

Ollscoil na hÉireann, Corcaigh
National University of Ireland, Cork



TISSUE IDENTIFICATION FOR SURGICAL GUIDANCE USING BIOPHOTONICS

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for the degree of
Doctor of Philosophy

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TISSUE IDENTIFICATION FOR SURGICAL GUIDANCE USING BIOPHOTONICS

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“The duty of the man who investigates the writings of scientists, if learning the truth is his goal, is to make himself an enemy of all that he reads, and attack it from every side. He should also suspect himself as he performs his critical examination of it, so that he may avoid falling into either prejudice or leniency.”

*Hasan Ibn al-Haytham
Father of modern optics
c. 965 – c. 1040
Kitāb al-Manāzīr (Book of Optics)*

ABSTRACT

Optical spectroscopic techniques provide powerful non-invasive means for probing biological tissues to reveal their structural, functional and molecular characteristics. Their utilization spans diagnostic, therapeutic and monitoring regimes. Advancing these capabilities, this work establishes a unified framework for optical tissue identification and surgical guidance by integrating diagnostics, analytical modeling, phantom development and minimally invasive biomarker assessment.

The work begins with continuous wave (CW) diffuse reflectance spectroscopy applied for early detection of malignancy in the oral cavity. This study demonstrates that spectral signature can sensitively capture biochemical changes to differentiate diseased tissues from healthy. Motivated by the clinical promise of CW methods but recognizing limitations in quantitative and depth resolved characterization, an analytical hybrid model combining continuous wave and photon time of flight (pToF) spectroscopy was subsequently developed. It offers simplicity and portability of CW systems with depth sensitivity of time-resolved measurements to overcome the bulky hardware and long acquisition times typically required for broadband pToF systems. The resulting optical parameters enable real time interpretation of key physiological markers such as blood content and oxygenation, supporting informed diagnostic decision making. To support therapeutic applications, a hybrid solid-liquid anthropomorphic phantom was developed to replicate optical and anatomical features simultaneously. This complex model addresses the long-standing need for anatomically accurate optical phantom models for optimizing interstitial photodynamic therapy dosimetry. Extending optical assessment beyond direct tissue interrogation, Raman spectroscopic analysis of blood plasma was explored as a minimally invasive means to detect systemic biochemical alterations associated with localized tissue disease. Correlations between plasma derived spectral signatures and paired tissue biopsies demonstrate feasibility of optical blood based biomarkers for monitoring and patient stratification without the need for repeated tissue sampling.

Collectively, these explorations establish a comprehensive framework for optical tissue characterization that spans clinical deployment, analytical modeling, phantom validation and minimally invasive diagnostics. This work contributes to the advancement of precision healthcare, while paving the way for future interdisciplinary developments in biophotonics.

LIST OF PUBLICATIONS

This thesis is based on the following research papers, which will be referenced using Roman numerals in the text. The works of Paper **III**, Paper **IV**, Paper **V**, Paper **VI** and Paper **VII** have been published, while Paper **I** and Paper **II** are still in pipeline.

I Diagnostic potential of diffuse reflectance spectroscopy and feature selection in identifying oral potentially malignant disorders

S. Maryam, M. D. Ghauri, E. Fahy, S. Innocente, R. Burke, A. Visentin, R. Riordain, L. Feeley, S. Andersson-Engels, R. Gautam and K. Komolibus.
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II Hybrid multi-distance continuous wave and discrete-wavelength time-domain analytical model for broadband optical characterization

M. D. Ghauri, S. K. Kothuri, P. Lanka, M. S. Nogueira, S. Andersson-Engels and S. Konugolu.
XYZ **00(0)**, 000-000 (xxxx).

III Hybrid heterogeneous phantoms for biomedical applications: a demonstration to dosimetry validation

M. D. Ghauri, S. Šušnjar, C. N. Guadagno, S. Bhattacharya, B. Thomasson, J. Swartling, R. Gautam, S. Andersson-Engels and S. Konugolu.
Opt Express. **15(2)**, 863-874 (2024).

