain cancer [70]. These systems have a restricted field of view (FOV), i.e. $<4 \text{ mm}^2$, which require numerous measurements to cover the entire surgical area [152]. It was demonstrated by our group in 2016, that coherent fiber bundles, which relay images from one end to the other coherently, can be used for Raman imaging probes for the purpose of wide field and line scanning collection in biological tissue [84]. The most recent system had a FOV of 14 mm^2 and spectral resolution of 6 cm^{-1} . It was shown that acquisition times of 700 to 900 ms per

line were required to discriminate, based on tissue Raman band ratios, porcine adipose from muscle tissue as well as bovine white and grey matter [150]. Raman imaging over a large FOV was also validated *in vivo* in mice with the use of injected nanoparticles for signal enhancement [153]. However, there remains a lack of Raman imaging system with large FOV for *in vivo* human clinical use. The only Raman imaging system with a large measurement area (1 cm^2) that was developed and tested for a human *in vivo* application is reported in Schmälzlin, E. *et al.* (2018) for skin cancer [152] although no cancer margin detection was demonstrated. As is the case for other Raman imaging systems, it is not sterilizable and it remains cumbersome with a head diameter of 20 mm mounted on a larger square head mount making it unsuitable for surgical guidance.

Here we present the design and characterization of a novel Raman line scanning system developed with the aim of being integrated in a human clinical study. The line scanning handheld imaging probe has a spatial resolution of $250 \text{ }\mu\text{m}$ and a spectral resolution of 6 cm^{-1} allowing Raman imaging to be performed over a FOV of 95 mm^2 . The probe is sterilizable and acquires spectra in the fingerprint region from 400 to 1900 cm^{-1} . It is designed to accommodate an apparatus for contact measurement with a head diameter of 20 mm while offering non-contact measurements with a working distance of 40 mm. A bright field channel allows visualization of tissue structures under white-light reflectance. The probe also uses a flexible coherent fiber bundle for collection and is the first to use a flexible fiber bundle for custom laser profile excitation, e.g. a scanned laser-line. The system optical design and corresponding performance metrics, i.e. laser line profile and optical transfer functions, employed for design validation are first presented. Raman hyperspectral images of porcine adipose and muscle tissue were acquired, and band assignments are presented with their corresponding biological interpretation in terms of molecular bonds. The system's ability to discriminate between tissue types is then demonstrated using support vector machine (SVM) technology. A detailed photonic budget analysis is finally presented comparing the system against a proven handheld single-point surgical-guidance Raman system [107]. The future development steps required to achieve *in vivo* human tissue imaging are finally discussed to obtain a comparable level of Raman signal-to-noise ratio within an acceptable clinical timeframe.

4.4 Methods

4.4.1 Imaging system

The line scanning system consists of three main parts (Figure 4-1): an excitation branch, an imaging probe and a collection branch. Each component of the system is controlled via custom software developed using LabVIEW (National Instruments, USA) and a graphical user interface. Components including the laser, the broadband source, a motorized linear translation stage, a galvanometer, a charge-coupled device (CCD) camera and a color (RGB) camera, are synchronized with the help of a digital acquisition device (USB-6351, National Instruments, USA).

The excitation branch (Figure 4-1(c)) consists of light sources for Raman spectroscopy or bright-field imaging, a series of optics and a 2-D translation stage (A-LSQ075A-E01, Zaber, Canada). The excitation branch generates either a scanning laser-line or a wide field white light illumination injected in a $4 \times 4 \text{ mm}^2$ imaging fiber bundle (Schott, USA). The laser source is a custom 785 nm continuous-wave laser (Innovative Photonic Solutions, USA) with 1.8 W power, a spectral width $<0.2 \text{ nm}$ and a numerical aperture (NA) of 0.5. The broadband source consists of a 3000 K light emitting diode (LED) (MWWHL4, Thorlabs, USA) that is turned off during Raman measurements to avoid contamination. Optics (cylindrical lenses and dichroic mirror) are used to generate a laser line on the surface of an imaging fiber bundle, with the secondary role of coupling the 785 nm laser-line and wide field white light sources into the fiber bundle. Finally, the imaging bundle is mounted onto a motorized translation stage responsible repositioning the bundle surface relative to the laser-line thus, ensuring line scanning in the excitation branch.

The imaging probe (Figure 4-1(b)) consists of a series of illumination optics and imaging optics. Illumination optics include an excitation fiber bundle to reproduce the laser excitation pattern from the excitation branch end of the excitation bundle to the probe end and a laser line filter centered at 785 nm (LL01-785-12.5, Semrock, USA) with a transmission spectral range from 425 nm to 600 nm. The excitation pattern (laser-line or widefield) is then projected through the probe illumination optics onto the sample at a working distance of 40 mm. The imaging optics includes a series of optics designed to collect white light (400 – 650 nm) as well as fluorescence and Raman scattering (810 – 922 nm) through another fiber bundle. A dichroic mirror allows for a

maximum reflection of the laser source onto the sample while maximizing fluorescence/Raman collection efficacy. The dichroic ensures a portion of broadband light (50%) is reflected on the sample and collected for bright field imaging. The probe was designed to accommodate a sterilizable and detachable nose with a Raman grade MgF_2 window (i.e. with no Raman peaks between 400 and 1900 cm^{-1} and low intrinsic fluorescence) (Batch #61973, Crystran, USA). This was designed for future in vivo applications for which ensuring tissue contact might be more practical. In the overall optical design, mobile parts, electronics and mechanical components were avoided to facilitate cleaning, sterilization and ensure patient safety. The probe (Figure 4-1(a)) weighs 0.6 kg without the fiber bundles and has a volume of approximately 4.5 (width) x 7 (height) x 18 (length) cm.

The collection branch (Figure 4-1(d)) is composed of a collection fiber bundle, a series of optics, a dichroic mirror (FLD 748 DSP, Iridian, Canada) mounted on a galvanometer scanner (6870M, Novanta, USA), a spectrometer (HT, EmVision, USA), a cooled CCD camera (Newton 920, Oxford Instruments, USA) and a high sensitivity complementary metal oxide semiconductor (CMOS) RGB camera (DCC1240C, Thorlabs, USA). The collection fiber bundle transmits the bright field image and fluorescence/Raman signal collected by the imaging probe to the collection branch. From the bundle collection end, the optics relay the collected image onto the dichroic mirror galvanometer through which visible light is transmitted through a series of optics into the RGB camera for bright field imaging. Raman scattering (810 – 922 nm, or 400 – 1900 cm^{-1}) is reflected into the Raman spectrometer and spectral components are separated onto the cooled (-70 °C) camera sensor. The image captured by the sensor thus possesses a spatial axis (y axis) and spectral axis (λ axis). The rotation of the mounted dichroic mirror controls which area of the imaging probe's FOV (x axis) is aligned with the spectrometer slit and is synchronized with the excitation branch's translation stage position during measurements.

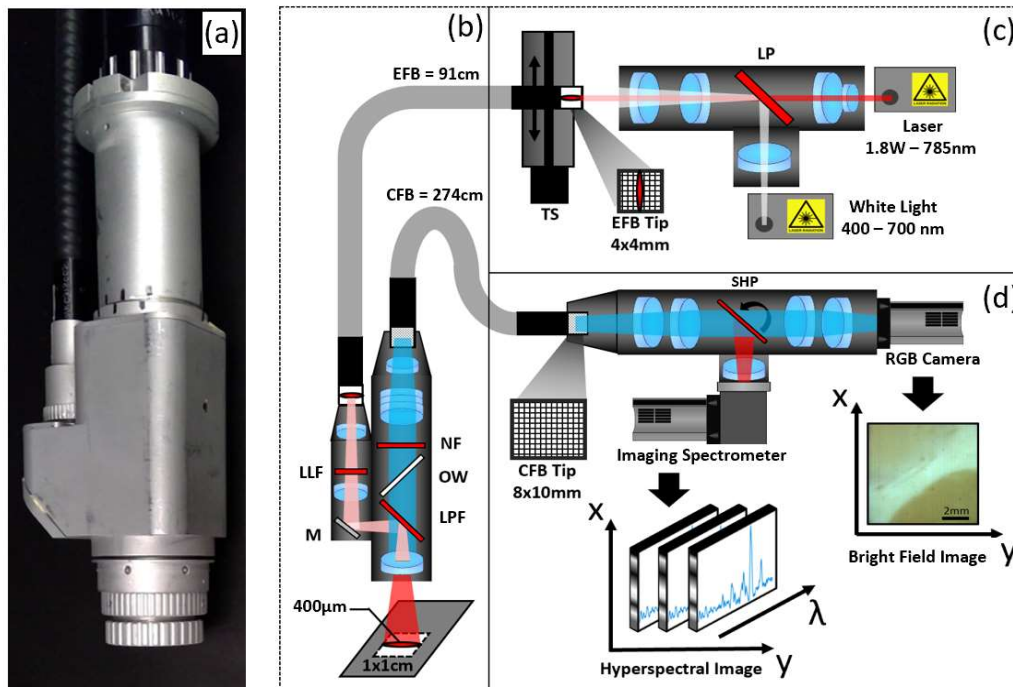


Figure 4-1: (a) Handheld Raman imaging probe and schematic of the imaging system consisting of: (b) the imaging probe with its excitation fiber bundle (EFB), a series of optics (lase line filter (LLF), mirror (M) and long pass filter (LPF)) to propagate white light and laser light onto the sample to interrogate the tissue, a series of optics (LPF, optical window (OW) and notch filter (NF)) to collect the generated Raman signal and reflected white light, and a collection fiber bundle (CFB); (c) the excitation branch generating a line-shaped laser excitation at the entrance of the EFB mounted on a translation stage (TS) while also shining white light into the EFB; (d) the collection branch capturing white light and Raman signal from the collection fiber bundle (CFB), separating the two optical components with a scanning high pass filter (SHP), obtaining a white light image on an RGB camera while extracting spectral features with an imaging spectrometer. All illustrated lenses are spherical unless labelled LH for cylindrical lenses with their curvature in the horizontal direction or LV for cylindrical lenses with their curvature in the vertical direction.

4.4.2 Optical design

Specific lens sizes and focal lengths were determined via the optical design software OpticStudio® (Zemax, USA) to meet requirements in each of the system subcomponents (i.e. excitation branch, imaging probe and collection branch). Off-the-shelf components were selected when applicable. The excitation branch requirements involved producing a uniform $100\text{ }\mu\text{m}$ (x axis) x 4 mm (y axis) laser excitation profile at the entrance of a $4\text{ x }4\text{ mm}$ excitation bundle, from a multimode fiber of $400\text{ }\mu\text{m}$ core diameter with 0.22 NA to be relayed through the $4\text{ x }4\text{ mm}^2$ excitation fiber bundle into the imaging probe. The laser line profile relative intensity was first simulated with the optical design software and was then measured at the face of the excitation fiber bundle with an RGB camera.

The imaging probe optical design was subdivided into two parts: the illumination branch and the imaging branch although each had some optical components in common. Lenses and their positions within the illumination and imaging branches were selected according to the resulting optical transfer function (OTF), evaluated through the optics of each branch, from sample to bundle face, across the circumradius of each branch's fiber bundle face ($4\text{ x }4\text{ mm}^2$ for illumination branch and $10\text{ x }10\text{ mm}^2$ for imaging branch). The normalized OTF was evaluated using the optical design software and used to optimize the spatial resolutions (along the x and y directions) of the system. The optimal optical configurations were those for which the OTF –for specific spatial resolutions and wavelengths– were above 0.5 across the entire circumradius of the fiber bundles. The illumination branch's targeted spatial resolution for 785 nm was set at 2 cycles/mm or $250\text{ }\mu\text{m}$. No resolution limit was set for visible light (400 – 650 nm) illumination since the excitation branch would generate wide field broadband illumination of the sample. The imaging branch's target spatial resolution for bright field imaging (400 – 650 nm) was set at 10 cycles/mm or $50\text{ }\mu\text{m}$ while the minimum resolution for Raman fingerprint imaging (800 – 940 nm) was set at 2 cycles/mm or $250\text{ }\mu\text{m}$. An additional design criterion for the imaging branch consisted of maintaining an >70% relative illumination profile across the circumradius of the FOV of the imaging probe.

The collection branch requirements included maintaining the bright field image spatial resolution relayed by the collection fiber bundle of at least 10 cycles/mm or $50\text{ }\mu\text{m}$ onto the sensor of the RGB camera while also maintaining the relayed Raman image's spatial resolution of 2

cycles/mm or 250 μm onto the 75 μm (x axis) x 7.2 mm (y axis) slit of the Raman spectrometer. The selected optics were deemed acceptable if the OTF for the above-mentioned spatial resolutions, and their corresponding wavelengths, was above 0.5 for the entire circumradius of the white light.

4.4.3 Raman imaging of biological samples

The biological sample used for this study was a pork chop from a butcher shop, 1 cm thick, flat and with a visually clear margin between adipose and muscle tissue at its surface. The tissue was placed over low Raman activity aluminum slide (Miro5011, Anomet, Canada) to prevent any background generated from under the sample. Raman images were acquired in three separate areas of the sample: muscle, adipose and on a margin essentially composed of a mixture of adipose and muscle tissue. Bright field images of the sample were visually inspected and all pixels were labeled as either pure adipose, pure muscle or tissue margin. The sample was humidified with buffered blood bank saline (312-651, Thermo Scientific, USA) drops between measurements. Bright field images before and after line scanning were acquired to confirm sample margins had not shifted during measurements nor had the sample's color changed, which would have been indicative of tissue damage.

The measurement parameters are presented in Table 4-1. The entire FOV of the system (95 mm^2) was imaged by scanning a total of 40 lines on the sample. Binning over 6 pixels along the spatial axis of the CCD (y axis) was done to increase signal to noise ratio (SNR), resulting in a 250 μm effective spatial resolution. Translation distance between line measurements was 250 μm . Binning along the spectral axis (3 pixels) also increased SNR with minor degradation of the spectral resolution. The resulting hyperspectral cube dimensions from line scanning were of 40 (x axis) x 42 (y axis) x 341 (λ axis). The laser line profile was of a 400 μm x 9.5 mm with a total power of 230 mW corresponding to an average intensity of 61 kW/m^2 at the sample surface. The luminescence (i.e. Raman and background fluorescence resulting from 785 nm laser line illumination) from 400 to 1900 cm^{-1} (810 to 922 nm) was collected for each line during a 5 second exposure time. An external broadband light LED was used to uniformly illuminate the sample. The bright field images were used as a reference to place the sample at the focus of the imaging system. The broadband source, along with any other sources of ambient light, were turned off throughout the duration of the line scanning measurements.

Table 4-1: Raman imaging system technical parameters selected for the ex vivo experiments.

	Specifications
Field of View	95 mm ²
Working Distance	40 mm
Number of Lines	40 lines (9.5 mm x 400 μ m each)
Translation Distance	250 μ m between line measurements
Spatial Resolution NIR	<i>x</i> axis: 250 μ m, <i>y</i> axis: 250 μ m
Spectral Resolution NIR	6 cm ⁻¹ (at 1085 cm ⁻¹)
Number of Pixels	<i>x</i> axis: 40, <i>y</i> axis: 42
Spectral Range	λ axis: 400 – 1900 cm ⁻¹
Exposure Time	Adjustable but 5 seconds per line Total: 200 seconds in this study
Spatial Resolution Bright Field	50 μ m

4.4.4 Spectral data pre-processing

The data pre-processing pipeline begins with applying a median filter to each spectrum of the image to eliminate cosmic rays. Following this, a Raman shift (λ axis) calibration is performed from a reference measurement of acetaminophen which has a very well characterized Raman spectrum with low background and is one of the Raman shift standards identified in the American Society for Testing and Materials's (ASTM) Standard Guide for Raman Shift Standards for Spectrometer Calibration (ASTM E1840). This calibration is achieved by performing a cubic-spline interpolation using the 6 most prominent peaks from the acetaminophen Raman fingerprint spectrum. The spectra are then corrected for system response and relative intensity variation across the image with a smoothed reference measurement on a NIST standard for 785 nm excitation (SRM 2241, NIST, USA). The NIST reference measurement was smoothed using a 2D gaussian filter of 2nd order to reduce artifacts introduced by scratches on the NIST standard. Spectra at the borders of the corrected hyperspectral cube were rejected due to border artifacts generated by the gaussian filtered system response. A 5th order polynomial fit is then applied on each system-corrected spectrum to approximate the background before removing it with a rolling-ball algorithm [184] using a ball size of 16. Spectra are then normalized using the standard normal variate (SNV) method prior to use for training, validating and testing a classifier to distinguish adipose from muscle tissue.

4.4.5 Classification

As a proof of concept for tissue margin detection, two classification algorithms were used: SVM and Random Forest (RF). The classifiers were trained and validated to perform pixel-wise classification (i.e. no spatial features were used, only spectral ones). The training set consisted of half an image of adipose tissue (20 lines) and half an image of muscle tissue (20 lines). The validation set was composed of the remaining half of the image of adipose tissue (20 lines), the remaining half of the image of muscle tissue (20 lines) and the entire image of a tissue margin (40 lines). The Raman images of only adipose or muscle tissue consist of an average of 10 acquisitions (total acquisition time of 2000 seconds) at the same spatial location to maximize signal-to-noise ratio (SNR). The margin image (Figure 4-6(a)) consists of a single Raman image acquisition (total acquisition time of 200 seconds) where the margin location is centered in the system FOV to ensure an even class distribution. Assigning class labels to images of solely porcine adipose or muscle tissue was straightforward since each image contained one class, whereas labeling of the tissue margin was executed visually using tissue color and structure changes across the bright field image. In total, the training set consists of 1482 spectra (741 for each class) and the validation set consists of 2964 spectra (741 for each class and 845 (adipose) + 637 (muscle) from the margin image). Including data from adipose, muscle and margin images into the validation set forces the models to learn to classify spectra coming from single class images (i.e. only adipose or muscle tissue) and from margin images for which there might be inter-class cross-talk.

The classification models were built by constructing the following pipeline: feature selection, feature standardization and classifier development. The feature selection step allows reducing the dimensionality of the dataset to simplify and improve the classifier performance. Here, a linear SVM penalized with the L1-norm regularization (L1-SVM) [185] was used as a feature selector. L1-SVMs have sparse solutions: many weights assigned to the features are zero. Features having non-zero weights were kept for model building. Then, features were independently standardized by subtracting their mean and dividing by their standard deviation. Feature standardization is an important preprocessing step because machine learning models can behave poorly if the features do not more or less look like normally distributed data [186]. The final step of the pipeline is the classification model (SVM or RF). SVMs with two different kernels were

explored: the linear and the radial basis function (RBF) kernels, where the optimized hyperparameters consisted of the regularization parameter (C) with the values $C = [0.001, 0.01, 0.1, 1, 10]$ evaluated for both kernels and the gamma parameter (γ) with the values $\gamma = [0.001, 0.01, 0.1, 1, 10]$ evaluated for the RBF kernel. The RF optimized hyperparameters that were evaluated were the number of trees ($n_estimators$) using the values: $n_estimators = [100, 200, 300]$, and the sub-sample size ($max_samples$) using the values: $max_samples = [0.6, 0.7, 0.8]$. A grid search was performed to obtain the optimal hyperparameters for both models. These hyperparameters consisted of parameters given to models to control the learning process (i.e. to avoid under/overfitting). During the grid search, for a given set of hyperparameters, the model was trained on the training data and then validated on the validation set. Optimal hyperparameters were chosen based on the validation accuracy of both models. The machine learning algorithms employed for this classifier-generating pipeline were retrieved from the Python library Scikit-learn [116]. The model was trained and validated with a training and validation set independent from each other.

The model having the optimal validation accuracy was kept and its pixel-wise predictions on the margin image were computed along with the corresponding class probabilities of each pixel to visually interpret the classification results. Class probabilistic outputs were computed using Platt scaling [187] resulting in probability scores varying from 0.5 to 1.0 where 1.0 corresponds to an absolute class membership probability while 0.5 is the lowest class membership probability. The algorithms used for class probability assignment were retrieved the Python library Scikit-learn [116]. No testing set was available in this study.

4.5 Results

4.5.1 Optical design

The optical design of each subcomponent of the imaging system was evaluated individually: the excitation branch, the imaging probe and the collection probe. Figure 4-2 presents the laser line profile generated by the excitation branch through the excitation fiber bundle. The laser line profile was simulated according to the optical configuration within the excitation branch showing the theoretical excitation laser line profile at the distal end of the fiber bundle with an x axis (line

width) of $160\ \mu\text{m}$ and a y axis (line height) of 4 mm. Measured normalized intensity profile was obtained for the x axis and the y axis resulting in a laser line width of $160\ \mu\text{m}$ and line height of 3.8 mm. Line width and line height are characterized according to their measured full width at half maximum. There is a slight disparity of intensity distribution at the extremities of the laser line height between the real and simulated laser profiles.

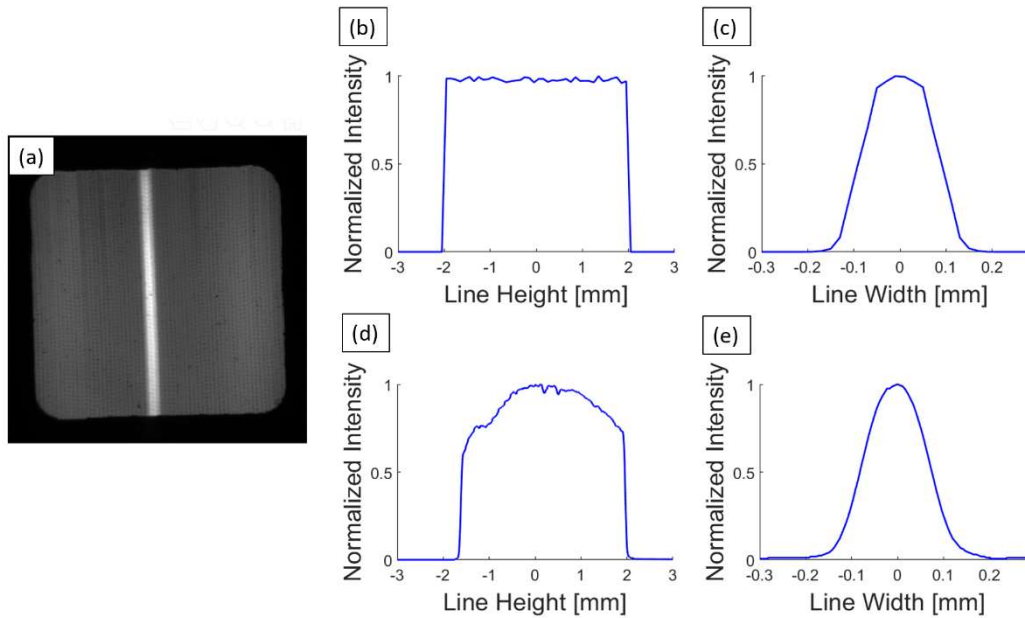


Figure 4-2: (a) Laser line profile relayed through the excitation fiber bundle and imaged distal face of the excitation fiber bundle. Simulated laser line profile generated by the excitation branch at the distal end of the 4 x 4 mm fiber bundle on the x-axis (b) and on the y-axis (c). Measured laser line profile on the x-axis (d) and on the y-axis (e).

The optical transfer function (OTF) was evaluated for 5 optical configurations (Figure 4-3(a)). Figure 4-3(b) presents the OTF of the imaging probe from the imaging plane to the excitation fiber bundle face across its circumradius for a spatial resolution of 2.00 cycles/mm (or $250\ \mu\text{m}$) and an illumination wavelength of 785 nm. The OTF of the imaging probe from the imaging plane to the collection fiber bundle face across its circumradius is shown for Figure 4-3(c) IR light (800-950 nm) at a spatial resolution of 2 cycles/mm (or $250\ \mu\text{m}$) and Figure 4-3(d) visible light (400-650 nm) at a spatial resolution of 10 cycles/mm (or $50\ \mu\text{m}$). Figure 4-3(e) presents the OTF of the

collection branch from the collection bundle face to the RGB camera for visible light (400-650 nm) at a spatial resolution of 10 cycles/mm (or 50 μm) (black). Figure 4-3(f) shows the OTF from the collection bundle face to the spectrometer slit for IR light (800-950 nm) at a spatial resolution of 2 cycles/mm (or 250 μm). Tangential and sagittal polarisation of light is presented for each OTF simulated. OTF modulus remained above 0.5 in most optical configurations for their respective target spatial resolutions.

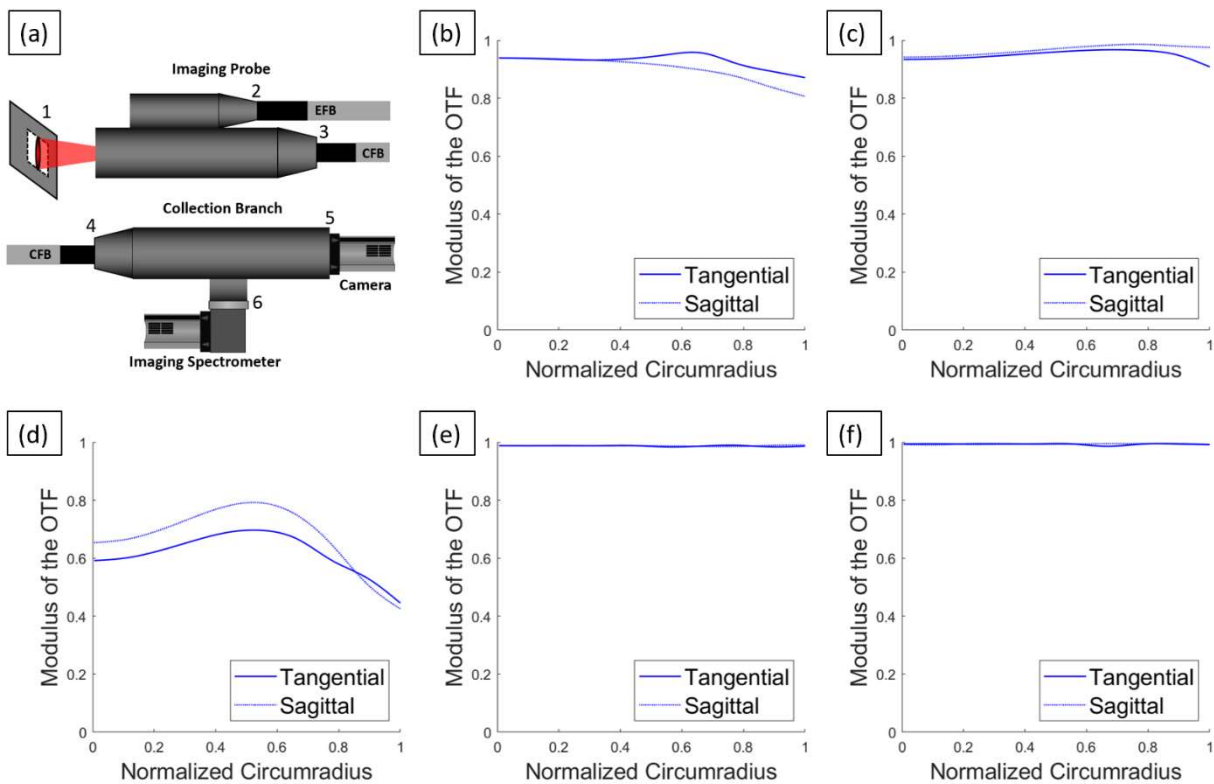


Figure 4-3: (a) Schematic of the imaging probe and collection branch illustrating components marking the start and end of the optical path selected for optical transfer function (OTF) simulation. (b) OTF of the imaging probe from 1-2 for a spatial resolution of 2.00 cycles/mm and for an illumination wavelength of 785 nm. (c)-(d) OTF from 1-3 for (c) IR light (800-950 nm) at a spatial resolution of 2 cycles/mm and (d) visible light (400-650 nm) at a spatial resolution of 10 cycles/mm. (e) OTF from 4-5 for visible light (400-650 nm) at a spatial resolution of 10 cycles/mm (black) and (f) from 4-6 for IR light (800-950 nm) at a spatial resolution of 2 cycles/mm.

4.5.2 Raman Imaging of Biological Samples

Figure 4-4 shows a photograph of the porcine sample interrogated with the imaging system: a bright field image, an uncorrected (no calibration, nor pre-processing applied) line scan luminescence (Raman and background fluorescence resulting from 785 nm laser line illumination) intensity image, an estimated line scan background intensity image and a line scan Raman intensity image. Each intensity value within the intensity images corresponds to the integral over the entire spectral range of the system. While no tissue contrast is perceived in the overall luminescence and the background intensity images, there is a visually distinct contrast of Raman scattering intensity between porcine adipose and muscle tissue.

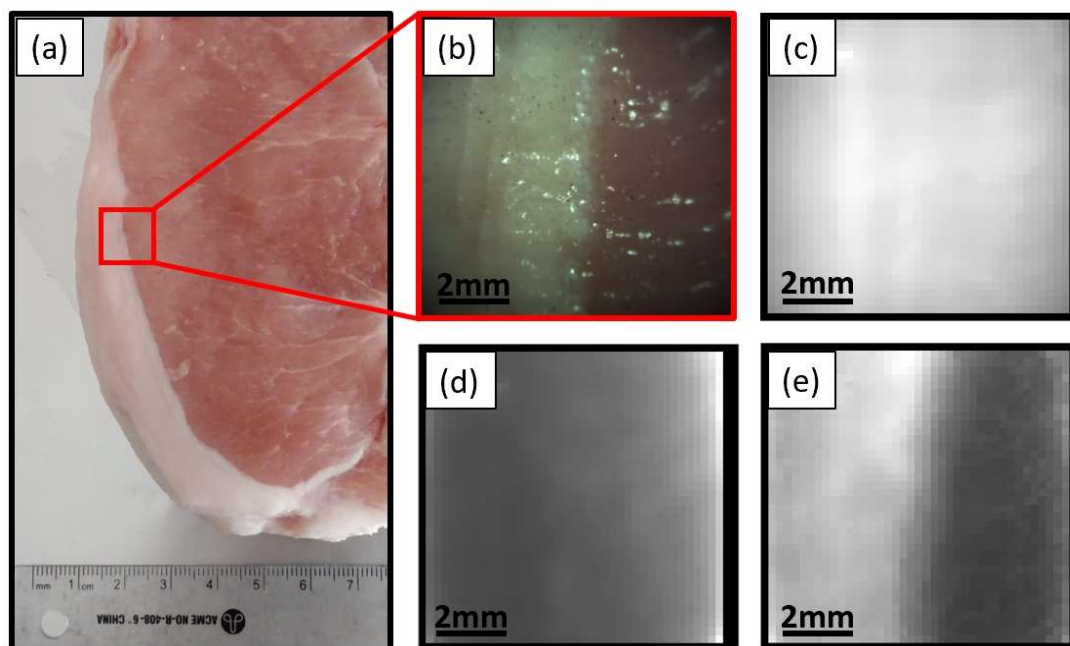


Figure 4-4: Study of the imaging system using a pork chop. (a) Photograph of the full pork chop, (b) bright field image of the FOV, (c) uncorrected line scan luminescence (Raman and background fluorescence resulting from 785 nm laser line illumination) intensity image (d) line scan background intensity image and (e) line scan Raman intensity image. Each intensity value within the intensity images corresponds to the integral over the entire spectral range of the system. Black column on the right of (b)-(d) correspond to pixels with relative illumination intensity <40%.

Figure 4-5 presents the average Raman spectra and standard deviation of the training data set for this study showing common and distinct spectral features (i.e. Raman bands) associated with protein and lipid content for each tissue type. Several spectral features and their biological relevance are shown in Table 4-2. From these spectra, several classifiers were trained, and the spectral features most important for tissue discrimination were extracted from the L1-SVM feature selector.

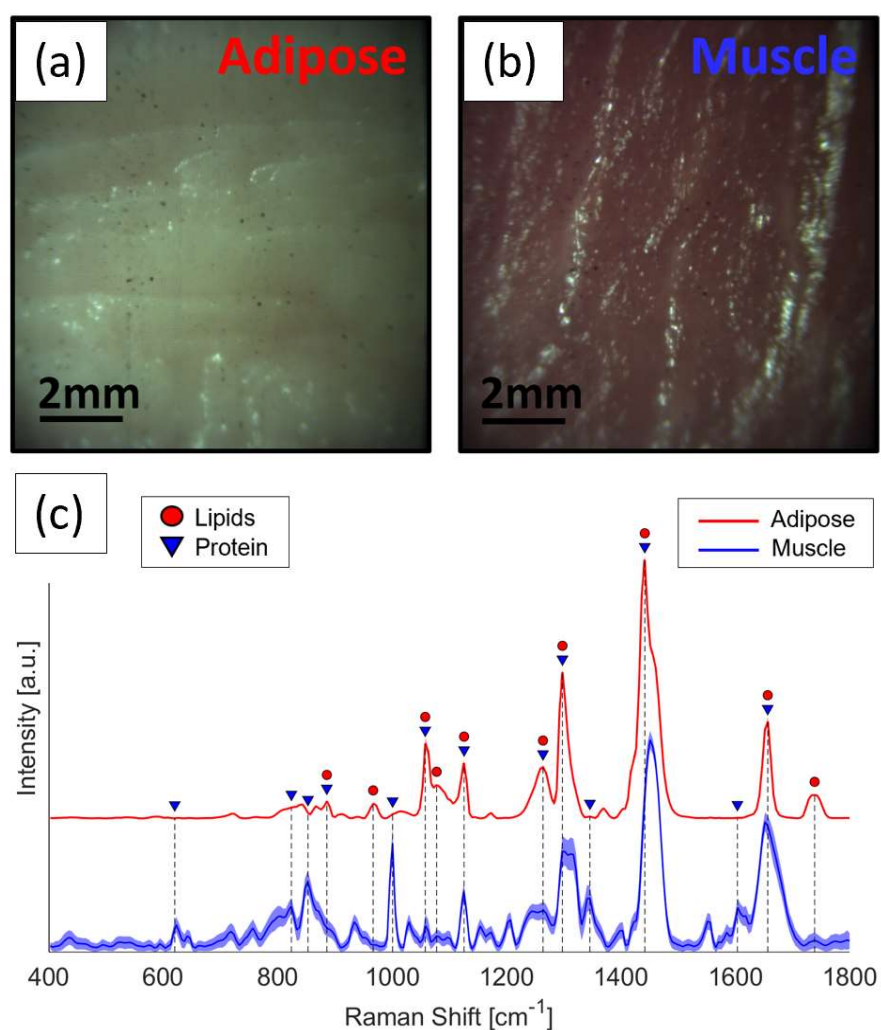


Figure 4-5: Training set imaged samples. (a) Bright field image of porcine adipose tissue, (b) bright field image of porcine muscle and (c) corresponding averaged spectrum and standard deviation (shading) of each tissue obtained following 10 repeat

measurements. Blue triangles designate Raman bands associated with protein content while red circles designate Raman bands mostly associated with lipid content. An arbitrary vertical offset was added to the adipose tissue mean spectrum and standard deviation to facilitate the visualization the spectra from both tissues. Variations across adipose tissue spectra are too small to observe the standard deviation (red shading) in the figure.

Table 4-2: Prominent Raman features for porcine muscle and adipose tissue. Bands highlighted in boldface correspond to peaks most relevant for tissue classification.

Raman Band (cm ⁻¹)	Molecular Bonds	Molecular Family	Reference
620	C-C twist of phenylalanine	Protein	[188], [189]
824	C-C stretch of proline and hydroxyproline	Protein	[188], [189]
853	C-C stretch of proline and tyrosine ring	Protein	[188], [189]
886	CH ₂ rocking	Lipid & protein	[188], [189]
967	CH ₂ out of plane bend & C-C wagging	Lipids	[188], [190]
1001	Phenylalanine breathing mode	Protein	[188], [189]
1063	C-C anti-symmetric stretch & C-N Stretch	Lipid & protein	[188], [190]
1082	C-C stretch & C-N stretch	Lipid	[188], [190]
1126	C-C symmetric stretch & C-N stretch	Lipid & protein	[188], [189], [190]
1264	=CH ₂ in plane deformation & amide III band	Lipid & protein	[188], [189], [190]
1298	CH ₂ twist	Lipid & protein	[188], [189], [190]
1346	CH deformation & CH ₂ /CH ₃ wagging	Protein	[188], [189]
1442	CH₂ symmetric deformation, CH₂/CH₃ antisymmetric deformation & CH₂ Bending	Lipid & protein	[188], [189], [190]
1604	C=O stretch of Amide I & C=C bending mode in tyrosine and phenylalanine	Protein	[188], [189]
1657	Amid I band, C=C stretch & C=O stretch	Lipid & protein	[188], [189], [190]
1739	C=O ester stretch	Lipid	[188], [190]

4.5.3 Margin identification

A Raman image was then acquired on a tissue margin between adipose and muscle tissue (Figure 4-6(a)). The corresponding mean spectra and standard deviation for regions in both tissue types were extracted (Figure 4-6(b)). This measurement revealed spectra in muscle tissue were not entirely consistent with the training data set introduced in Section 4.5.2. Pearson coefficients were calculated to evaluate the correlation between the average spectra of pure tissue types in the training set in Figure 4-6(c) with those of adipose and muscle tissue of the margin in Figure 4-6(b). The Pearson coefficients for adipose tissue within the margin compared with pure muscle and pure adipose tissue were 0.776 and 0.995, respectively, while those for muscle within the margin compared with pure muscle and pure adipose tissue were 0.889 and 0.955, respectively. This indicates that the muscle spectra within margins correlate more with the spectra of the pure adipose tissue.

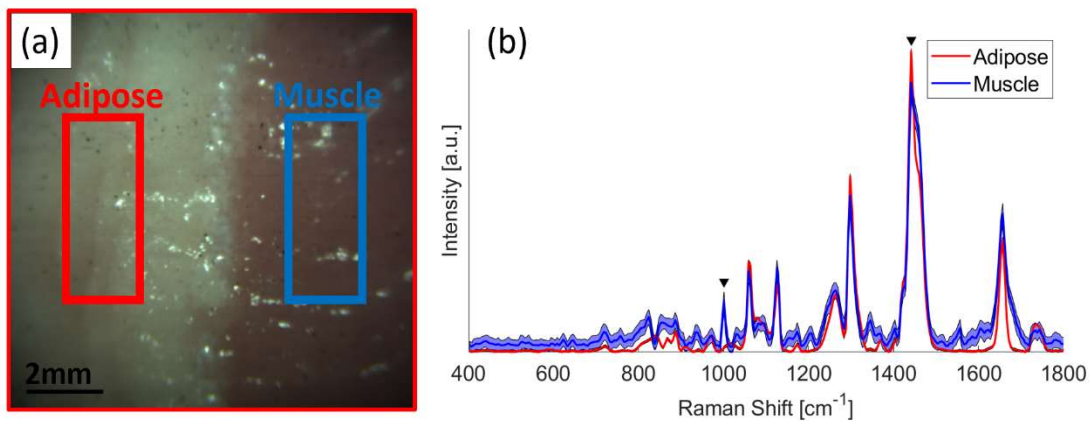


Figure 4-6: (a) Bright field image of the sample tissue margin and (b) corresponding mean and standard deviation (shading) of 126 Raman spectra for pork adipose tissue (red) and muscle (blue). Rectangle area corresponds to the selected areas from which the spectra were selected. Black triangles correspond to the two most important features for tissue discrimination identified by the L1-SVM feature selection tool.

Following the training dataset standardization, the feature selection step retained only three non-zero-weighted features (i.e. Raman bands): 1001, 1437 and 1442 cm^{-1} . These features were then used to train the classification models. The optimized SVM and RF models achieved accuracy scores of 0.990 and 0.967 on the validation set, respectively. Because it had the highest validation accuracy score, the SVM, with hyperparameters $C = 0.1$ and $\gamma = 10$, was chosen as the model to generate and illustrate the prediction results and their respective class probabilities over the tissue margin in Figure 4-7. The accuracy, sensitivity and specificity of the classification model to discriminate adipose tissue from muscle within the margin are 0.980, 0.996 and 0.958, respectively.

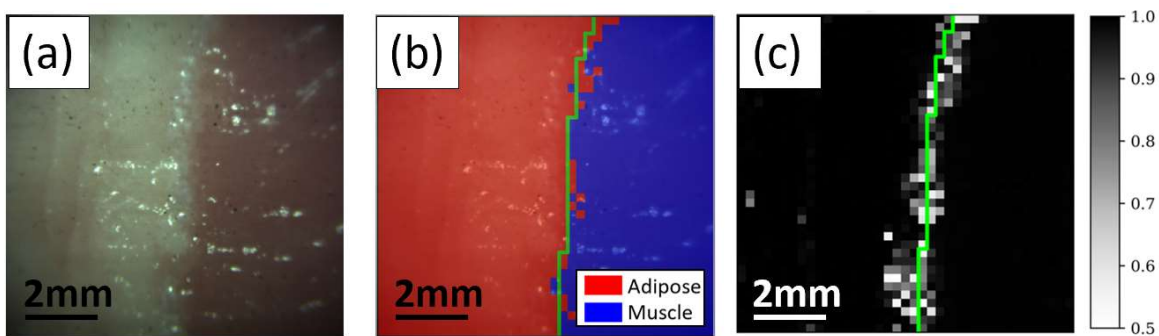


Figure 4-7: (a) Bright field image of the sample tissue margin, (b) classification map with red indicating predicted adipose tissue and blue indicating predicted muscle tissue and (c) classification confidence map ranking from 0.5 (lowest confidence) to 1.0 (highest confidence). The green line shows the ground truth for the tissue margin location.

4.6 Discussion

4.6.1 Optical design

An apparent weakness of the system consists in the resulting line profile at the face of the excitation fiber bundle, for which the laser line width (160 μm) is 60% larger than what was targeted (100 μm). The optics for the excitation branch were designed to accommodate a multimode fiber input of 0.22 NA and 400 μm core, however, the only available laser at the time had a 0.5 NA and a multimode core of 400 μm which resulted in a larger line width spread in the laser line profile. The impact of this line spread is the generation of a laser line with a width of 400 μm at the sample

(after a magnification of 2.5 by the imaging probe optics) as opposed to a targeted width at the sample of 250 μm . A larger laser line produces more in depth signal from neighboring tissue which translates into unwanted noise. Additionally, it was measured that a total of 87% of the laser power is lost through the optics of the excitation branch and the imaging probe due to this difference in fiber specifications. These limitations are expected to be rectified with the integration of a 785 nm laser designed to relay 5 W of power through a 0.22 NA multimode fiber of 400 μm .

A further limitation to the system is the use of fiber bundles for both excitation and collection. Although fiber bundles offer the much-needed range of motion of an imaging probe designed for a clinical context, they present significant optical losses (55 to 65%), first through the limited effective collection area of the several thousand fibers within the fiber bundles and second, through the propagation losses within the bundle itself which are dependent on bundle length. However, the ability to generate the laser line scanning at the excitation branch through fiber bundles offers the advantage of not having any electrical nor moving components in the imaging probe therefore significantly reducing risks to the patient and allowing for a more robust probe design. Flexibility versus optical losses is a trade-off deemed necessary for user practicality in a surgical context and therefore requires significant efforts to mitigate additional optical losses.

Off-the-shelf lenses were selected when available with two custom lenses manufactured for the imaging probe design. The aluminum probe casing was fabricated to accommodate the selected optics. The resulting probe size and weight of 0.6 kg could be considered cumbersome for some clinical applications especially if the probe is to be handheld. Lens size customization will contribute to reducing the probe body's footprint while a plastic mold approach may reduce overall probe dimensions constrained by machining tolerances and component assembly required by a metal casing. The mounting of the probe on a custom apparatus which is itself mounted on a surgical table (e.g. with a Mayfield® skull clamp) could provide a suitable alternative to a lighter handheld probe.

The resolution criterion through the OTF for illumination and collection across the different parts of the system was easily met across the entire circumradius of the different components with the exception of bright field imaging spatial resolution within the imaging probe where the criterion

was not quite met in corners of the FOV. However, the reduction of spatial resolution in the corners of the FOV in bright field imaging does not hinder the ability to visualize tissue structures.

4.6.2 Raman imaging of biological samples

Raman spectra in both adipose tissue and muscle tissue were collected totaling 5040 spectra. In the averaged spectra recovered from adipose and muscle tissue, 16 prominent Raman bands were identified along with their biological relevance. The spectra and selected bands showed strong correspondence to previous work in the literature [150], [189], [190], [191] where several Raman bands (620 , 824 , 853 , 1001 and 1604 cm^{-1}) are prominent and are associated to the high protein content of muscle while others (967 , 1082 , 1298 , 1346 and 1739 cm^{-1}) to the high lipid content of adipose tissue. Many Raman bands (886 , 1063 , 1126 , 1264 , 1442 and 1657 cm^{-1}) are prominent in both tissue types with varying relative intensities. 16 prominent Raman bands were identified although the signal to noise ratio for each averaged spectrum allowed for the identification and interpretation of many more Raman bands. These Raman bands were not presented in this work since its aim was to present the imaging system's ability to acquire high SNR Raman spectra within tissue and use few Raman features for tissue discrimination within an image. Within the entire FOV, spectra were acquired with negligible variance in the highly scattering adipose tissue while muscle tissue spectra showed slightly more variance which is indicative of a difference of overall SNR for identical acquisition parameters. Comparing adipose and muscle tissue spectra, we observe several distinct high SNR Raman bands, such as the phenylalanine band at 1001 cm^{-1} which was only present in muscle tissue and the 1442 cm^{-1} band with a higher relative intensity in adipose tissue. These molecular contrasts expressed in the Raman spectra suggest a low-complexity classifier should successfully discriminate these tissue types.

4.6.3 Margin identification

While spectra were significantly distinct when line scanning pure tissue types, it was not the case when the imaged tissue contained margins, i.e. several tissue types within one image. It was calculated that the mean spectra of adipose and muscle tissue interrogated within a tissue margin both correlated much stronger with spectra acquired in pure adipose images. This can be attributed to the orientation of the line measurement in accordance to the tissue margin (in this case

perpendicular to the margin), which introduces an in-depth contribution of signal due to the penetration depth of scattered light within tissue (laser & tissue luminescence) and the optical properties of these tissue [192]. The line profile of the laser generates in-depth signal within the sample that is ultimately collected across the detection line of the system. This is a phenomenon exploited in spatially offset Raman spectroscopy (SORS) when specific in-depth interrogation is the goal but, in this study, it is an undesirable confounding factor. One notable contribution to unwanted in-depth signal originates from using a wider laser line profile (400 μm) than that of the collection line (104 μm). The larger the mismatch between the illumination and collection area, the larger will be the contribution from in-depth tissue to the overall Raman signal. It was reported that adipose tissue possesses a very strong Raman cross-section [193] and is a higher scattering tissue than muscle for the range of 810 to 922 nm [194], which would explain the high SNR observed in adipose tissue and its significant spatially offset signal recovered across the detection line in the margin image. If line scanning was performed parallel to the margin presented in Fig. 6 such cross contamination of signal would not have been so apparent. Even so, it was shown that validation results for pure tissue and tissue margin, i.e. accuracy, specificity and sensitivity, all exceeded values of 97% with only three selected Raman bands identified to achieve these results: 1001 (phenylalanine breathing mode) and, 1437 and 1442 cm^{-1} (CH_2 symmetric deformation and CH_2/CH_3 antisymmetric deformation). Although only three bands were selected by the feature selector, this does not exclude the biological relevance and the pertinence of other Raman bands identified in Section 4.6.2 for adipose and muscle tissue discrimination. It does however signify that, from the data available, we can generate a very simple yet effective classifier using only these 3 Raman features although it is likely that including more spectral features would help in providing accurate tissue class predictions in spectra with an inferior SNR. This classification model could further be simplified by acknowledging that 1437 and 1442 cm^{-1} , are both indicative of the same biological content of tissue. Considering the prevalence of phenylalanine in muscle in opposition to its absence in adipose tissue and the large cross section of the long hydrocarbon chains in adipose tissue, rich in CH_2 symmetric deformation and CH_2/CH_3 antisymmetric deformation, these extracted Raman bands are biologically relevant for tissue discrimination using molecular imaging. The consideration of these tissue specific bands and successful classification validation shows that in a worst-case scenario, i.e. much contribution of signal from neighboring tissue, a robust classifier

can still successfully discriminate tissue types in macroscopic line scanning with very few Raman bands.

The implementation of a class probabilities map in Figure 4-7(c) visually and clearly presents the location of the tissue margin. The biological relevance of this class probability map is that in measurement conditions where tissues can be discriminated effectively with a classifier, there is a diffuse area corresponding to the margin between tissue types for which a well-trained classifier will gradually have more difficulty in making a prediction from one tissue to the next, due in part, to a contribution of Raman scattering that is gradually occurring from a new class of tissue. As the predicted pixel gets closer to the margin, the prediction probability for that class will drop towards its minimum of 50 % until the predicted class changes to the new tissue type, gradually increasing as the measurement is further from the margin and as the signal occurs only from the other tissue type. In fact, we observe that in both tissue types, the classifier confidence is overall near perfect when moving away from the margin. It is acknowledged that visual identification of the margin location based on tissue color and texture does not take into account the thickness or the heterogeneity of the tissue margin (i.e. mixture of adipose and muscle tissue) in the imaged sample and only allows for an approximation of the margin location. Histological analysis of the sample would provide the precise margin size and cellular content crucial for clinical applications but, it was not considered relevant for the conclusions presented in this work. It is estimated visually that the margin presented in Figure 4-7 is <1 mm in width which corresponds to <4 pixels in the image. This is consistent with the probabilities map of Figure 4-7(c) highlighting the margin location with an average width of 4 pixels having a prediction confidence <90 %.

Finally, this classification example was purposefully tested with tissue classes trivial to discriminate, adipose vs. muscle tissue, to illustrate functionality of the system and an example of margin recognition capability in only one sample. Using the same sample to interrogate only one area of adipose and muscle tissue, as well as a tissue margin presents limitations in creating a machine learning classifier which generalizes well to other samples. In this study, measurements resulted in relatively low spectra variance within tissue types and across all Raman images. This led to the creation of classifiers with high validation scores (i.e. SVM and RF accuracy respectively 99.0% and 96.7%) indicating that only 3 bands (i.e. 1001, 1437 and 1442 cm^{-1}) can be used to discriminate adipose and muscle tissue. Raman spectra of porcine tissue from previous publications

also present absence of 1001 cm^{-1} phenylalanine band in adipose tissue [150], [190], [191] suggesting our selected features and model could be generalizable to other samples. This will be confirmed in a future study following system improvements. In tissues that present more subtle differences in Raman SNR and band locations such as in diffuse glioma, more imaged samples for training and testing are necessary to capture inter-sample and inter-subject variability.

4.6.4 Photonic budget limitation of the system

The results of this study have been observed in a novel line scanning system which was initially designed to obtain comparable signal levels to a single point probe system employed in surgical guidance for glioma surgery [107]. Although preliminary data to identify tissue margins is promising, it was observed that the photonic budget (total Raman photons collected) of the line scanner is likely insufficient to generate spectra with sufficiently high SNR spectra for margin identification in tissue with more subtle molecular difference such as brain in an adequate timeframe (<10 mins). Table 4-3 illustrates the key differences in photonic budget for an equal interrogation surface, between the line scanner and the above-mentioned point system, explaining the performance differences. It is apparent that, in current conditions, the photonic budget collection of the line-scanner when compared to that of the point probe is approximately 9600 % inferior for the same interrogated unit area although the line scanner acquires 500 % more-unit area per line. While fiber bundle-related losses and the number of collecting fibers (where collecting fibers refer to the collection geometry of the point probe in which multiple fibers participate in the collection of the same detection area) are inherent to these two systems, it is estimated that by implementing a more powerful fiber-coupled 785 nm laser (5 W) with a NA of 0.22 matching the NA of the excitation branch we will decrease power losses and increase the total laser intensity at the sample by >600 %. By additionally customizing the spectrometer to obtain an NA which matches that of the collection branch, it is expected that these modifications will result in a realistic increase of >1200 % in the collected Raman signal for the same acquisition time and interrogated unit area. A high-power laser (5 W at 785 nm) may cause concern for a clinical setting however, what is most important is that laser exposure to tissue never exceeds the maximum permissible exposure (MPE) for skin set by the American National Standards Institute (ANSI) Z136 laser safety standards. A higher laser intensity offers the opportunity to quickly scan the target surface multiple

times leaving ample time between scans for tissue to cool down and thus avoid thermal damage. Further modifications including increasing the numerical aperture of the system to 0.36 ($f\# = 1.4$) by means of a larger non-contact probe with NIR collection independent of fiber bundles may reduce photon budget difference between the single point system and the line scanner to only 50 %. With an added change in detection line dimensions, from $104\ \mu\text{m} \times 9.5\ \text{mm}$ to $400\ \mu\text{m} \times 10\ \text{mm}$, such a line scanner would interrogate the equivalent of 20 point-probe measurements for every line.

Table 4-3: Parameter differences between Raman line scanning and Raman point systems comparing the overall photon collection budget for an identical interrogated area and acquisition time.

Parameters	Point System	Line Scanning System	Ratio Point/Line
Laser Intensity on sample	38.2 W/cm ²	6.1 W/cm ²	6.3
Fiber bundle transmission	N/A	37.5 %	2.67
Collection branch NA	0.22	0.159	1.96
Number of collecting fibers	7 x 41.7 %*	1	2.92
Photon budget	-	-	96

*The point probe contains 7 collection fibers which have an effective collection area of 41.7%.

4.7 Conclusion

This article reports the development of a novel, label-free, large field of view line scanning Raman system, created to address the clinical need of molecular image-guided surgery. The layout of the system, and its optical design, are detailed and a proof of functionality on porcine tissue is presented. The system can acquire 1680 high SNR Raman spectra in the fingerprint region over a field of view nearing $1\ \text{cm}^2$ in less than 3 minutes in tissue. With data from pure porcine adipose and muscle tissue, SVM and RF classifiers were trained and validated on a tissue margin with >97 % accuracy, showing the potential of this molecular imaging system to locate tissue margins based

on machine learning approaches. Feature selection led to generating simplified classification models using only 3 Raman bands to discriminate porcine adipose from muscle tissue. A detailed discussion addressed the necessary modifications to take this system to a clinical study in human patients to evaluate its potential in locating disease margins in vivo. The authors of this work have no relevant conflicts of interest to disclose.

4.7.1 Acknowledgements

This work is supported by the Discovery Grant program from Natural Sciences and Engineering Research Council of Canada (NSERC), the Collaborative Health Research Program (CIHR and NSERC), the Institute for Data Valorization (IVADO) and the Ministry of Education and Innovation of Quebec (MEI). This research was also undertaken thanks, in part, to funding from the Canada First Research Excellence Fund through the TransMedTech Institute.

CHAPTER 5 ARTICLE 2: A CLINICAL RAMAN SPECTROSCOPY IMAGING SYSTEM AND SAFETY REQUIREMENTS FOR IN SITU INTRAOPERATIVE TISSUE CHARACTERIZATION

THE ANALYST – ROYAL SOCIETY OF CHEMISTRY

Published on April 6th, 2023

François Daoust^{1,2}, Hugo Tavera^{1,2}, Frédérick Dallaire^{1,2}, Patrick Orsini³, Keven Savard³, Jacques Bismuth³, Philippe Mckoy³, Israel Veilleux^{1,2}, Kevin Petrecca⁴ and Frédéric Leblond^{1,2}

¹ Polytechnique Montreal, Department of Engineering Physics, Montreal, QC H3T 1J4, Canada

² Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montréal, QC H2X 0A9, Canada

³ Optech, LaSalle, QC H8N 2J4, Canada

⁴ Department of Neurology and Neurosurgery, Montreal Neurological Institute-Hospital, McGill University, Montreal, QC H3A 2B4, Canada

5.1 Background

In Chapter 4 was presented the development of a novel Raman imaging system for the purpose of machine learning-based tissue assessment. In addition to this, a proof-of-concept study in animal tissue was shown in combination with a machine learning based classification pipeline. The Raman imaging system was confirmed to be a promising tool for tissue characterization and discrimination during surgery. To test the system in a clinical setting, it was necessary to transform the system into a clinical prototype capable of performing *in situ* tissue interrogation in an operational setting. In this chapter, we present the evolution of the Raman imaging system towards this clinical prototype. This work was completed in accordance with Objective 2 of the dissertation: *Enhance the Raman imaging system to create a safe and portable clinical prototype suitable for in situ intraoperative tissue characterization*. We first present the work towards transforming the Raman imaging system into a clinical prototype that could perform intraoperative tissue imaging *in vivo* and *ex vivo*. A complete safety assessment of the newly developed clinical prototype is presented

and discussed. Finally, an upgraded acquisition workflow including machine learning pipeline developed for tissue margin detection, to be employed by this clinical prototype is presented.

5.2 Abstract

Raman spectroscopy imaging is a technique that can be adapted for intraoperative tissue characterization to be used for surgical guidance. Here we present a macroscopic line scanning Raman imaging system that has been modified to ensure suitability for intraoperative use. The imaging system has a field of view of $1 \times 1 \text{ cm}^2$ and acquires Raman fingerprint images of 40×42 pixels, typically in less than 5 minutes. The system is mounted on a mobile cart, it is equipped with a passive support arm and possesses a removable and sterilizable probe muzzle. The results of a proof of concept study are presented in porcine adipose and muscle tissue. Supervised machine learning models (support vector machines and random forests) were trained and they were tested on a holdout dataset consisting of 7 Raman images (10,080 spectra) acquired in different animal tissues. This led to a detection accuracy $>96\%$ and prediction confidence maps providing a quantitative detection assessment for tissue border visualization. Further testing was accomplished on a dataset acquired with the imaging probe's contact muzzle and tailored classification models showed robust classifications capabilities with specificity, sensitivity and accuracy all surpassing 95% with a support vector machine classifier. Finally, laser safety, biosafety and sterilization of the system was assessed. The safety assessment showed that the system's laser can be operated safely according to the American National Standards Institute's standard for maximum permissible exposures for eyes and skin. It was further shown that during tissue interrogation, the temperature-history in cumulative equivalent minutes at 43°C ($CEM43^\circ\text{C}$) never exceeded a safe threshold of 5 min.

5.3 Introduction

Cancer resection surgery is a frontline treatment during which surgeons identify bulk tumour visually and by palpation. Frozen section analyses and medical imaging modalities –e.g., magnetic resonance imaging (MRI)– can help to more precisely characterize the extent of cancer spread, oftentimes associated with pathological manifestations that are visually imperceptible. Although emerging techniques such as intraoperative MRI (iMRI) led to significant advances in surgical

oncology, they are not readily accessible in hospitals, mainly due to their high cost and the increased surgery time associated with their use. Moreover, iMRI can be limited in its ability to detect subtle biomolecular and morphological alterations associated with oncogenic processes initiated close to the borders of a tumour, thereby limiting the surgeon's capacity to maximize the volume of resected cancer[31], [172]. On the other hand, pathology analysis of frozen sections is commonly used but they require on average 20 minutes, which can limit the frequency of their use during a procedure[195], [196]. Failing to locate and completely remove tumours often results in follow-up repeat surgery from disease recurrence with concomitant negative impact on patient outcomes.

Amongst technologies that aim to guide surgeons in locating the full extent of cancer *live* during surgery is Raman spectroscopy. Label-free spontaneous Raman spectroscopy can reveal biomolecular information in the form of a tissue spectral fingerprint. That fingerprint can be matched with a known reference set by histology using machine learning methods, resulting in predictive mathematical models[197], [198]. Several in-human studies were conducted using this technology to characterize tumour tissue in breast[199], brain[38], [39], [200], colon[201], skin[202], [203], [204] and prostate[205], [206]. Raman-based systems could be available for surgical guidance in hospitals within the next decade[207].

Although intraoperative Raman spectroscopy was mainly developed for millimetre-scale single-point detection, some reports point towards larger field-of-view Raman imaging at mesoscopic scales as a promising surgical guidance modality. This technique would have all the advantages of single-point Raman spectroscopy, albeit with the added benefit of providing molecular information over a larger surface area[144], [208]. Large-area Raman spectroscopy could improve in situ biomolecular characterization of subtle oncogenic processes at the interface between cancer and normal tissue, providing surgeons with the ability to more accurately identify and visualize diffuse tumour borders[36], [145]. For example, these technologies could help solve crucial clinical problems, including the detection of cancer infiltrations during glioblastoma surgery[26] and the detection of microscopic ductal carcinoma in situ (DCIS) lesions during breast conservation surgery[146]. Research laboratories have developed label-free large field of view Raman imaging systems for intraoperative surgical margin assessment in skin[152] and oral cancer[182]. Important

limitations of these systems were their inability to achieve intraoperative measurements over a large field-of-view within a timeframe minimizing disruption of the surgical workflow.

Our group previously developed a large-angle handheld Raman imaging line-scanning system[209]. The device could image over a field-of-view of 1 cm² at a spatial resolution of 0.25 millimeters (pixel size). Briefly, it had a light detection branch composed of a spectrophotometer, a charged-coupled device (CCD) camera and a galvanometer coupled to custom optics to detect over a line scanned over a specimen. Line excitation was achieved with a 785 nm laser based on custom optics and a translation stage to allow surface scanning. The excitation and detection lines were spatially co-located. The system also included a brightfield white-light source and standard red-green-blue (RGB) camera to allow video-rate wide-field visualization of the specimen. Device performance was preliminarily demonstrated on animal tissue (porcine) and a machine learning data pipeline was developed to detect a tissue margin modelled by high adipose (fat) and high-protein tissue (muscle).

The system lacked several key features making it unsuitable for intraoperative use. Limitations included: the absence of a sterilization procedure, prohibitively long acquisition times in biological tissue and bulkiness resulting in lack of mobility. Further, the imaging probe could not be manoeuvred in space because of its weight and the lack of a flexible holding arm. Several safety considerations were also not addressed, including laser exposure limits and biocompatibility. Moreover, the imaging system was used to produce classification models trained and validated on only a limited number of porcine samples (3 samples), which prevented testing on an independent hold-out data subset.

In this paper we present an upgraded Raman imaging instrument suitable for intraoperative use. The system was modified to ensure patient and user safety, portability and probe head 3-D manoeuvrability, as well as tissue imaging times compatible with clinical use (<5 minutes per image). A crucial improvement was the development of a contact measurement sterilizable sub-unit (muzzle) flattening the interrogated tissue surface to ensure optimal positioning at the focal plane. Another key system upgrade was the integration a 3D-articulated arm and custom supporting the imaging probe. The new system was tested by acquiring a large dataset of porcine tissue images

(21 samples) from which classification models were trained and evaluated using an independent hold-out testing set.

5.4 Experimental

5.4.1 Clinical prototype Raman imaging system

The system achieved line scanning over a field of view of 1 cm^2 , allowing acquisition of Raman spectra over the spectral range $400 - 2100 \text{ cm}^{-1}$ with a spectral resolution of 8 cm^{-1} (Figure 5-1). Spatially co-located bright field images (i.e., white light images) with a spatial resolution of $50 \text{ }\mu\text{m}$ can be acquired to guide where Raman imaging is performed based on visually identifiable tissue landmarks. Laser line excitation at 785 nm and bright field illumination are relayed through an imaging fibre optics bundle (Schott, USA) to an imaging probe head. The images are relayed to the system's cameras through a second flexible imaging bundle. The use of imaging bundles enables the user to manoeuvre the probe at the desired location, alleviating the need for moving parts in the probe head itself. Line scanning is achieved using cylindrical lenses transforming the laser beam into a line followed by transmission into the excitation imaging bundle. A translation stage controls which area of the imaging bundle is illuminated while the detection bundle and the imaging probe optics relay the light reemitted following line excitation to a spectrophotometer. The on-sample excitation line dimension is $400 \text{ }\mu\text{m} \times 10 \text{ mm}$. The Raman collection line dimension is $200 \text{ }\mu\text{m} \times 10 \text{ mm}$ and its on-sample location is collocated with the excitation line using a mirror mounted on a galvanometer (Novanta Photonics, USA) placed in the optical path leading to the spectrophotometer.

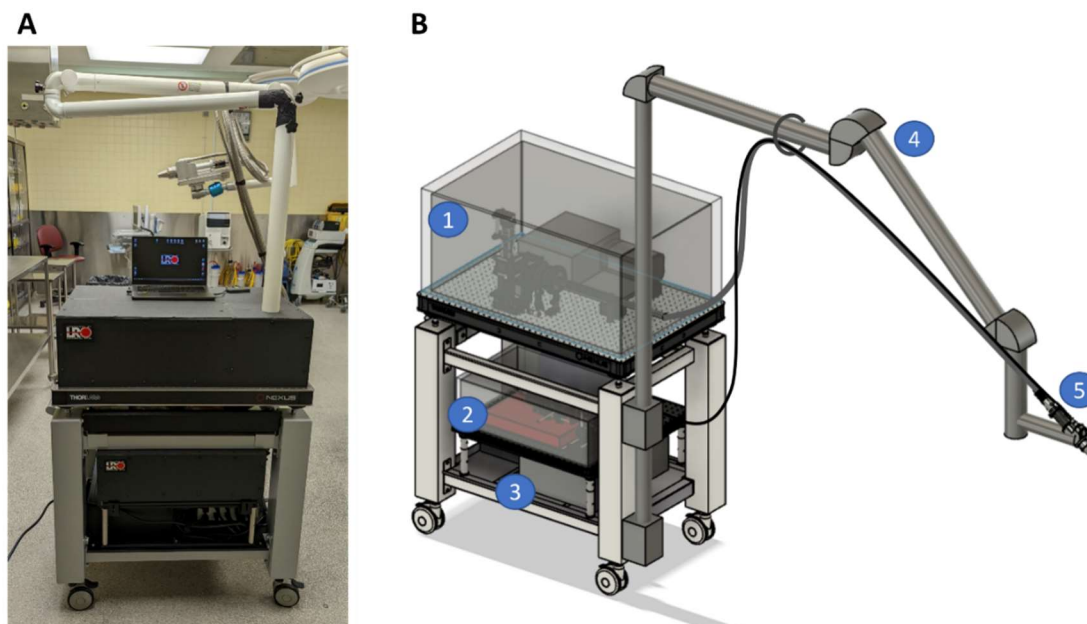


Figure 5-1: (A) Cart-mounted clinical prototype of a Raman imaging system designed for intraoperative clinical measurements with (B) the computer aided design of the imaging system illustrating the location of several parts: (1) the collection branch covered by a light tight enclosure, (2) the excitation branch covered by its light tight enclosure, (3) the medical isolation transformer, (4) the articulated arm and (5) the imaging probe with its contact measurement muzzle.

Optical design changes were made to improve performance of the system compared with our group's prior version. First, the system's spectrophotometer was replaced by a custom-designed spectrograph (slit width: 1 cm, NA 0.22; EH 0001 model, EmVision, USA) with an entrance slit of 200 μm (previously 75 μm) which increased light throughput by a factor of 2.7 with minimum impact on the spectral resolution (8 cm^{-1} instead of 6 cm^{-1}). A second design change consisted of using a more powerful 785 nm multimode laser (5 W instead of 1.8 W, Innovative Photonic Solutions, USA) with a lower numerical aperture (NA 0.39 instead of NA 0.5). Finally, the lens responsible for focusing the light reemitted from the sample onto the new spectrometer was changed for optimal etendue matching based on ray tracing simulations (OpticStudio®, Zemax, USA). This increased light detection efficiency by 42 % at the centre of the image and by 26 % closer to its borders.

In addition to the previously mentioned upgrades, several features were added to the Raman imaging system to make it suitable for use in an operating room. The prototype was mounted on a compact breadboard cart (POC001, Thorlabs, USA) to ensure mobility. Light tight enclosures made of black cardboard (TB4, Thorlabs, USA) were used for the light collection and excitation sub-units, preventing laser light from inadvertently endangering staff while keeping any ambient light from contaminating the collected signal. A medical-grade isolation transformer (MEDBOX-1200, Amgis LLC, USA) was installed to protect the system and the users from electrical power surges and faulty components. An articulated support arm (BRAPT7880, Bracci & Dispositivi, Italy) was also integrated into the system with the purpose of maintaining the imaging probe in place. This articulated arm extended over 1.5 m to keep the imaging system body out of the way of clinical staff and, when applicable, away from the sterile field (e.g., for use in neurosurgery). The arm also allowed the user to position the probe close to the patient and to hold it in place during a measurement. The articulated arm had the added benefit of supporting the fibre optics bundles along its frame to reduce strain. Two draped lead bricks (18 kg each) were added as counterweights to prevent the system from tipping over when the articulated arm is fully extended. A custom probe mount was designed to secure the imaging probe to the articulating arm while allowing fine angle and position adjustments. This custom mount includes a manual translation stage with 8 cm travel distance for the probe head.

Two probe muzzles were designed that can be connected to the imaging probe using spring plungers. One muzzle was designed to allow contact measurements and one for non-contact measurements. The working distance of the non-contact measurements muzzle is 40 mm. Both muzzle units were made of sterilizable and biocompatible materials since they can come in direct contact with a patient. They were composed of three different parts: a main body, an optical window, an adhesive to keep the two pieces attached to one another. The probe muzzle body was made of 304 stainless steel which is commonly used for clinical applications due to its resistance to corrosion, its biocompatibility, and its tolerance to most sterilization methods. The front window of the probe muzzle was either made of an MgF_2 or CaF_2 Raman grade optical window (Crystran, United Kingdom). These materials have low inelastic scattering signal (from 400 to 2100 cm^{-1}) and low fluorescence signal when excited at 785 nm. Finally, the adhesive used to attach the optical window to the probe muzzle body consisted of a high temperature resistant (up to $350\text{ }^\circ\text{C}$) epoxy

(EPO-TEK® 353ND) designed for fibre optics and medical applications. The epoxy was cured at 160°C for 1 hour. For future probe muzzles dedicated to intraoperative in situ imaging, a medical variant of this epoxy (EPO-TEK® MED-353ND: ISO 10993-tested, certified biocompatible) could be employed. All probe muzzle materials are known to be compatible with conventional sterilization cycles of autoclave steam, ETO gas, gamma radiation and vaporized hydrogen peroxide gas plasma. A sterilization procedure, shown in the supplementary material, included sterilizing the probe muzzle and covering the imaging probe and articulated arm with a sterile surgical drape. Figure 5-2 shows the imaging probe with the contact measurement muzzle mounted on the articulating arm.

The system was controlled via custom software developed using LabVIEW (National Instruments, USA). In case of emergency, a button was installed that allows the operator to immediately shutdown the laser.

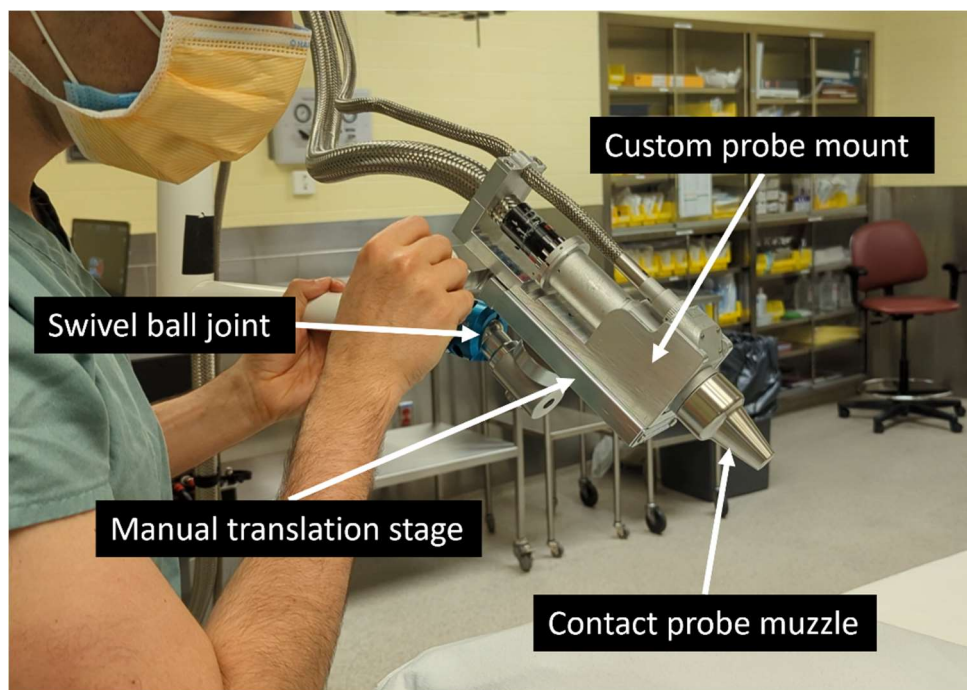


Figure 5-2: Imaging probe with its attached probe muzzle for contact measurements, mounted on an articulated support arm allowing the user to move the probe with ease.

A manual translation stage with 8 cm travel distance lets the user position the probe ensuring contact with the tissue area targeted for imaging.

5.4.2 Spectral data pre-processing

Cosmic rays were removed using a 2D median filter. Luminescence measured during an image acquisition in mid-air was subtracted from all raw spectra. This luminescence is additive in nature and mostly comes from the focusing achromatic triplet in the Raman imaging probe. Following this, spectral misregistration (also known as “smile”)[210] correction and wavenumber calibration were applied based on a reference measurement of acetaminophen powder. The spectral misregistration was corrected using 4 steps: 1) locate the pixel positions of the 12 most prominent acetaminophen Raman peaks in the computed average of spectra from the centre of the CCD (corresponds to 40% of all spectra where wavelength shift is visually imperceptible), then for every spectrum in the acetaminophen Raman image: 2) locate the pixel positions of the nearest corresponding peaks, 3) compute a second-degree polynomial fit, and, 4) apply a linear interpolation. Spectral misregistration correction resulted in a common wavenumber axis for all spectra within an image. The wavenumber calibration was completed using a cubic-spline interpolation based on the 5 prominent peaks (651.6, 857.9, 1168.5, 1323.9 and 1609.0 cm^{-1}) from the acetaminophen Raman fingerprint spectrum. Relative intensity correction of each spectrum was achieved using a reference Raman image measurement on a NIST reference material (SRM 2241, NIST, USA). Artifacts that appeared on the reference measurement caused by scratches on the NIST material were filtered with a 2nd order 2D gaussian filter. A novel baseline removal algorithm named BubbleFill was used to isolate the inelastic signature from other contributions to the signal[211]. The resulting Raman spectra were then processed using standard-normal-variate (SNV) normalization.

5.4.3 Imaging of biological tissue

The biological specimens in this study were porcine tissue from 3 different pigs, herein labelled pigs #1, #2 and #3. Specifically, 2 porcine loin samples were used per pig and each set of loins were purchased at different butcher shops. This choice was made for preliminary proof-of-principle studies because of accessibility, the well-established Raman signature of muscle and adipose

tissue[191], [212] as well as the visually apparent tissue borders between adipose and muscle tissue, used here as a toy model for tumour borders. Each pig sample was approximately 2 cm thick with a 10 cm diameter and was placed over black aluminum foil tape (T205-2.0, Thorlabs, USA) to minimize background luminescence from the sample support. The tissue was imaged using the parameters shown in Table 5-1. Three different areas per specimen were selected based on visual inspection: pure adipose, pure muscle and adipose-muscle tissue interface. In total, 21 images were acquired: 6 in adipose tissue, 6 in muscle tissue and 9 included an adipose-muscle tissue interface. In addition, 3 images were acquired using the contact measurements probe muzzle, one in pure adipose, one in pure muscle and one including a tissue interface. For each Raman image, a co-located bright field image was acquired.

Table 5-1 : Raman imaging system parameters selected for the *ex vivo* tissue experiments.

	Specifications
Field of view	1 cm ²
Working distance	40 mm (non-contact) 0 mm (with contact muzzle)
Number of excitation lines	40 lines (10 mm x 400 μ m each)
Distance between scanned excitation lines	250 μ m
Pixel Size	x-axis: 200 μ m, y-axis: 250 μ m
Spectral Resolution NIR	8 cm ⁻¹ (at 1085 cm ⁻¹)
Number of Pixels	x-axis: 40, y-axis: 42, λ -axis: 1024
Spectral Range	λ -axis: 400 – 2100 cm ⁻¹
Exposure Time	7.5 seconds per line Total: 5 min in this study
Spatial Resolution (field of view): bright field mode	50 μ m (1 cm ²)

The imaging FOV of 1 cm² was covered by scanning 40 lines per image. Pixel binning along the spatial axis of the camera CCD (y-axis) was used to increase signal to noise ratio (SNR), which effectively increased the y-axis pixel size to 250 μ m. The spatial offset between subsequent line measurements was 250 μ m. The dimension of each Raman image was thus 40 (x-axis) $\times$ 42 (y-axis) $\times$ 1024 (wavenumber axis). Six columns of pixels on the image borders were always discarded

due to low photon detection efficiency in those regions. This resulted in Raman images 36 x 40 spatial pixels, for a total of 1,440 spectra. The laser line excitation profile was 400 μm x 10 mm with a total power of 905 mW corresponding to an average on-sample intensity of 22.6 W/cm². The luminescence (i.e., Raman and background fluorescence resulting from 785 nm excitation) from 400 to 2100 cm⁻¹ (810 to 940 nm) was collected for each line during a 7.5 s exposure time. Bright field images allowed placement of the sample surface at the focal plane of the system. The broadband source was turned off during line-scanning Raman spectroscopy imaging. Every measurement was conducted within a custom-designed light tight enclosure to prevent ambient light sources from contaminating spectroscopic signals.

5.4.4 Classification

Two classification algorithms, support vector machines (SVM) and random forests (RF), were investigated. The classifiers were trained, validated, and tested to perform pixel-wise classification (i.e., no spatial features were used, only spectral features associated with Raman band intensities). Tissue labels were assigned visually to each imaged pixel using the collocated bright field images. The training set consisted of all 7 Raman images (10,080 spectra) from pig #1. The validation set consisted of all 7 images (or 10,080 spectra) from pig #2. Finally, the testing set consisted of the remaining 7 images (10,080 spectra) from pig #3. The training, validation and testing sets were independent from each other, and all contained 2 images of only muscle tissue, 2 images of only adipose tissue and 3 images containing adipose-muscle tissue interfaces. An additional testing set (4,320 spectra) was obtained from the 3 additional Raman images acquired in pig #3 with the contact measurement probe muzzle. This testing set consisted of one Raman image from a tissue interface, one image from adipose tissue and one image from muscle tissue.