IV Two-stage diffuse fluorescence tomography for monitoring of drug distribution in photodynamic therapy of tumors

S. Šušnjar, M. D. Ghauri, B. Thomasson, S. Konugolu, S. Andersson-Engels, J. Swartling and N. Reistad.
J Biomed Opt. **30(1)**, 015003 (2025).

V Resorbable optical fibers for interstitial photodynamic therapy — assessment of photosensitizer spatial distribution in tumors

J.T. Pandayil, S. Šušnjar, M. D. Ghauri, S. Konugolu, J. Swartling, D. Janner, N. Boetti and N. Reistad.
J Biomed Opt. **30(5)**, 058001 (2025).

VI Mobile multi-configuration clinical translational Raman system for oral cancer application

S. Maryam, S. Konugolu, M. D. Ghauri, E. Fahy, M. S. Nogueira, H. Lu, F. Beffara, G. Humbert, R. Riordain, P. Sheahan, R. Burke, K. W. Kho, R. Gautam and S. Andersson-Engels.
Opt Express. **30(10)**, 16572-16584 (2022).

VII Biomolecular Fingerprint of Crohn's Disease Using Raman Spectroscopy

M. D. Ghauri, M. V. Fernandez, G. Santacroce, S. Chanda, I. Zammarchi, I. Capobianco, S. Konugolu, S. Ghosh, K. Komolibus, S. Andersson-Engels, M. Iacucci and R. Gautam.
J Biophotonics. **19(1)**, e70219 (2026).

ABBREVIATIONS

CARS	Coherent Anti-Stroke Raman Scattering
CCD	Charge Coupled Device
CV	Coefficient of Variation
CW	Continuous wave
DA	Diffusion Approximation
DFT	Diffuse Fluorescence Tomography
DPF	Differential Path Length
DRS	Diffuse Reflectance Spectroscopy
FD	Frequency Domain
HPLC	High-Performance Liquid Chromatography
iPDT	Interstitial Photodynamic Therapy
IR	Infrared
IRF	Instrumental Response Function
MBLL	Modified Beer-Lambert Law
ML	Machine Learning
MRI	Medical Resonance Imaging
NIR	Near infrared
OD	Optical Density
PCR	Polymerase Chain Reaction
PMT	Photomultiplier
PS	Photosensitizer
pToF	Photon Time of Flight
RTE	Radiative Transport Equation
ROI	Region of Interest
RS	Raman Spectroscopy
SERS	Surface-Enhanced Raman Scattering
SD	Source Detector
SiPM	Silicone Photomultiplier
SORS	Spatially Offset Raman Spectroscopy
SNR	Signal-to-noise ratio
SPADs	Single-Photon Avalanche Diodes
STL	Stereolithography
TCSPC	Time-Correlated Single-Photon Counting
TD	Time Domain
TR	Time Resolved
UV	Ultraviolet light spectrum
VIS	Visible light spectrum

CONTENTS

Abstract	v
Introduction	1
0.1 Clinical Motivation for Tissue Identification	1
0.1.1 Histopathology: The Gold Standard	1
0.1.2 Real-Time Tissue Identification	1
0.1.3 Longitudinal and Minimally Invasive Disease Monitoring . .	2
0.2 Tissue Optical Properties	2
0.3 Role of Tissue Mimicking Phantoms in Biophotonics	3
0.4 Role of Biofluids for Minimally Invasive Disease Monitoring	3
0.5 Scope of the Thesis	3
0.6 Objectives	3
0.7 Structure of the Thesis	4
1 Background	7
1.1 Light Matter Interactions	7
1.1.1 Reflection	7
1.1.2 Absorption	8
1.1.3 Fluorescence	8
1.1.4 Scattering	9
1.2 Light propagation in Tissues	11
1.2.1 Potential and Challenges	11
1.3 Optical Sensing Techniques	12
1.3.1 Continuous wave diffuse reflectance spectroscopy	12
1.3.2 Photon Time-of-flight spectroscopy	13
1.3.3 Raman spectroscopy	14
1.4 Summary	14
2 Light Propagation Models	15
2.1 Theoretical Models of Light Propagation in Biological Tissues	15
2.1.1 Radiative Transport Equation	15
2.1.2 Diffusion Approximation	16
2.1.3 Modified Beer Lambert Law	17
2.1.4 Measurement of Tissue Optical Properties	18
2.1.5 Potential and Challenges	18
2.2 Summary	19
3 Tissue Mimicking Phantoms	21
3.1 Design Considerations for Optical Phantoms	21
3.1.1 Liquid Phantoms	21
3.1.2 Solid Phantoms	22
3.1.3 Tuning Optical Properties	22
3.2 Mimicking Tissue Heterogeneity	22
3.3 3D Printing Anthropomorphic Phantoms	23
3.4 Solid-Liquid Phantoms for realizing Anthropomorphic Features	23
3.5 Diffuse Fluorescence Tomography	24
3.6 Challenges	25
3.7 Summary	25
4 Instrumentation and Experimental Setup	27
4.1 Hybrid CW ToF Instrumentation	27
4.1.1 Probe Design	27
4.1.2 Continuous Wave Systems	28
4.1.3 Photon Time of Flight Systems	28
4.2 Raman Spectroscopy Instrumentation	30
4.2.1 Core Components	30
4.2.2 Sample Preparation	32
4.2.3 Substrate Selection	33
4.2.4 Experimental Challenges	33

4.3	Summary	34
5	Biofluid Analysis	35
5.1	Blood and its Biomedical Relevance	35
5.2	Potential of Raman Spectroscopy for Blood Analysis	35
5.3	Blood Preparation for Raman Acquisition	36
5.4	Raman Signal Interpretation	36
5.4.1	Data Treatment	36
5.4.2	Data Analysis	39
5.5	RS Biofluid Experimental Challenges	40
5.6	Summary	40
6	Tissue Characterization	41
6.1	Diffuse Optics	41
6.2	Methods for Extracting Optical Properties	41
6.2.1	Continuous Wave (CW)	42
6.2.2	Time Domain (TD)	42
6.2.3	Frequency Domain (FD)	43
6.3	System Characterization and Performance Assessment	43
6.4	Potential and Challenges	43
6.5	Summary	44
7	Conclusions	45
7.1	Retrieval of Tissue Optical properties	45
7.2	Tissue Mimicking Phantoms	46
7.3	Biofluid Spectral Analysis	46
7.4	Integrated Perspective	47
7.5	Summary	47
	Comments on the Papers	49
	Reflection on My Doctoral Training	53
	Acknowledgements	55
	Funding Info	57
	References	59

Papers

I	Diagnostic potential of diffuse reflectance spectroscopy and feature selection in identifying oral potentially malignant disorders	75
II	Hybrid multi-distance continuous wave and discrete-wavelength time-domain analytical model for broadband optical characterization	105
III	Hybrid heterogeneous phantoms for biomedical applications: a demonstration to dosimetry validation	123
IV	Two-stage diffuse fluorescence tomography for monitoring of drug distribution in photodynamic therapy of tumors	137
V	Resorbable optical fibers for interstitial photodynamic therapy — assessment of photosensitizer spatial distribution in tumors	165
VI	Mobile multi-configuration clinical translational Raman system for oral cancer application	179
VII	Biomolecular Fingerprint of Crohn's Disease Using Raman Spectroscopy	191

INTRODUCTION

Biophotonics is an interdisciplinary field that combines photonics and life sciences to observe, analyze and manipulate biological systems. It has enabled the development of powerful tools that are now central to biomedical research and clinical practice [1]. Advanced imaging modalities such as optical coherence tomography and fluorescence microscopy along with therapeutic techniques like photodynamic therapy and optogenetics, illustrate the breadth and impact of light-based technologies across modern healthcare applications [2–5]. This thesis explores the transformative potential of biophotonics, with a particular focus on tissue identification and presents a roadmap for developing biophotonic tools from today’s proof-of-concept research systems into clinically accepted tools.

0.1 Clinical Motivation for Tissue Identification

Tissue identification refers to structural, biochemical and functional characterization of biological tissues [6]. It plays a central role in clinical diagnosis, disease grading/staging and personalized treatment planning [7]. Accurate visualization and assessment of tissues is essential in clinical practice for diagnostic, therapeutic and monitoring purposes [8].