The classification data pipeline consisted of manual feature selection followed by feature standardization and classifier development. Manual feature extraction (i.e., handpicking of prominent Raman peaks following visual inspection of the spectra in the training set) took precedence over automated feature extraction (e.g., principal component analysis [PCA]) since it lets human understanding of the discrimination task lead the selection of the features.

In total, 8 Raman features were manually selected for classification from the training dataset. The features selected had a visually apparent peak prominence for at least one tissue type and had a relevant biological assignment for its tissue type. For example, Raman features associated with lipid content were chosen as markers of adipose tissue and features associated with protein content as markers of muscle tissue. All selected features were for peaks with a Raman shift $\geq 1000 \text{ cm}^{-1}$ due to lower SNR below that range. Both SVM and RF classifiers were trained on the training set from pig #1 using a grid search over optimization parameters (hyperparameters). Linear and Gaussian kernels were considered for SVM models where the regularization parameter C was varied between 0.001 and 1 and the kernel coefficient parameter γ was varied between 0.001 and 10. Random forest models were trained with either 100, 200, or 300 trees, each single tree being trained with maximum 60%, 70%, or 80% of the training samples. The combination of parameters produced a total of 24 SVM models and 9 RF models. These models were then used to predict adipose and muscle tissue spectra on the validation set (pig #2).

The RF and SVM models having the highest validation accuracy in pig #2 were tested on the independent datasets from pig #3 (i.e. 7 images acquired without a probe muzzle and 3 images acquired with the probe muzzle). Pixel-wise predictions were computed along with their corresponding probabilities and were shown next to their respective bright field images to allow visually interpretation of the classification results. Class probabilistic outputs were computed using Platt scaling, which effectively quantifies model prediction confidence[213]. Probability scores varied from 0.5 to 1.0 where 1.0 corresponded to the highest confident class membership probability. A value of 0.5 was the lowest class membership probability. Algorithms from the Python library Scikit-learn were used[116].

5.4.5 Clinical safety assessment

The imaging probe muzzle serves as an intermediary between the probe body and the patient. Because the probe body will never be sterilized, there remains a risk of infection if there is not a proper seal between the probe muzzle window and the probe muzzle body. Three contact measurements probe muzzles were manufactured and their hermiticity was evaluated using a helium leak testing device (979 Helium Leak Detector, Varian, USA) combined with a custom-made silicon base (Figure 5-3). To test the probe muzzle resistance to heat such as in a steam

autoclave, one probe muzzle (Serial 00002) underwent a dry and high temperature cycle: 22 °C – 145 °C – 22 °C with 1 °C increments and 60 min at peak temperature, and its hermeticity was once again measured.

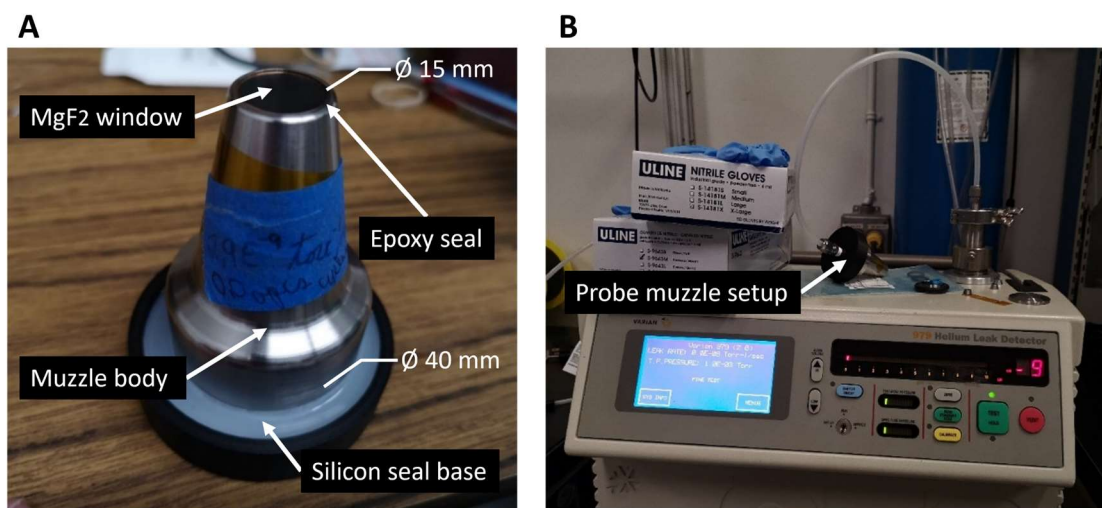


Figure 5-3 : (A) Contact measurements probe muzzle mounted on a hermetic silicon base for a helium leak test and (B) leak test setup using a helium leak detector.

Laser exposure risk to the eyes was evaluated for the system's operator and the other operating room staff (e.g., nurses, research staff and anaesthesiologists). With an output power of 0.905 W at 785 nm per line, the imaging system emits enough laser light to cause permanent damage to the retina. The beam intensity was computed for various distances from the probe tip and the maximum permissible exposure (MPE) for eyes was computed according to the Z136.1 ANSI Laser safety standard[214] using the equations shown in the supplemental material. The maximum beam power was also experimentally measured at different distances from the imaging probe, using an optical power meter (PM100D, Thorlabs, USA) and a thermal power sensor (S425C, Thorlabs, USA) having a 2.54 cm diameter input aperture.

Accidental laser exposure risk to skin for operating staff and patients was evaluated using the MPE for skin from the ANSI Z136.1 standard. The risk involving a continuous wave near-infrared (NIR) laser consists of thermal effects to tissue (e.g., coagulation, cauterizing and burning). Risk assessment was done near the area with the highest laser intensity during measurements, i.e., at the imaging probe focal plane. The maximum power (per line) in this study was 0.905 W at 785 nm.

The total output energy was computed for a total imaging time of 7.5 s averaged over a circular area of 3.5 mm diameter as per the ANSI Z136.1 standard's guidelines. Equations and detailed results for MPE in skin with the imaging system are shown in the supplemental material.

Intentionally administered laser radiation for diagnostic applications may exceed the MPE if the risk is warranted according to ANSI Z136.3 laser safety in healthcare[215]. The MPE was therefore exceeded during Raman measurements. However, the thermal risk to interrogated tissue was assessed by measuring the temperature–time history of the tissue surface during laser exposure with the cumulative thermal dose CEM43°C. The latter is an accepted metric for assessing thermal dose and risk of thermal damage in tissues and is referenced to evaluate the safety of heat exposure in humans and animals in hyperthermia therapy[216], radiotherapy[217], and ultrasound[218]. CEM43°C is computed using the measured time-dependant tissue temperature profile, i.e. $T(t)$ [219],

$$CEM43^{\circ}C = \int_0^t R^{(43-T(t))} dt \quad (5-1)$$

where R is a coefficient with values $R(T < 43^{\circ}C) = 1 / 4$ and $R(T \geq 43^{\circ}C) = 1 / 2$. Tissue damage was never observed for CEM43°C < 5 min in large animals (i.e., cats, goats, dogs, pigs and humans). Lowest values for which damages were observed in large animals was in dog brain for CEM43°C = 7.5 min[220]. It was also reported that in magnetic resonance radiofrequency exposure, a CEM43°C = 9 min appeared to be an acceptable thermal dose threshold for most patients[221].

Here, three different biological samples were studied ex vivo. Specifically, temperature profiles (surface temperature) were measured for porcine adipose tissue, porcine muscle tissue, and 5 mL of rat blood. This was done using a thermal camera (E45 ThermoCAM, FLIR, USA) and continuous-wave laser exposition of 60 s exposure (785 nm, 0.905 W per line). To emulate a real-world clinical setting, the measured temperature difference was added to the known average basal body temperature in humans (i.e., 36.9 °C) and the resulting temperature profile $T(t)$ was used to calculate the normalized thermal dose from equation (5-1). The animal ethics protocol was approved by the Institutional Animal Care Committee of the *Centre de recherche du Centre*

hospitalier de l'Université de Montréal in accordance with the Canadian Council on Animal Care guidelines.

5.5 Results

5.5.1 Raman tissue imaging

The dataset consisted of 21 images (30,240 spectra) from 3 pigs, containing images of adipose tissue, muscle tissue and the interface of these tissues. The bright field images and the corresponding labels are presented in Figure 5-4. The total class distribution was balanced with 5,011 spectra from adipose tissue and 5,096 spectra from muscle tissue. The average Raman spectra, raw spectra and standard deviation for each class are presented in Figure 5-5 (A), showing visually distinguishable molecular contrast between adipose and muscle tissue. The prominent molecular contrast is due to the difference in relative concentrations of lipids and proteins. As observed in the variance from Figure 5-5 (A), for identical acquisition parameters, spectra from muscle tissue have a lower signal to noise ratio (SNR) than those of adipose tissue.

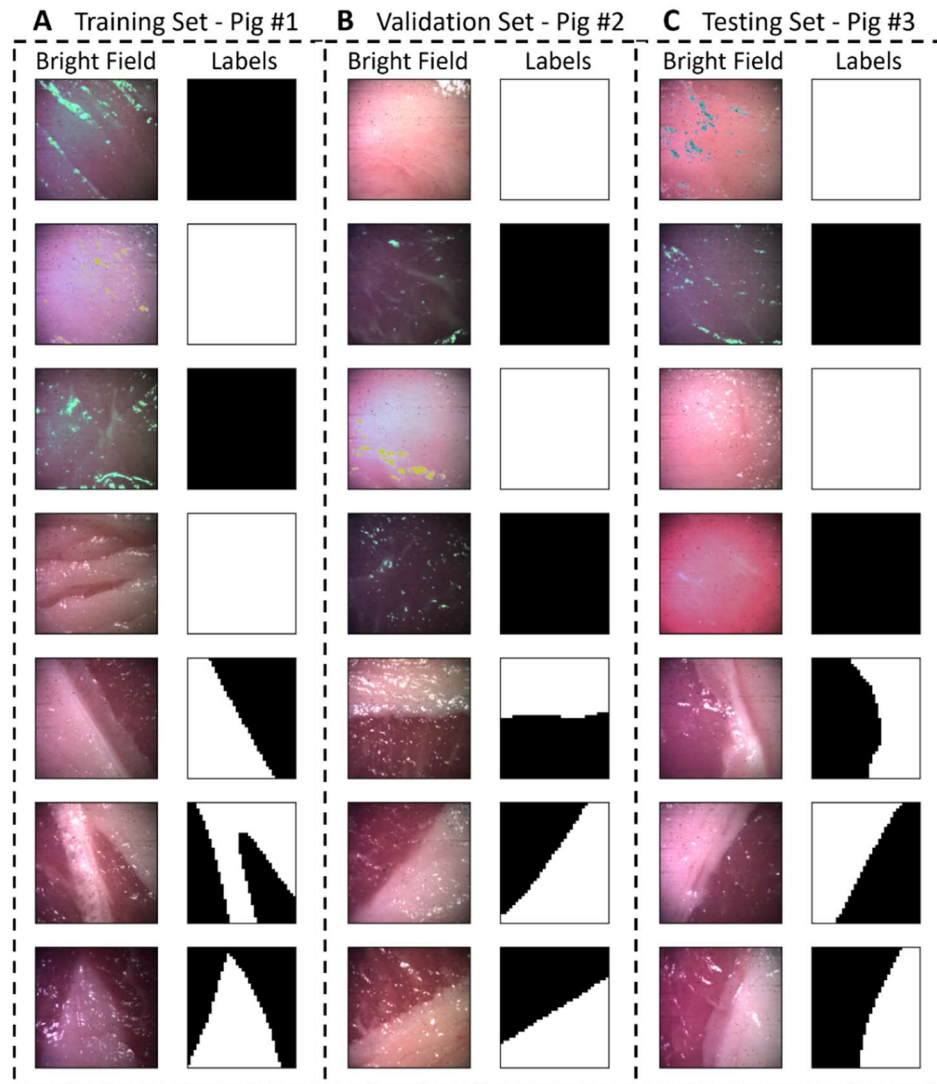


Figure 5-4: Study non-contact dataset consisting of (A) the training set (7 measurements from pig #1), (B) the validation set (7 measurements from pig #2) and (C) the testing set (7 measurements from pig #3). For each subdivision of the data set, bright field images of porcine adipose and muscle tissue and their corresponding visually selected labels are shown. Black labels correspond to muscle tissue while white labels correspond to adipose tissue. Each square delimits a field of view of roughly $10 \times 10 \text{ mm}^2$. In some bright field images, false colours including green tint were caused by an automatic white balance error introduced by the system's software.

A second dataset consisting of 3 images (4,320 spectra) from pig #3 contained one image from an adipose-muscle tissue interface, one image of adipose tissue and one image of muscle tissue, all acquired with the contact measurements muzzle. The average Raman spectra, raw spectra and standard deviation for adipose and muscle spectra acquired with the contact muzzle are shown in Figure 5-5 (B). Specific Raman band assignments and biomolecular interpretation can be found in the supplemental material.

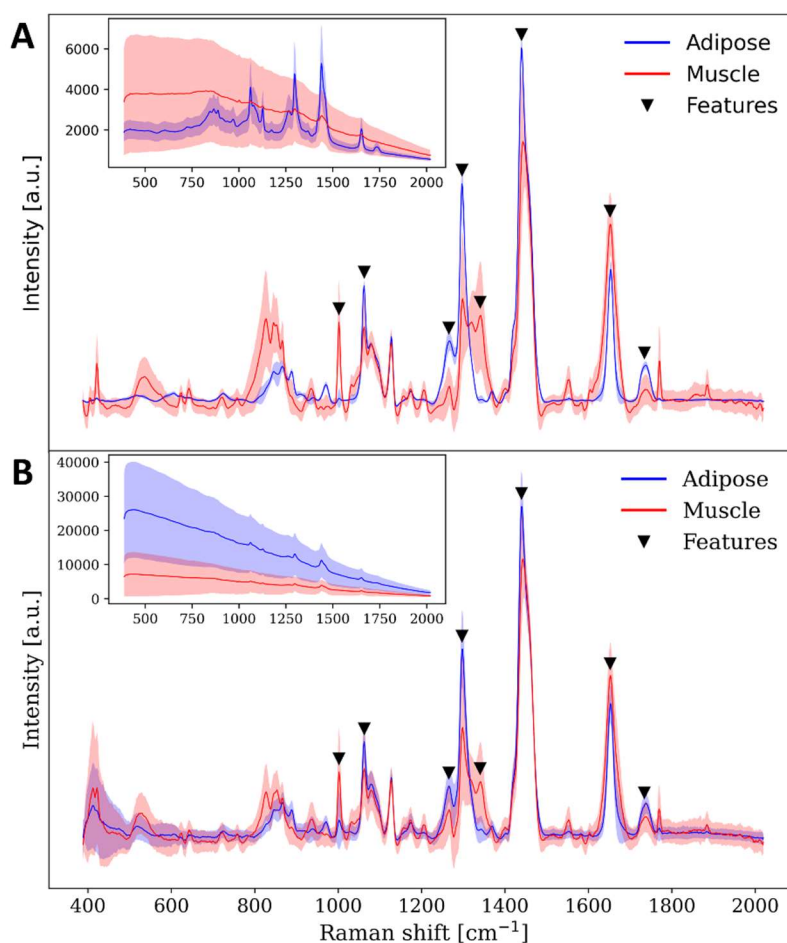


Figure 5-5 : Mean Raman spectra (solid line) and its 1x standard deviation (shaded area) for adipose (blue) and muscle (red) tissue of (A) the training dataset and (B) the contact muzzle testing set. For (A) and (B), the upper left spectra correspond to the mean raw luminescence counts and its 1x standard deviation for the same tissue types. Inverted black triangles correspond to the Raman features manually selected from the training data set for the classification.

5.5.2 Classification & tissue border detection

The SVM model with the highest validation accuracy had a Gaussian kernel with regularization parameter $C = 0.1$ and kernel coefficient $\gamma = 0.1$. The best performing RF model had 300 trees with each tree being trained with a maximum of 70% of all training instances. The best performing SVM and RF models had a training accuracy of 95% and 99%, respectively. Their validation accuracy was 98% for both SVM and RF. The features that were manually selected from the training set were the Raman peaks at 1004, 1062, 1265, 1295, 1340, 1459, 1652 and 1734 cm^{-1} .

Using the best trained/validated SVM model on the testing set resulted in prediction accuracy, specificity, and sensitivity of 96%, 94%, and 99%, respectively. Using the best trained RF model on the testing set resulted in prediction accuracy, specificity, and sensitivity of 96%, 95% and 98%, respectively.

Figure 5-6 illustrates the first test set (i.e., 7 Raman images acquired with no probe muzzle): bright field images, their corresponding labels, the pixel-based predictions, and their respective probability assignments. A probability value of 1 (black) signifies absolute certainty of prediction while a value of 0.5 (white) signifies a random prediction. Classification on Raman images from the probe equipped with the contact muzzle resulted in prediction accuracy, specificity, and sensitivity of 97%, 98% and 97% when using the best SVM model and 94%, 100% and 89% when using the best RF model.

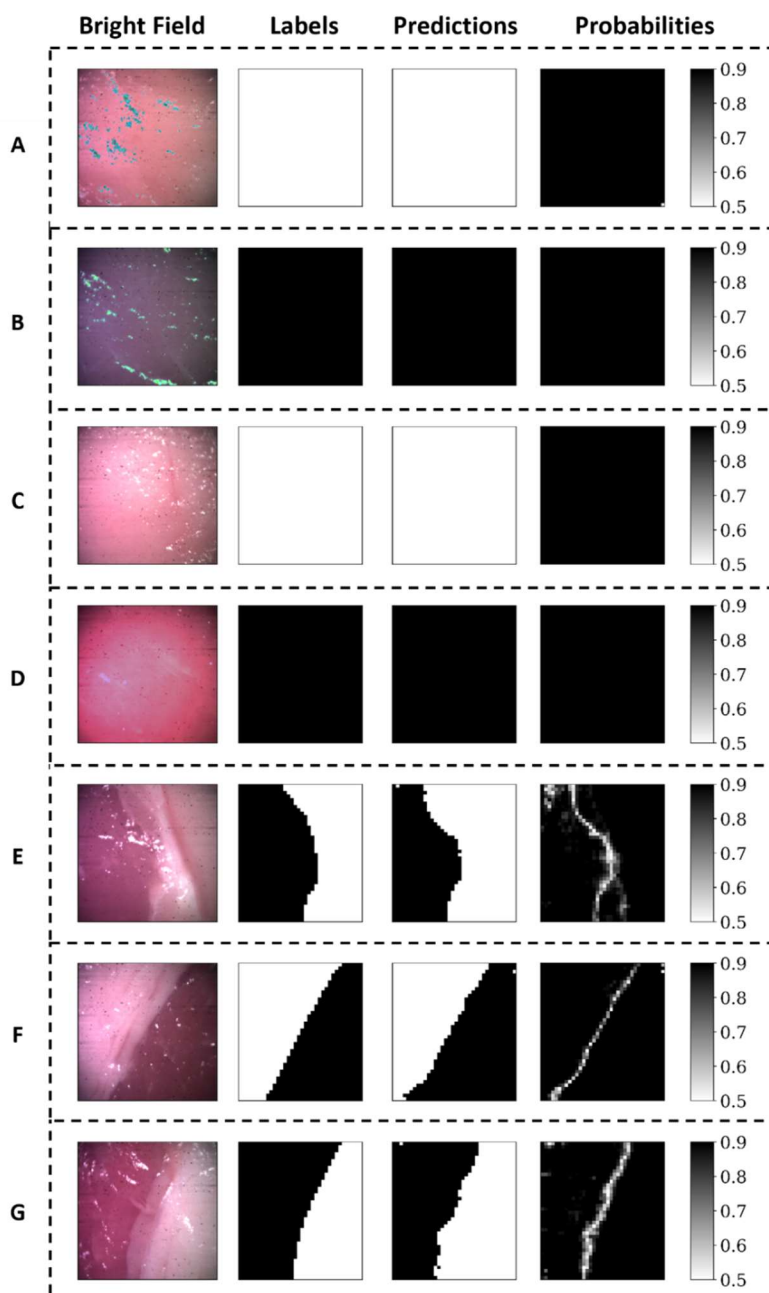


Figure 5-6 : (A-G) Raman images from the non-contact testing set (pig #3) with, from left to right respectively, the bright field image of the interrogated tissue, its corresponding labels (white for adipose tissue and black for muscle tissue), the classification predictions from the best performing SVM model and the assigned prediction probability. A prediction probability of 0.9 (black) indicates near certain prediction while a probability of 0.5 (white) corresponds to random prediction.

5.5.3 Clinical safety assessment

Leak tests from Table 5-2 show that the seal satisfies the 5×10^{-8} atm·cc/s rejection limit set by the Department of Defense Test Method Standard (MIL-STD-883E - Method 1014.10) which is referenced for medical applications. Following the dry and high temperature cycle: 22 °C – 145 °C – 22 °C, the measured leak rate of Serial 00002 was 1.32×10^{-9} atm·cc/s.

Table 5-2 : Helium leak test results validating muzzle hermiticity.

Muzzle Serial No.	Optical window material	Leak rate [atm·cc/s]
00001	MgF ₂	5.80×10^{-9}
00002	MgF ₂	3.82×10^{-9}
00003	CaF ₂	5.01×10^{-9}

Results in Table 5-3 from calculated and measured laser intensities are showing which distances are safe to observe the tip of the probe for a duration exceeding 10 seconds with a maximum power output of 0.905 W. The laser beam exiting the imaging probe has a significant divergence created by the probes focussing lens. Optical simulations of the imaging system with OpticStudio® (Zemax, USA) have shown this beam has a rectangular shape and a divergence of 5.6° along the beam *y*-axis (10 mm) and 8.8° along the beam *x*-axis (400 μm). Due to this large divergence the output laser beam rapidly loses intensity the further away the beam is from the focussing plane (i.e., 40 mm from the imaging probe or 0 mm from the contact muzzle tip).

Calculated and measured laser intensities, along with known laser beam divergence suggest that for an exposure time < 10 s, the safe distance of exposure for eyes is ≥ 16 cm from the contact muzzle tip or ≥ 20 cm from the imaging probe. For an accidental exposure time of < 1 s, the safe distance of exposure for eyes becomes ≥ 11 cm from the muzzle tip and ≥ 15 cm from the imaging probe.

Table 5-3 : MPE for eyes calculated and measured according to distance from the probe nose tip for an exposure time >10 s.

Distance from contact muzzle tip [cm]	Measured Power [W]	Measured Intensity [W/cm ²]	Computed Intensity [W/cm ²]	Below MPE eyes for >10 s?
0	0.905	2.26×10^1	2.26×10^1	No
7.7	0.878	1.7×10^{-1}	4.2×10^{-1}	No
23.2	0.359	7.09×10^{-2}	7.7×10^{-2}	Yes
65.2	0.046	9.1×10^{-3}	1.2×10^{-2}	Yes
90.7	0.030	5.9×10^{-3}	6.6×10^{-3}	Yes

Results from Table 5-4 suggest that accidental laser exposure to skin over 0.4 s per line at the probe tip exceeds the MPE. MPE computations also suggest that accidental skin exposure ≥ 10 cm from the probe tip is well below the MPE. The thermal study showed that recorded temperature differences for laser exposure up to 60 seconds in muscle tissue and adipose tissue (Figure 5-7) never exceeded 1.5 °C and 1.0 °C, respectively. Considering the maximum induced temperature difference (recorded during 60 s) in live animal or human tissue which would have an average basal temperature of 36.9 °C, this corresponds to a $CEM43^\circ C = 1.7 \times 10^{-3}$ min and 8.5×10^{-4} min for $t = 1$ min for muscle and adipose tissue respectively which in both cases are considered safe thermal doses. When exposing 5 mL of stagnant blood to the maximum laser exposure of the system, temperature differences significantly increased and $CEM43^\circ C > 5$ min occurred for an exposure time > 10.5 s. For laser exposure time = 7.5 s in blood, $CEM43^\circ C = 0.93$ min which is well within a safe threshold of $CEM43^\circ C = 5$ min. Thermal doses evaluated in muscle tissue, adipose tissue and stagnant blood suggest the laser exposure parameters of the system (i.e., 0.905 W power and 7.5 s per line exposure time) are safe for tissues.

Table 5-4 : Excitation line laser intensity at the tissue surface for different exposure times and a power of 0.9 W compared to the MPE of skin of 3.27 W/cm²

Exposure time [s]	Intensity permitted [W/cm ²]	Below MPE skin
0.3	4.01	Yes
0.4	3.23	Yes
0.5	2.74	No
1	1.63	No
5	0.49	No

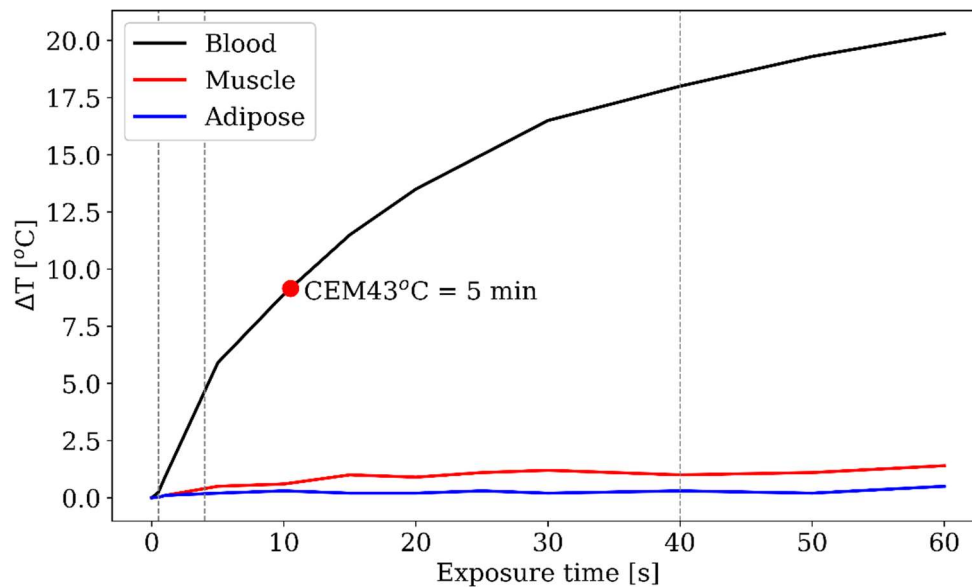


Figure 5-7 : Temperature difference following maximum laser exposure to blood (black), muscle (red) and adipose (blue) tissue for a total exposure time of 60 seconds. Each dotted line represents from left to right, 1x, 10x and 100x the skin MPE respectively. Only laser exposure to blood would exceed a safe limit of CEM43°C = 5 min after an exposure time of 10.5 s if adding the recorded temperature difference to a basal body temperature of 36.9 °C.

5.6 Discussion

5.6.1 Raman imaging of biological tissue

One aspect of the work aimed at acquiring sufficiently large datasets to demonstrate the new Raman imaging device could be used towards the development of generalizable tissue classification machine learning models. This was achieved by creating a dataset from 6 porcine loin samples from 3 pigs for a total of 21 Raman and bright field images. Labelling of images proved challenging in some images where diffuse adipose tissue was observed. Without a more accurate method to precisely identify tissue lipid and protein content, (e.g., histology) it is probable that some diffuse areas were mislabeled.

Discriminating adipose versus muscle tissue was used as a model for proof-of-principle studies although it bears limited clinical relevance to the intended use, i.e., cancer detection. In fact, discriminating cancer from normal tissue typically results in spectral fingerprint differences more subtle than the changes detected between adipose and muscle tissue. However, the molecular content detected with Raman spectroscopy in those two tissue types cover all main peaks encountered in surgical applications, specifically Raman bands associated with lipids, proteins as well as aromatic amino acids.

Increase of background was observed when comparing contact with non-contact measurements acquired with the system. This was attributed to the highly fluorescent epoxy used to seal the probe muzzle. Although no fluorescence was observed when acquiring Raman images with the muzzle in mid-air, this epoxy was exposed to excitation light when acquiring measurements in a scattering medium. The system is then sensitive enough to collect the backscattered fluorescence generated in the epoxy. This added background signal did not impede tissue discrimination based on Raman spectra in adipose and muscle tissue. However, it is expected that for discrimination of tissues with much more subtle molecular differences such as normal versus cancer, the decrease in SNR may prevent accurate discrimination. For oncological applications, a change of epoxy in the probe muzzle is therefore warranted for contact measurements. Even so, when attempting to discriminate tissue types using Raman spectroscopy, we can circumvent some signal differences introduced by

environmental factors (e.g., operating room lights) or by an apparatus, such as probe muzzle, by carefully selecting Raman features and classification parameters, as discussed below.

5.6.2 Classification and tissue border detection

As specified in the classification results, training accuracy for the best performing SVM and RF models reached 95% while validation accuracy exceeded 97%. The slightly lower training accuracy is attributed to Raman images in the training set containing some diffuse tissue borders and muscle tissue with high lipid content. Those may have been mislabeled or may present much more subtle molecular contrast not considered by the classifier. This did not prevent the trained model from discriminating adipose from muscle tissue with a high classification accuracy of 96% for both the SVM and RF models in an independent hold-out testing set.

Additionally, the model performed well in identifying tissue borders through classification probability assignment in Figure 5-6. In fact, all predictions were attributed a high prediction probability of success except for clearly defined lines delineating the border between the two classes of tissue. These delineations correspond quite well to the location of the tissue interface identified in the labelling. It is indeed expected that at a tissue interface, the signal recovered will originate from a mixture of both classes of tissue which will lead to a reduced prediction confidence. The clinical implication from these predictions along with prediction probability across the boundaries are significant since tumor boundaries may be well-defined (e.g., benign tumors) or diffuse (e.g., malignant tumors). With a well-trained classifier for tumor detection, well-defined boundaries will appear as only being a few pixels wide while diffuse tumor boundaries will appear as a gradient of prediction probability that can extend up to several millimeters. We can observe, from the bright field images and corresponding prediction probabilities of tissue borders in Figure 5-6, that even in the trivial case of adipose versus muscle tissue, not all tissue boundaries are the same pixel width. As such, this study presents an example of how prediction probabilities in Raman imaging can show the general location of tissue boundaries but can also show the local invasiveness (e.g., infiltrations of cancer cells) at tissue borders. Such information presented could indicate to surgeons if they are working with diffuse tumors or not. This could be used as an aid to decision when assessing how much tissue needs to be removed.

Contact measurements with the probe muzzle used as a second testing dataset did not result in a significant degradation of predictive accuracy for both the SVM and RF models (i.e. for SVM: 97% with muzzle versus 96% without muzzle and for RF: 94% with muzzle vs 96% without muzzle). The feature selection of prominent and relevant Raman peaks was successful in creating classifiers that circumvented a large degradation of SNR, introduced by the probe muzzle, to discriminate tissues with large molecular contrasts (i.e., adipose and muscle tissue). However, the same strategy may be employed to circumvent a smaller instrument response introduced by an improved probe muzzle, in a discrimination task involving tissues with more subtle molecular differences (e.g. cancer versus normal).

5.6.3 Clinical safety assessment

The measured leak rate of the probe muzzle (serial 00002) following a dry and heat cycle was 1.32×10^{-9} atm·cc/s. This result showed no degradation of the seal and a leak rate that remained satisfactory according to the rejection limit in MIL-STD-883E - Method 1014.10. This small leak test study gives confidence in the method of assembly of the probe nose, its design and its hermiticity. In the aim of developing a commercial product, such hermiticity will need to be tested in dozens of manufactured copies of the probe muzzle.

Regarding laser exposure risk to eyes, the MPE calculations suggest that an accidental laser exposure from the imaging probe poses only a risk to individuals in proximity of the imaging probe (i.e., the surgeon or any personnel handling the probe). An operator would need to directly observe the tip of the probe, at a distance below 20 cm, while the laser is activated at maximum power. In a clinical setting, this would be unlikely since Raman image measurements, with laser power activated, are to be initiated only when the probe is placed in contact with or near contact facing the surgical cavity. Standard operating procedures such as keeping the laser off using a turnkey during imaging probe setup and activating the laser only when system is in place and ready for acquisition would help mitigate this risk. Finally, there is always the option of providing laser safety goggles for surgeons during the use of such a device. We consider the risk for eye exposure to the patient to be negligible since patients are completely covered by opaque surgical drapes during major surgical procedures while also being sedated.

According to Z136.1 ANSI laser safety standards, results from Table 5-4 suggest that an accidental exposure to skin in immediate proximity of the probe tip needs to be avoided. As with accidental eye exposure, standard operating procedures in an operating room and staff training would make such an accidental exposure highly unlikely.

As for intentional laser exposure to tissue, the MPE for skin was exceeded up to a factor of 18 in this study yet no tissue damage was observed in the form of tissue discoloration or tissue burning. The cumulative thermal dose $CEM_{43^{\circ}C}$ was used as a metric for evaluating dangerous laser exposure to tissues in a clinical setting. Temperature differences observed in Figure 5-7 over a 60 s laser exposure would not expose live muscle or adipose tissue to a thermal dose exceeding $CEM_{43^{\circ}C} = 5$ min. This assessment has its limits since live tissue will have circulation of whole blood which has an absorption coefficient $\mu_a = 0.34 - 0.54 \text{ mm}^{-1}$ at 785 nm[222]. This absorption will increase temperature locally and increase the thermal dose on the interrogated area. Laser exposure to stagnant rat blood showed a difference of temperature that would exceed $CEM_{43^{\circ}C} > 5$ min following ≥ 11 s of laser exposure from the system if added to the average basal temperature of $36.9^{\circ}C$ of mammals. Knowing that blood circulates through live tissue and dissipates heat, laser exposure to stagnant blood presents a worst-case scenario for a temperature increase following laser exposure at 785 nm. We add that anaesthesia of patients during surgical procedures usually causes a decrease in body temperature ($< 36.0^{\circ}C$)[223]. The permissible thermal dose through laser exposure with the system would therefore be greater during surgical procedures. With these results, it is expected that in live tissues during surgery, temperature differences and total heat exposure to tissue following the system's laser exposure will be between what was observed in adipose/muscle tissue and stagnant blood.

We conclude that, in accordance with the literature, we can safely and intentionally exceed the MPE for skin set by the ANSI standard by a factor of $10.5 \text{ s} / 0.4 \text{ s} = 26$ when performing Raman imaging with this system in live tissue. We are further confident with this assessment since our experiment was conducted in a worst-case scenario due to the absence of blood flow in the interrogated blood sample of this study, as opposed to live tissue, where heat transfer occurs by convection from blood perfusion[224]. Additionally, a cooling effect from cold air blown onto the tissue could help control local temperature increase such as in some laser therapies in skin[225]. The observed temperature differences observed are therefore much higher than one would expect

in an in vivo setting. When intentionally exceeding the ANSI MPE for skin in a clinical setting, we recommend monitoring the surface temperature to assess and ensure that heat generated by laser exposure to tissues never exceeds known safe CEM43°C.

5.7 Conclusions

This report presents an upgraded large field of view macroscopic line-scan Raman imaging system which has been modified into a clinical prototype ready for intraoperative tissue imaging. A dataset of bright field and Raman images was generated from porcine tissue allowing for the training, validation, and testing of robust classifiers. Clear contrast was observed in the spectra acquired in adipose and muscle tissue. Additionally, similar molecular contrast was present between adipose and muscle tissue imaged with and without the system's contact muzzle, which suggests a classification model could be generalizable to both cases. Biologically relevant Raman features were selected from the training set for classification of adipose and muscle tissue in porcine loins. SVM and RF classifiers were trained and validated, and the best performing SVM and RF models produced classification accuracies >94 % on two separate independent testing sets of porcine loins (contact and non-contact measurements). Finally, safety assessments were conducted for biosafety and laser safety. It was shown that the sterilizable probe muzzle poses minimal biosafety risk to patients for intraoperative use while the system's laser can be used safely when observing standard operating procedures in the operating room while also respecting the $CEM43^{\circ}C = 5$ min thermal dose limit.

5.7.1 Author Contributions

François Daoust: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Original Draft, Writing – Review and Editing, Visualization. **Hugo Tavera:** Conceptualization, Methodology, Software. **Frédéric Dallaire:** Methodology, Formal analysis. **Patrick Orsini:** Conceptualization, Methodology, Project administration. **Keven Savard:** Conceptualization, Methodology, Visualization. **Jacques Bismuth:** Conceptualization, Methodology, Software. **Philippe Mckoy:** Conceptualization, Methodology. **Israel Veilleux:** Software, Resources. **Kevin Petrecca:** Supervision, Project administration, Funding

acquisition, Review & Editing. Frédéric Leblond: Supervision, Project administration, Funding acquisition, Visualization, Writing – Review & Editing.

5.7.2 Conflicts of interest

Kevin Petrecca and Frédéric Leblond are co-founders of Reveal Surgical, a medical device company that seeks to commercialize the Raman spectroscopy system for real-time detection of tissue abnormalities.

5.7.3 Acknowledgements

This work is supported by the Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Collaborative Health Research Program (Canadian Institutes of Health Research and NSERC), and the TransMedTech Institute. This work is also supported by the Alliance program from NSERC in collaboration with the company Reveal Surgical.

5.8 Supplementary Material

5.8.1 Installation of a surgical drape on the clinical prototype for intraoperative imaging

Once a sterilized probe muzzle is recovered, a sterile drape is installed. A sterile C-Arm drape (1013 Steri-Drape™, 3M, USA) is used to cover the probe body (see Figure 5-8), the articulated arm and the fiber bundles which cannot be sterilized. This is achieved by first securing the sterilized probe nose to the sterile drape with a tight seal (i.e. with a sterile tape or rubber band) as shown in Figure 5-9 (A-B). The sterile drape is then passed on to a circulating nurse which will cover the probe body, articulating arm and fiber bundle Figure 5-9 (C). The probe nose is then fixed to the probe body by press fit with an audible ‘click’ sound Figure 5-9 (D). Ball springs on the probe body help keep the probe nose in place. The surgical drape is then secured at the probe body, the articulated arm and the fiber bundles using commercially available sterile tape and/or rubber bands Figure 5-9 (E-F).



Figure 5-8 : Raman imaging probe in a clinical setting with a sterile drape covering the probe body and the articulated arm. The surgical drape is secured using sterile rubber bands and sterile tape. The probe muzzle is sterilized, ready to be inserted in a patient surgical cavity and it presents a minimal infection risk to the patient.

5.8.2 Imaging system parameters for laser safety assessment

To evaluate the maximum permissible exposure (MPE) with a laser for eyes and skin, several parameters were recovered from the imaging system technical specifications. These technical specifications are listed in Table 5-5 and are the parameters used for all MPE calculations. MPE equations, parameters and correction factors were taken from ANSI Z136.1 Laser safety standard.



Figure 5-9 : Imaging probe drape covering procedure consisting of 6 steps: (A) recover the sterilized probe nose and remove the sterile drape from its packaging, (B) secure the probe nose to the sterile drape, (C) cover the probe body and articulated arm with the sterile drape, (D) secure the probe nose to the probe body, (E) secure the sterile drape to the probe body using sterile tape or rubber bands and (F) secure the sterile drape to the articulating arm and fiber bundles.