0.1.1 Histopathology: The Gold Standard

Histopathological analysis serves as the gold standard for tissue evaluation in clinical practice [9]. Examining excised tissue specimens allows for microscopic detection of cellular abnormalities, enabling definitive diagnosis and disease grading [10]. However, histopathology has certain limitations. It relies primarily on visible morphological changes, which may take time to develop [11, 12] and is also subject to inter-observer variability, introducing a degree of diagnostic subjectivity [13–15]. These can contribute to diagnostic delays and carries risk of misdiagnoses, both of which carry significant consequences for patient management. Additionally, because biopsy procedures are invasive, they can place a significant burden on patients. These limitations highlight the need for alternate approaches that enable earlier, more objective and less invasive tissue assessment.

0.1.2 Real-Time Tissue Identification

Histopathology is inherently a post-excision diagnostic modality and therefore cannot support real-time tissue identification. However, many clinical decisions depend on immediate tissue characterization. For example in surgical oncology intraoperative identification of tissue boundaries between tumor and healthy tissue is essential for guiding resection strategies in real time [16]. Reliance on frozen-section analysis or histopathological delays often limits the surgeon’s ability to make real-time decisions. This could lead to either incomplete excision or unnec-

essary removal of healthy tissue with consequences for tumor recurrence and postoperative functional morbidity respectively [17]. Similar challenges arise in interventional procedures such as photodynamic therapy, where therapeutic efficacy depends on accurate dosimetry and real-time feedback within the tissue. In cases involving deep or inaccessible organs, biopsy may be impractical necessitating non-invasive alternatives for tissue characterization [18]. These clinical scenarios present unmet need for methods capable of providing rapid, quantitative and minimally invasive assessment of tissue at the point of care.

0.1.3 Longitudinal and Minimally Invasive Disease Monitoring

Beyond initial diagnosis and procedural guidance, effective patient management often requires the ability to monitor tissue state over time. Chronic inflammatory conditions such as Crohn's disease is one such example where the disease follows a relapsing–remitting pattern [19]. Repeated endoscopic biopsy is invasive, burdensome and unsuitable for frequent monitoring. Similarly, therapy response assessment could immensely benefit from tools that can detect subtle biochemical and structural changes earlier than conventional evaluations.

0.2 Tissue Optical Properties

The way light behaves in biological tissues depends on its intrinsic optical properties, including absorption coefficient, scattering coefficient and anisotropy factor. These properties determine light-tissue interactions such as absorption, reflection, scattering, fluorescence and photothermal effects [20]. Biophotonic techniques take advantage of these interactions to reveal detailed structural, functional and biochemical information that can guide clinical decisions [21]. Understanding these optical parameters is crucial for designing systems that deliver light accurately, perform reliably and minimize unwanted effects [22]. Additionally, correlating these parameters with pathological changes can provide insights into disease mechanisms, support early detection, help stratify patients and facilitate monitoring treatment responses [23–25]. Therefore, interpreting optical measurements quantitatively relies heavily on knowing the intrinsic optical properties of tissues.

Beyond generating diagnostically relevant signals, the same optical properties also influence the strength of the detected signal by attenuating the incident and remitted light [26]. Hence determining light penetration depth and consequently the tissue volume being probed [26]. This dual aspect of producing signals used for tissue characterization and simultaneously defining the tissue volume probed, adds significant complexity to the accurate assessment of optical measurements. Obtaining reliable broadband measurements of these optical properties *in vivo* is still challenging. This is mainly because of spectral complexity of biological tissues and the limitations of individual optical modalities. Developing methods that recover broadband optical properties robustly under clinically realistic conditions is therefore crucial for advancing biophotonic diagnostics and therapeutics. The real-time and quantitative tissue characterization would strengthen the role of biophotonics for precision healthcare.

0.3 Role of Tissue Mimicking Phantoms in Biophotonics

While tissue studies provide the gold standard for understanding disease pathology, they come with significant ethical, logistical and safety challenges including procurement, handling and storage. To address this, tissue-mimicking phantoms have become invaluable tools in biomedical optics research and development [27]. These phantoms can be developed in a way to replicate the optical, mechanical, acoustic or thermal properties of biological tissues [28–30]. They are widely used for calibration, preliminary system testing, performance comparison and therapeutic planning [31]. The closer a phantom mimics the properties of real tissue, the more effective it becomes in facilitating translational research and clinical application.

0.4 Role of Biofluids for Minimally Invasive Disease Monitoring

Beyond modelling and phantom studies, biofluids such as blood, saliva, urine and others are emerging as a minimally invasive resources for disease monitoring. These fluids contain vast amount of metabolites, proteins and other biomolecules which change in response to disease activity [32]. Thus providing a promising avenue for monitoring systemic biochemical signatures reflecting local tissue pathology without the need for repeated invasive sampling. Other advantages include ease of access, increased patient comfort, repeatable sampling and potential for early detection even before structural changes occur. Biofluid biomarkers are already used as the standard of care for various conditions e.g blood for complete blood counts, tumor markers and inflammatory markers [33], saliva for hormonal and infectious disease testing [34], urine for renal function and metabolic disorders [35] etc. By integrating biofluid analysis with traditional histopathology, clinicians obtain a more comprehensive view of the disease without the need for invasive sampling. Optical spectroscopic methods, particularly Raman spectroscopy, offer the capability to detect molecular alterations associated with disease activity, providing a minimally invasive route for longitudinal assessment. Developing reliable optical biomarkers for monitoring holds potential for more timely interventions and personalized treatment adaptation.

0.5 Scope of the Thesis

The clinical and technological challenges outlined above highlight the need for minimally invasive methods for tissue identification. This thesis investigates the potential of optical techniques to address diagnostic, therapeutic and monitoring needs in tissue characterization. It develops a streamlined framework for objective and quantitative optical tissue assessment that integrates measurements based on sample measurements and tissue-mimicking phantoms. The framework is subsequently applied across several biophotonic applications presented in this thesis, demonstrating its versatility and translational potential.

0.6 Objectives

The central hypothesis of this work is that appropriately designed optical techniques can capture physiologically and pathologically relevant information from tissue and body fluids for diagnostic, therapeutic and monitoring purposes. Fig. 0.1 illustrates these objectives pictorially. The broad objectives of this thesis were as follows:

- Exploring optical techniques for accurately characterizing key tissue chromophores and supporting clinical diagnostic decision-making.
- Assessing the possibility of incorporating structural complexity of human anatomy into optical phantoms for improving tissue mimicking capabilities for therapeutic planning and system validation.
- Investigating biofluid based optical spectroscopy as a minimally invasive method for indirectly assessing pathological and physiological state of tissue for monitoring and patient stratification.

The responses to these objectives are thesis contributions that have been disseminated through journal publications and conference presentations.

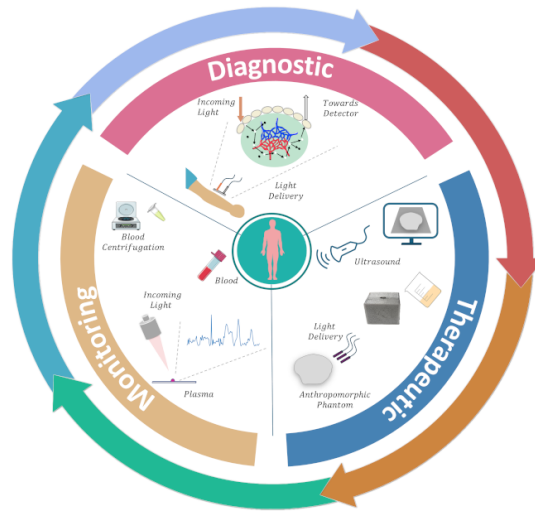


Figure 0.1: Objectives overview: Exploring optical techniques for tissue diagnostics, therapy and monitoring.

0.7 Structure of the Thesis

The thesis is organized into the following chapters:

Chap. 1 – Background: This chapter outlines fundamental principles behind light matter interactions and the potential they hold for optical sensing. An overview of various optical sensing techniques is provided to establish the context for the work presented in this thesis.