From the imaging system parameters in Table 5-5, we first calculate the intensity at maximum power I_{max} of the imaging system:

$$I_{max} = \frac{P_{max}}{x_0 \cdot y_0} \left[\frac{W}{cm^2} \right] = \frac{9.05 \times 10^{-1}}{4.00 \times 10^{-2} \cdot 1.00} \left[\frac{W}{cm^2} \right] = 22.6 \left[\frac{W}{cm^2} \right] \quad (5-2)$$

Table 5-5 : Imaging system parameters for MPE calculations

Parameter name	Parameter symbol	Value	Units
Excitation wavelength	λ	785	nm
Maximum laser power	P_{max}	905	mW
Horizontal laser beam dimension at focus	x_0	400	μm
Vertical laser beam dimension at focus	y_0	1.0	cm
Horizontal beam full angle	α_x	154	mrad
Vertical beam full angle	α_y	98	mrad

According to Table 8a. in ANSI Z136.1 laser safety standard, the beam intensity is to be averaged over a limiting aperture of 3.5 mm when evaluating laser hazard in skin. We therefore calculate I_{exp} as follows:

$$I_{exp} = \frac{I_{max} \cdot x_0 \cdot y_a}{0.175^2 \cdot \pi} \left[\frac{W}{cm^2} \right] = \frac{22.6 \cdot 4.00 \times 10^{-2} \cdot 0.35}{0.175^2 \cdot \pi} \left[\frac{W}{cm^2} \right] = 3.29 \left[\frac{W}{cm^2} \right] \quad (5-3)$$

where y_a is the height of the laser line profile considered for the calculation of laser hazard over the limiting aperture area (Figure 5-10).

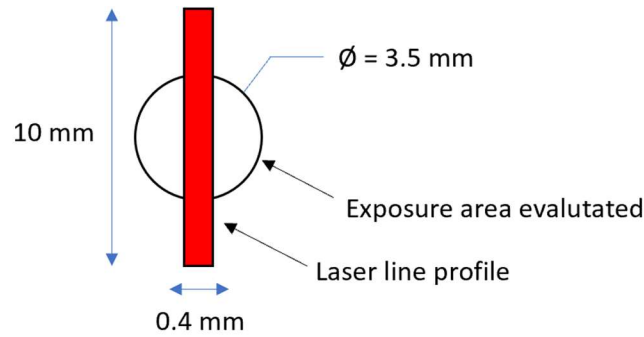


Figure 5-10 : Laser line profile dimensions of the imaging system compared with the exposure area (i.e. limiting aperture) evaluated in maximum permissible exposure calculations.

5.8.3 Maximal permissible exposure to skin calculation

From ANSI Z136.1 Table 7b: MPE for skin exposure to a laser for wavelength from $\lambda = 785$ nm we extract the equation to calculate the MPE for skin for $t < 10$ s and $t > 10$ s.

$$MPE_{skin}(t < 10 \text{ s}) = 1.1 \cdot C_A \cdot t^{0.25} \left[\frac{\text{J}}{\text{cm}^2} \right] \quad (5-4)$$

$$MPE_{skin}(t > 10 \text{ s}) = 0.2 \cdot C_A \left[\frac{\text{W}}{\text{cm}^2} \right] = 0.30 \left[\frac{\text{W}}{\text{cm}^2} \right] \quad (5-5)$$

where t is the exposure time and C_A is a correction factor:

$$C_A = 10^{0.002(\lambda - 700)} = 10^{0.002(785 - 700)} = 1.48 \quad (5-6)$$

From these equations, we evaluate what is the maximum permissible laser intensity for different exposure times (see Table 5-6).

Table 5-6 : Laser MPE for skin using the Raman imaging system.

Exposure Time [s]	MPE [J/cm ²]	MPE Intensity [W/cm ²]
0.3	1.20	4.01
0.4	1.29	3.23
0.5	1.37	2.74
1	1.63	1.63
2	1.93	0.97
3	2.14	0.71
4	2.30	0.58
5	2.43	0.49
6	2.55	0.42
7	2.65	0.38
8	2.74	0.34
9	2.82	0.31
≥10	2.89	0.30

From Table 5-6 and I_{exp} calculated, we evaluate that a laser exposure time exceeding 0.4 s is not permissible for skin.

5.8.4 Maximal permissible exposure to eyes calculation

The laser beam exiting the Raman imaging probe is not collimated and has a divergence which quickly reduces the laser intensity the further away the beam is from the imaging probe muzzle tip. The quick drop of laser intensity may become safe from a certain distance from the imaging probe muzzle tip. We therefore calculate the beam dimensions x_L and y_L at the distance L from the probe muzzle tip:

$$x_L = 2 \cdot \left(L \cdot \tan\left(\frac{\alpha_x}{2}\right) + \frac{x_0}{2} \right) \quad (5-7)$$

$$y_L = 2 \cdot \left(L \cdot \tan\left(\frac{\alpha_y}{2}\right) + \frac{y_0}{2} \right) \quad (5-8)$$

From the new laser beam dimensions, we evaluated the laser intensity I_L to which an observer would be exposed at a distance L from the probe muzzle tip:

$$I_L = I_{max} / (x_L \cdot y_L) \quad (5-9)$$

The intensity of the laser beam from the tip of the imaging probe muzzle according to distance is shown in Table 5-7.

Table 5-7 : Intensity of laser beam evaluated for several distances from the tip of the imaging probe muzzle.

Distance from tip of the probe muzzle	Max Intensity
0	22.63
1	4.25
2	2.18
3	1.39
4	0.99
5	0.75
6	0.59
7	0.48
8	0.40
9	0.34
10	0.29
11	0.25
12	0.22
13	0.20
14	0.17
15	0.16
16	0.14
17	0.13
18	0.12

We then calculate the MPE threshold for eyes and compare it to the results in table S3. Since the source is extended and shaped as a rectangle, we use the extended source parameters and correction factors for a wavelength of 785 nm presented in the ANSI Z136.1 Table 6b. From this table we calculate the correction factor CE :

$$CE = \frac{\alpha_x * (\alpha_{max} + \alpha_y)}{(2 * \alpha_{max} * \alpha_{min})} = 101 \quad (5-10)$$

where α_{min} and α_{max} are parameters and $\alpha_{min} = 1.5$ mrad and $\alpha_{max} = 100$ mrad. The exposure duration (time) beyond which extended source MPEs based upon thermal injury are expressed as

a constant irradiance: $T_2 = 100$ s. Finally, we calculate the MPE for extended source ocular exposure to a laser beam for a wavelength of 785 nm using table 5f from the ANSI Z136.1 laser safety standard. For MPE for eyes with $10^{-6} < t < T_2$

$$MPE_{eyes}(t) = 1.8 * C_A * C_E * t^{0.75} * 10^{-3} \left[\frac{J}{cm^2} \right] \quad (5-11)$$

We evaluate the MPE for eyes for exposure times of 1 s and 10 s respectively:

$$\begin{aligned} MPE_{eyes}(t = 1 \text{ s}) &= 1.8 * C_A * C_E * t^{0.75} * 10^{-3} = 0.27 \left[\frac{J}{cm^2} \right] = \\ &> 0.27 \left[\frac{W}{cm^2} \right] \end{aligned} \quad (5-12)$$

$$MPE_{eyes}(t = 10 \text{ s}) = 1.5 \left[\frac{J}{cm^2} \right] \Rightarrow 0.15 \left[\frac{W}{cm^2} \right] \quad (5-13)$$

According to Table S3, distances ≥ 11 cm from the probe muzzle tip are safe for laser exposures to the eyes for exposures of ≤ 1 s. Additionally, distances ≥ 16 cm from the probe muzzle tip are safe for laser exposures to the eyes for laser exposures of ≤ 10 s.

5.8.5 Prominent Raman features in porcine muscle and adipose tissue.

Listed in Table 5-8 can be found the prominent Raman bands used for the tissue classification in the study, their assigned molecular bonds and respective molecular family [191], [209], [212].

Table 5-8 : Prominent Raman features for porcine muscle and adipose tissue.

Raman Band (cm^{-1})	Molecular Bonds	Molecular Family
1004	Phenylalanine breathing mode	Protein
1062	C-C anti-symmetric stretch & C-N Stretch	Lipid & protein
1265	=CH ₂ in plane deformation & amide III band	Lipid & protein
1295	CH ₂ twist	Lipid & protein

1340	CH deformation & CH ₂ /CH ₃ wagging	Protein
1459	CH ₂ symmetric deformation, CH ₂ /CH ₃ antisymmetric deformation & CH ₂ Bending	Lipid & protein
1652	Amid I band, C=C stretch & C=O stretch	Lipid & protein
1734	C=O ester stretch	Lipid

CHAPTER 6 ARTICLE 3: INTRAOPERATIVE WHOLE-SPECIMEN RAMAN SPECTROSCOPY IMAGING DETECTS HIGH CANCER CELL DENSITY CLUSTERS IN GLIOBLASTOMA

SCIENTIFIC REPORTS – SPRINGER NATURE

Submitted for review on March 30th, 2024

François Daoust^{1,2}, Frédérick Dallaire^{1,2}, Hugo Tavera^{1,2}, Katherine Ember^{1,2},
Marie-Christine Guiot³, Kevin Petrecca⁴ and Frédéric Leblond^{1,2,5}

¹ Polytechnique Montreal, Department of Engineering Physics, Montreal, QC H3T 1J4, Canada

² Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montréal, QC H2X 0A9, Canada

³ Division of Neuropathology, Department of Pathology, Montreal Neurological Institute-Hospital, McGill University, Montreal, QC H3A 2B4, Canada

⁴ Montreal Neurological Institute-Hospital, McGill University, Montreal, QC H3A 2B4, Canada

⁵ Institut du cancer de Montréal, Montreal, QC, H2X 0A9, Canada

6.1 Background

In Chapter 5 was presented the transformation of the Raman imaging system into a clinical prototype capable of *in situ* tissue imaging within an operating theatre. The system was packaged and mounted onto a cart to be suitably moved within a hospital. Its safety was robustly assessed, and the workflow of the device was improved to ensure machine learning could be reliably used to characterize tissues and locate tissue margins. With the system ready to be tested intraoperatively, it was time to proceed to testing the system in a real intraoperative setting.

In this chapter, the first intraoperative pilot study in human patients was conducted with the clinical prototype imaging system. The study involved fresh brain specimens from eight patients suffering from glioblastoma. The work presented in the chapter addresses the third objective of this dissertation: Objective 3: *Evaluate the feasibility, practicality, and performance of the clinical*

prototype in a pilot study on resected tumoral tissue from 8 patients affected by brain tumours. For this chapter, results of the pilot study are presented and show the systems ability to characterize the molecular signature of large freshly resected specimens. The imaging system was augmented with a machine learning model trained and tested on a reference point Raman system (i.e. the Sentry from Reveal Surgical) to discriminate glioblastoma from normal brain. The predictions of tumor in brain specimens were assessed with histopathology and proved to be accurate in predicting the likelihood for specimens to contain cancer. Finally, the pilot study illustrates how Raman imaging over 1 cm² could be used to assess surgical margins intraoperatively and could provide actionable information to surgeons to guide them during cancer resection.

6.2 Abstract

Raman spectroscopy is a technique that uses laser light to gain molecular information without causing damage. The method can be used *in vivo* or *ex vivo* and does not require contrast agents. Clinically, the method has primarily been used in the form of a laser-based probe which analysis only a single point within tissue. Here we report the development of a whole-specimen spectroscopic imaging system for detection of residual cancer cells on the surface of surgical specimens. A cancer detection machine learning (ML) model trained from data acquired using a single-point probe (24 glioblastoma patients) was created. This ML model was used to produce cancer prediction maps of the specimens that were validated with histology. The field-of-view of the system covers a 1 cm² square area, allowing the technology to be used for residual cancer detection at surgical margins. This pilot study presents results from specimens from 8 glioblastoma patients. The instrument was able to detect clusters of cancer cells from the Raman peaks associated with the amino acid phenylalanine and tryptophan as well as the relative concentration of lipids and proteins associated with deformations of the CH₂ and CH₃ molecular bonds. The technique can be applied to common cancers for which margins examination is done include breast cancer, prostate cancer, lung cancer, and gynecological cancers.

6.3 Introduction

Approximately 45 million surgical procedures occur each year and surgical intervention is a mainstay treatment for many cancers [226]. The goal of surgical oncology is to investigate the

cancer, remove tumors, ensure a safe margin of surrounding tissue while preserving as much healthy tissue and organ function as possible. However, there is a lack of tools for guidance during the surgical procedure [227], particularly tools that can give real-time feedback without an arduous staining process or administration of contrast agents [42].

Primary tumor removal remains at the core of surgical oncology and cancer treatment [228]. Here, the original cancerous lesion is removed, and for many cancers the surgical margins are examined by histopathology after surgery to ensure that there are no residual cancer cells left behind. Residual tumor is indicative of a positive surgical margin, and often necessitates a second surgery to reduce risk of recurrence [229], [230]. However, histopathology is difficult to achieve intraoperatively as it is time-consuming [231], [232] and requires trained pathologists, who are exceedingly in demand [233]. For brain cancers, margin assessment is often done by post-operative magnetic resonance imaging (MRI) scans, by which point residual tumor may necessitate a follow-up surgery, which is expensive, time consuming and carries further risk to the patient [234], [235].

For brain cancers, it is especially important to find a balance between complete tumor removal and minimizing impact to normal tissue [20], [236], [237]. Time from surgery to tumor recurrence, termed progression free survival, and life-expectancy of patients with gliomas negatively correlate with the volume of tumor remaining after surgery [238]. Furthermore, residual tumor may necessitate another surgery [239], [240]. However, resections that extend beyond tumor margins into the adjacent brain can lead to neurological deficits, worsening patients' quality of life and overall survival [13]. These negative outcomes could be mitigated by the development of surgical tools that distinguish tumor tissue from normal brain tissue from resected specimens.

The optical tool Raman spectroscopy is a possible solution for real-time tissue analysis and cancer margin detection [241], [242]. Raman spectroscopy is an analytical technique that uses laser light to gain molecular information about a sample. The inelastic (Raman) scattering of laser light by a sample is dependent on vibrational states and thus the structure and bonding of molecules. As molecular composition of biological samples can alter in disease states such as cancer and ischemia, samples may be classified based on their Raman spectral profiles. The energetic differences between the incident near-infrared light and the inelastically scattered light can be plotted on the x -axis of a Raman spectrum whilst the intensity of Raman scattering at a particular

wavenumber is given on the y -axis. The peak position of the x -axis gives information about molecular identity whilst the peak height gives information about relative molecular concentrations. A single Raman peak may have contributions from different types of biomolecules if they have molecular bonds with similar vibrational states.

We have already developed a Raman spectroscopy system for detection of brain tumors during surgery [107], [108]. This system has proven able to detect glioblastoma, meningioma, and brain metastases bulk tumor intraoperatively with greater than 90% accuracy based on data from 67 cases in multiple clinics [243]. Whilst this was achieved rapidly and, in a reagent-free way, this “single-point” device gains molecular information from a relatively small portion of tissue (0.2cm^2) with no imaging capability. To gain biomolecular information from larger tumor areas and to create a molecular map of tumor margins, a wide-field system was developed. This allows illumination and molecular analysis of an area of 1cm^2 [209], [244].

Here, we present the development of a system for detection of residual cancer cells on the surface of surgical specimens. At the core of the device is a whole-specimen Raman spectroscopy unit for real-time reagent-free molecular analysis. This relies on a cancer detection machine learning model which was trained *in vivo* on the pre-existing single-point system data from 24 patients undergoing glioblastoma surgery [243]. Equipped with this cancer detection model, the new, large field-of-view Raman imaging device was tested on brain specimens from 8 glioblastoma patients, including tumor and non-tumor tissue. The technique was compared with gold standards and can in principle be generalized to any cancer type [241], [245], [246], [247].

6.4 Results

6.4.1 Hyperspectral Raman imaging instrument

A Raman spectroscopy imaging system was developed to detect residual cancer cells in the surgical margin. The device was tested on surgically excised tumor specimens, including specimens from within the bulk tumor and those from the edge of the tumor (peripheral samples). The instrument included a portable imaging probe with the largest field-of-view (FOV) ever achieved for a biomedical Raman imaging system: a total FOV of 1cm^2 [209], [244]. The cart-mounted system was portable and allowed its deployment directly into the operating room for testing in fresh brain

specimens from glioblastoma patients. Tumor was resected during neurosurgery and resected specimens were immediately analyzed by Raman imaging. Spectral images and co-located brightfield images were acquired for each specimen, both with a square-shaped field-of-view of 1 cm x 1 cm. The Raman images contained 1,440 pixels (40 x 36 pixels each of size 200 μm x 250 μm). Each pixel was associated with one individual Raman spectrum of resolution 6 cm^{-1} with a spectral domain ranging from 400 to 1800 cm^{-1} .

Each spectrum contained biomolecular tissue information from more than 20 distinguishable peaks of known biomolecular origin (Figure 6-1). All peaks were associated with different molecular bonds. Intensity of Raman scattering is directly proportional to the concentration of molecules within a sample [248]. Therefore, the intensity of one biomolecular peak relative to all other peaks in a single spectrum was used as a quantitative readout of the concentration of different families of biomolecules. The molecular origin of the peaks could be traced back to protein backbones, aromatic amino acids (phenylalanine, tryptophane, tyrosine), different classes of lipids, and the structural units of nucleic acids [188], [249].

6.4.2 Cancer detection machine learning model

A glioblastoma detection machine learning model (herein referred to as the “cancer detection model”) was created using data from a well-validated “single-point system”. The single-point system illuminates a spot of tissue 0.2 cm^2 in area and obtains a single Raman spectrum from the region under interrogation. There is no two-dimensional imaging capability in this system. Spectroscopic data was acquired independently using this system in the scope of a multi-center clinical study [243] (Methods). The cancer detection model was trained using support vector machines (SVM). It was trained/validated and tested using 352 spectra from 24 patients (glioblastoma: $n = 248$, normal brain: $n = 104$). All spectra were acquired *in vivo* during tumor resection and prior to tissue removal. The reported glioblastoma detection sensitivity, specificity and accuracy were all 91%.

The predictive model was integrated into the data processing and operating software of the new, large field-of-view Raman imaging instrument, hereafter named the “Raman imaging system”. Each time a Raman image was acquired from an excised brain specimen using the Raman

imaging system, the cancer detection model was directly applied to each individual spectrum within that image. Therefore, each image pixel had an output consisting of a predictive metric between 0 and 1. Values closer to 0 were more likely taken from tissue associated with *Normal brain*, while values closer to 1 had spectral features consistent with *Cancer* tissue. Each image was hence a map of the *Cancer Likelihood* metric, showing the spatial distribution within specimens in terms of *Normal brain* or *Cancer* likelihood (6.6 Methods).

Only three Raman peaks were used within the cancer detection model: 1004 cm^{-1} , 1340 cm^{-1} , and $1430\text{-}1460\text{ cm}^{-1}$ (Figure 6-1). Other studies have demonstrated the phenotypic relevance of these spectral regions and explained their biological origin. The Raman peak at 1004 cm^{-1} is related to the concentration of phenylalanine [188], [250], [251] and the inelastic scattering signal in the spectral region around 1340 cm^{-1} is associated with collagen and tryptophan [188], [252]. The peak covering the $1430\text{-}1460\text{ cm}^{-1}$ spectral domain contains information on the relative concentration of lipids to proteins and is related to the deformations of the CH_2 and CH_3 molecular bonds [188], [251].

6.4.3 Patient specimens and histology

Between one and three tumor specimens ($1\text{-}5\text{ cm}^3$) were analyzed from five glioblastoma patients scheduled from tumor resection surgery at the Montreal Neurological Institute and Hospital. These were prepared as in the Methods and imaged using the Raman imaging system. In total, nine specimens were analyzed, each labeled with a code consisting of a patient identification number (P5 to P9) and a set of letters (SPA, SPB or SPC) identifying each individual specimen acquired from the same patient (Table 6-1). A brightfield image of each specimen was acquired (Figure 6-2: first column) and a digitized H&E histology map generated for each specimen (Figure 6-2: second column), which was co-located with the Raman images by a trained neuropathologist (Methods).

The *Cancer Likelihood* images obtained from the Raman imaging system are shown in Figure 6-2: third column. A *Cellular Density Map* was produced from each H&E image that quantified the number of cells for each imaged pixel (Figure 6-2, fourth column; Methods). Cellularity colormaps were produced by counting the number of nuclei per unit area in the stained histology section.

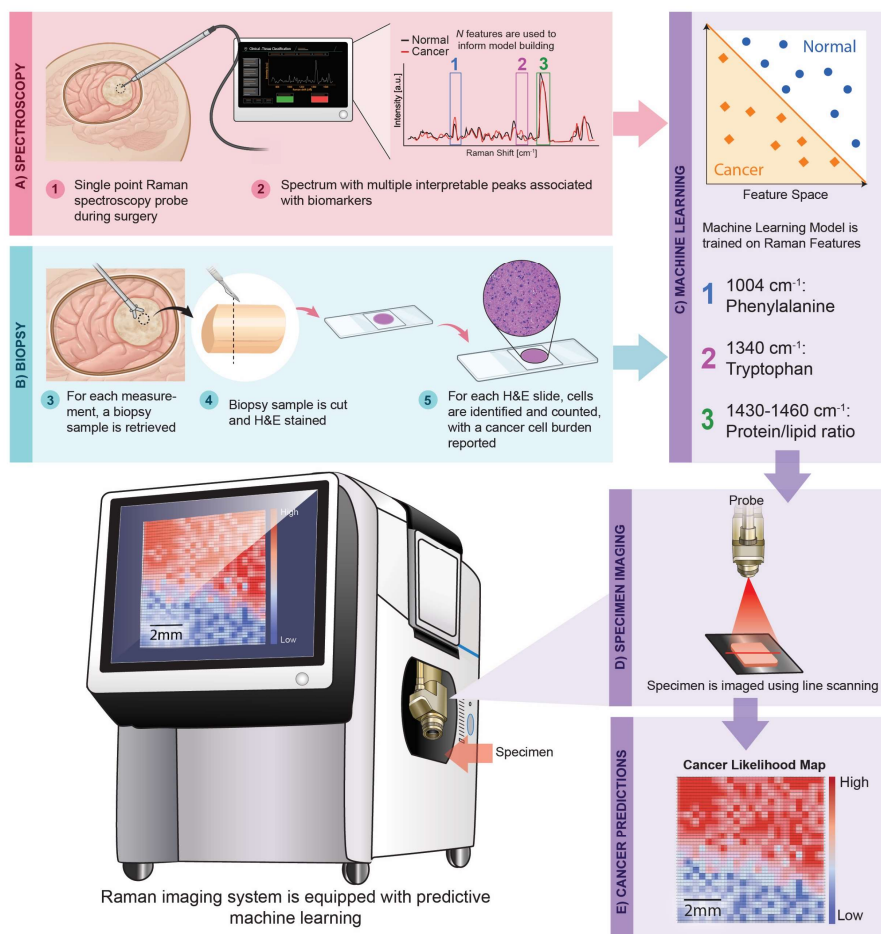


Figure 6-1: Graphical depiction of the workflow leading to cancer prediction maps of specimens resected from glioblastoma patients that were imaged using the Raman spectroscopy imaging system. A) The experimental workflow showcases how glioblastoma and normal brain Raman spectra are collected. Spectral fingerprint measurements are acquired using a single point probe during neurosurgery. B) Biopsy workflow showing how histopathological data is linked with each spectral measurement. A biopsy is recovered immediately following the Raman measurement, the specimen is prepared and then annotated by a neuropathologist. C) Shows the combination of Raman single point spectra, with prevalent Raman peaks, and the corresponding histological labels which allow the creation of machine learning classifiers based on only a select number of differentiating Raman features. In this

context, the phenylalanine band (1004 cm^{-1}), the tryptophan band (1340 cm^{-1}) and the protein/lipid band ($1430\text{-}1460\text{ cm}^{-1}$) are used to create a cancer (i.e. glioblastoma) prediction model. In D), the imaging system uses line scanning to image a freshly resected brain specimen. Finally, E) the cancer prediction model is applied to each pixel on the Raman image to recover a cancer likelihood map which depicts areas of dense tumor (red), areas of normal tissue (blue) and diffuse areas in between (white) associated with tumor infiltration.

Each specimen was classified by the neuropathologist as belonging to one of four histological categories. Specimens P7 SPA-SPC were labeled *All Tumor* because they were composed of only cancer tissue. Specimens P5 SPA-B and P6 SPA were labeled *All Tumor with Extensive Necrosis* because necrosis was observed over approximately 20 % of the surface area. The remaining three specimens were either associated with a low density of cancer cells spread throughout the tissue (*Low Cellularity Tumor*: P9 SPA-B) or consisted of normal brain mixed with tissue having a low density of cancer cells (*Normal with Infiltration*: P8 SPB).

Table 6-1. Information from retained *ex vivo* specimens, histological diagnosis, number of pixels retained in the Raman image and machine learning (ML) predictions. PX refers to a patient where X is the patient's identifying number and SPY refers to a specimen where Y is the sample's identifying letter.

Specimen ID	Sex	Age	Histology label	Cancer likelihood metric (avg $\pm$ std)	Cellular density [cells/mm ²] (avg $\pm$ std)
P5 SPA	F	83	All tumor with extensive necrosis	0.88 ± 0.19	600 ± 600
P5 SPB	F	83	All tumor with extensive necrosis	0.96 ± 0.10	1000 ± 600
P6 SPA	M	83	All tumor with extensive necrosis	0.98 ± 0.07	2500 ± 1200
P7 SPA	F	67	All tumor	0.98 ± 0.08	3000 ± 800
P7 SPB	F	67	All tumor	0.99 ± 0.04	3400 ± 700
P7 SPC	F	67	All tumor	0.98 ± 0.09	2400 ± 800
P8 SPB	M	61	Normal with infiltration	0.30 ± 0.19	900 ± 300
P9 SPA	F	59	Low cellularity tumor	0.52 ± 0.30	1500 ± 300
P9 SPB	F	59	Low cellularity tumor	0.56 ± 0.28	1400 ± 300

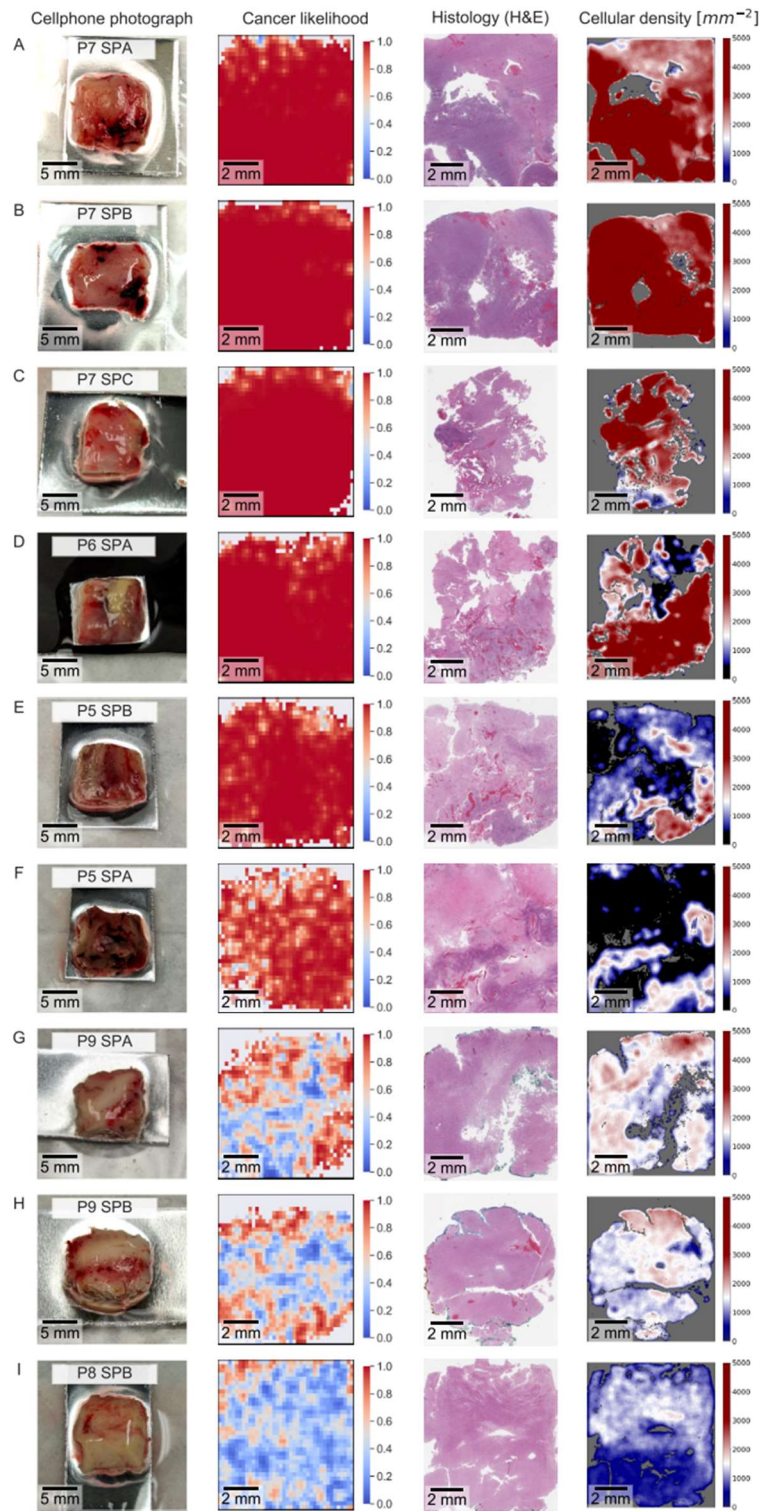


Figure 6-2: Glioblastoma specimen shown in decreasing order of quantity of predicted cancer pixels (cancer likelihood >0.8) with corresponding photograph, machine

learning predictions from single point Raman spectroscopy (SPRS), the haematoxylin and eosin (H&E) stained digital scan and cellular density map. Cancer likelihood predictions gave a score from 0.0 (blue) to 1.0 (red) where 1.0 or red corresponds to a high probability of cancer cells and 0.0 corresponds to a low probability of cancer (normal tissue). Cellular density maps were produced by counting the number of nuclei per mm^2 with black areas labelled as necrosis.

6.4.4 Biomolecular information content and inter-system spectral discrepancies

The dataset consisted of 10,600 spectra acquired from 9 specimens and the cancer detection model was applied to each spectrum. All spectra that had a *Cancer Likelihood* value higher than 0.8 were assigned to the *Cancer* category, whilst all other spectra were assigned to the *Normal brain* category. Overall, the classifier predicted that 7,730 pixels (72.9% of all image data points) were associated with glioblastoma, and 2,870 pixels (27.1% of all image data points) were associated with *Normal brain*.

A qualitative comparison of the spectra acquired with the Raman imaging and single-point systems demonstrated that the detected biomolecular features were consistent between the two systems. The mean *Cancer* spectrum from the imaging system strongly overlapped with the mean glioblastoma spectrum from the single-point system dataset (Figure 6-3A). The mean *Normal brain* imaging spectrum strongly overlapped with the mean normal brain spectrum taken using the single-point system (Figure 6-3B). For both systems, the prominent Raman peaks observed for the glioblastoma and *Normal brain* categories were at 1004, 1064, 1090, 1128, 1300, 1340, 1430-1460, 1620 and 1660 cm^{-1} (Figure 6-3: red, green, and blue highlights). The Raman peak around 1064 cm^{-1} is associated with the carbon skeletal stretching of lipids [94] while the inelastic scattering signal at 1128 cm^{-1} is associated with the CH_2 wagging mode found in lipids [94]. The peak at 1004 cm^{-1} was linked with the phenylalanine tissue content and the Raman band around 1300 cm^{-1} was linked to CH_2 twist and wag as well as with the Amide III band of proteins [94]. Similarly, the band at 1660 cm^{-1} was associated with the Amide I band of proteins, more specifically sourced from the carbon-oxygen double bond in peptide bonds [94]. The band around 1340 cm^{-1} was linked with tryptophan and CH_2/CH_3 deformations in lipids and protein. The band around 1620 cm^{-1} was

associated with contributions from the aromatic amino acids: tyrosine, tryptophane and phenylalanine [253], [254], [255]. The band located in the 1430-1460 cm^{-1} range included several vibrational modes involving carbon and hydrogen linked to protein and lipid content [255], [256].

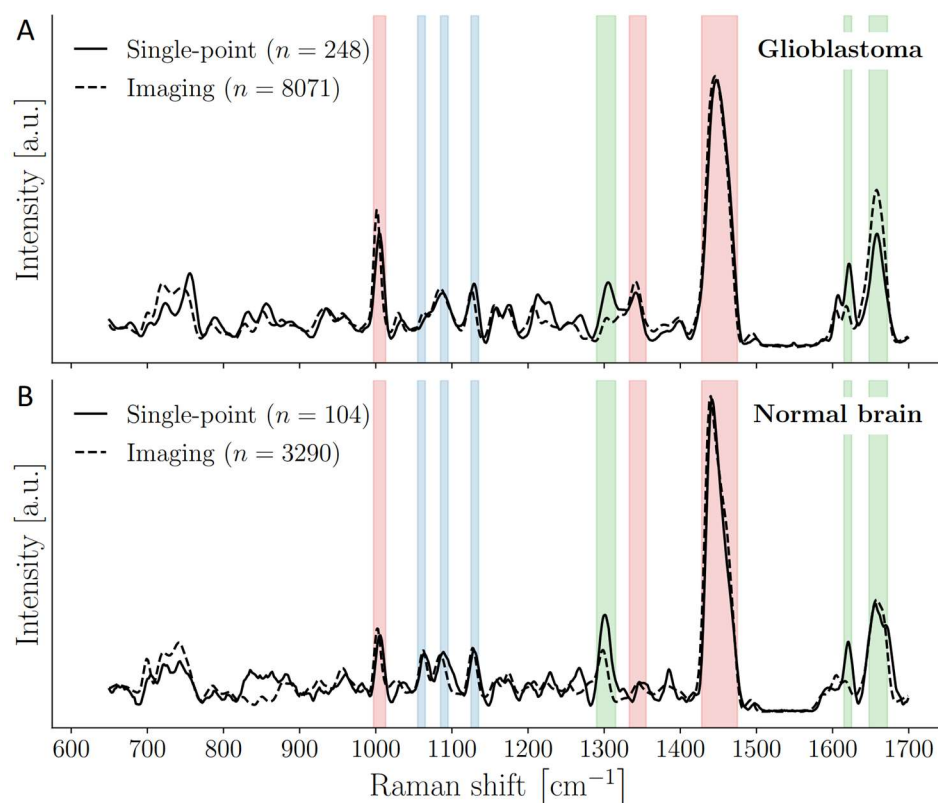


Figure 6-3: Overlay of the average fingerprint spectra from the single-point (solid) and imaging (dashed) dataset. Blue bands correspond to regions showing similar signatures between the two systems, red regions represent bands that were used during the classification, and green regions represent bands showing differences between single-point and imaging systems.

Whilst the spectra detected with both systems showed the same Raman bands, differences in peak intensities were observed between the two instrument configurations. These occurred within the protein bands around 1300, 1620 and 1660 cm^{-1} (Figure 6-3: red highlights). The single-point measurements were taken *in vivo*, whilst the imaging measurements were taken *ex vivo*. Therefore, the differences could be attributed to tissue deterioration (e.g., denaturation of the proteins) from environmental changes. These differences may also be linked to the automated

application of the fluorescence background removal algorithm (Methods), which may have caused overfitting under some peaks. Importantly, none of the spectral features utilized by the cancer detection machine learning model were associated with the bands that had been identified as due to intrinsic differences between the Raman systems (Figure 6-3: green highlights).

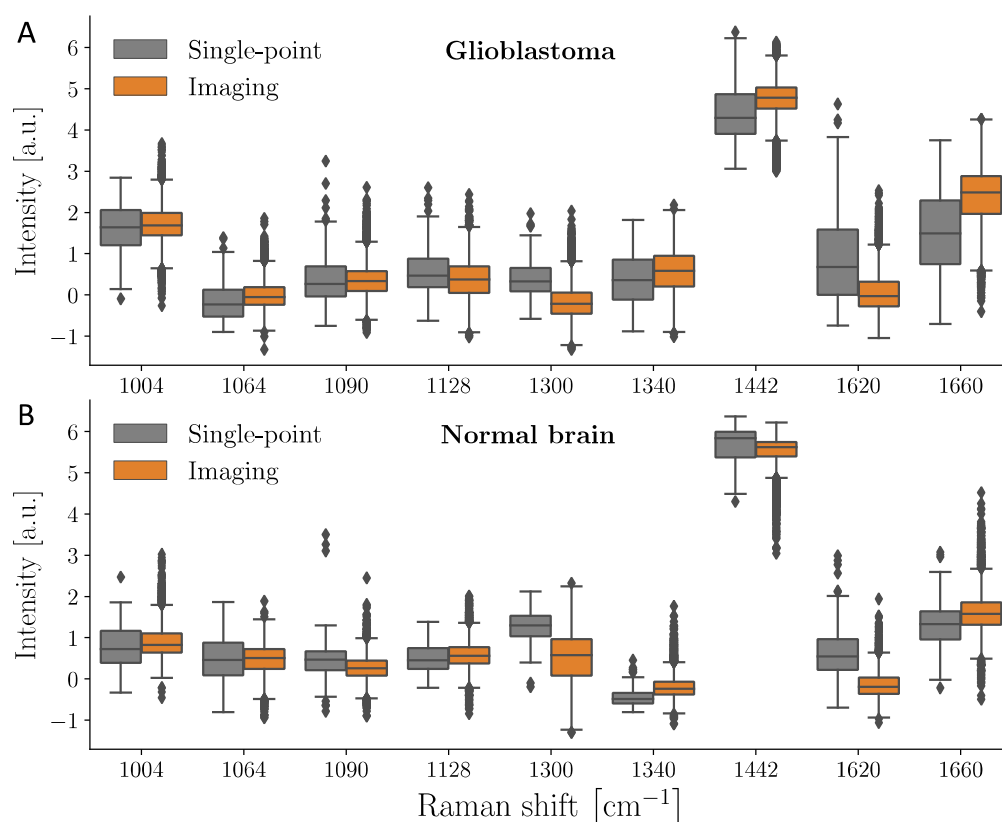


Figure 6-4: Differences between the intensity of inelastic scattering bands of spectra acquired with the single-point probe system and the Raman imaging system. Boxplots associated with the Raman band intensity of all spectra at 1004, 1064, 1090, 1128, 1300, 1340, 1442, 1620 and 1660 cm^{-1} .

6.4.5 Consistency between machine learning predictions and histology

The Raman imaging system was able to correctly detect the different types of tumorous and non-tumorous tissue that were sampled by the neurologist. The predictive power of the cancer detection machine learning model was evaluated using the co-located histology and cellular density maps as gold standard (Figure 6-2). Specimens P7 SPA, P7 SPB and P7 SPC were classified as pure tumor

when using the gold standard techniques. The cellular density maps exhibited hypercellularity i.e. cell density higher than in normal tissue, and often linked with tumor tissue. Histopathology revealed multiple other features consistent with glioblastoma, including microvascular proliferation and pseudopalisading necrosis. This latter feature is a hallmark of glioblastoma associated with cells migrating from the necrotic tumor core. The Raman imaging system succeeded in predicting that these specimens contained cancer tissue across the whole field-of-view. The average *Cancer Likelihood* was high at 0.98 with a small standard deviation (0.07) that was consistent with a mostly homogenous specimen.