Chap. 2 – Light Propagation Models: This chapter details the theoretical models of light propagation in biological tissues and the associated potential and challenges. This chapter provides a foundational ground for research presented in Paper II and Paper I.

Chap. 3 – Tissue Mimicking Phantoms: This chapter discusses tissue mimicking optical phantoms and the various types commonly used in biophotonic research. It also highlights integration of anthropomorphic features to replicate various anatomical structures and the associated fabrication challenges of such phantoms. This work lays the foundation of a complex anthropomorphic optical phantom presented in Paper III. This phantom is further utilized in Paper IV and V.

Chap. 4 – Instrumentation and Experimental Setup: This chapter provides a detailed description of instrumental setups and the key design consideration utilized for work presented in this thesis. The

first part of the chapter is dedicated to continuous-wave (CW) and photon time-of-flight (pToF) measurement systems utilized in Paper **II** and Paper **I** while the second part is dedicated to the Raman spectroscopic systems presented in Paper **VI** and Paper **VII**.

Chap. 5 – Biofluid Analysis: This chapter details the potential of utilizing biofluids to gain insights into the physiological and pathological states of the body. It further discusses study design, data acquisition and spectral data treatment for interpretation for Raman spectral measurements. The approach and procedures detailed here have been utilized for research presented in Paper **VII**.

Chap. 6 – Tissue Characterization: This chapter discusses well established techniques for characterization tissue optical properties including Continuous Wave (CW), Time Domain (TD), Frequency-Domain (FD). These have been utilized for research presented in Paper **II**.

Conclusions: This chapter concludes this thesis by summarizing key findings along with recommendations for future research.

These chapters are followed by publications. Finally, a personal reflection at the end of the thesis provides insight into the learning process and professional development that accompanied this work.

BACKGROUND

This chapter presents background material for a better understanding of the thesis. It begins with overview of light-matter interactions followed by a focus on light-tissue interactions. Then major optical spectroscopic sensing techniques including continuous wave diffuse reflectance spectroscopy (CW), photon time-of-flight (pToF) and Raman spectroscopy (RS) are introduced. The limitations and challenges associated with each technique are also discussed.

1.1 Light Matter Interactions

Light-matter interactions are one of the most common phenomena observed throughout the universe. Human ability to perceive these interactions in the visible regime is what allows us to see the world around us. The interactions of light with matter are governed by a range of complexities all the way from wavelength and intensity of the incoming light to the properties of the matter itself. Understanding these interactions forms the basis of numerous diagnostic, imaging and sensing techniques used across disciplines including physics, chemistry, biology and medicine.

1.1.1 Reflection

Reflection of light is a fundamental optical phenomenon that occurs when light encounters an interface and is redirected to the original medium. However, when the interface represents a change in refractive index¹ between the two mediums, a portion of light is not reflected but rather refracted. The direction of refraction is governed by Snell's law [36]:

$$n_1 \sin(\theta_i) = n_2 \sin(\theta_r)$$

where n_1 and n_2 are the refractive indices of the first and second medias respectively and θ_i and θ_r represent the angles of incidence and refraction measured from the normal.

When a light ray traveling through a medium with a higher refractive index reaches the boundary with a medium of lower refractive index at an angle greater than the critical angle² θ_c . Then instead of refracting into the second medium, the light is entirely reflected back into the original medium. This is the key principle behind the operation of optical fibers.

¹Refractive index is a fundamental property of material that quantifies the change in speed of light in a material as compared to in a vacuum.

²The angle of incidence where the angle of refraction is exactly 90°

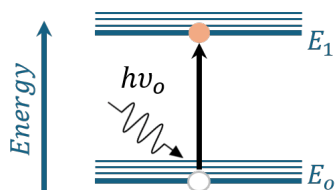


Figure 1.1: Photon absorption excites the molecule from ground state (E_0) to a higher energy state (E_1).

1.1.2 Absorption

Absorption of light is another fundamental process in which the energy of incident photons is taken up by the atoms, molecules or electrons within a material. This absorbed energy typically leads to electronic excitations. In a semiclassical picture, absorption is only possible when energy of the photon is equal or greater than the energy difference, ΔE , between the ground states (E_0) and the excited states (E_1). Fig. 1.1 illustrates a molecule absorbing the incoming photon and transitioning to excited state.

The absorption coefficient, μ_a [cm^{-1}], is a measure of how far light can propagate inside a medium before undergoing an absorption event. A higher absorption coefficient indicates stronger attenuation of light due to absorption so shorter penetration depth. In clear homogeneous materials such as in Fig. 1.2, absorption of light obeys Beer-Lambert law, the intensity of transmitted light (I) decreases exponentially with the product of the absorption coefficient and the path length (d) through the absorbing material. Mathematically expressed as [37]

$$I = I_0 e^{-\mu_a d} \quad (1.1)$$

$$A = \log\left(\frac{I_0}{I}\right) = \epsilon_\lambda C d \quad (1.2)$$

where ϵ_λ [$cm^{-1} \cdot M^{-1}$], is termed as extinction coefficient. It determines how strongly light can be absorbed at a particular wavelength. While C [$mol \cdot L^{-1}$] is the molar concentration of the absorbers in the medium.

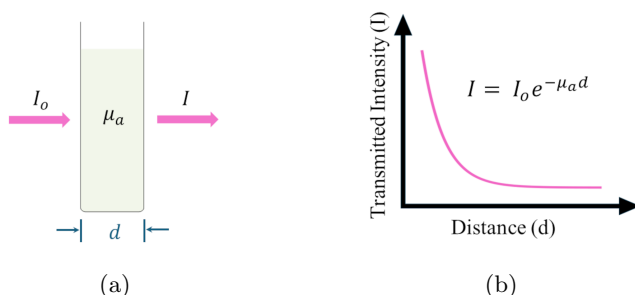


Figure 1.2: (a) Light passing through a cuvette. (b) Transmitted light as a factor of absorption coefficient and path length.

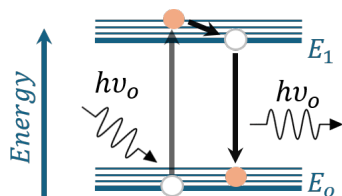


Figure 1.3: Photon emission as a result of relaxation of molecule from higher energy state (E_1) to ground state (E_0) to a.

The ability of the Beer-Lambert law to correlate attenuation of light with properties of the absorbing medium enables non-destructive characterization of the medium. This provides Beer-Lambert law a foundational value in a number of fields including spectroscopy.

1.1.3 Fluorescence

As already discussed in 1.1.2, following absorption the relaxation process can result in re-emission at different wavelengths as shown in Fig. 1.3. Depending on the absorber, this can lead to light emission. This phenomenon is known as fluorescence and the molecules responsible are referred to as fluorophores. The emitted fluorescence is typically of lower energy as compared to excitation light due to energy loss during internal conversion and vibrational relaxation [38]. The spectral characteristics of fluorescence emission can provide information about the internal molecular environment of the medium.

1.1.4 Scattering

Scattering of light is another fundamental phenomenon. When light passes through an inhomogeneous medium, the variations in refractive index cause it to change direction. As the light undergoes multiple scattering events its original path gets significantly altered. This results in a scattered and often diffuse propagation. In contrast to absorption, scattering is merely redirection of light as shown in Fig. 1.4.

In a continuous medium, scattering particles are assumed to be randomly distributed. The scattering coefficient, μ_s [cm^{-1}], describes the likelihood per unit distance that a photon will undergo a scattering event as it travels through a medium. A higher scattering coefficient indicates that photons are more likely to deviate from their original path. A related parameter is mean free path length, l_s [cm^{-1}], which is defined as the distance traveled by a photon before being scattered. A comparison between μ_s with μ_a is used to determine whether the material is scattering-dominated or absorption-dominated.

There are two general classes of scattering: elastic scattering and inelastic scattering.

Elastic Scattering

A scattering event in which the energy of the incident photon is preserved after scattering, i.e. $\Delta E = 0$, is referred to as elastic scattering. Depending upon the size of the scatter it can be subclassified into Rayleigh scattering and Mie Scattering.