Specimens P5 SPA, P5 SPB and P6 SPA were also all pure tumor, however these samples were rich in necrosis (cell death). This is often seen in the core of the tumor. All samples exhibited hypercellularity within some regions of the specimens, whilst other regions had additional histological features typical of glioblastoma, including microvascular proliferation and extensive areas of necrosis. Necrotic areas were associated with a low cell density, confirmed by the absence of cellular architecture and nuclei on the histology maps. These histological features indicate that the specimens were taken from the necrotic core of the tumor. Despite the difference in morphology and cell density compared to the P7 tumor features, the Raman imaging system correctly predicted that most of the area of these specimens were cancer tissue. However, the specimens had a slightly lower average *Cancer Likelihood* metric and were more heterogenous when compared to P7 samples. The average *Cancer Likelihood* for these samples was 0.94 with a standard deviation of 0.13. This slightly lower *Cancer Likelihood* was likely because the surface area of the specimens was associated with a larger fraction of necrotic tissue. The initial cancer detection model from the study with the single point system did not contain spectra from necrotic tissue.

The histological features associated with specimens P9 SPA and P9 SPB were different compared with specimens from P5, P6 and P7. These specimens were from the periphery of the tumor. They exhibited hypercellularity in some areas but only limited microvascular proliferation with no areas associated with necrosis nor pseudopalisading necrosis. In addition, a reduction in cellularity compared to other tumor specimens was observed in both P9 SPA and P9 SPB that was associated with an organized tissue architecture characteristic of normal brain. These features are consistent with specimens resected at the periphery of the tumor, i.e. towards the outer edge the MRI-enhancing ring of the glioblastoma. The Raman images are consistent with what would be

expected from samples with a mixture of normal and cancer cells. The average *Cancer Likelihood* for these two specimens was 0.54 with a standard deviation of 0.29, consistent with a highly heterogeneous specimen. The Raman images showed areas of predicted cancer mixed with normal brain areas, as seen using the gold standard techniques.

The last specimen considered –P8 SPB– mostly consisted of normal brain having histology features consistent with an organized cellular architecture. It also had a cellularity of 2000 cells/mm² that was low compared to all other specimens. The specimen only had a few signs of tissue infiltration with cancer cells, low mitotic activity, and a low level of nuclear atypia. It had none of the distinguishing features found in glioblastoma such as microvascular proliferation and necrosis. The average *Cancer Likelihood* was very low at 0.30 with a standard deviation consistent with moderate tissue heterogeneity 0.19.

6.4.6 Biomolecular alterations between different anatomical regions

The Raman imaging system used different biomolecular features to identify different tumor and non-tumor pathologies. An analysis was performed to determine precisely which biomolecular features were characteristic of each type of pathology. Firstly, specimens were divided into four categories based on their suspected anatomical origin and the histology analyses. The 3,464 spectra from the samples P5 SPA, P5 SPB, P6 SPA were labeled *Tumor Core*. Specimens P7 SPA, P7 SPB and P7 SPC were associated with the MRI-enhancing ring of the tumor with the 4,079 spectra labeled *Tumor Interphase*. Spectra from P8 SPB, P9 SPA and P9 SPB that had a *Cancer Likelihood* metric larger than 0.8 ($n = 528$) were labeled *Tumor periphery – Cancer* while all other spectra were labeled *Tumor periphery – Normal brain* ($n = 3,290$). Of note, those spectra were not all histologically confirmed to be normal brain, but they were all predicted to belong to the normal brain category based on the cancer detection machine learning model. Despite this limitation, these spectra will be used as baseline to assess biomolecular changes between specimens having different histology features.

The average spectrum for each tissue category was computed along with the inter-measurement standard deviation within each class (Figure 6-5A). The difference between the spectra for *Tumor core*, *Tumor interphase* and *Tumor periphery – Cancer* compared to the baseline

normal brain spectrum (*Tumor periphery – Normal brain*) was also computed (Figure 6-5B). These *difference spectra* allowed identification of the main Raman-predicted biomolecular changes between different types of glioblastoma tissue and tissue that had a more organized cellular architecture.

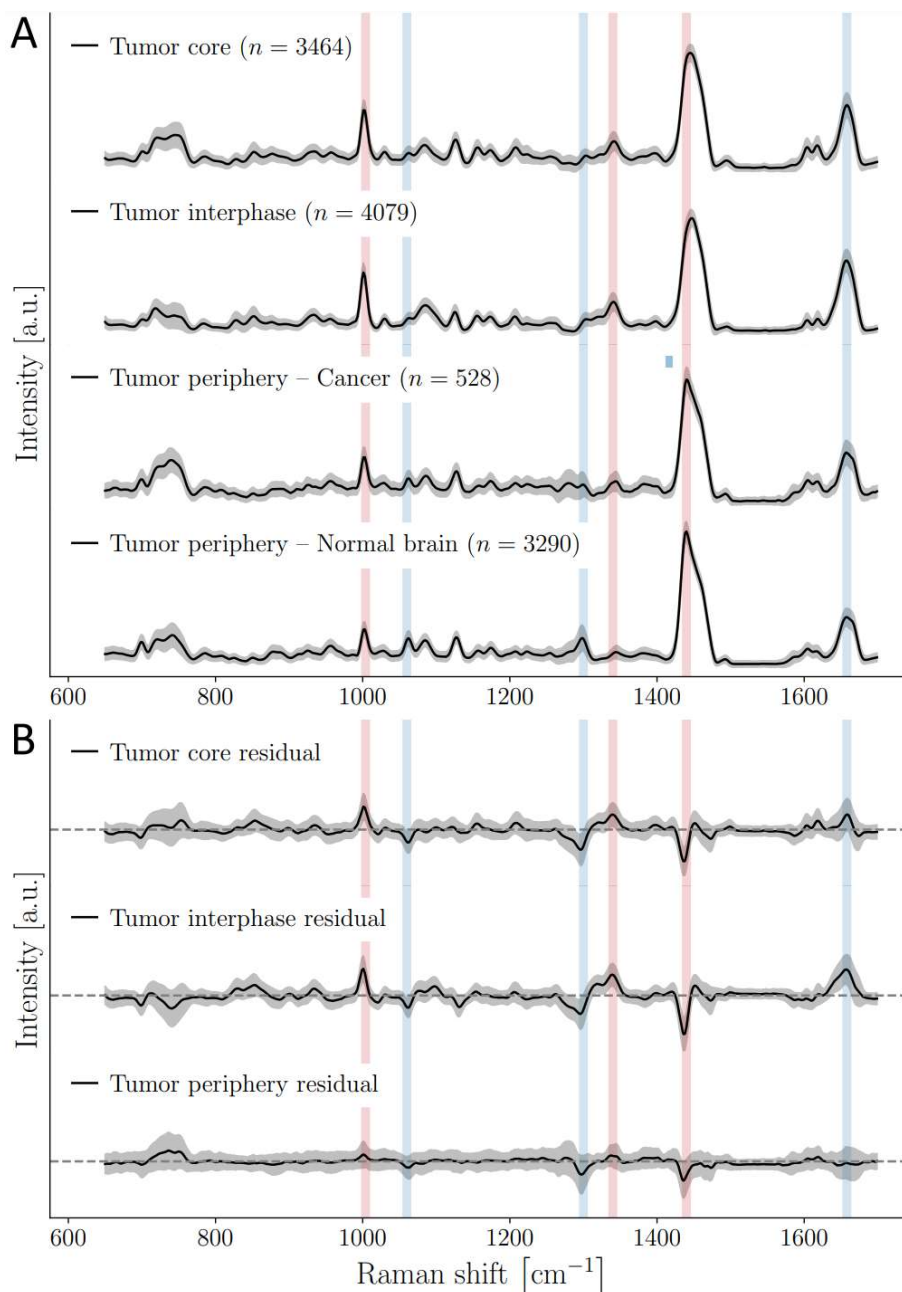


Figure 6-5: A) Average Raman spectra (solid line) and standard deviation (shading) of specimens recovered from the tumor core, the tumor interphase and the tumor

periphery shown beside the average Raman spectra and standard deviation of all normal-predicted spectra using the single point Raman machine learning model. B) Average difference and standard deviation between the Raman signature of specimens from the tumor core, tumor interphase, tumor periphery and the normal-predicted spectra. The dashed grey line corresponds to 0. The spectral features used in the cancer prediction model are highlighted in red and other spectral features which differ significantly (i.e. at least one standard deviation) between tumor and normal spectra are highlighted in blue.

There were no significant differences observed within the Raman bands used by the cancer detection machine learning model (1004 cm^{-1} , 1340 cm^{-1} , 1442 cm^{-1}) between specimens from the *Tumor core* – which had extensive areas of necrosis – and those from the *Tumor interphase*. This meant the model was not able to distinguish cancer from necrotic tissue. However, there were spectral regions that qualitatively showed spectral differences between *Tumor core* and *Tumor interphase*. Those regions included, for example, the low wavenumber range below 800 cm^{-1} and molecular bonds associated with phospholipids and nucleic acids in the peaks around 1100 cm^{-1} .

There were however significant differences between *Tumor core/Tumor interphase*, *Tumor periphery – Cancer* and *Tumor periphery – Normal brain* for the Raman bands used by the cancer detection model. All observed changes were consistent with a gradual decrease of the ratio of lipids to proteins, as well as the proteins and amino acid concentration, when moving away from the *Tumor core/Tumor interphase* region towards areas showing fewer distinguishing features of cancer. This is manifested by an increase – in cancer tissue – of the intensity of the Raman peaks associated with phenylalanine (1004 cm^{-1}) and tryptophan (1340 cm^{-1}), combined with a decrease of lipid to protein ratio computed from the $1430\text{--}1460\text{ cm}^{-1}$ band. Other protein bands showed a marked decrease between cancer and normal brain, including at 1300 cm^{-1} and 1660 cm^{-1} .

6.5 Discussion

Predicting the presence of cancer cells from large field-of-view Raman imaging was achieved, allowing sub-millimeter resolution maps of glioblastoma specimens to be produced. The agreement between histology and the Raman-predicted *Cancer Likelihood* maps suggested the method could

effectively assess margins in a real-world intraoperative context. Furthermore, the device detected cancer tissue from three molecular bands. Relative to normal brain, glioblastoma exhibited a lower lipid to protein ratio based on spectral features extracted from the spectral range 1430-1460 cm^{-1} . Glioblastoma also showed an overexpression of phenylalanine (1004 cm^{-1}) as well as collagen and tryptophan (1340 cm^{-1}). These trends were consistent across specimens, including pure tumor associated a high density of cancer cells, specimens presenting with a mixture of high-density cancer cell clusters and necrosis, as well as non-necrotic tissue associated with a low density of cancer cells.

The cancer detection machine learning model used to produce the *Cancer Likelihood* maps was trained using a different instrument, in the scope of an independent multicentric study. This model was not able to distinguish areas associated with a high density of cancer cells from regions with necrotic areas. However, this did not imply there were no spectral differences between those different tissue types. Rather, this meant the subset of spectral features used to train the cancer detection model were present in tumor tissue regardless of necrosis presence. In fact, spectral changes were visually detected between tumor specimens that included necrotic tissue and those that did not. Necrotic areas showed elevated inelastic scattering signal within the 700-750 cm^{-1} band associated with phospholipids, and a decrease of the peaks around 1150 cm^{-1} that was predominantly associated with phospholipids and nucleic acids. This suggested that – given a sufficiently large dataset was assembled in the scope of a new clinical study – it could be possible to train a new machine learning model to detect necrotic tissue.

This observation could have profound implications for the future of technology. Other clinical studies could be designed aimed at developing more complex machine learning models detecting more subtle phenotypes. For example, detecting the invasive gradient of glioblastoma is important in glioma surgery [257]. A model could be developed aiming at detecting subtle tissue changes associated with cellular proliferation beyond the MRI-enhancing ring of a glioblastoma. This was not attempted as part of the current study because detecting invasive glioblastoma cells with high accuracy is difficult with conventional histology techniques. There is, in fact, no gold standard for this because there are no definitive histological or protein markers of glioblastoma cells. Using standard technologies, it is thus not possible to count invasive glioblastoma cells with

clinical grade accuracy, unlike astrocytoma and oligodendrogliomas in which single cells can be detected with an antibody to the IDHR132H mutation [131].

Unlocking new applications will require improvements in system design leading to enhanced signal-to-noise ratio, i.e. *super pixels* associated with less stochastic noise. Here the system was only able to acquire high signal-to-noise ratio for a subset of Raman bands, principally the three bands used to train the cancer detection model. This was mainly due to the 30-minute limit to imaging time for integration into the workflow. There were also light losses due to a 2 m coherent imaging bundle used in the detection path of the system [209], [244]. The imaging bundle was imposed by prior design constraints requiring the system to acquire signal at-a-distance through a hand-held imaging probe *in situ* within the surgical cavity. This aspect of the design could be relaxed for *ex vivo* margins assessment application, improving light collection efficiency by at least a factor 10X. Other aspects reducing imaging times – that would not require any modifications of the hardware – could include the reduction of the spatial resolution along the scanning axis of the system by a factor 2, with limited impact in the ability of the system to detect the whole surface area of the specimen. A re-design of the system would thus allow the imaging time achieved in the current work to be reduced to 90 seconds. Further, keeping the same imaging time as in this current work – 30 minutes on average – would lead to a signal-to-noise ratio increase of 4.5X, improving overall signal quality to increase the number of spectral features available to develop new more complex machine learning models.

One of the most enticing aspects of the current work is the application of the system to other types of cancer surgery. For example, a workflow could be developed in breast surgical oncology allowing lumpectomy specimens to be imaged during a surgical excision procedure. If residual cancer was detected on the specimen, the surgeon could then resect extra tissue. This could improve outcomes by reducing disease recurrence whilst minimizing damage to healthy tissue. The same approach could apply in radical prostatectomy procedures either performed laparoscopically or using a robotic platform [206]. Pilot clinical studies have already been performed using a hand-held single-point Raman spectroscopy probe. These demonstrate cancer detection in multiple organs including breast [258], prostate [259], [260], [261], lung [262], the ovaries, and endometrium [263].

6.6 Methods

6.6.1 Raman imaging system

Raman images of the specimens were acquired using the system published by Daoust et al. (2023) [244]. Briefly, the imaging system was cart-mounted and equipped with an imaging probe connected to a coherent fiber optics imaging bundle of length ~ 2 m. The system was operated in non-contact mode with a working distance of 40 mm between the front lens and the specimen surface. Images consisted of 40 lines projected from a 785 nm laser that was scanned across a square field-of-view of side dimension 1 cm. Each line had a 200 μm thickness along the scanning axis and 10 mm along the transverse direction. Images were formed by scanning the line with 0.25 mm steps. Spatial binning of the pixels on the charge-coupled device (CCD) sensor was implemented resulting in Raman images composed of *super pixels* that had a square shape with a side dimension of 0.25 mm. The resulting dimension of each Raman image was 40 pixels along the scanning axis, 42 pixels along the transverse axis and 1,024 light intensity values along the wavenumber (cm^{-1}) axis.

Spectra were produced through spectral separation of light using a high-resolution diffraction grating spectrometer coupled to a cooled CCD camera. The spectral range of each spectrum was 400 to 2100 cm^{-1} (810 to 940 nm) with a spectral resolution of approximately 4 cm^{-1} . Laser light power at the specimen was 905 mW when measured for an entire line, resulting in an average intensity of 22.6 W/cm^2 . The exposure time for each line ranged from 4 to 15 seconds. The later was optimized to ensure the full dynamical range of the CCD sensor was occupied to maximize the signal-to-noise ratio (SNR). The camera sensor was cooled to -60 $^{\circ}\text{C}$ prior to each measurement. Each measurement consisted of a signal averaged over $N = 4$ to 10 repetitions (i.e., accumulations) to further improve the SNR. The total imaging time per specimen was on average 30 minutes. The system also allowed brightfield images to be acquired that were collocated with the hyperspectral Raman images.

6.6.2 Patient selection, specimen preparation and histology analyses

Eight patients were recruited that had a diagnosis of glioblastoma and were scheduled for a tumor resection procedure at the Montreal Neurological Institute and Hospital (McGill University Health Center). All participants signed a written Information and Consent Form. Sufficient information was provided to allow each patient to make an informed decision about their participation in the study.

A procedure was developed that allowed specimens to be harvested and prepared before being imaged with the Raman spectroscopy imaging system (Figure 6-6). Specimens were recovered during surgery that were destined for biological waste and that had a volume ranging between 1 and 4 cm³. The sample was immediately soaked in phosphate buffered solution (312-651, Thermo Scientific, USA) and subsequently placed in a sterile petri dish. The specimens were then sliced using a sterile razor blade to form two or three smaller flat samples of approximate size 10 mm x 10 mm x 3 mm. Each specimen was then placed on an aluminum surface (Miro5011, Anomet, Canada) because this material has limited inelastic scattering signal in the 400 to 2100 cm⁻¹ wavenumber range. A phosphate buffer solution was regularly sprayed onto the specimen during the imaging session to keep it moistened and avoid dehydration.

Raman imaging was performed as described above in a vacant area next to the patient during surgery. After the imaging session was completed, a procedure was applied to each specimen to ensure its spatial orientation was registered. This was necessary to ensure Raman and white light images could be spatially collocated with histopathology images. Each specimen was first immersed in paraformaldehyde 10 % for 18 to 24 hours. The borders of each square-shaped specimen were then inked following three steps: 1) the fixing agent acetic acid 5 % was applied all borders, 2) four different colors of ink were applied on each side of the specimen (top: blue, right: red, bottom: green, left: yellow or orange), 3) the ink was left to dry for five minutes. Each specimen was then placed in a microtissue pathology cassette and submerged for another 24 hours in fresh paraformaldehyde 10 % before undergoing paraffin-embedding. Slices were then obtained that covered the whole top surface of the resulting formalin-fixed paraffin-embedded (FFPE) specimen. Each slice with an area consistent with the field-of-view of the Raman imaging system was stained with hematoxylin and eosin (H&E) and digitally scanned. Recombining all images

resulted in a histopathology map co-located with Raman and white light images that was analyzed by a neuropathologist.

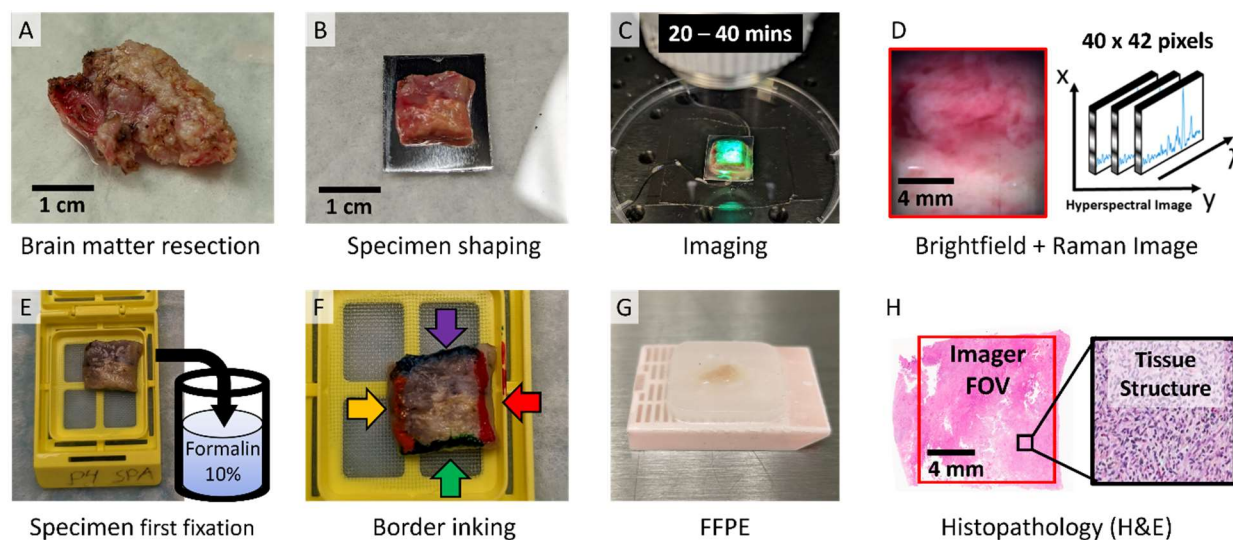


Figure 6-6: Raman imaging workflow for brain specimen imaging and producing assessment. (A) Brain matter is resected during surgery and (B) shaped into square specimens of 10 mm x 10 mm x 3 mm. (C) Specimens are imaged for 20 to 40 min and (D) a collocated brightfield image and a 40 x 42 hyperspectral Raman image are produced. (E) Specimens then undergo a first 18 to 24 h fixation in paraformaldehyde 10% before undergoing (F) border inking for orientation identification. Finally, specimens undergo regular standard tissue preparation (G) consisting of formaldehyde fixation, paraffin-embedding (FFPE). In (H), the paraffin block is sliced, the slices are hematoxylin and eosin stained and scanned digitally for pathological annotations. The digital scans are oriented with the Raman images using the ink visible in the digital scan.

Cellular density maps were calculated from the digital histology scans of the H&E-stained brain specimens slices using the open-source software for bioimage analysis: QuPath 0.5.0 [22]. QuPath's *Cell Detection* function was first used to locate all cells within the digital scan using the default values set by the software. Then, the *Create Density Maps* function was used to produce a cellular density image, i.e. a lower resolution image where each pixel value in the image corresponds to the density of cells surrounding the pixel. Each pixel value was determined by

counting the number of cells surrounding the pixel within a radius of 282 μm and dividing this cell count by the surface area considered (i.e. 0.25 mm^2). All cellular density values of pixels located in blank areas of the histology scan (i.e. empty areas with no visible cells or tissue) were set to 0. Python 3.11.3 with Scikit-image 0.20.0 were finally used to generate the cellular density maps with colors showcasing areas of necrosis in black, higher cellularity (>1500 cells/ mm^2) in red and lower cellularity (<1500 cells/ mm^2) in blue.

6.6.3 Spectral pre-processing

Data analysis and spectral pre-processing were done using Python 3.9.5 with Scikit-Learn 1.2.2 [116] in combination with code from a public repository [264]. The following data pre-processing steps were applied to all spectra associated with the hyperspectral images: 1) filtering using a two-dimensional median filter to remove occasional cosmic ray events, 2) wavenumber correction using a reference acetaminophen measurement, 3) averaging over N repeat measurements, 4) instrument response correction from a NIST 785 nm Raman standard measurement, 5) removing the system intrinsic background signal using measurements with no sample within the imaging focal plane, 6) baseline subtraction using the BubbleFill algorithm [264] with a minimum ‘bubble’ diameter of 40 cm^{-1} , 7) truncation of spectra to the range 650–1700 cm^{-1} and interpolation in 1 cm^{-1} steps, 8) standard normal variate (SNV) normalization. The data from step 2, step 4 and step 5 were associated with images covering the same field-of-view as the specimen images. This allowed calibration to be achieved on a pixel-by-pixel basis. Further, spectral smoothing was done by averaging each pixel with its closest neighbours: 2 pixels for the corners, 3 pixels for the borders, and 4 pixels otherwise.

Finally, a metric was computed for each spectrum that allowed to evaluate their spectral quality [264]. The quality factor (QF) was used to automatically dismiss poor quality spectra that were plagued with high levels of stochastic noise. Only spectra from images with $\text{QF} \geq 0.4$ were retained in this study. The QF was defined as the average signed squared intensity:

$$\text{QF} = \frac{1}{L} \sum_i^L \text{sgn}(r_i) \cdot r_i^2 \quad (6-1)$$

where L is the number of spectral bins composing each SNV-normalized spectrum and r_i is the i^{th} spectral element.

6.6.4 Cancer detection machine learning model

The spectral pre-processing procedure led to images that were each composed of more than 1,150 Raman spectra. A cancer detection machine learning model was produced using data acquired independently with a different system. That model was applied to each spectrum associated with hyperspectral data acquired using the Raman imaging system. Briefly, inelastic scattering spectra of glioblastoma and normal brain were recovered from a previous study that was conducted using a single-point Raman spectroscopy probe instrument [243]. *In situ* spectroscopic measurements were acquired *in vivo* with a sterilizable handheld point probe system. One biopsy specimen was extracted along with each spectroscopic measurement ensuring spatial collocation. The probe interrogated a surface area of approximately 0.25 mm^2 and the size of the biopsy specimen was approximately 1-2 mm. The specimens were formalin-fixed paraffin-embedded. A section of the resulting FFPE tissue was sliced, stained (H&E) and digitized for histology analyses. Acquisition parameters with the probe system were: 1) excitation wavelength: 785 nm, 2) exposure time: 100 ms, 3) number of repeat measurements: 4 - 15, 4) laser intensity on the brain: 75 mW, 5) spectral range: $400 - 2100 \text{ cm}^{-1}$ (810 - 940 nm). Each spectrum was labelled either as glioblastoma or *Normal brain* by a neuropathologist. The dataset consisted of 104 normal brain spectra and 248 glioblastoma spectra from a total of 24 patients.

A cancer detection classifier was produced from a dimensionally reduced dataset that was limited to the spectral fingerprint region previously shown to have the most contrast between glioblastoma and the *Normal brain* category [265]: 1) the phenylalanine band at 1004 cm^{-1} , 2) the tryptophan band region located at 1340 cm^{-1} , 3) the lipid-protein band centered around $1430\text{-}1460 \text{ cm}^{-1}$. Specifically, all spectral features were associated with the spectral bins in the regions $997\text{--}1013 \text{ cm}^{-1}$, $1333\text{--}1355 \text{ cm}^{-1}$ and $1428\text{--}1475 \text{ cm}^{-1}$, resulting in a total of 85 features. The feature space was further reduced to less than 20 individual features using a support vector machine (SVM) with a linear kernel and L1 regularization (L1-SVM) [266]. This technique is a Lasso regression that limited the number of features to the ones presenting the most significant contribution to inter-class separation.

The cancer detection model was then trained from the subset of features using a linear SVM algorithm. Hyperparameters –both for the feature selection procedure described above and for the classification algorithms– were optimized using a grid search approach. The L1-SVM regularization parameter C was varied between 0.005 and 0.05, the number of features between 5 and 20, and the classification regularization parameter C between 0.01 and 0.5. A class weight parameter corresponding to the normal/cancer class ratio was used to account for the unbalance between the two classes and avoid classification biases. The performance of the trained model was assessed through five-fold cross-validation where model predictions were compared to the histopathological predictions. A receiver operating characteristic (ROC) analysis was used to determine the set of hyperparameters yielding the best performance. The model trained using this set of hyperparameters corresponded to the cancer detection model applied to each *super pixel* with the hyperspectral Raman images.

6.6.5 Cancer likelihood maps of Raman images

After all spectral pre-processing steps were applied, the 85 features used to train the cancer detection model were computed for all super pixels contained within the Raman images. The model was then applied to the pixels associated with all specimen images. The output of the model was then used to generate the *Cancer Likelihood* maps. The output was a probability between 0 and 1 with values close to 0 associated with *Normal brain* and those closer to 1 to *Cancer*. A threshold optimizing sensitivity and specificity was obtained from a ROC analysis and used to classify the pixels in binary categories: spectra with a probability lower than this threshold (0.8) were classified as *Normal brain*, the other ones as *Cancer*.

The average of the *Cancer Likelihood* metric and the cellular density metric were computed over the surface of each specimen, along with their fluctuations measured by the standard deviation within each image (Table 6-1). All other information (white-light image, digitized H&E histology map, cellular density image) was displayed, ranking the specimens in decreasing order of the average cellular density computed from the cellular density maps (Figure 6-2: Glioblastoma specimen shown in decreasing order of quantity of predicted cancer pixels (cancer likelihood >0.8) with corresponding photograph, machine learning predictions from single point Raman spectroscopy (SPRS), the haematoxylin and eosin (H&E) stained digital scan and cellular density

map. Cancer likelihood predictions gave a score from 0.0 (blue) to 1.0 (red) where 1.0 or red corresponds to a high probability of cancer cells and 0.0 corresponds to a low probability of cancer (normal tissue). Cellular density maps were produced by counting the number of nuclei per mm² with black areas labelled as necrosis.). This allowed differences in spatial distribution of cancer cells across different specimens to be more easily visualized and compared.

6.7 Data availability

The main data supporting the findings of this study are available within the Article and its Supplementary Information. The raw data generated during the study is too large to be publicly shared, yet it is available for research purposes from the corresponding author upon reasonable request.

6.8 Code availability

Source code used in this work is available for non-commercial purposes from the corresponding author on request.

6.9 Contributions

François Daoust: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Visualization, Writing – Original Draft, Writing – Review and Editing. **Frédéric Dallaire:** Methodology, Formal analysis, Visualization, Writing – Original Draft, Writing – Review and Editing. **Hugo Tavera:** Conceptualization, Methodology, Data Curation, Software, Visualization, Writing – Original Draft. **Katherine Ember:** Conceptualization, Writing – Original Draft, Writing – Review and Editing. **Marie-Christine Guiot,** Methodology, Investigation, Formal analysis, Resources. **Kevin Petrecca:** Methodology, Investigation, Resources, Supervision, Project administration, Funding acquisition, Writing – Review and Editing. **Frédéric Leblond:** Methodology, Supervision, Project administration, Funding acquisition, Visualization, Writing – Original Draft, Writing – Review & Editing.

6.10 Ethics declaration

Conflicts of interest: Kevin Petrecca and Frédéric Leblond are co-founders of Reveal Surgical, a medical device company that seeks to commercialize a Raman spectroscopy system for intraoperative cancer detection.

Human participants consent and ethics: Informed and written consent was obtained from all participants. The studies were approved by the Ethics Committees of the Montreal Neurological Institute and Hospital.

CHAPTER 7 GENERAL DISCUSSION

The aim of this thesis was to produce a clinical prototype Raman imaging system for the purpose of characterizing tissue during glioma resection surgery. To achieve this, a novel proof of concept Raman large FOV line scanning system was designed, fabricated, and tested in animal tissue. Afterwards, several upgrades were brought to the system to make it suitable for a clinical setting, ensuring safety for users and patients. Finally, a clinical study was accomplished in a hospital setting to test the novel system in *ex vivo* brain specimens from human patients suffering from brain cancer. In this chapter, we discuss the objectives of the dissertation by examining the Raman system's proof of concept design and fabrication, its conversion into a clinical prototype and its introduction into a clinical setting. We then discuss the COVID-19 pandemic's impact on the project and the limitations of the work presented in this dissertation. We conclude the chapter with the future scopes of work regarding the Raman imaging system.

7.1 Raman line scanning system design and fabrication

Objective 1: Design and develop a large field of view ($\sim 1 \text{ cm}^2$) proof of concept Raman imaging system for machine-learning-based molecular tissue characterization. ✓

Sub-Objective 1.1: Design and fabricate a Raman line scan imaging system for molecular tissue characterization. ✓

A novel Raman imaging system was conceived with a hyperspectral line scanning modality coupled with bright field imaging. The novelty of the system included its ability to capture large Raman images over 1 cm^2 in tissues using a flexible and robust handheld imaging probe. This system and its capabilities were presented in depth in Chapters 4 through 7. From a bench top proof-of-concept Raman imaging system to the final line-scanning system developed for a pilot study, several iterations of the system were made to bring the system closer towards its final goal of characterizing tissues intraoperatively. In Table 7-1, the progression of technical specifications towards the target specifications for each iteration of the imaging system are presented, from a previously developed bench top imaging system proof of concept from St-Arnaud et al. (2018)

[150], to the finalized clinical prototype system from Chapters 5 and 6. These differences outline a constant progression towards an imaging system suitable for a clinical setting (i.e. practical and safe) with a robust machine-learning workflow for tissue border detection. By the end of the doctoral work, the initially defined specifications of the system were achieved.

Table 7-1 : Technical specification changes between each iteration of the Raman imaging system, from the proof of concept to the finalized clinical prototype.

Raman Band (cm⁻¹)	Bench Top System [150]	Chapter 4	Chapter 5-7	Targeted Specifications
Probe numerical aperture	0.26	0.159	0.22	0.22
FOV [cm ²]	0.14	0.95	1.0	≥1.0
NIR pixel size (x axis; y axis) [μm]	75 x 33	100 x 250	200 x 250	~250 x 250
NIR spectral resolution [cm ⁻¹]	6	6	8	≤8
Laser intensity at sample [W/cm ²]	3.4	6.1	22	25
Bright field resolution [μm]	N/A	50	50	50
Contact measurement available	No	Yes	Yes	Yes
Portable in clinical setting	No	No	Yes	Yes

Sub-Objective 1.2: Characterize the molecular content of animal tissue using the Raman line scan system. ✓

In Chapter 4, porcine adipose and muscle tissues were imaged from pork loins. 1680 Raman spectra per image were acquired and illustrated the system's ability of capturing hyperspectral Raman fingerprint spectra of tissue over an area of ~1 cm². 4,446 spectra were captured in adipose and muscle tissue. The Raman pre-processing workflow proved effective to extract only the Raman scattering from the tissue luminescence. Adipose and muscle tissue molecular band assignments were presented and were shown to be consistent with the literature. It was also shown that only two Raman bands 1004 cm⁻¹ (phenylalanine) and 1440 cm⁻¹ (lipids and protein) could be targeted by

machine learning to discriminate adipose from muscle tissue in porcine loins, which provides a much simpler explanation of the classifier's behaviour.

Sub-Objective 1.3: Evaluate the system's ability to locate tissue borders with machine-learning-based classifiers. ✓

Classification models (SVM and RF) were generated and validated from normalized Raman spectra of porcine adipose and muscle tissue. Classifier validation showed that predictions could be accomplished with an accuracy, sensitivity and specificity over 97%. Furthermore, predictions were accomplished using only two Raman bands, automatically selected with a feature selection algorithm, implying the imaging system and classification workflow could further benefit from the inclusion of more Raman features for more complex learning tasks. Additionally, a tissue border was imaged with predictions as well as Platt scaling probability assignment made, showing that the tissue border could be located by observing a decrease of prediction confidence at the tissue border of the captured image. The pixel-width associated with tissue borders could be used to characterize the degree of invasiveness of tissues which is expected to be quite significant in glioma surgery. This invasiveness would translate in a gradual transition of prediction confidence in the Raman images when looking at the glioma tumor periphery.

7.2 From proof of concept to clinical prototype Raman imaging system

Objective 2: Enhance the Raman imaging system proof of concept to create a safe and portable clinical prototype suitable for *in situ* intraoperative tissue characterization. ✓

Sub-Objective 2.1: Develop and validate the capability of the system to conduct *ex vivo* (i.e. outside of the patient) and *in vivo* (i.e. within the patient) measurements in humans within an operating room. ✓

In Chapter 5, system hardware upgrades were introduced to enable the imaging system to be deployed in a clinical setting. These upgrades include mounting the system on a mobile cart capable of being transported in a hospital. Another upgrade consisted in installing a passive support arm to maintain the probe in position. Having a support arm allows for easier manipulation of the imaging

probe and even a controlled movement of the probe towards tissue to be imaged. The system was brought to the MNI in the operating theater and flawlessly navigated its environment. An additional upgrade consisted of developing a sterilization workflow including developing two probe muzzles: for contact and non-contact measurements. The muzzles were developed to ensure they could be sterilized according to standard clinical operating procedures. They were also designed to accommodate the installation of a commercial grade sterile drape which covers the imaging probe, protecting its optical components from bodily fluids while also protecting the patient from exposure to microorganisms and reducing risks of infection. This engineering work led to a version of the prototype that not only allowed data acquisition of higher scientific significance through clinical studies, but also improved credibility of the technology to users, collaborators, and potential entities interested in commercialisation of such a device.

Sub-Objective 2.2: Optimize and validate the clinical prototype's imaging workflow (i.e. acquisition parameters, pre-processing and machine-learning) in a large dataset (>20 samples) from animal tissue. ✓

A tissue optics study in 21 porcine samples from 3 pigs demonstrated a robust and improved imaging workflow capable of discriminating different tissue types while clearly showing tissue borders with probabilistic prediction assignments. The workflow showed generalizable and robust pre-processing and machine learning methodologies which led to a specificity, a sensitivity and an accuracy all exceeding 94% over an independent testing data set composed of 10,080 spectra. The tested set contained three images of tissue borders for which tissue classes were visually assessed and predicted, and tissue borders were adequately highlighted by Platt Scaling assignment. The optimized and validated workflow illustrates, in a toy dataset of porcine tissue, that this workflow could be suitable for characterizing tissue and locating tissue margins in a clinical setting.

Sub-Objective 2.3: Assess the safety of the system for users and patients in a clinical setting. ✓

In its current form, the imaging system appears to be a safe-to-use research prototype for *in situ* investigational studies in humans including *in vivo* use. Key standard operating procedures have been discussed regarding laser safety for tissue exposure and eye exposure (i.e. ANSI's MPE)

which will always need to be respected to minimize risk of injury to users and patients. These procedures include limiting the exposure time to the interrogated tissue to 7,5 seconds per line to respect cumulative temperature dose CEM43°C, training clinicians in using the probe safely to avoid accidental eye exposure <16 cm and sterilizing the probe nose. Helium leak tests were conducted to validate the hermicity of the imaging probe's contact and non-contact muzzles, hence ensuring no transfer of microorganisms could occur between the patient and the imaging probe and thus increase the risk of infection to the patient during *in vivo* use. A sterile drape installation procedure was elaborated based on existing clinical instrumentation procedures and validated by future clinical users. With safety assessed, the imaging system was ready to be introduced for intraoperative measurements (i.e. *ex vivo* and *in vivo*) following approval from a hospital's research ethics board.

7.3 Raman imaging system tested in a clinical setting

Objective 3: Evaluate the feasibility, practicality, and performance of the clinical prototype in a pilot study on resected tumoral tissue from patients affected by brain tumours. ✓

The cart-mounted clinical prototype was tested in a clinical setting during a pilot study in patients suffering from glioblastoma. Eight glioblastoma patients requiring cancer resection surgery were recruited for this study. Brain specimens were resected and recovered for Raman imaging which enabled the spectral characterization of nineteen specimens. Using a machine learning model trained on glioblastoma and normal brain spectra acquired with a single point Raman spectroscopy system, predictions of likelihood of cancer were made on all pixels of each Raman images. These predictions led to color maps which illustrate the presence and location of cancer within the specimens. With histopathology matching the Raman-based predictions, it was confirmed that Raman imaging can help locate cancer even in areas of the normal brain with lower densities of cancer cells. It was further shown that only three Raman bands (i.e. 1004, 1342 and 1430-1460 cm^{-1}) are enough to predict the location of residual cancer cells.

With this small pilot study, we were able to show that the imaging system can be deployed in an operational theatre, can reliably reproduce Raman signatures of human tissues and that this signature can be used to predict and locate cancer in brain tissue. Although acquisition times for

each specimen were between 20 and 40 minutes, a strategy to reduce these acquisition times was presented and provided means to ensure the method is feasible and practical for locating glioma during surgery. The final objective of the study was therefore addressed successfully.

7.4 The COVID-19 Pandemic and a novel Raman-based saliva test

Significant time and resources for this thesis were lost due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or COVID-19 pandemic. Laboratories at CRCHUM and Polytechnique Montreal were shut down for 4 months starting March 2020 and laboratory access was significantly limited for the following 8 months. This made equipment difficult to access and forced a pause in the development and testing of the imaging system in a clinical environment. Additionally, clinical studies were sporadically interrupted, several months at a time, between March 2020 and June 2022 due to waves of COVID-19 outbreaks. In parallel, REBs were focused primarily on COVID-19 related studies, as part of emergency efforts to fight the global pandemic. They were thus slowed in their examination of surgery-related research projects and accumulated a significant backlog of projects awaiting examination. Such conditions created a significant challenge to plan and execute clinical studies in humans. For these reasons, the pilot studies in the *ex vivo* tissue human brain was delayed by one year and an *in vivo* pilot study showcasing another intraoperative use of the imaging system was not executed.

With efforts towards my thesis paused from March 2020 to September 2020, I joined and co-led an initiative to develop a rapid screening test for COVID-19 in saliva using Raman spectroscopy. The project successfully led to the creation of a novel rapid COVID-19 salivary test using Raman spectroscopy which is presented in the *Journal of Biomedical Optics* [267]. This work also allowed the co-development of one patent pending invention [268]. Since September 2020, I have been actively involved in an initiative in the LumedLab to screen for diseases (e.g. cancers and respiratory viruses) in saliva and other biofluids. In September 2022, I co-founded a company named *Exclaro* (14336186 CANADA CORP.) for which I am its current Chief Executive Officer (CEO). This company aims to detect pathogens and diseases using Raman spectroscopy in human biofluids. This company possesses 6 co-founders, is incubated in one of the top 10 university incubators in the world (Centech, Montreal, Canada) and is conducting its pre-seed funding round as of April 2024.

7.5 Thesis work limitations

Although all objectives of this dissertation were met, some limitations to the work presented need to be discussed. These limitations are described below so readers may be aware of the main challenges that remain to be addressed to successfully deploy Raman imaging for commercial use in a clinical environment.

One significant limitation in the work presented regards the impact of using long acquisition times to capture one Raman image (up to 40 minutes). Lengthy measurements not only cause specimens to degrade through dehydration and oxidation, but they also disrupt the workflow of clinical procedures which rely on quick feedback to maximize surgical outcomes. Indeed, feedback provided by collaborating surgeons suggest this imaging system would not be practical for intraoperative margin assessment unless an acquisition time <5 min could be achieved. However, long Raman image measurements were required to obtain sufficient Raman signals to characterize and discriminate tissues such as glioblastoma. Several techniques and hardware modifications are suggested in Chapters 5 and 6 as well as in Appendix A, to lower the required acquisition times to <5 min.

The second limitation we discuss relates the omission of spatial information contained in Raman and bright field images when generating classification models and predictions. Predictions in tissue specimens were all performed using pixel-wise classification models applied on individual Raman spectra only. Neighboring pixels in an image are correlated and this spatial information could be used to increase the accuracy and sensitivity of Raman-based predictions. Additionally, the bright field imaging modality could also augment predictions by providing supplementary structural information (e.g. visible necrotic tissue and large blood vessels) which may help improve predictions. The image data collected for this dissertation remains available for future studies that aim to examine the impact of this structural information on Raman-based predictions. It is expected however that exploiting image features for prediction tasks will require a significantly larger image dataset (i.e. >100 annotated images) to be conducted effectively.

The third weakness regarding the accomplished work is the lack of an investigational study showcasing Raman imaging *in vivo*. Although significant work was done to prepare the system for an *in vivo* pilot study, including a sterilization procedure, its practicality and its impact on an *in*

vivo workflow remains to be tested. Indeed, some design considerations, such as creating a sterilizable contact muzzle for the imaging probe and creating a flexible imaging probe with imaging fiber bundles were meant for a probe that would be placed in contact with live brain tissue. We envision two hurdles that need to be addressed before conducting a successful *in vivo* study: 1) reducing the acquisition time for tissue interrogation to <5 min and 2) replacing the epoxy within the probe's contact apparatus with epoxy exhibiting much less background fluorescence than what was observed in Chapter 5.

7.6 Future scope of work with the Raman imaging system

The Raman imaging system was developed to be versatile and used to interrogate different tissues (e.g. brain tumor and porcine tissue) in different environments (e.g. *in vivo* and *ex vivo*). Knowing the acquisition times for brain specimens can be significantly reduced (discussed in Chapters 5 and 6, and Appendix A), the versatility and speed of Raman large field of view imaging offers many research and commercial opportunities. It is for this reason that there have already been other studies conducted with the system which have been submitted for publication [269], [270]. Currently, there are three research studies planned which will use the imaging system: *in vivo* glioma study at MNI, *in vivo* brain study in epileptic mice at CRCHUM and *ex vivo* breast tissue imaging at McGill University Health Centre. Along with additional upcoming studies, several scopes of work will ensue to further improve the system's capabilities. The future scopes of work expected with the Raman imaging system, shown in Figure 7-1, are described below and further detailed in Appendix A:

1. Clinical studies with the system are on-going with colleagues and are planned for several disease types (i.e. epilepsy, breast cancer and *in vivo* brain cancer). With this system, it is expected that more studies will follow in other clinical and research settings requiring quick intraoperative tissue characterization. LumedLab of Polytechnique Montreal is planning a total of 3 separate clinical studies with the imaging system. Two *in vivo* pilot studies are currently under review by the REBs of CRCHUM and MUHC for investigational studies in breast (N = 20) and brain (N = 20) cancer respectively. With these studies, the imaging system's feasibility for *in vivo* measurements will be validated thus enabling future studies showcasing the ability of the system to guide cancer resection. Currently, an *ex vivo* study

in mice using the imaging system to detect the location of epileptic focus is underway at CRCHUM.

2. Optomechanical redesigning will aim to improve ergonomics, brightfield imaging and practical use of the system while maintaining system performance.
3. Hardware upgrades such as laser and camera replacements will look to optimize the system's collection and acquisition times for tissue characterization. Cost-effective hardware changes are also expected to guide the device towards a commercially viable product.
4. Image registration improvements are required to account for non-affine tissue deformations that occur during tissue resection and sample preparation required for histopathological assessment and labelling. Improved image registration will allow for more accurate tissue characterization, tissue discrimination and margin assessment.
5. Image pre-processing improvements are also expected regarding system correction response, baseline correction, image processing and Raman feature extraction, with the purpose of generating higher quality data to be employed in AI models.
6. An imaging biopsy device will need to be designed and coupled with the imaging system to adequately extract and label tissues characterized intraoperatively which will serve as training samples for AI-based tissue classification. This imaging biopsy would serve to minimize tissue deformations and track sample changes (e.g. sample rotation and shrinking) from sample interrogation *in situ* to sample histopathological examination. This device will help ensure that a detailed histological labelling of heterogeneous tissue can be adequately linked to the corresponding Raman image measurement which will also be important to validate the system's predictions during surgical guidance.
7. Deep learning classification: The spatial information from Raman and bright field images combined with the very large number of spectra acquired in one Raman image (e.g.: 1680 spectra per image) require sophisticated AI methods to be adequately exploited. The large number of Raman spectra (i.e.: <30 000) acquired in the *ex vivo* brain study makes it appropriate to exploit deep learning for the purpose of tissue discrimination. Alternatively,

Raman and bright field image spatial content can be exploited by complex image classification architectures such as CNNs.

8. Additional imaging modalities (e.g. SORS for in-depth imaging and Multi-Spectral Raman Spectroscopy (MSRS) for faster imaging) can be integrated in the system with little to no modifications in hardware and software. Adding modalities may help to further characterize tissue, reduce acquisition time in the operative room, enhance classification performance and characterize tissue in depth (e.g. up to 5 mm in depth with SORS).

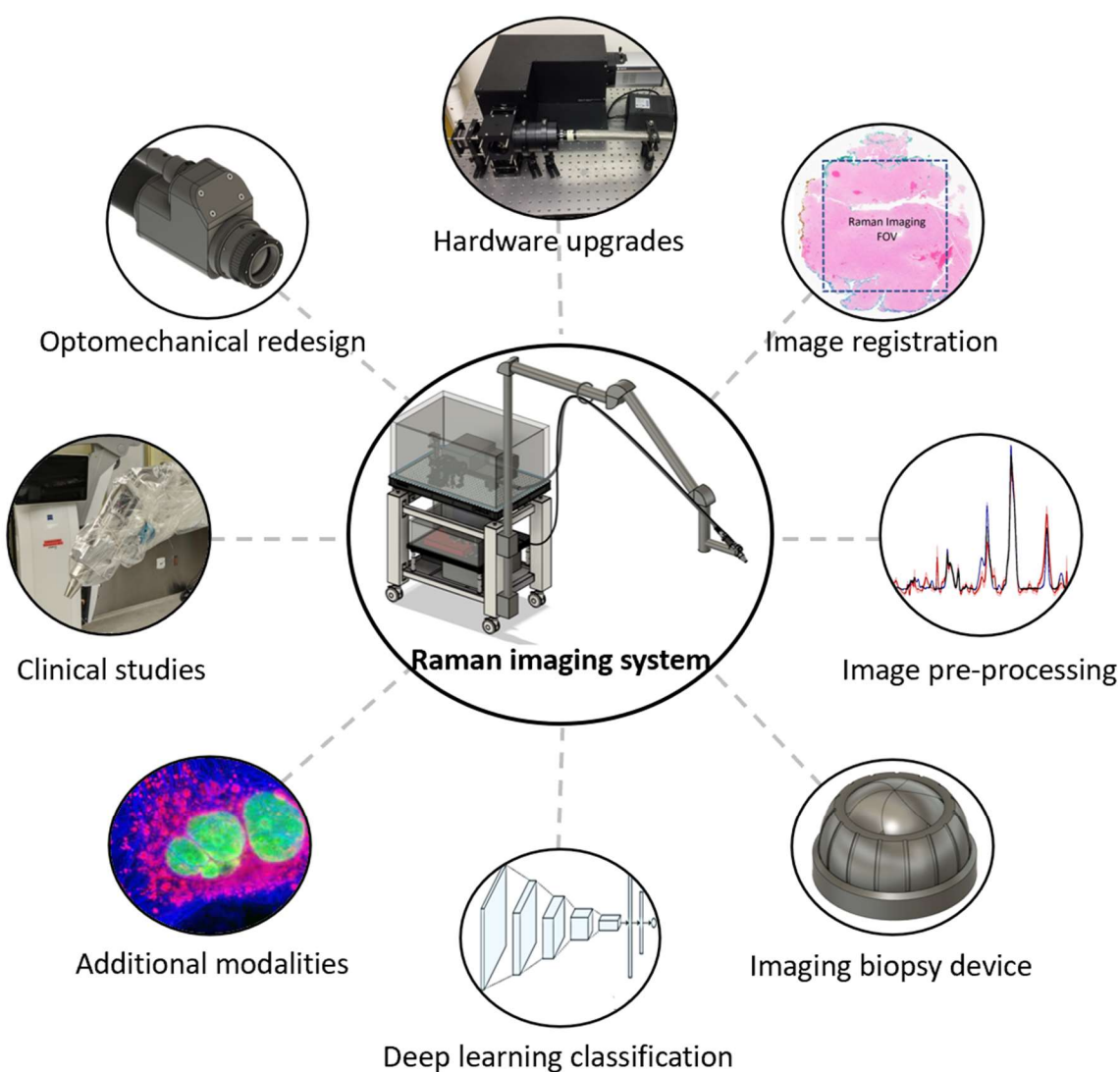


Figure 7-1: Future scope of work expected with the Raman imaging system.

CHAPTER 8 CONCLUSION AND RECOMMENDATIONS

A Raman imaging system for *in situ* characterization was developed and tested in a clinical setting. The line scan imaging system's optomechanical design was the first built of its kind having a large field of view of 1 cm² and capable of *in vivo* measurement of specimens. Its design included several innovations which led to two granted patents, and which proved capable of characterizing tissues during surgical procedures. This work led to the publication of two scientific articles and a third scientific manuscript submitted for publication.

The first original aspect of this project was the development of the first ever macroscopic Raman image capable of *in vivo* tissue characterization of human tissue. This is significant as it opens the possibility of recovering vast amounts of *in vivo* molecular specific information of human tissue in combination with spatial context for a better understanding of human physiology. The second original contribution comes from introducing the first ever macroscopic Raman imaging system to be used in a surgical procedure. This is a major milestone in the clinical adoption of Raman imaging for surgical guidance of any pathology, while offering the possibility for the acquisition of rich data for AI-based applications in oncology and for further safe removal of diseased tissue during glioma surgery. The third original aspect is that it is the first Raman imaging system which uses an externally trained machine learning model to accurately predict tumor locations in fresh human specimens. This finding is significant since it suggests that deploying new Raman spectroscopy systems may be facilitated by utilizing previously trained, validated, and tested AI-based classifiers instead of requiring completely new data acquisition studies.

The clinical impact of this work is evident in the way it has the potential of providing non-destructive and non-invasive optical diagnostic information for tumor visualisation during surgery for further safe resection of cancer cells. Since there is a relation between the amount of cancer resected during glioblastoma surgery and patient outcome, there is a net benefit to patients of providing the surgeon this additional information. Seeing how some procedures require a postoperative MRI to verify if there is a remaining tumor mass rapidly detecting positive margins during surgery can reduce the need for second surgeries and their associated costs. The introduction of Raman imaging in the context of neurosurgery is also a gateway to address other pathologies for which cancer margins are difficult to identify rapidly with current technology. Such pathologies include melanomas, prostate cancer, ovarian cancer, and breast cancer. The system's versatility

and its corresponding clinical protocol for image registration make it easily integrated in any open surgeries.

One additional impact of the project is the commercial opportunity in the eventual success of the system's clinical adoption. Many companies currently aim to commercialize surgical guidance equipment that make use of optical technologies. Such examples include the fluorescence imaging system FireflyTM by Intuitive Surgical and the SentryTM Raman system from company ODS Medical. These indicate a market for in-surgery guidance of cancer resection. The imaging system was developed for this clinical need, keeping patient and clinician safety in mind while also developing a practical visual assessment of tissue disease state. The system's ability to characterize other tissue types makes it versatile and applicable to other pathologies. This can enable the system to be deployed to address a larger proportion of the oncology market. Finally, with intellectual property already generated and patented, commercial value of this technology is increased.

Although the impact of this work is significant, there remains much work to ensure Raman imaging is effective to assist clinicians during surgical procedures. The focus of future efforts should aim to bring Raman imaging towards two objectives: 1) acquiring Raman images as quickly as possible with the aim of achieving real time characterization of tissues and 2) acquiring Raman images over the largest field of view possible to assess large surface areas of tissues at once. These two aims are crucial to ensure clinicians are willing to adopt the technology into their fast-paced workflow. The recommended way to achieve this real time large field of view Raman imaging is to develop new Raman systems which focus on acquiring Raman signal on a very select number of Raman bands. This method is underway in the LumedLab [269].

REFERENCES

- [1] J. Liang *et al.*, “Prognostic factors of patients with Gliomas – an analysis on 335 patients with Glioblastoma and other forms of Gliomas,” *BMC Cancer*, vol. 20, no. 1, p. 35, 2020, doi: 10.1186/s12885-019-6511-6.
- [2] Mesfin F. B. and Al-Dhahir M. A., *Gliomas*. Treasure Island: StatPearls Publishing, 2023.
- [3] M. Progress, P. Y. Wen, and S. Kesari, “Malignant Gliomas in Adults,” 2008.
- [4] Q. T. Ostrom *et al.*, “CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2007–2011,” *Neuro Oncol*, vol. 16, no. suppl_4, pp. iv1–iv63, Oct. 2014, doi: 10.1093/neuonc/nou223.
- [5] P. Wesseling and D. Capper, “WHO 2016 Classification of gliomas,” *Neuropathol Appl Neurobiol*, vol. 44, no. 2, pp. 139–150, Feb. 2018, doi: <https://doi.org/10.1111/nan.12432>.
- [6] A. F. Tamimi and M. Juweid, *Glioblastoma*. Exon Publications, 2017.
- [7] S. Cha, “Update on Brain Tumor Imaging: From Anatomy to Physiology,” *American Journal of Neuroradiology*, vol. 27, no. 3, pp. 475 LP – 487, Mar. 2006.
- [8] C.-C. Ko *et al.*, “Differentiation between Glioblastoma Multiforme and Primary Cerebral Lymphoma: Additional Benefits of Quantitative Diffusion-Weighted MR Imaging,” *PLoS One*, vol. 11, p. e0162565, Sep. 2016, doi: 10.1371/journal.pone.0162565.
- [9] T. J. Brown *et al.*, “Association of the Extent of Resection With Survival in Glioblastoma: A Systematic Review and Meta-analysis,” *JAMA Oncol*, vol. 2, no. 11, pp. 1460–1469, Nov. 2016, doi: 10.1001/jamaoncol.2016.1373.
- [10] H. Jiang *et al.*, “Classification of Progression Patterns in Glioblastoma: Analysis of Predictive Factors and Clinical Implications,” *Front Oncol*, vol. 10, 2020, [Online]. Available: <https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2020.590648>

- [11] M. Rapp, J. Baernreuther, B. Turowski, H.-J. Steiger, M. Sabel, and M. A. Kamp, "Recurrence Pattern Analysis of Primary Glioblastoma," *World Neurosurg*, vol. 103, pp. 733–740, 2017, doi: <https://doi.org/10.1016/j.wneu.2017.04.053>.
- [12] A. Claes, A. J. Idema, and P. Wesseling, "Diffuse glioma growth: a guerilla war," *Acta Neuropathol*, vol. 114, no. 5, pp. 443–458, 2007, doi: [10.1007/s00401-007-0293-7](https://doi.org/10.1007/s00401-007-0293-7).
- [13] N. Sanai and M. S. Berger, "Glioma extent of resection and its impact on patient outcome.," *Neurosurgery*, vol. 62, no. 4, pp. 753–766, 2008, doi: [10.1227/01.NEU.0000310769.20996.BD](https://doi.org/10.1227/01.NEU.0000310769.20996.BD).
- [14] M. Glas *et al.*, "Residual tumor cells are unique cellular targets in glioblastoma," *Ann Neurol*, vol. 68, no. 2, pp. 264–269, Aug. 2010, doi: <https://doi.org/10.1002/ana.22036>.
- [15] A. J. Gogos, J. S. Young, R. A. Morshed, S. L. Hervey-Jumper, and M. S. Berger, "Awake glioma surgery: technical evolution and nuances," *J Neurooncol*, vol. 147, no. 3, pp. 515–524, 2020, doi: [10.1007/s11060-020-03482-z](https://doi.org/10.1007/s11060-020-03482-z).
- [16] S. J. Han *et al.*, "Subcortical stimulation mapping of descending motor pathways for perirolandic gliomas: assessment of morbidity and functional outcome in 702 cases," *J Neurosurg*, vol. 131, no. 1, pp. 201–208, 2019, doi: <https://doi.org/10.3171/2018.3.JNS172494>.
- [17] H. Duffau *et al.*, "Usefulness of intraoperative electrical subcortical mapping during surgery for low-grade gliomas located within eloquent brain regions: functional results in a consecutive series of 103 patients," *J Neurosurg*, vol. 98, no. 4, pp. 764–778, 2003, doi: <https://doi.org/10.3171/jns.2003.98.4.0764>.
- [18] G. E. Keles, D. A. Lundin, K. R. Lamborn, E. F. Chang, G. Ojemann, and M. S. Berger, "Intraoperative subcortical stimulation mapping for hemispheric perirolandic gliomas located within or adjacent to the descending motor pathways: evaluation of morbidity and assessment of functional outcome in 294 patients," *J Neurosurg*, vol. 100, no. 3, pp. 369–375, 2004, doi: <https://doi.org/10.3171/jns.2004.100.3.0369>.
- [19] M. Rahman *et al.*, "The effects of new or worsened postoperative neurological deficits on survival of patients with glioblastoma," *Journal of Neurosurgery JNS*, vol. 127, no. 1, pp. 123–131, 2017, doi: <https://doi.org/10.3171/2016.7.JNS16396>.

- [20] K. Petrecca, M.-C. Guiot, V. Panet-Raymond, and L. Souhami, "Failure pattern following complete resection plus radiotherapy and temozolomide is at the resection margin in patients with glioblastoma," *J Neurooncol*, vol. 111, no. 1, pp. 19–23, 2013, doi: 10.1007/s11060-012-0983-4.
- [21] C. Senft, A. Bink, K. Franz, H. Vatter, T. Gasser, and V. Seifert, "Intraoperative MRI guidance and extent of resection in glioma surgery: a randomised, controlled trial," *Lancet Oncol*, vol. 12, no. 11, pp. 997–1003, 2011, doi: [https://doi.org/10.1016/S1470-2045\(11\)70196-6](https://doi.org/10.1016/S1470-2045(11)70196-6).
- [22] T. Noh, M. Mustroph, and A. J. Golby, "Intraoperative Imaging for High-Grade Glioma Surgery," *Neurosurg Clin N Am*, vol. 32, no. 1, pp. 47–54, 2021, doi: <https://doi.org/10.1016/j.nec.2020.09.003>.
- [23] F. Prada *et al.*, "Intraoperative Cerebral Glioma Characterization with Contrast Enhanced Ultrasound," *Biomed Res Int*, vol. 2014, no. 484261, 2014.
- [24] L.-G. Cheng *et al.*, "Intraoperative Contrast Enhanced Ultrasound Evaluates the Grade of Glioma," *Biomed Res Int*, vol. 2016, 2016.
- [25] A. R. Asthagiri, N. Pouratian, J. Sherman, G. Ahmed, and M. E. Shaffrey, "Advances in Brain Tumor Surgery," *Neurol Clin*, vol. 25, no. 4, pp. 975–1003, 2007, doi: <https://doi.org/10.1016/j.ncl.2007.07.006>.
- [26] W. Stummer, U. Pichlmeier, T. Meinel, O. D. Wiestler, F. Zanella, and H.-J. Reulen, "Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial," *Lancet Oncol*, vol. 7, no. 5, pp. 392–401, May 2006, doi: 10.1016/S1470-2045(06)70665-9.
- [27] C. G. Hadjipanayis, W. Stummer, and J. P. Sheehan, "5-ALA fluorescence-guided surgery of CNS tumors," *J Neurooncol*, vol. 141, no. 3, pp. 477–478, 2019, doi: 10.1007/s11060-019-03109-y.
- [28] P. Abraham *et al.*, "Cost-effectiveness of Intraoperative MRI for Treatment of High-Grade Gliomas," *Radiology*, vol. 291, no. 3, pp. 689–697, Mar. 2019, doi: 10.1148/radiol.2019182095.

- [29] A. Pichierri, M. Bradley, and V. Iyer, “Intraoperative Magnetic Resonance Imaging–Guided Glioma Resections in Awake or Asleep Settings and Feasibility in the Context of a Public Health System,” *World Neurosurg* *X*, vol. 3, p. 100022, 2019, doi: <https://doi.org/10.1016/j.wnsx.2019.100022>.
- [30] B. K. R. Munkvold, A. S. Jakola, I. Reinertsen, L. M. Sagberg, G. Unsgård, and O. Solheim, “The Diagnostic Properties of Intraoperative Ultrasound in Glioma Surgery and Factors Associated with Gross Total Tumor Resection,” *World Neurosurg*, vol. 115, pp. e129–e136, 2018, doi: <https://doi.org/10.1016/j.wneu.2018.03.208>.
- [31] A. Nabavi *et al.*, “Five-aminolevulinic acid for fluorescence-guided resection of recurrent malignant gliomas: A phase II study,” *Neurosurgery*, vol. 65, no. 6, pp. 1070–1076, 2009, doi: 10.1227/01.NEU.0000360128.03597.C7.
- [32] G. Abdalla, A. Hammam, M. Anjari, D. F. D’Arco, and D. S. Bisdas, “Glioma surveillance imaging: current strategies, shortcomings, challenges and outlook,” *BJR|Open*, vol. 2, no. 1, p. 20200009, Nov. 2020, doi: 10.1259/bjro.20200009.
- [33] R.-O. Mirimanoff, “High-grade gliomas: reality and hopes,” *Chin J Cancer*, vol. 33, no. 1, pp. 1–3, Jan. 2014, doi: 10.5732/cjc.013.10215.
- [34] I. W. H. Chung and S. Eljamel, “Risk factors for developing oral 5-aminolevulinic acid-induced side effects in patients undergoing fluorescence guided resection,” *Photodiagnosis Photodyn Ther*, vol. 10, no. 4, pp. 362–367, 2013, doi: <https://doi.org/10.1016/j.pdpdt.2013.03.007>.
- [35] E. Suero Molina, S. Schipmann, and W. Stummer, “Maximizing safe resections: the roles of 5-aminolevulinic acid and intraoperative MR imaging in glioma surgery—review of the literature,” *Neurosurg Rev*, vol. 42, no. 2, pp. 197–208, 2019, doi: 10.1007/s10143-017-0907-z.
- [36] D. DePaoli *et al.*, “Rise of Raman spectroscopy in neurosurgery: a review,” *J Biomed Opt*, vol. 25, no. 5, pp. 1–36, May 2020, doi: 10.1117/1.JBO.25.5.050901.
- [37] M. Jermyn *et al.*, “Intraoperative brain cancer detection with Raman spectroscopy in humans,” *Sci Transl Med*, vol. 7, no. 274, pp. 274ra19 LP-274ra19, Feb. 2015, doi: 10.1126/scitranslmed.aaa2384.

- [38] M. Jermyn *et al.*, “Highly accurate detection of cancer in situ with intraoperative, label-free, multimodal optical spectroscopy,” *Cancer Res*, vol. 77, no. 14, pp. 3942–3950, 2017, doi: 10.1158/0008-5472.CAN-17-0668.
- [39] J. Desroches *et al.*, “A new method using Raman spectroscopy for in vivo targeted brain cancer tissue biopsy,” *Sci Rep*, vol. 8, no. 1, p. 1792, 2018, doi: 10.1038/s41598-018-20233-3.
- [40] C.-S. Liao *et al.*, “In Vivo and in Situ Spectroscopic Imaging by a Handheld Stimulated Raman Scattering Microscope,” *ACS Photonics*, vol. 5, no. 3, pp. 947–954, Mar. 2018, doi: 10.1021/acsphotonics.7b01214.
- [41] R. Galli *et al.*, “Identification of distinctive features in human intracranial tumors by label-free nonlinear multimodal microscopy,” *J Biophotonics*, vol. 12, no. 10, p. e201800465, Oct. 2019, doi: <https://doi.org/10.1002/jbio.201800465>.
- [42] N. Ogrinc, P. Saudemont, Z. Takats, M. Salzert, and I. Fournier, “Cancer Surgery 2.0: Guidance by Real-Time Molecular Technologies,” *Trends Mol Med*, vol. 27, no. 6, pp. 602–615, 2021, doi: <https://doi.org/10.1016/j.molmed.2021.04.001>.
- [43] C. V RAMAN, “A Change of Wave-length in Light Scattering,” *Nature*, vol. 121, no. 3051, p. 619, 1928, doi: 10.1038/121619b0.
- [44] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson, *Molecular biology of the cell*, vol. 3. Garland New York, 1994.
- [45] R. F. Begley, A. B. Harvey, and R. L. Byer, “Coherent anti-Stokes Raman spectroscopy,” *Appl Phys Lett*, vol. 25, no. 7, pp. 387–390, Oct. 1974, doi: 10.1063/1.1655519.
- [46] E. Smith and G. Dent, *Modern Raman spectroscopy: a practical approach*. John Wiley & Sons, 2019.
- [47] R. Salzer and H. W. Siesler, *Infrared and Raman spectroscopic imaging*. John Wiley & Sons, 2014.
- [48] M. Jermyn *et al.*, “A review of Raman spectroscopy advances with an emphasis on clinical translation challenges in oncology,” *Phys Med Biol*, vol. 61, no. 23, pp. R370–R400, Dec. 2016, doi: 10.1088/0031-9155/61/23/R370.

- [49] J. Yao and L. V Wang, "Sensitivity of photoacoustic microscopy," *Photoacoustics*, vol. 2, no. 2, pp. 87–101, 2014, doi: <https://doi.org/10.1016/j.pacs.2014.04.002>.
- [50] C. Boudoux, *Fundamentals of Biomedical Optics*, First Edit. Pollux Editions, 2017.
- [51] M. Y. Berezin and S. Achilefu, "Fluorescence Lifetime Measurements and Biological Imaging," *Chem Rev*, vol. 110, no. 5, pp. 2641–2684, May 2010, doi: 10.1021/cr900343z.
- [52] J. R. Lakowicz, "Introduction to Fluorescence," in *Principles of Fluorescence Spectroscopy*, J. R. Lakowicz, Ed., Boston, MA: Springer US, 1999, pp. 1–23. doi: 10.1007/978-1-4757-3061-6_1.
- [53] T. Nagaya, Y. A. Nakamura, P. L. Choyke, and H. Kobayashi, "Fluorescence-Guided Surgery," *Front Oncol*, vol. 7, p. 314, 2017, doi: 10.3389/fonc.2017.00314.
- [54] C. Beleites and R. Salzer, "Assessing and improving the stability of chemometric models in small sample size situations," *Anal Bioanal Chem*, vol. 390, no. 5, pp. 1261–1271, 2008, doi: 10.1007/s00216-007-1818-6.
- [55] J. Popp *et al.*, "Label-free molecular imaging of biological cells and tissues by linear and non-linear Raman spectroscopic approaches," *Angewandte Chemie International Edition*, pp. 4392–4430, 2016, doi: 10.1002/anie.201607604.
- [56] S. Koljenovic *et al.*, "Tissue characterization using high wave number Raman spectroscopy," *J Biomed Opt*, vol. 10, no. 3, p. 031116, May 2005, doi: 10.1117/1.1922307.
- [57] S. Yang, "High-Wavenumber Raman Analysis," in *Recent Developments in Atomic Force Microscopy and Raman Spectroscopy for Materials Characterization*, C. S. Pathak and S. Kumar, Eds., Rijeka: IntechOpen, 2021, p. Ch. 6. doi: 10.5772/intechopen.100474.
- [58] S. Dochow *et al.*, "Etaloning, fluorescence and ambient light suppression by modulated wavelength Raman spectroscopy," *Biomed Spectrosc Imaging*, vol. 1, pp. 383–389, 2012, doi: 10.3233/BSI-120031.
- [59] J. Zhao, H. Lui, D. I. McLean, and H. Zeng, "Automated Autofluorescence Background Subtraction Algorithm for Biomedical Raman Spectroscopy," *Appl Spectrosc*, vol. 61, no. 11, pp. 1225–1232, 2007, [Online]. Available: <https://opg.optica.org/as/abstract.cfm?URI=as-61-11-1225>

- [60] K. St-arnaud, “Développement d’une sonde portable d’imagerie Raman pour guider l’exérèse de tumeur,” *Memoire, Polytechnique Montreal*, 2017.
- [61] S. J. Choquette, E. S. Etz, W. S. Hurst, D. H. Blackburn, and S. D. Leigh, “Relative Intensity Correction of Raman Spectrometers: NIST SRMs 2241 through 2243 for 785 nm, 532 nm, and 488 nm/514.5 nm Excitation,” *Appl Spectrosc*, vol. 61, no. 2, pp. 117–129, Feb. 2007, doi: 10.1366/000370207779947585.
- [62] W. Liu *et al.*, “Snapshot depth sensitive Raman spectroscopy in layered tissues,” *Opt Express*, vol. 24, no. 25, pp. 28312–28325, 2016, doi: 10.1364/OE.24.028312.
- [63] J.-P. Tremblay, “Études de sources de bruit et de facteurs confondants sur la mesure de spectres raman de tissus biologiques,” 2018. [Online]. Available: <https://publications.polymtl.ca/3104/>
- [64] J. Desroches *et al.*, “Raman spectroscopy in microsurgery: impact of operating microscope illumination sources on data quality and tissue classification,” *Analyst*, vol. 142, no. 8, pp. 1185–1191, 2017, doi: 10.1039/C6AN02061E.
- [65] M. Jermyn *et al.*, “Neural networks improve brain cancer detection with Raman spectroscopy in the presence of light artifacts,” in *SPIE 9690, Clinical and Translational Neurophotonics; Neural Imaging and Sensing; and Optogenetics and Optical Manipulation, 96900B*, SPIE, 2016.
- [66] F. Légaré, C. L. Evans, F. Ganikhanov, and X. S. Xie, “Towards CARS Endoscopy,” *Opt Express*, vol. 14, no. 10, pp. 4427–4432, 2006, doi: 10.1364/OE.14.004427.
- [67] B. G. Saar, R. S. Johnston, C. W. Freudiger, X. S. Xie, and E. J. Seibel, “Coherent Raman scanning fiber endoscopy,” *Opt Lett*, vol. 36, no. 13, pp. 2396–2398, 2011, doi: 10.1364/OL.36.002396.
- [68] S. Feng *et al.*, “Surface-enhanced Raman spectroscopy of saliva proteins for the noninvasive differentiation of benign and malignant breast tumors,” *Int J Nanomedicine*, pp. 537–547, 2015.
- [69] S. Li *et al.*, “Noninvasive prostate cancer screening based on serum surface-enhanced Raman spectroscopy and support vector machine,” *Appl Phys Lett*, vol. 105, no. 9, p. 091104, Sep. 2014, doi: 10.1063/1.4892667.

- [70] M. Ji *et al.*, “Rapid, Label-Free Detection of Brain Tumors with Stimulated Raman Scattering Microscopy,” *Sci Transl Med*, vol. 5, no. 201, pp. 201ra119 LP-201ra119, Sep. 2013, doi: 10.1126/scitranslmed.3005954.
- [71] D. Fu *et al.*, “Quantitative Chemical Imaging with Multiplex Stimulated Raman Scattering Microscopy,” *J Am Chem Soc*, vol. 134, no. 8, pp. 3623–3626, Feb. 2012, doi: 10.1021/ja210081h.
- [72] C.-S. Liao *et al.*, “Spectrometer-free vibrational imaging by retrieving stimulated Raman signal from highly scattered photons,” *Sci Adv*, vol. 1, no. 9, p. e1500738, Apr. 2024, doi: 10.1126/sciadv.1500738.
- [73] H. Kano, H. Segawa, P. Leproux, and V. Couderc, “Linear and nonlinear Raman microspectroscopy: History, instrumentation, and applications,” *Opt Rev*, vol. 21, no. 6, pp. 752–761, 2014, doi: 10.1007/s10043-014-0123-9.
- [74] M. Fleischmann, P. J. Hendra, and A. J. McQuillan, “Raman spectra of pyridine adsorbed at a silver electrode,” *Chem Phys Lett*, vol. 26, no. 2, pp. 163–166, 1974, doi: [https://doi.org/10.1016/0009-2614\(74\)85388-1](https://doi.org/10.1016/0009-2614(74)85388-1).
- [75] R. Pilot, R. Signorini, C. Durante, L. Orian, M. Bhamidipati, and L. Fabris, “A Review on Surface-Enhanced Raman Scattering,” *Biosensors (Basel)*, vol. 9, no. 2, 2019, doi: 10.3390/bios9020057.
- [76] N. J. Halas, S. Lal, W.-S. Chang, S. Link, and P. Nordlander, “Plasmons in Strongly Coupled Metallic Nanostructures,” *Chem Rev*, vol. 111, no. 6, pp. 3913–3961, Jun. 2011, doi: 10.1021/cr200061k.
- [77] P. G. Etchegoin and E. C. Le Ru, “A perspective on single molecule SERS: current status and future challenges,” *Physical Chemistry Chemical Physics*, vol. 10, no. 40, pp. 6079–6089, 2008, doi: 10.1039/B809196J.
- [78] S.-Y. Ding, E.-M. You, Z.-Q. Tian, and M. Moskovits, “Electromagnetic theories of surface-enhanced Raman spectroscopy,” *Chem Soc Rev*, vol. 46, no. 13, pp. 4042–4076, 2017, doi: 10.1039/C7CS00238F.
- [79] W. Stummer *et al.*, “Intraoperative Detection of Malignant Gliomas by 5-Aminolevulinic Acid-induced Porphyrin Fluorescence,” *Neurosurgery*, vol. 42, no. 3, 1998, [Online].

Available:

https://journals.lww.com/neurosurgery/fulltext/1998/03000/intraoperative_detection_of_malignant_gliomas_by.17.aspx

- [80] S. Schlücker, M. D. Schaeberle, S. W. Huffman, and I. W. Levin, “Raman Microspectroscopy: A Comparison of Point, Line, and Wide-Field Imaging Methodologies,” *Anal Chem*, vol. 75, no. 16, pp. 4312–4318, Aug. 2003, doi: 10.1021/ac034169h.
- [81] T. W. Bocklitz, S. Guo, O. Ryabchykov, N. Vogler, and J. Popp, “Raman Based Molecular Imaging and Analytics: A Magic Bullet for Biomedical Applications!?,” *Anal Chem*, vol. 88, no. 1, pp. 133–151, Jan. 2016, doi: 10.1021/acs.analchem.5b04665.
- [82] A. Papour, J. H. Kwak, Z. Taylor, B. Wu, O. Stafsudd, and W. Grundfest, “Wide-field Raman imaging for bone detection in tissue,” *Biomed Opt Express*, vol. 6, no. 10, pp. 3892–3897, 2015, doi: 10.1364/BOE.6.003892.
- [83] J. Ramsey, S. Ranganathan, R. L. McCreery, and J. Zhao, “Performance Comparisons of Conventional and Line-Focused Surface Raman Spectrometers,” *Appl Spectrosc*, vol. 55, no. 6, pp. 767–773, 2001, [Online]. Available: <https://opg.optica.org/as/abstract.cfm?URI=as-55-6-767>
- [84] K. St-Arnaud *et al.*, “Wide-field spontaneous Raman spectroscopy imaging system for biological tissue interrogation,” *Opt. Lett.*, vol. 41, no. 20, pp. 4692–4695, 2016, doi: 10.1364/OL.41.004692.
- [85] M. J. Pelletier, “Quantitative Analysis Using Raman Spectrometry,” *Appl Spectrosc*, vol. 57, no. 1, pp. 20A–42A, Jan. 2003, doi: 10.1366/000370203321165133.
- [86] G. W. Auner *et al.*, “Applications of Raman spectroscopy in cancer diagnosis,” *Cancer and Metastasis Reviews*, vol. 37, no. 4, pp. 691–717, 2018, doi: 10.1007/s10555-018-9770-9.
- [87] R. Gautam, S. Vanga, F. Ariese, and S. Umapathy, “Review of multidimensional data processing approaches for Raman and infrared spectroscopy,” *EPJ Tech Instrum*, vol. 2, no. 1, p. 8, 2015, doi: 10.1140/epjti/s40485-015-0018-6.

- [88] A. Beljebbar, S. Dukic, N. Amharref, and M. Manfait, “Ex vivo and in vivo diagnosis of C6 glioblastoma development by Raman spectroscopy coupled to a microprobe,” *Anal Bioanal Chem*, vol. 398, no. 1, pp. 477–487, 2010, doi: 10.1007/s00216-010-3910-6.
- [89] N. Bergner *et al.*, “Hyperspectral unmixing of Raman micro-images for assessment of morphological and chemical parameters in non-dried brain tumor specimens,” *Anal Bioanal Chem*, vol. 405, no. 27, pp. 8719–8728, 2013, doi: 10.1007/s00216-013-7257-7.
- [90] H. Abramczyk and A. Imiela, “The biochemical, nanomechanical and chemometric signatures of brain cancer,” *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 188, pp. 8–19, 2018, doi: <https://doi.org/10.1016/j.saa.2017.06.037>.
- [91] C. Krafft *et al.*, “Advances in optical biopsy – correlation of malignancy and cell density of primary brain tumors using Raman microspectroscopic imaging,” *Analyst*, vol. 137, no. 23, pp. 5533–5537, 2012, doi: 10.1039/C2AN36083G.
- [92] R. P. Aguiar, L. Silveira, E. T. Falcão, M. T. T. Pacheco, R. A. Zângaro, and C. A. Pasqualucci, “Discriminating Neoplastic and Normal Brain Tissues in Vitro Through Raman Spectroscopy: A Principal Components Analysis Classification Model,” *Photomed Laser Surg*, vol. 31, no. 12, pp. 595–604, Nov. 2013, doi: 10.1089/pho.2012.3460.
- [93] E. Kaur *et al.*, “Unique spectral markers discern recurrent Glioblastoma cells from heterogeneous parent population,” *Sci Rep*, vol. 6, no. 1, p. 26538, 2016, doi: 10.1038/srep26538.
- [94] É. Lemoine *et al.*, “Feature engineering applied to intraoperative in vivo Raman spectroscopy sheds light on molecular processes in brain cancer: a retrospective study of 65 patients,” *Analyst*, vol. 144, no. 22, pp. 6517–6532, 2019, doi: 10.1039/C9AN01144G.
- [95] J. Trevisan, P. P. Angelov, P. L. Carmichael, A. D. Scott, and F. L. Martin, “Extracting biological information with computational analysis of Fourier-transform infrared (FTIR) biospectroscopy datasets: current practices to future perspectives,” *Analyst*, vol. 137, no. 14, pp. 3202–3215, 2012, doi: 10.1039/C2AN16300D.
- [96] X. Yuan and R. A. Mayanovic, “An Empirical Study on Raman Peak Fitting and Its Application to Raman Quantitative Research,” *Appl Spectrosc*, vol. 71, no. 10, pp. 2325–2338, Aug. 2017, doi: 10.1177/0003702817721527.

- [97] K. Tanahashi *et al.*, “Assessment of Tumor Cells in a Mouse Model of Diffuse Infiltrative Glioma by Raman Spectroscopy,” *Biomed Res Int*, vol. 2014, p. 860241, 2014, doi: 10.1155/2014/860241.
- [98] A. W. Auner, R. E. Kast, R. Rabah, J. M. Poulik, and M. D. Klein, “Conclusions and data analysis: a 6-year study of Raman spectroscopy of solid tumors at a major pediatric institute,” *Pediatr Surg Int*, vol. 29, no. 2, pp. 129–140, 2013, doi: 10.1007/s00383-012-3211-6.
- [99] O. Uckermann *et al.*, “IDH1 mutation in human glioma induces chemical alterations that are amenable to optical Raman spectroscopy,” *J Neurooncol*, vol. 139, no. 2, pp. 261–268, 2018, doi: 10.1007/s11060-018-2883-8.
- [100] L. J. Livermore *et al.*, “Raman spectroscopy to differentiate between fresh tissue samples of glioma and normal brain: a comparison with 5-ALA–induced fluorescence-guided surgery,” *Journal of Neurosurgery JNS*, pp. 1–11, 2020, doi: <https://doi.org/10.3171/2020.5.JNS20376>.
- [101] M. B. Fenn, P. Xanthopoulos, G. Pyrgiotakis, S. R. Grobmyer, P. M. Pardalos, and L. L. Hench, “Raman Spectroscopy for Clinical Oncology,” *Advances in Optical Technologies*, vol. 2011, p. 213783, 2011, doi: 10.1155/2011/213783.
- [102] Y. Zhou *et al.*, “Human brain cancer studied by resonance Raman spectroscopy,” *J Biomed Opt*, vol. 17, no. 11, p. 116021, Nov. 2012, doi: 10.1117/1.JBO.17.11.116021.
- [103] N. Bergner *et al.*, “Identification of primary tumors of brain metastases by Raman imaging and support vector machines,” *Chemometrics and Intelligent Laboratory Systems*, vol. 117, pp. 224–232, 2012, doi: <https://doi.org/10.1016/j.chemolab.2012.02.008>.
- [104] R. Stables *et al.*, “Feature driven classification of Raman spectra for real-time spectral brain tumour diagnosis using sound,” *Analyst*, vol. 142, no. 1, pp. 98–109, 2017, doi: 10.1039/C6AN01583B.
- [105] D. G. Leslie *et al.*, “Identification of Pediatric Brain Neoplasms Using Raman Spectroscopy,” *Pediatr Neurosurg*, vol. 48, no. 2, pp. 109–117, Nov. 2012, doi: 10.1159/000343285.

- [106] Z. Xu, Y. Chen, M. R. Gartia, J. Jiang, and G. L. Liu, "Surface plasmon enhanced broadband spectrophotometry on black silver substrates," *Appl Phys Lett*, vol. 98, no. 24, pp. 1–4, 2011, doi: 10.1063/1.3599551.
- [107] J. Desroches *et al.*, "Characterization of a Raman spectroscopy probe system for intraoperative brain tissue classification," *Biomed Opt Express*, vol. 6, no. 7, pp. 2380–2397, 2015, doi: 10.1364/BOE.6.002380.
- [108] M. Jermyn *et al.*, "Intraoperative brain cancer detection with Raman spectroscopy in humans," *Sci Transl Med*, vol. 7, no. 274, pp. 274ra19-274ra19, 2015, doi: 10.1126/scitranslmed.aaa2384.
- [109] M. Jermyn *et al.*, "Raman spectroscopy detects distant invasive brain cancer cells centimeters beyond MRI capability in humans," *Biomed Opt Express*, vol. 7, no. 12, pp. 5129–5137, Nov. 2016, doi: 10.1364/BOE.7.005129.
- [110] S. Malek, F. Melgani, and Y. Bazi, "One-dimensional convolutional neural networks for spectroscopic signal regression," *J Chemom*, vol. 32, no. 5, p. e2977, May 2018, doi: <https://doi.org/10.1002/cem.2977>.
- [111] S. L. Neal, "Multivariate Analysis of Mixed Lipid Aggregate Phase Transitions Monitored Using Raman Spectroscopy," *Appl Spectrosc*, vol. 72, no. 1, pp. 102–113, Sep. 2017, doi: 10.1177/0003702817729347.
- [112] C. Cui and T. Fearn, "Modern practical convolutional neural networks for multivariate regression: Applications to NIR calibration," *Chemometrics and Intelligent Laboratory Systems*, vol. 182, pp. 9–20, 2018, doi: <https://doi.org/10.1016/j.chemolab.2018.07.008>.
- [113] J. Acquarelli, T. van Laarhoven, J. Gerretzen, T. N. Tran, L. M. C. Buydens, and E. Marchiori, "Convolutional neural networks for vibrational spectroscopic data analysis," *Anal Chim Acta*, vol. 954, pp. 22–31, 2017, doi: <https://doi.org/10.1016/j.aca.2016.12.010>.
- [114] T. Hastie, R. Tibshirani, and J. Friedman, "Linear Methods for Classification," in *The Elements of Statistical Learning: Data Mining, Inference, and Prediction*, T. Hastie, R. Tibshirani, and J. Friedman, Eds., New York, NY: Springer New York, 2009, pp. 101–137. doi: 10.1007/978-0-387-84858-7_4.

- [115] C. Cortes and V. Vapnik, "Support-vector networks," *Mach Learn*, vol. 20, no. 3, pp. 273–297, 1995, doi: 10.1007/BF00994018.
- [116] F. Pedregosa *et al.*, "Scikit-learn: Machine Learning in Python," *Journal of Machine Learning Research*, vol. 12, no. 85, pp. 2825–2830, 2011, [Online]. Available: <http://jmlr.org/papers/v12/pedregosa11a.html>
- [117] Y. LeCun, Y. Bengio, and G. Hinton, "Deep learning," *Nature*, vol. 521, no. 7553, pp. 436–444, 2015, doi: 10.1038/nature14539.
- [118] G. R. Criscuolo, "The genesis of peritumoral vasogenic brain edema and tumor cysts: a hypothetical role for tumor-derived vascular permeability factor.," *Yale J Biol Med*, vol. 66, no. 4, p. 277, 1993.
- [119] F. B. Furnari *et al.*, "Malignant astrocytic glioma: genetics, biology, and paths to treatment," *Genes & Development*, vol. 21, no. 21, pp. 2683–2710, Nov. 2007, [Online]. Available: <http://genesdev.cshlp.org/content/21/21/2683.abstract>
- [120] D. Sunit and M. P. A, "Angiogenesis in Glioblastoma," *New England Journal of Medicine*, vol. 369, no. 16, pp. 1561–1563, Apr. 2024, doi: 10.1056/NEJMcibr1309402.
- [121] D. Hambardzumyan and G. Bergers, "Glioblastoma: defining tumor niches," *Trends Cancer*, vol. 1, no. 4, pp. 252–265, 2015.
- [122] A. A. Abd-Elghany *et al.*, "Radiological characteristics of glioblastoma multiforme using CT and MRI examination," *J Radiat Res Appl Sci*, vol. 12, no. 1, pp. 289–293, 2019, doi: <https://doi.org/10.1080/16878507.2019.1655864>.
- [123] W. Stummer, H. Stepp, O. D. Wiestler, and U. Pichlmeier, "Randomized, Prospective Double-Blinded Study Comparing 3 Different Doses of 5-Aminolevulinic Acid for Fluorescence-Guided Resections of Malignant Gliomas.," *Neurosurgery*, vol. 81, no. 2, pp. 230–239, Aug. 2017, doi: 10.1093/neuros/nyx074.
- [124] A. Horská and P. B. Barker, "Imaging of Brain Tumors: MR Spectroscopy and Metabolic Imaging," *Neuroimaging Clin N Am*, vol. 20, no. 3, pp. 293–310, 2010, doi: <https://doi.org/10.1016/j.nic.2010.04.003>.

- [125] D. A. Orringer *et al.*, “Rapid intraoperative histology of unprocessed surgical specimens via fibre-laser-based stimulated Raman scattering microscopy,” *Nat Biomed Eng*, vol. 1, no. 2, p. 0027, 2017, doi: 10.1038/s41551-016-0027.
- [126] T. C. Hollon *et al.*, “Rapid Intraoperative Diagnosis of Pediatric Brain Tumors Using Stimulated Raman Histology,” *Cancer Res*, vol. 78, no. 1, pp. 278–289, Jan. 2018, doi: 10.1158/0008-5472.CAN-17-1974.
- [127] T. P. Plesec and R. A. Prayson, “Frozen Section Discrepancy in the Evaluation of Central Nervous System Tumors,” *Arch Pathol Lab Med*, vol. 131, no. 10, pp. 1532–1540, Oct. 2007, doi: 10.5858/2007-131-1532-FSDITE.
- [128] V. Nanarng, S. Jacob, D. Mahapatra, and J. E. Mathew, “Intraoperative diagnosis of central nervous system lesions: Comparison of squash smear, touch imprint, and frozen section,” *J Cytol*, vol. 32, no. 3, 2015, [Online]. Available: https://journals.lww.com/jocy/fulltext/2015/32030/intraoperative_diagnosis_of_central_nervous_system.2.aspx
- [129] M. Thotakura, N. Tirumalasetti, and R. Krishna, “Role of Ki-67 labeling index as an adjunct to the histopathological diagnosis and grading of astrocytomas,” *J Cancer Res Ther*, vol. 10, no. 3, 2014, [Online]. Available: https://journals.lww.com/cancerjournal/fulltext/2014/10030/role_of_ki_67_labeling_index_as_an_adjunct_to_the.34.aspx
- [130] A. K. Roshandel *et al.*, “The predictive capability of immunohistochemistry and DNA sequencing for determining TP53 functional mutation status: a comparative study of 41 glioblastoma patients,” *Oncotarget*, vol. 10, no. 58, p. 6204, 2019.
- [131] Y. Hai *et al.*, “IDH1 and IDH2 Mutations in Gliomas,” *New England Journal of Medicine*, vol. 360, no. 8, pp. 765–773, Mar. 2024, doi: 10.1056/NEJMoa0808710.
- [132] C. L. Gladson, R. A. Prayson, and W. M. Liu, “The pathobiology of glioma tumors,” *Annual Review of Pathology: Mechanisms of Disease*, vol. 5, pp. 33–50, 2010.
- [133] D. N. Louis *et al.*, “The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary,” *Acta Neuropathol*, vol. 131, no. 6, pp. 803–820, 2016, doi: 10.1007/s00401-016-1545-1.

- [134] S. N. Kalkanis *et al.*, “Raman spectroscopy to distinguish grey matter, necrosis, and glioblastoma multiforme in frozen tissue sections,” *J Neurooncol*, vol. 116, no. 3, pp. 477–485, 2014, doi: 10.1007/s11060-013-1326-9.
- [135] R. Kast *et al.*, “Identification of regions of normal grey matter and white matter from pathologic glioblastoma and necrosis in frozen sections using Raman imaging,” *J Neurooncol*, vol. 125, no. 2, pp. 287–295, 2015, doi: 10.1007/s11060-015-1929-4.
- [136] K. Gajjar *et al.*, “Diagnostic segregation of human brain tumours using Fourier-transform infrared and/or Raman spectroscopy coupled with discriminant analysis,” *Analytical Methods*, vol. 5, no. 1, pp. 89–102, 2013, doi: 10.1039/C2AY25544H.
- [137] M. Ji *et al.*, “Detection of human brain tumor infiltration with quantitative stimulated Raman scattering microscopy,” *Sci Transl Med*, vol. 7, no. 309, pp. 309ra163–309ra163, Oct. 2015, doi: 10.1126/scitranslmed.aab0195.
- [138] F.-K. Lu *et al.*, “Label-free neurosurgical pathology with stimulated Raman imaging,” *Cancer Res*, vol. 76, no. 12, pp. 3451–3462, 2016.
- [139] M. Jermyn *et al.*, “Highly Accurate Detection of Cancer & In Situ with Intraoperative, Label-Free, Multimodal Optical Spectroscopy,” *Cancer Res*, vol. 77, no. 14, pp. 3942 LP – 3950, Jul. 2017, doi: 10.1158/0008-5472.CAN-17-0668.
- [140] J. Desroches *et al.*, “Development and first in-human use of a Raman spectroscopy guidance system integrated with a brain biopsy needle,” *J Biophotonics*, vol. 12, no. 3, p. e201800396, Mar. 2019, doi: <https://doi.org/10.1002/jbio.201800396>.
- [141] É. Lemoine *et al.*, “Feature engineering applied to intraoperative in vivo Raman spectroscopy sheds light on molecular processes in brain cancer: a retrospective study of 65 patients,” *Analyst*, vol. 144, no. 22, pp. 6517–6532, 2019, doi: 10.1039/C9AN01144G.
- [142] M. Köhler, S. Machill, R. Salzer, and C. Krafft, “Characterization of lipid extracts from brain tissue and tumors using Raman spectroscopy and mass spectrometry,” *Anal Bioanal Chem*, vol. 393, no. 5, pp. 1513–1520, 2009, doi: 10.1007/s00216-008-2592-9.
- [143] C. Krafft, S. B. Sobottka, G. Schackert, and R. Salzer, “Near infrared Raman spectroscopic mapping of native brain tissue and intracranial tumors,” *Analyst*, vol. 130, no. 7, pp. 1070–1077, 2005, doi: 10.1039/B419232J.

- [144] I. P. Santos *et al.*, “Raman spectroscopy for cancer detection and cancer surgery guidance: translation to the clinics,” *Analyst*, vol. 142, no. 17, pp. 3025–3047, 2017, doi: 10.1039/C7AN00957G.
- [145] S. Hu, H. Kang, Y. Baek, G. El Fakhri, A. Kuang, and H. S. Choi, “Real-Time Imaging of Brain Tumor for Image-Guided Surgery,” *Adv Healthc Mater*, vol. 7, no. 16, p. 1800066, Aug. 2018, doi: <https://doi.org/10.1002/adhm.201800066>.
- [146] B. W. Maloney, D. M. McClatchy, B. W. Pogue, K. D. Paulsen, W. A. Wells, and R. J. Barth, “Review of methods for intraoperative margin detection for breast conserving surgery,” *J Biomed Opt*, vol. 23, no. 10, p. 100901, Oct. 2018, doi: 10.1117/1.JBO.23.10.100901.
- [147] A. Lukic *et al.*, “Endoscopic fiber probe for nonlinear spectroscopic imaging,” *Optica*, vol. 4, no. 5, pp. 496–501, 2017, doi: 10.1364/OPTICA.4.000496.
- [148] A. Lombardini *et al.*, “High-resolution multimodal flexible coherent Raman endoscope,” *Light Sci Appl*, vol. 7, no. 1, p. 10, 2018, doi: 10.1038/s41377-018-0003-3.
- [149] P. Zirak *et al.*, “Invited Article: A rigid coherent anti-Stokes Raman scattering endoscope with high resolution and a large field of view,” *APL Photonics*, vol. 3, no. 9, p. 092409, Aug. 2018, doi: 10.1063/1.5027182.
- [150] K. St-Arnaud *et al.*, “Development and characterization of a handheld hyperspectral Raman imaging probe system for molecular characterization of tissue on mesoscopic scales,” *Med Phys*, vol. 45, no. 1, pp. 328–339, Jan. 2018, doi: 10.1002/mp.12657.
- [151] E. Schmälzlin *et al.*, “Raman imaging with a fiber-coupled multichannel spectrograph,” *Sensors (Switzerland)*, vol. 14, no. 11, pp. 21968–21980, 2014, doi: 10.3390/s141121950.
- [152] E. Schmälzlin *et al.*, “Nonscanning large-area Raman imaging for ex vivo/in vivo skin cancer discrimination,” *J Biomed Opt*, vol. 23, no. 10, p. 1, 2018, doi: 10.1117/1.jbo.23.10.105001.
- [153] S. E. Bohndiek *et al.*, “A small animal Raman instrument for rapid, wide-area, spectroscopic imaging,” *Proceedings of the National Academy of Sciences*, vol. 110, no. 30, pp. 12408 LP – 12413, Jul. 2013, doi: 10.1073/pnas.1301379110.

- [154] C. Krafft *et al.*, “Perspectives, potentials and trends of ex vivo and in vivo optical molecular pathology,” *J Biophotonics*, vol. 11, no. 1, p. e201700236, Jan. 2018, doi: 10.1002/jbio.201700236.
- [155] F. Leblond, K. St-Arnaud, and F. Daoust, “Methods and systems for imaging a sample using Raman spectroscopy,” US11105682B2, 2021 [Online]. Available: <https://lens.org/149-115-466-793-756>
- [156] F. Leblond, F. Daoust, and D. Sandryne, “Methods for performing a Raman spectroscopy measurement on a sample and Raman spectroscopy systems,” US11796475B2, 2023 [Online]. Available: <https://lens.org/172-581-486-829-068>
- [157] F.-W. Kreth *et al.*, “Gross total but not incomplete resection of glioblastoma prolongs survival in the era of radiochemotherapy,” *Ann Oncol*, vol. 24, no. 12, pp. 3117–3123, Dec. 2013, doi: 10.1093/annonc/mdt388.
- [158] M. Lacroix *et al.*, “A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival,” *J Neurosurg*, vol. 95, no. 2, pp. 190–198, 2001, doi: 10.3171/jns.2001.95.2.0190.
- [159] F. G. M. Taylor *et al.*, “Preoperative high-resolution magnetic resonance imaging can identify good prognosis stage I, II, and III rectal cancer best managed by surgery alone: a prospective, multicenter, European study,” *Ann Surg*, vol. 253, no. 4, pp. 711–719, Apr. 2011, doi: 10.1097/SLA.0b013e31820b8d52.
- [160] T. Shien *et al.*, “Primary tumor resection improves the survival of younger patients with metastatic breast cancer,” *Oncol Rep*, vol. 21, no. 3, pp. 827–832, 2009, doi: 10.3892/or_00000291.
- [161] L. Tao, C. Yuan, Z. Ma, B. Jiang, and D. Xiu, “Surgical resection of a primary tumor improves survival of metastatic pancreatic cancer: a population-based study,” *Cancer Manag Res*, vol. 9, pp. 471–479, Oct. 2017, doi: 10.2147/CMAR.S145722.
- [162] M. S. Moran *et al.*, “Society of Surgical Oncology–American Society for Radiation Oncology Consensus Guideline on Margins for Breast-Conserving Surgery With Whole-Breast Irradiation in Stages I and II Invasive Breast Cancer,” *Journal of Clinical Oncology*, vol. 32, no. 14, pp. 1507–1515, Feb. 2014, doi: 10.1200/JCO.2013.53.3935.

- [163] I. D. Nagtegaal, C. A. M. Marijnen, E. K. Kranenbarg, C. J. H. van de Velde, and J. H. J. M. van Krieken, "Circumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit.," *Am J Surg Pathol*, vol. 26, no. 3, pp. 350–357, Mar. 2002, doi: 10.1097/00000478-200203000-00009.
- [164] S.-J. Chang, R. E. Bristow, and H.-S. Ryu, "Impact of Complete Cytoreduction Leaving No Gross Residual Disease Associated with Radical Cytoreductive Surgical Procedures on Survival in Advanced Ovarian Cancer," *Ann Surg Oncol*, vol. 19, no. 13, pp. 4059–4067, 2012, doi: 10.1245/s10434-012-2446-8.
- [165] J. M. Noh *et al.*, "Clinical significance of post-surgical residual tumor burden and radiation therapy in treating patients with lacrimal adenoid cystic carcinoma," *Oncotarget*, vol. 7, no. 37, pp. 60639–60646, Sep. 2016, doi: 10.18632/oncotarget.10259.
- [166] M. Mimouni *et al.*, "Reexcision for positive margins in breast cancer: A predictive score of residual disease," *Surg Oncol*, vol. 24, no. 3, pp. 129–135, 2015, doi: <https://doi.org/10.1016/j.suronc.2015.08.003>.
- [167] M. L. Piper *et al.*, "Success rates of re-excision after positive margins for invasive lobular carcinoma of the breast," *NPJ Breast Cancer*, vol. 5, no. 1, p. 29, 2019, doi: 10.1038/s41523-019-0125-7.
- [168] S. M. Chang *et al.*, "Perioperative complications and neurological outcomes of first and second craniotomies among patients enrolled in the Glioma Outcome Project.," *J Neurosurg*, vol. 98, no. 6, pp. 1175–1181, Jun. 2003, doi: 10.3171/jns.2003.98.6.1175.
- [169] V. Vendrely *et al.*, "Stratégies de préservation d'organe dans le traitement des cancers du rectum," *Cancer/Radiothérapie*, vol. 19, no. 6, pp. 404–409, 2015, doi: <https://doi.org/10.1016/j.canrad.2015.05.009>.
- [170] C. G. Hadjipanayis, G. Widhalm, and W. Stummer, "What is the Surgical Benefit of Utilizing 5-Aminolevulinic Acid for Fluorescence-Guided Surgery of Malignant Gliomas?," *Neurosurgery*, vol. 77, no. 5, pp. 663–673, Nov. 2015, doi: 10.1227/NEU.00000000000000929.

- [171] C. Krafft *et al.*, “Label-Free Molecular Imaging of Biological Cells and Tissues by Linear and Nonlinear Raman Spectroscopic Approaches,” *Angewandte Chemie International Edition*, vol. 56, no. 16, pp. 4392–4430, Apr. 2017, doi: 10.1002/anie.201607604.
- [172] H. Dickhaus, K. Ganser, A. Staubert, and M. Bonsanto, “Quantification of brain shift effects by MR-imaging,” *Engineering in Medicine and Biology Society*, vol. 00, no. C, pp. 0–3, 1997, doi: 10.1109/IEMBS.1997.757652.
- [173] K. Petrecca, M.-C. Guiot, V. Panet-Raymond, and L. Souhami, “Failure pattern following complete resection plus radiotherapy and temozolomide is at the resection margin in patients with glioblastoma,” *J Neurooncol*, vol. 111, no. 1, pp. 19–23, 2013, doi: 10.1007/s11060-012-0983-4.
- [174] C. W. Barth and S. L. Gibbs, “Fluorescence Image-Guided Surgery - a Perspective on Contrast Agent Development,” *Proc SPIE Int Soc Opt Eng*, vol. 11222, p. 112220J, Feb. 2020, doi: 10.1117/12.2545292.
- [175] S. K. Majumder, M. D. Keller, F. I. Boulos, M. C. Kelley, and A. Mahadevan-Jansen, “Comparison of autofluorescence, diffuse reflectance, and Raman spectroscopy for breast tissue discrimination,” *J Biomed Opt*, vol. 13, no. 5, pp. 1–11, Sep. 2008, doi: 10.1117/1.2975962.
- [176] Z. Huang *et al.*, “Integrated Raman spectroscopy and trimodal wide-field imaging techniques for real-time in vivo tissue Raman measurements at endoscopy,” *Opt Lett*, vol. 34, no. 6, pp. 758–760, 2009, doi: 10.1364/OL.34.000758.
- [177] S. P. Singh, A. Sahu, A. Deshmukh, P. Chaturvedi, and C. M. Krishna, “In vivo Raman spectroscopy of oral buccal mucosa: a study on malignancy associated changes (MAC)/cancer field effects (CFE).,” *Analyst*, vol. 138, no. 14, pp. 4175–4182, Jul. 2013, doi: 10.1039/c3an36761d.
- [178] W. C. Zúñiga *et al.*, “Raman Spectroscopy for Rapid Evaluation of Surgical Margins during Breast Cancer Lumpectomy,” *Sci Rep*, vol. 9, no. 1, p. 14639, 2019, doi: 10.1038/s41598-019-51112-0.

- [179] C. Krafft *et al.*, “Advances in optical biopsy--correlation of malignancy and cell density of primary brain tumors using Raman microspectroscopic imaging,” *Analyst*, vol. 137, no. 23, pp. 5533–5537, Dec. 2012, doi: 10.1039/c2an36083g.
- [180] Z. Liao, M. G. Lizio, C. Corden, H. Khout, E. Rakha, and I. Notingher, “Feasibility of integrated high-wavenumber Raman imaging and fingerprint Raman spectroscopy for fast margin assessment in breast cancer surgery,” *Journal of Raman Spectroscopy*, vol. n/a, no. n/a, Jun. 2020, doi: 10.1002/jrs.5937.
- [181] X. Feng *et al.*, “Biophysical basis of skin cancer margin assessment using Raman spectroscopy,” *Biomed Opt Express*, vol. 10, no. 1, pp. 104–118, Jan. 2019, doi: 10.1364/BOE.10.000104.
- [182] E. M. Barroso *et al.*, “Water Concentration Analysis by Raman Spectroscopy to Determine the Location of the Tumor Border in Oral Cancer Surgery,” *Cancer Res*, vol. 76, no. 20, pp. 5945 LP – 5953, Oct. 2016, doi: 10.1158/0008-5472.CAN-16-1227.
- [183] F.-K. Lu *et al.*, “Label-free DNA imaging in vivo with stimulated Raman scattering microscopy,” *Proceedings of the National Academy of Sciences*, p. 201515121, Aug. 2015, doi: 10.1073/pnas.1515121112.
- [184] R. Perez-Pueyo, M. J. Soneira, and S. Ruiz-Moreno, “Morphology-based automated baseline removal for Raman spectra of artistic pigments,” *Appl Spectrosc*, vol. 64, no. 6, pp. 595–600, Jun. 2010, doi: 10.1366/000370210791414281.
- [185] D. Lopez-Martinez, “Regularization approaches for support vector machines with applications to biomedical data,” *ArXiv*, 2017.
- [186] S. Raschka, “About Feature Scaling and Normalization and the effect of standardization for machine learning algorithms,” Jul. 2014, doi: 10.13140/2.1.4245.1849.
- [187] J. Platt, “Probabilistic Outputs for Support Vector Machines and Comparisons to Regularized Likelihood Methods,” *Adv. Large Margin Classif.*, vol. 10, Jun. 2000.
- [188] A. C. S. Talari, Z. Movasaghi, S. Rehman, and I. ur Rehman, “Raman Spectroscopy of Biological Tissues,” *Appl Spectrosc Rev*, vol. 50, no. 1, pp. 46–111, Jan. 2015, doi: 10.1080/05704928.2014.923902.

- [189] J. R. Beattie, S. E. J. Bell, C. Borggaard, and B. W. Moss, “Preliminary investigations on the effects of ageing and cooking on the Raman spectra of porcine longissimus dorsi,” *Meat Sci*, vol. 80, no. 4, pp. 1205–1211, 2008, doi: <https://doi.org/10.1016/j.meatsci.2008.05.016>.
- [190] M. MOTOYAMA, I. NAKAJIMA, H. OHMORI, G. WATANABE, and K. SASAKI, “A Raman Spectroscopic Method of Evaluating Fat Crystalline States and Its Application in Detecting Pork Fat,” *Japan Agricultural Research Quarterly: JARQ*, vol. 52, no. 1, pp. 17–22, 2018, doi: [10.6090/jarq.52.17](https://doi.org/10.6090/jarq.52.17).
- [191] D. T. Berhe, C. E. Eskildsen, R. Lametsch, M. S. Hviid, F. van den Berg, and S. B. Engelsens, “Prediction of total fatty acid parameters and individual fatty acids in pork backfat using Raman spectroscopy and chemometrics: Understanding the cage of covariance between highly correlated fat parameters,” *Meat Sci*, vol. 111, pp. 18–26, 2016, doi: <https://doi.org/10.1016/j.meatsci.2015.08.009>.
- [192] P. Matousek *et al.*, “Subsurface Probing in Diffusely Scattering Media Using Spatially Offset Raman Spectroscopy,” *Appl Spectrosc*, vol. 59, no. 4, pp. 393–400, 2005.
- [193] P. Matousek and N. Stone, “Recent advances in the development of Raman spectroscopy for deep non-invasive medical diagnosis,” *J Biophotonics*, vol. 6, no. 1, pp. 7–19, Jan. 2013, doi: [10.1002/jbio.201200141](https://doi.org/10.1002/jbio.201200141).
- [194] S. L. Jacques, “Optical properties of biological tissues: a review,” *Phys Med Biol*, vol. 58, no. 11, pp. R37–R61, 2013, doi: [10.1088/0031-9155/58/11/r37](https://doi.org/10.1088/0031-9155/58/11/r37).
- [195] L. Loh *et al.*, “Use of Intraoperative Frozen Section in the Surgical Management of Patients with Nonmelanoma Skin Cancer.,” *J Skin Cancer*, vol. 2021, p. 4944570, 2021, doi: [10.1155/2021/4944570](https://doi.org/10.1155/2021/4944570).
- [196] J. M. Laakman *et al.*, “Frozen Section Quality Assurance: Using Separate Frozen Section Slide Preparation Times and Interpretative Time Measurements to Improve Process,” *Am J Clin Pathol*, vol. 156, no. 3, pp. 461–470, Sep. 2021, doi: [10.1093/ajcp/aqaa259](https://doi.org/10.1093/ajcp/aqaa259).
- [197] M. Diem *et al.*, “Molecular pathology via IR and Raman spectral imaging.,” *Journal of biophotonics*, vol. 6, no. 11–12. Germany, pp. 855–886, Dec. 2013. doi: [10.1002/jbio.201300131](https://doi.org/10.1002/jbio.201300131).

- [198] H. J. Byrne *et al.*, “Spectropathology for the next generation: Quo vadis?,” *Analyst*, vol. 140, no. 7, pp. 2066–2073, 2015, doi: 10.1039/C4AN02036G.
- [199] K. Hanna, E. Krzoska, A. M. Shaaban, D. Muirhead, R. Abu-Eid, and V. Speirs, “Raman spectroscopy: current applications in breast cancer diagnosis, challenges and future prospects,” *Br J Cancer*, vol. 126, no. 8, pp. 1125–1139, 2022, doi: 10.1038/s41416-021-01659-5.
- [200] T. Hollon and D. A. Orringer, “Label-free brain tumor imaging using Raman-based methods,” *J Neurooncol*, vol. 151, no. 3, pp. 393–402, 2021, doi: 10.1007/s11060-019-03380-z.
- [201] H. Noothalapati, K. Iwasaki, and T. Yamamoto, “Non-invasive diagnosis of colorectal cancer by Raman spectroscopy: Recent developments in liquid biopsy and endoscopy approaches,” *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 258, p. 119818, 2021, doi: <https://doi.org/10.1016/j.saa.2021.119818>.
- [202] H. Lui, J. Zhao, D. McLean, and H. Zeng, “Real-time Raman spectroscopy for in vivo skin cancer diagnosis,” *Cancer Res*, vol. 72, no. 10, pp. 2491–2500, May 2012, doi: 10.1158/0008-5472.CAN-11-4061.
- [203] J. Zhao, H. Zeng, S. Kalia, and H. Lui, “Using Raman Spectroscopy to Detect and Diagnose Skin Cancer In Vivo,” *Dermatol Clin*, vol. 35, no. 4, pp. 495–504, 2017, doi: <https://doi.org/10.1016/j.det.2017.06.010>.
- [204] I. P. Santos *et al.*, “Improving clinical diagnosis of early-stage cutaneous melanoma based on Raman spectroscopy,” *Br J Cancer*, vol. 119, no. 11, pp. 1339–1346, 2018, doi: 10.1038/s41416-018-0257-9.
- [205] F. Gaba, W. J. Tipping, M. Salji, K. Faulds, D. Graham, and H. Y. Leung, “Raman Spectroscopy in Prostate Cancer: Techniques, Applications and Advancements,” *Cancers (Basel)*, vol. 14, no. 6, 2022, doi: 10.3390/cancers14061535.
- [206] M. Pinto *et al.*, “Integration of a Raman spectroscopy system to a robotic-assisted surgical system for real-time tissue characterization during radical prostatectomy procedures,” *J Biomed Opt*, vol. 24, no. 2, pp. 1–10, Feb. 2019, [Online]. Available: <https://doi.org/10.1117/1.JBO.24.2.025001>

- [207] S. Tanwar, S. K. Paidi, R. Prasad, R. Pandey, and I. Barman, “Advancing Raman spectroscopy from research to clinic: Translational potential and challenges,” *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 260, p. 119957, 2021, doi: <https://doi.org/10.1016/j.saa.2021.119957>.
- [208] A. N. Ramya, J. S. Arya, M. Madhukrishnan, S. Shamjith, M. S. Vidyalekshmi, and K. K. Maiti, “Raman Imaging: An Impending Approach Towards Cancer Diagnosis,” *Chem Asian J*, vol. 16, no. 5, pp. 409–422, Mar. 2021, doi: <https://doi.org/10.1002/asia.202001340>.
- [209] F. Daoust *et al.*, “Handheld macroscopic Raman spectroscopy imaging instrument for machine-learning-based molecular tissue margins characterization,” *J Biomed Opt*, vol. 26, no. 2, pp. 1–18, Feb. 2021, doi: 10.1117/1.JBO.26.2.022911.
- [210] N. Yokoya, N. Miyamura, and A. Iwasaki, “Detection and correction of spectral and spatial misregistrations for hyperspectral data using phase correlation method,” *Appl. Opt.*, vol. 49, no. 24, pp. 4568–4575, Aug. 2010, doi: 10.1364/AO.49.004568.
- [211] G. Sheehy *et al.*, “Open-sourced Raman spectroscopy data processing package implementing a baseline removal algorithm validated from multiple datasets acquired in human tissue and biofluids,” *J Biomed Opt*, vol. 28, no. 2, p. 25002, Feb. 2023, doi: 10.1117/1.JBO.28.2.025002.
- [212] A. Beganović, L. M. Hawthorne, K. Bach, and C. W. Huck, “Critical Review on the Utilization of Handheld and Portable Raman Spectrometry in Meat Science,” *Foods*, vol. 8, no. 2. 2019. doi: 10.3390/foods8020049.
- [213] J. Platt, “Probabilistic outputs for support vector machines and comparisons to regularized likelihood methods,” *Advances in large margin classifiers*, vol. 10, no. 3, pp. 61–74, 1999.
- [214] American National Standards Institute, “ANSI Z136.1: American National Standard for Safe Use of Lasers,” 2014.
- [215] American National Standards Institute, “ANSI Z136.3-2018 Safe Use Of Lasers In Health Care,” 2018.
- [216] E. L. Jones *et al.*, “Randomized trial of hyperthermia and radiation for superficial tumors,” *J Clin Oncol*, vol. 23, no. 13, pp. 3079–3085, May 2005, doi: 10.1200/JCO.2005.05.520.

- [217] N. R. Datta *et al.*, “Quantification of thermal dose in moderate clinical hyperthermia with radiotherapy: a relook using temperature–time area under the curve (AUC),” *International Journal of Hyperthermia*, vol. 38, no. 1, pp. 296–307, Jan. 2021, doi: 10.1080/02656736.2021.1875060.
- [218] R. M. Jones *et al.*, “Accumulated thermal dose in MRI-guided focused ultrasound for essential tremor: repeated sonications with low focal temperatures,” *Journal of Neurosurgery JNS*, vol. 132, no. 6, pp. 1802–1809, 2020, doi: 10.3171/2019.2.JNS182995.
- [219] S. A. Sapareto and W. C. Dewey, “Thermal dose determination in cancer therapy.,” *Int J Radiat Oncol Biol Phys*, vol. 10, no. 6, pp. 787–800, Jun. 1984, doi: 10.1016/0360-3016(84)90379-1.
- [220] A. B. HARRIS, L. ERICKSON, J. H. KENDIG, S. MINGRINO, and S. GOLDRING, “Observations on selective brain heating in dogs.,” *J Neurosurg*, vol. 19, pp. 514–521, Jun. 1962, doi: 10.3171/jns.1962.19.6.0514.
- [221] G. C. van Rhoon, T. Samaras, P. S. Yarmolenko, M. W. Dewhurst, E. Neufeld, and N. Kuster, “CEM43°C thermal dose thresholds: a potential guide for magnetic resonance radiofrequency exposure levels?,” *Eur Radiol*, vol. 23, no. 8, pp. 2215–2227, Aug. 2013, doi: 10.1007/s00330-013-2825-y.
- [222] N. Bosschaart, G. J. Edelman, M. C. G. Aalders, T. G. Van Leeuwen, and D. J. Faber, “A literature review and novel theoretical approach on the optical properties of whole blood,” *Lasers Med Sci*, vol. 29, no. 2, pp. 453–479, 2014, doi: 10.1007/s10103-013-1446-7.
- [223] R. Matika, M. Ibrahim, and A. Patwardhan, “The importance of body temperature: An anesthesiologist’s perspective,” *Temperature*, vol. 4, no. 1, pp. 9–12, Mar. 2017, doi: 10.1080/23328940.2016.1243509.
- [224] M. A. Ansari, M. Erfanzadeh, and E. Mohajerani, “Mechanisms of Laser-Tissue Interaction: II. Tissue Thermal Properties.,” *J Lasers Med Sci*, vol. 4, no. 3, pp. 99–106, 2013.
- [225] A. Das, A. Sarda, and A. De, “Cooling Devices in Laser therapy.,” *J Cutan Aesthet Surg*, vol. 9, no. 4, pp. 215–219, 2016, doi: 10.4103/0974-2077.197028.
- [226] NIHR Global Health Research Unit on Global Surgery, “Quality and outcomes in global cancer surgery: protocol for a multicentre, international, prospective cohort study

- (GlobalSurg 3),” *BMJ Open*, vol. 9, no. 5, p. e026646, May 2019, doi: 10.1136/bmjopen-2018-026646.
- [227] P. Dell’Oglio *et al.*, “Precision surgery,” *Am J Nucl Med Mol Imaging*, vol. 12, no. 2, pp. 74–80, Apr. 2022.
- [228] J. D. Phillips and S. L. Wong, “Patient-Reported Outcomes in Surgical Oncology: An Overview of Instruments and Scores,” *Ann Surg Oncol*, vol. 27, no. 1, pp. 45–53, 2020, doi: 10.1245/s10434-019-07752-7.
- [229] G. Houvenaeghel *et al.*, “Positive or close margins: Reoperation rate and second conservative resection or total mastectomy?,” *Cancer Manag Res*, vol. 11, pp. 2507–2516, 2019, doi: 10.2147/CMAR.S190852.
- [230] A. K. Schmitz *et al.*, “Second-look surgery after pediatric brain tumor resection – Single center analysis of morbidity and volumetric efficacy,” *Brain and Spine*, vol. 2, p. 100865, 2022, doi: <https://doi.org/10.1016/j.bas.2022.100865>.
- [231] R. J. Buesa, “Histology: a unique area of the medical laboratory,” *Ann Diagn Pathol*, vol. 11, no. 2, pp. 137–141, 2007, doi: <https://doi.org/10.1016/j.anndiagpath.2007.01.002>.
- [232] M. Titford and B. Bowman, “What May the Future Hold for Histotechnologists?,” *Lab Med*, vol. 43, no. suppl_2, pp. e5–e10, Nov. 2012, doi: 10.1309/LMXB668WDCBIAWJL.
- [233] D. M. Metter, T. J. Colgan, S. T. Leung, C. F. Timmons, and J. Y. Park, “Trends in the US and Canadian Pathologist Workforces From 2007 to 2017,” *JAMA Netw Open*, vol. 2, no. 5, pp. e194337–e194337, May 2019, doi: 10.1001/jamanetworkopen.2019.4337.
- [234] R. M. Young, A. Jamshidi, G. Davis, and J. H. Sherman, “Current trends in the surgical management and treatment of adult glioblastoma,” *Ann Transl Med*, vol. 3, no. 9, Jun. 2015, doi: 10.3978/j.issn.2305-5839.2015.05.10.
- [235] O. Eidel *et al.*, “Tumor Infiltration in Enhancing and Non-Enhancing Parts of Glioblastoma: A Correlation with Histopathology,” *PLoS One*, vol. 12, no. 1, pp. e0169292–, Jan. 2017, [Online]. Available: <https://doi.org/10.1371/journal.pone.0169292>

- [236] M. Rahman *et al.*, “The effects of new or worsened postoperative neurological deficits on survival of patients with glioblastoma,” *Journal of Neurosurgery JNS*, vol. 127, no. 1, pp. 123–131, 2017, doi: <https://doi.org/10.3171/2016.7.JNS16396>.
- [237] S. J. Han *et al.*, “Subcortical stimulation mapping of descending motor pathways for perirolandic gliomas: assessment of morbidity and functional outcome in 702 cases,” *J Neurosurg*, vol. 131, no. 1, pp. 201–208, 2019, doi: <https://doi.org/10.3171/2018.3.JNS172494>.
- [238] T. J. Brown *et al.*, “Association of the Extent of Resection With Survival in Glioblastoma: A Systematic Review and Meta-analysis,” *JAMA Oncol*, vol. 2, no. 11, pp. 1460–1469, Nov. 2016, doi: [10.1001/jamaoncol.2016.1373](https://doi.org/10.1001/jamaoncol.2016.1373).
- [239] M. Weller, T. Cloughesy, J. R. Perry, and W. Wick, “Standards of care for treatment of recurrent glioblastoma-are we there yet?,” *Neuro-Oncology*, vol. 15, no. 1, pp. 4–27, Jan. 2013, doi: [10.1093/neuonc/nos273](https://doi.org/10.1093/neuonc/nos273).
- [240] A. Tosoni, E. Franceschi, R. Poggi, and A. A. Brandes, “Relapsed Glioblastoma: Treatment Strategies for Initial and Subsequent Recurrences,” *Curr Treat Options Oncol*, vol. 17, no. 9, p. 49, 2016, doi: [10.1007/s11864-016-0422-4](https://doi.org/10.1007/s11864-016-0422-4).
- [241] D. DePaoli *et al.*, “Rise of Raman spectroscopy in neurosurgery: a review,” *J Biomed Opt*, vol. 25, no. 5, p. 050901, May 2020, doi: [10.1117/1.JBO.25.5.050901](https://doi.org/10.1117/1.JBO.25.5.050901).
- [242] S. Murugappan, S. A. M. Tofail, and N. D. Thorat, “Raman Spectroscopy: A Tool for Molecular Fingerprinting of Brain Cancer,” *ACS Omega*, vol. 8, no. 31, pp. 27845–27861, Aug. 2023, doi: [10.1021/acsomega.3c01848](https://doi.org/10.1021/acsomega.3c01848).
- [243] K. Ember *et al.*, “In Situ Brain Tumor Detection Using the Raman Spectroscopy Sentry System – Results of a Multicenter Study,” *Communications Medicine*, May 2024.
- [244] F. Daoust *et al.*, “A clinical Raman spectroscopy imaging system and safety requirements for in situ intraoperative tissue characterization,” *Analyst*, vol. 148, no. 9, pp. 1991–2001, 2023, doi: [10.1039/D2AN01946A](https://doi.org/10.1039/D2AN01946A).
- [245] W. C. Zúñiga *et al.*, “Raman Spectroscopy for Rapid Evaluation of Surgical Margins during Breast Cancer Lumpectomy,” *Sci Rep*, vol. 9, no. 1, p. 14639, 2019, doi: [10.1038/s41598-019-51112-0](https://doi.org/10.1038/s41598-019-51112-0).

- [246] D. Lunter, V. Klang, D. Kocsis, Z. Varga-Medveczky, S. Berkó, and F. Erdő, “Novel aspects of Raman spectroscopy in skin research,” *Exp Dermatol*, vol. 31, no. 9, pp. 1311–1329, Sep. 2022, doi: <https://doi.org/10.1111/exd.14645>.
- [247] M. Paraskevaïdi *et al.*, “Clinical applications of infrared and Raman spectroscopy in the fields of cancer and infectious diseases,” *Appl Spectrosc Rev*, vol. 56, no. 8–10, pp. 804–868, Nov. 2021, doi: [10.1080/05704928.2021.1946076](https://doi.org/10.1080/05704928.2021.1946076).
- [248] B. C. Windom and D. W. Hahn, “Raman Spectroscopy,” in *Encyclopedia of Tribology*, Q. J. Wang and Y.-W. Chung, Eds., Boston, MA: Springer US, 2013, pp. 2742–2747. doi: [10.1007/978-0-387-92897-5_1216](https://doi.org/10.1007/978-0-387-92897-5_1216).
- [249] N. Kuhar, S. Sil, T. Verma, and S. Umapathy, “Challenges in application of Raman spectroscopy to biology and materials,” *RSC Adv*, vol. 8, no. 46, pp. 25888–25908, 2018, doi: [10.1039/C8RA04491K](https://doi.org/10.1039/C8RA04491K).
- [250] B. Hernández, F. Pflüger, S. G. Kruglik, and M. Ghomi, “Characteristic Raman lines of phenylalanine analyzed by a multiconformational approach,” *Journal of Raman Spectroscopy*, vol. 44, no. 6, pp. 827–833, Jun. 2013, doi: <https://doi.org/10.1002/jrs.4290>.
- [251] R. P. Aguiar, E. T. Falcão, C. A. Pasqualucci, and L. Silveira, “Use of Raman spectroscopy to evaluate the biochemical composition of normal and tumoral human brain tissues for diagnosis,” *Lasers Med Sci*, vol. 37, no. 1, pp. 121–133, 2022, doi: [10.1007/s10103-020-03173-1](https://doi.org/10.1007/s10103-020-03173-1).
- [252] H. Takeuchi, “Raman structural markers of tryptophan and histidine side chains in proteins,” *Biopolymers*, vol. 72, no. 5, pp. 305–317, Jan. 2003, doi: <https://doi.org/10.1002/bip.10440>.
- [253] A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, and M. Baranska, “Raman spectroscopy of proteins: a review,” *Journal of Raman Spectroscopy*, vol. 44, no. 8, pp. 1061–1076, Aug. 2013, doi: <https://doi.org/10.1002/jrs.4335>.
- [254] G. Zhu, X. Zhu, Q. Fan, and X. Wan, “Raman spectra of amino acids and their aqueous solutions,” *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 78, no. 3, pp. 1187–1195, 2011, doi: <https://doi.org/10.1016/j.saa.2010.12.079>.

- [255] Z. Movasaghi, S. Rehman, and I. U. Rehman, “Raman Spectroscopy of Biological Tissues,” *Appl Spectrosc Rev*, vol. 42, no. 5, pp. 493–541, Sep. 2007, doi: 10.1080/05704920701551530.
- [256] K. Czamara, K. Majzner, M. Z. Pacia, K. Kochan, A. Kaczor, and M. Baranska, “Raman spectroscopy of lipids: a review,” *Journal of Raman Spectroscopy*, vol. 46, no. 1, pp. 4–20, Jan. 2015, doi: <https://doi.org/10.1002/jrs.4607>.
- [257] J. K. W. Gerritsen *et al.*, “Safe surgery for glioblastoma: Recent advances and modern challenges,” *Neurooncol Pract*, vol. 9, no. 5, pp. 364–379, Oct. 2022, doi: 10.1093/nop/npac019.
- [258] S. David *et al.*, “*In situ* Raman spectroscopy and machine learning unveil biomolecular alterations in invasive breast cancer,” *J Biomed Opt*, vol. 28, no. 3, p. 036009, Mar. 2023, doi: 10.1117/1.JBO.28.3.036009.
- [259] K. Aubertin *et al.*, “Mesoscopic characterization of prostate cancer using Raman spectroscopy: potential for diagnostics and therapeutics,” *BJU Int*, vol. 122, no. 2, pp. 326–336, Aug. 2018, doi: <https://doi.org/10.1111/bju.14199>.
- [260] F. Picot *et al.*, “Image-guided Raman spectroscopy navigation system to improve transperineal prostate cancer detection. Part 1: Raman spectroscopy fiber-optics system and *in situ* tissue characterization,” *J Biomed Opt*, vol. 27, no. 9, p. 095003, Sep. 2022, doi: 10.1117/1.JBO.27.9.095003.
- [261] D. Grajales *et al.*, “Image-guided Raman spectroscopy navigation system to improve transperineal prostate cancer detection. Part 2: *in-vivo* tumor-targeting using a classification model combining spectral and MRI-radiomics features,” *J Biomed Opt*, vol. 27, no. 9, p. 095004, Sep. 2022, doi: 10.1117/1.JBO.27.9.095004.
- [262] F. Leblond *et al.*, “Subsecond lung cancer detection within a heterogeneous background of normal and benign tissue using single-point Raman spectroscopy,” *J Biomed Opt*, vol. 28, no. 9, p. 090501, Sep. 2023, doi: 10.1117/1.JBO.28.9.090501.
- [263] S. David *et al.*, “Multispectral label-free Raman spectroscopy can detect ovarian and endometrial cancer with high accuracy,” *J Biophotonics*, vol. 15, no. 2, p. e202100198, Feb. 2022, doi: <https://doi.org/10.1002/jbio.202100198>.

- [264] G. Sheehy *et al.*, “Open-sourced Raman spectroscopy data processing package implementing a baseline removal algorithm validated from multiple datasets acquired in human tissue and biofluids,” *J Biomed Opt*, vol. 28, no. 2, p. 025002, Feb. 2023, doi: 10.1117/1.JBO.28.2.025002.
- [265] M. Kopec, M. Błaszczuk, M. Radek, and H. Abramczyk, “Raman imaging and statistical methods for analysis various type of human brain tumors and breast cancers,” *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 262, p. 120091, 2021, doi: <https://doi.org/10.1016/j.saa.2021.120091>.
- [266] A.-A. Grosset *et al.*, “Identification of intraductal carcinoma of the prostate on tissue specimens using Raman micro-spectroscopy: A diagnostic accuracy case–control study with multicohort validation,” *PLoS Med*, vol. 17, no. 8, pp. e1003281-, Aug. 2020, [Online]. Available: <https://doi.org/10.1371/journal.pmed.1003281>
- [267] K. J. Ember *et al.*, “Saliva-based detection of COVID-19 infection in a real-world setting using reagent-free Raman spectroscopy and machine learning,” *J Biomed Opt*, vol. 27, no. 2, pp. 1–24, Feb. 2022, doi: 10.1117/1.JBO.27.2.025002.
- [268] K. Ember, F. Daoust, N. Ksantini, N. Picot, F. Leblond, and D. Trudel, “Methods and systems for interrogating a drop of saliva using Raman spectroscopy,” WO 2022/261781 A1, 2022 [Online]. Available: <https://lens.org/192-856-710-619-416>
- [269] S. David *et al.*, “Towards Non-contact and Real-time Large Field-of-view Single-band Raman Spectroscopy Imaging in Brain Cancer Surgery,” *[Manuscript submitted for publication]*.
- [270] S. David *et al.*, “Machine learning and hyperspectral Raman imaging identify invasive breast cancer,” *[Manuscript submitted for publication]*.

APPENDIX A – PROPOSED SYSTEM OPTIMIZATIONS

Proposed system optimizations

Throughout the work and manuscripts presented in this dissertation, several means of improving the system were identified. Here are summarized the suggested modifications (from simple to more complex modifications) to improve the imaging system's acquisition speed, signal quality and practicality. Figure 8-1 illustrates the imaging probe's optomechanical design to assist in understanding some of the listed suggestions.

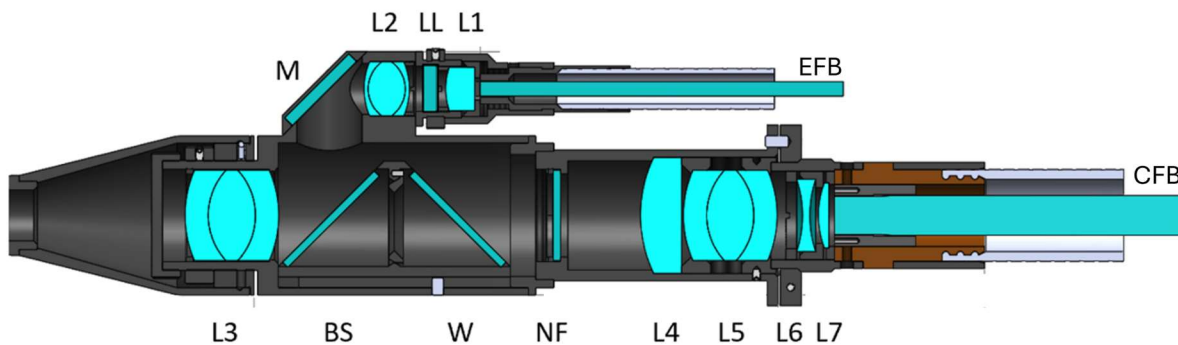


Figure 8-1 : Current imaging probe's optomechanical schematic showing all optical components and their labels (i.e. laser line filter (LL), lens (L1, L2... L7), beam splitter (BS), optical window (W), mirror (M), notch filter (NF)), excitation fiber bundle (EFB) and collection fiber bundle (CFB).

1. Laser line filter position: The first improvement proposed is to install the laser line filter (LL) between the mirror (M) and the beam splitter (BS) instead of its current location on Figure 8-1. This filter (LL01-785-12.5, Semrock, USA) is located where the excitation beam is divergent (i.e. not collimated) thus significantly clipping some of the 785 nm laser power. No indents nor mounting edges on the current probe design enable this filter to be placed in the preferred collimated pathway which led the decision for its current suboptimal

placement. A 35% difference of excitation laser power was measured at the probe tip due to this suboptimal filter location.

2. AR coatings on custom lenses: The second improvement to the imaging probe consists of applying (AR) coatings on the custom lenses L1 (AR coating 400 – 800 nm) and L7 (AR coating 400 – 1000 nm) shown on Figure 8-1. These are the only uncoated lenses in the probe, and each of their uncoated surfaces are responsible for a 4% decrease of light transmission due to refractive index differences with air (total 8% transmission loss for each lens). AR coatings on these lenses would increase by 8% the total laser power at the sample (coating of L1) and by 8% the luminescence collected by the system (coating of L7).
3. 785 nm laser NA match: The current custom 5 W 785 nm laser (Innovative photonics solutions, USA) has a 0.39 NA which is mismatched with the designed 0.22 NA of the excitation branch and imaging probe. Installing a laser with identical technical specifications and a matched NA of 0.22 would result in a gain ~35 % of laser power at the sample. This gain was calculated based on the measured power difference between laser's output and the excitation fiber bundle's proximal end.
4. Upgrade CCD camera for Raman imaging: The system's CCD camera (Andor Newton 920) has an average quantum efficiency (QE) of ~83 % between 800 nm (QE 96 %) and 940 nm (QE 70 %). A superior high NIR sensitivity camera (Blaze 400-HR/HRX, Princeton Instruments, USA) is available on the market and would offer a QE of >95 % over the entire spectral range of the Raman imaging spectrometer (i.e. 800 – 940 nm). This gain in QE would add an average of >14 % more collected Raman light with minimal changes required to the system's optomechanical design.
5. Install bright field illumination light ring: A bright field imaging modality was integrated to the system design to allow users to visualize tissue structures (e.g. tissue borders) and provide a frame of reference when imaging tissues. Albeit designed with a collinear bright field excitation source, all bright field images presented in the published and submitted manuscripts were acquired with bright field illumination from an exterior near vertical light source. A near vertical light source was employed since the system's collinear bright field illumination led to specular reflections. Additionally, when using the system's bright field illumination, observed images were green-dominated. This color imbalance was caused by

the system's laser-line filter (LL01-785-12.5, Semrock, USA) in the excitation optical path which filtered blue and red light from the bright field source, combined to the significant reflection of blue light (~80%) by the probe beam splitter (DI03-R785, Semrock, USA). Absence of intensity in the RGB camera's blue channel during colinear bright field imaging prevented any white balancing of images. Figure 8-2 shows a bright field image of a brain tumor border acquired with the system in three separate lighting conditions: the first using only an exterior near vertical bright field source, the second with the colinear and exterior bright field source and finally, a third with only the colinear source. From Figure 8-2, it is apparent that collinear bright field illumination results in suboptimal sample visualization.

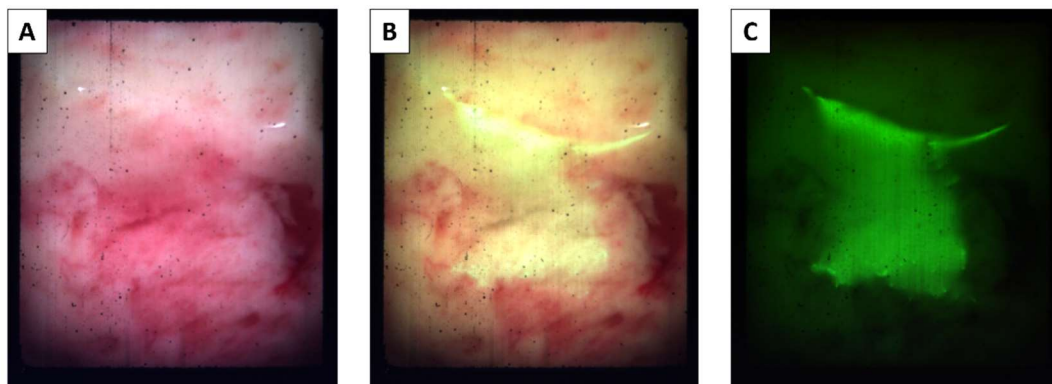


Figure 8-2: Bright field image of a brain tumor border acquired with A) angled bright light, B) angled and colinear bright light simultaneously and C) only colinear bright light. Collinear bright field illumination produces green dominated images with specular reflections.

Employing an exterior illuminating bright light is feasible for intraoperative measurements (e.g. with a surgical microscope) if conducted without the contact probe muzzle. However, a design modification is required for users looking to acquire bright field image while employing the contact probe muzzle.

To correct specular reflections and mitigate green dominant RGB images, one proposed design change to is to install an LED ring light (e.g. 100-009304, StarLight Opto-Electronics, Germany) at the tip of the imaging probe as shown in Figure 8-3. This light ring would provide near vertical and uniform bright field illumination of the sample with or without the contact muzzle. Carefully selected LEDs with no emission wavelengths above 800 nm (or covered with a high pass filter with <800 nm cut-off) would allow bright

field illumination and visualization of the sample while conducting Raman imaging. With this modification, the original bright field source may be removed or replaced by an alternative light source for a different modality (e.g. a blue LED for intrinsic fluorescence imaging).



Figure 8-3 : Proposed LED ring light location at the tip of the imaging probe for near vertical bright field illumination.

6. Replace focusing triplet lens: The achromatic triplet L3 (#67-422, Edmund Optics, USA) has an intrinsic luminescence response which is captured by the imaging system during Raman measurements. Replacing this lens with by one or many lenses with Raman grade material (i.e. material with minimal luminescence between 800 and 950 nm) could improve signal quality in measurements resulting in little signal (e.g. tissues with significant absorption). Optical simulations (OpticStudio®, Zemax, USA) were conducted by Optech to evaluate the impact of replacing of L3 with a single custom plano-convex Raman grade CaF_2 lens. These simulations demonstrated that this replacement would create chromatic aberrations that would prevent a correct focusing in NIR and bright field imaging. These aberrations could not be corrected without significant changes to the overall probe optomechanical design. Conclusions from these simulations suggest replacing L3 will require a customized Raman grade doublet or triplet lens and a significant optomechanical redesign of the imaging probe.
7. Replace contact muzzle epoxy: In Chapter 5, it was observed that the contact muzzle epoxy (EPO-TEK® 353ND, Epoxy Technology Inc., USA) generates significant background signal which is captured by the imaging system during measurements. This added background signal is accentuated in samples with higher scattering coefficients (e.g.

adipose tissue) and hinders Raman SNR especially in low SNR samples (e.g. human brain). An alternative, less fluorescent epoxy, is required to replace the current epoxy. A suggested alternative is the following epoxy (EPO-TEK® 320, Epoxy Technology Inc., USA). Background signal from the contact muzzle and leak tests will need to be evaluated to ensure this muzzle is also suitable for the clinical setting. An alternative optomechanical design of the probe muzzle may also be investigated to allow the muzzle window to be fixed to the muzzle body while maintaining hermiticity and reducing the exposure of the epoxy to the scattered laser during measurements.

8. Alternative optomechanical design for simpler optical alignment: One of the practical challenges of setting up the imaging system consists of setting a proper optical alignment throughout the system. This is particularly challenging to accomplish in the imaging probe where excitation and collection images need to be aligned in rotation (α), position (x and y axis), and focal plane (z axis). Rotation alignment ensures the excitation and collection lines are completely superimposed. Position alignment ensures the entire FOV is covered by both excitation and collection. Finally, focal plane ensures the excitation line is at its highest laser intensity and that the collected image quality is optimal. The current design would benefit from rotation mounts (e.g. RSP1/M, Thorlabs, USA) for both the excitation and collection fiber bundles at the imaging probe. These mounts would prevent the probe's focal plane from shifting while attempting to align the orientation of the excitation and collection fiber bundles. To facilitate position alignment, reinforcement of the excitation path of the imaging probe is required. The applied torque from the excitation bundle weight onto the imaging probe results in slight bending of the excitation optical assembly and a slight shift in the excitation image position. Such bending also introduces a slight angle in the excitation beam and a clear cut at one extremity of the laser line profile when using a sharp transition beam splitter (LPD02-785RU-25, Semrock, USA). Such a cut was corrected using a beam splitter with a more forgiving edge wavelength of 800 nm (DI03-R785, Semrock, USA). It is not clear however if any laser power is lost in the imaging probe due to clipping caused by this optomechanical flaw. Finally, the imaging probe would benefit from a quick mounting assembly for mounting and dismounting of the excitation and collection fiber bundles. This practical addition would aim to facilitate probe and system transportation and maintenance.

9. Reduced probe footprint and weight: An additional suggested modification to improve practicality of use consists of reducing the probe footprint and weight. The probe is bulky (4.5 (width) x 7 (height) cm), long (18 cm), heavy (0.6 kg without bundles) and has an even bigger footprint when mounted on its articulated arm. Two methods have been identified to reduce its footprint and weight. The first being a reduction in length in the collection pathway while ensuring minimal disruption to the bright field image resolution. Optical simulations suggest the window (W) does not contribute significantly to the visible and NIR spatial resolution and may be removed. The second method involves trimming the probe optomechanical design to remove excess material present on the body and surrounding the probe. The current design already includes significant material which may be trimmed without compromising the structural integrity of the probe. Finally, as a more radical approach, an optical redesign may be considered to shorten the probe's collecting optical pathway to a more practical length of <14 cm. This is expected to come at the cost of image quality in bright field imaging. Any reduction in probe footprint would also enable a reduction in footprint from the custom probe mount required to handle the probe with the passive support arm.
10. Reinforcement of the cart wheels: On two separate occasions, moving the system to a new hospital led to cart wheels breaking and a potential catastrophic system collapse. This occurred because the system's wheels (PSY140, Thorlabs, USA) are fragile and prone to breaking when attempting to cross uneven surfaces (e.g. water drains and door thresholds) with the full the weight of the system (estimated at 170 kg). The cart (POC001, Thorlabs, USA) is rated for a weight up to 320 kg however, it is apparent that its wheels require reinforcement to prevent the system from toppling over. No issues were encountered while the system was being utilized in two hospitals (MNI and MUCH) and the CRCHUM where no uneven surfaces were encountered.
11. Alternative 785 nm relative intensity standard for system response correction: Correcting the system response using the NIST 785 nm relative intensity standard (SRM 2241, NIST, USA) in Chapter 4 to 7 introduced several undesirable artefacts in pre-processed Raman images. Firstly, scratches on the surface of the standard (occurred due to wear and tear) introduced spatial artefacts across the image. Secondly, the similar size (10 x 10 mm) of the standard to the system's FOV created visible border effects (e.g. exaggerated system

response correction) on Raman images. Identifying an alternative reference material for system response correction is recommended to work with an immaculate surface quality with dimensions exceeding 10 x 10 mm. This is further needed since the SRM 2241 reference material is hard to come and appears to be no longer manufactured.

The proposed modifications 1 through 5 would improve light intensity at the sample by 257% (35% power loss due to laser line positioning, 35% power loss reduction due to laser NA matching and 8% power loss due to absence of AR lens coating) and would increase collection efficiency by 24% (8% collection loss from absence of AR lens coatings and 14% QE difference between the currently used and the proposed sensor). These proposed modifications would increase laser intensity at the sample and the system's collection efficiency resulting in an overall Raman photon count increase of >300%. The improvements would therefore enable reduced acquisition times and bring current brain characterisation exposure times from 20 – 40 min down to 6 – 13 min.

For additional improvements in excitation and collection efficiency, investigated either the impact of removing the imaging bundles or changing the image acquisition parameters is recommended. Light losses are dominated due to the use of imaging fiber bundles which have only 45% transmission per bundle. The imaging bundles give the system the flexibility of position a probe to measure human tissue *in vivo* during surgery, however, imaging bundles are not necessary if conducting solely *ex vivo* specimen examinations. Without imaging bundles, the acquisition times may be further reduced by a factor of 4.9 (55% loss per imaging bundle) which would bring each specimen analysis to 1.2 – 2.5 min which falls well below the maximum acquisition time of 5 min per specimen set by our collaborating clinicians. To perform *in vivo* acquisitions however, one alternative strategy may be to increase spatial binning to observe Raman signal over larger pixels (i.e. 200 x 500 μm instead of 200 x 250 μm) while also reducing the number of lines scanned by half (i.e. 20 lines over 1 cm instead of 40 lines). These parameters would increase the signal collected per pixel by 100% and reduce the total exposure time by 50%. Although a loss of spatial resolution would result from these parameters, total measurement times would decrease to 1.5 – 3.3 min which fall within the acceptable clinical timeframe for measurements.

Confounding luminescence from system components

The novel Raman imaging system was fabricated with carefully selected optics to optimize image resolution in the visible and to ensure a spatial resolution >100 μm for 800 – 950 nm (Raman

spectral range). However, it was observed that luminescence is generated from the focusing achromatic triplet (#67-422, Edmund Optics, USA) circled in Figure 8-4 (A) during Raman acquisitions. Albeit relatively small in intensity when compared to signal obtained in adipose tissue, this signal is apparent in tissues with low Raman SNR (e.g. human brain and muscle tissue). This spectral signature was no longer observed (i.e. only ready out noise was observed) when performing a measurement in the same conditions after having removed the focussing achromatic triplet. This luminescence signal is additive and accentuated when placing a mirror at the tip of the probe. Since this luminescence is not part of the optical transfer function of the system, it cannot be corrected using the NIST 785 nm relative intensity standard. The best method found was to acquire a measurement in air with the same acquisition parameters of tissue measurements and subtract the raw photonic counts from the tissue measurements. Although it does not eliminate this parasitic signal, it was observed it could reduce >50% of the unwanted signal from measurements.

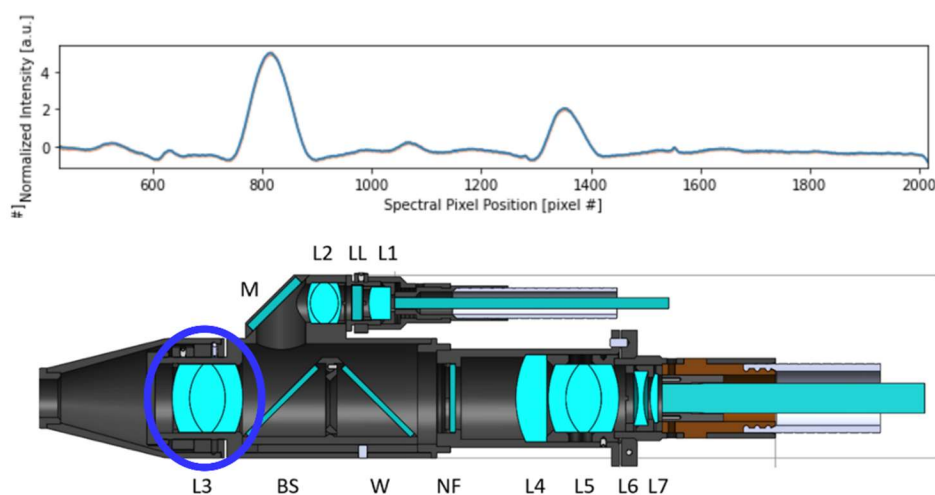


Figure 8-4: Normalized mean spectra acquired with imaging system in air showing the luminescence profile generated by the focusing achromatic lens of the imaging probe.

APPENDIX B – LIST OF SCIENTIFIC CONTRIBUTIONS & AWARDS

Patents [3]

- [1] F. Leblond, **F. Daoust**, and D. Sandryne, “Methods for performing a Raman spectroscopy measurement on a sample and Raman spectroscopy systems,” US11796475B2, 2023 [Online]. Available: <https://lens.org/172-581-486-829-068>
- [2] K. Ember, **F. Daoust**, N. Ksantini, et al., “Methods and systems for interrogating a drop of saliva using Raman spectroscopy,” PCT/CA 2022/ 050974, 2022, [PCT Patent Application].
- [3] F. Leblond, K. St-Arnaud, and **F. Daoust**, “Methods and systems for imaging a sample using Raman spectroscopy,” US11105682B2, 2021 [Online]. Available: <https://lens.org/149-115-466-793-756>

Scientific publications [9]

- [1] **Daoust F.**, Dallaire, F. Tavera, H., et al., “Intraoperative whole-specimen Raman spectroscopy imaging detects high cancer cell density clusters in glioblastoma”. Scientific Reports, (article submitted for publication).
- [2] David S., Ksantini N., Dallaire F., Ember K., **Daoust F.**, et al., “Towards Non-contact and Real-time Large Field-of-view Single-band Raman Spectroscopy Imaging in Brain Cancer Surgery”, Journal of Biomedical Optic, (article submitted for publication).
- [3] David S., Tavera H., Tran T., Dallaire F., **Daoust F.**, Mahfoud M., Tremblay F., Richer L., Meterissan S. and Leblond F., “Machine learning and hyperspectral Raman imaging identify invasive breast cancer”, Journal of Biomedical Optics Express, (article submitted for publication).
- [4] **F. Daoust et al.**, “A clinical Raman spectroscopy imaging system and safety requirements for in situ intraoperative tissue characterization,” *Analyst*, vol. 148, no. 9, pp. 1991–2001, 2023, doi: 10.1039/D2AN01946A.
- [5] K. J. Ember, **F. Daoust et al.**, “Saliva-based detection of COVID-19 infection in a real-world setting using reagent-free Raman spectroscopy and machine learning,” *J Biomed Opt*, vol. 27, no. 2, pp. 1–24, Feb. 2022, doi: 10.1117/1.JBO.27.2.025002.

- [6] A. Plante, F. Dallaire, A.-A. Grosset, T. Nguyen, M. Birlea, J. Wong, **F. Daoust et al.**, “Dimensional reduction based on peak fitting of Raman micro spectroscopy data improves detection of prostate cancer in tissue specimens,” *J Biomed Opt*, vol. 26, no. 11, pp. 1–14, Nov. 2021, doi: 10.1117/1.JBO.26.11.116501.
- [7] **F. Daoust et al.**, “Handheld macroscopic Raman spectroscopy imaging instrument for machine-learning-based molecular tissue margins characterization,” *J Biomed Opt*, vol. 26, no. 2, pp. 1–18, Feb. 2021, doi: 10.1117/1.JBO.26.2.022911.
- [8] F. Picot, **F. Daoust et al.**, “Data consistency and classification model transferability across biomedical Raman spectroscopy systems,” *Transl Biophotonics*, vol. 3, no. 1, p. e202000019, Mar. 2021, doi: <https://doi.org/10.1002/tbio.202000019>.
- [9] A.-A. Grosset, F. Dallaire, T. Nguyen, M. Birlea, J. Wong, **F. Daoust et al.**, “Identification of intraductal carcinoma of the prostate on tissue specimens using Raman micro-spectroscopy: A diagnostic accuracy case–control study with multicohort validation,” *PLoS Med*, vol. 17, no. 8, pp. e1003281-, Aug. 2020, [Online]. Available: <https://doi.org/10.1371/journal.pmed.1003281>

Conferences [13]

(*speaker)

- [1] **F. Daoust***, "Innovation technologique au cœur de la lutte contre le cancer," presented at the Université du Troisième Âge de Sherbrooke Conference, Regional Conference held in Beloeil, Canada, October 2022. (Oral presentation).
- [2] **F. Daoust***, "Innovation technologique au cœur de la lutte contre le cancer," presented at the Université du Troisième Âge de Sherbrooke Conference, Regional Conference held in Vaudreuil-Dorion, Canada, September 2022. (Oral presentation).
- [3] **F. Daoust***, F. Dallaire, F. Guiot, et al., "Large Field of View Raman Line Scan Imaging System for Intraoperative Brain Cancer Detection," presented at SPEC2022, International Conference held in Dublin, Ireland, June 2022. (Oral presentation).

- [4] **F. Daoust***, "Innovation technologique au cœur de la lutte contre le cancer," presented at the Université du Troisième Âge de Sherbrooke Conference, Regional Conference held in Châteauguay, Canada, April 2022. (Oral presentation).
- [5] **F. Daoust***, P. Orsini, J. Bismuth, et al., "Large field of view macroscopic Raman line-scanning imaging system for neuro-oncology applications," presented at SPIE Photonics West - BIOS, International Conference held in San Francisco, USA, February 2020. (Oral presentation).
- [6] **F. Daoust*** & F. Leblond, "La fin possible du cancer," presented at the Université du Troisième Âge de Sherbrooke Conference, Regional Conference held in Chambly, Canada, October 2019. (Oral presentation).
- [7] **F. Daoust*** & F. Leblond, "Raman Macroscopic Imaging to Guide Cancer Resection Surgery," presented at the 28th Annual Scientific Day of the Institut du cancer de Montréal, Regional Conference held in Montreal, Canada, January 2019. (Oral presentation).
- [8] F. Leblond, **F. Daoust***, A. Laurence, et al., "Multi-Modal Wide-Field Optical Spectroscopy Imaging Platform for Biological Tissue Data-Mining," presented at the Conference on Bio-Sensing and Imaging, Conference held in Florence, Italy, December 2018. (Oral presentation).
- [9] **F. Daoust*** & F. Leblond, "Development of a Wide-Field Raman Imaging System for Human Intraoperative Cancer Applications," presented at Innove-Action 2018 - Réinventer la santé, Provincial Conference held in Montreal, Canada, November 2018. (Oral presentation).
- [10] **F. Daoust*** & F. Leblond, "Macroscopic Raman Line-Scanning Imaging for Brain Tissue Margin Detection," presented at Innovation Day of the Polytechnique Montréal OSA/SPIE student chapter, Conference held in Montreal, Canada, September 2018. (Oral presentation).
- [11] **F. Daoust***, A. Laurence & F. Leblond, "Development of Medical Instruments Using Optical Spectroscopy to Guide Surgical Interventions," presented at the AQPMC Congress, Conference held in Montreal, Canada, September 2018. (Oral presentation).

- [12] **F. Daoust*** & F. Leblond, "Wide-Field Raman Imaging – Ex Vivo Brain Structure Margin Recognition," presented at the 2018 Photonics North Conference, International Conference held in Montreal, Canada, June 2018. (Oral presentation).
- [13] **F. Daoust***, M.-C. Guiot, K. Petrecca, & F. Leblond, "Wide field Raman Imaging System to Guide Brain Cancer Resection," presented at SPEC2018, International Conference held in Glasgow, United-Kingdom, June 2018. (Oral presentation).

Awards [8]

- | | | |
|-----|---|------|
| [1] | Entrepreneurship Excellence Bursary – Centech | 2023 |
| [2] | Relève Étoile Award – Quebec Research Fund | 2022 |
| [3] | Doctorate Travel Scholarship – CHUM Research Center | 2020 |
| [4] | Alexander Graham Bell Canada Graduate Scholarship -NSERC | 2020 |
| [5] | Postgraduate Doctoral Scholarship (PGS) – NSERC | 2019 |
| [6] | Oncology Entrepreneurship Bursary – Oncopole & IRICoR | 2019 |
| [7] | Top Student Speaker at the 28th Annual Scientific Day – Montreal Cancer Institute | 2019 |
| [8] | TransMedTech Bursary of Excellence – TransMedTech Institute | 2018 |