Mie scattering,³ describes scattering when the scatterers are similar or larger in size than the wavelength of the incident light [26]. It is used extensively for modeling light transport.

Rayleigh scattering⁴ on the other hand is a subset of Mie Scattering that becomes dominant when the scatterer are much smaller than the wavelength of incident light [26]. The intensity of the scattered light can be given by [39]:

$$I_s \propto \frac{1}{\lambda^4} \quad (1.3)$$

Mie scattering is highly anisotropic. This directional dependence is quantified by anisotropic factor, g , defined as the mean value of scattering angle and is given as [40]:

$$g = \langle \cos\theta \rangle \quad (1.4)$$

The anisotropic factor ranges between -1 and 1 where 0 describes isotropic scattering while -1 and 1 describe complete back scattering and complete forward scattering respectively, as illustrated in Fig. 1.6. To account for directional preference in light transport models, a new parameter reduced scattering coefficient, μ'_s [cm^{-1}] is defined, which simply relates μ_s and g and is given as

$$\mu'_s = \mu_s(1 - g) \quad (1.5)$$

By relating μ_s and g , μ'_s effectively transforms the complex angular scattering behavior into an isotropic approximation model, as illustrated

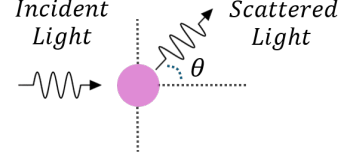


Figure 1.4: Light incident on a particle gets scattered in a random direction.

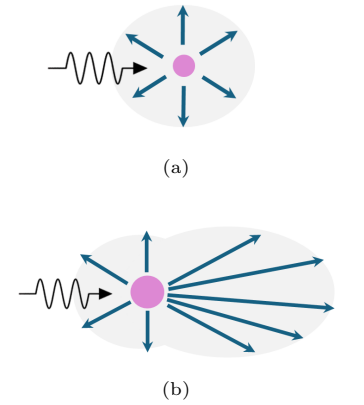


Figure 1.5: Light incident on a particle gets scattered (a) Rayleigh scattering. (b) Mie scattering.

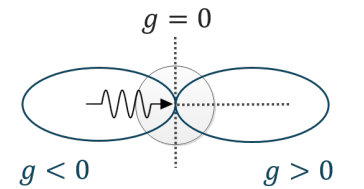


Figure 1.6: Depiction of scattering direction in-terms of anisotropic factor g .

³Named after Gustav Mie

⁴Named after Lord Rayleigh

in Fig. 1.7, that is more convenient for macroscopic modeling of light transport.

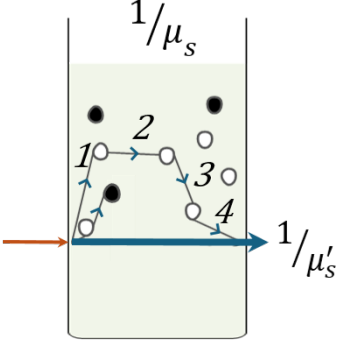


Figure 1.7: Representation of using one big step of μ'_s as compared to taking four small steps using μ_s for estimating light trajectory inside medium. The black dots represent absorption centers while the white scattering centers.

Inelastic Scattering

Inelastic scattering occurs where the scattered photon changes energy, i.e. $\Delta E \neq 0$, as compared to the incident photon. A major example of inelastic scattering is Raman scattering,⁵ where the change in energy between the incident photon and the scattered photon happens due to interaction with the medium. The energy shift corresponds to molecular vibrational modes, providing a spectral fingerprint unique to each molecule [41]. This energy shift is referred to as Raman shift, typically expressed in cm^{-1} . The relationship between Raman shift and wavelength is governed by the following equation:

$$\text{Raman Shift} = \frac{10^7}{\lambda_{\text{exc}}} - \frac{10^7}{\lambda} [\text{cm}^{-1}] \quad (1.6)$$

where λ_{exc} [nm] is the excitation wavelength and λ [nm] is the scattering photon wavelength.

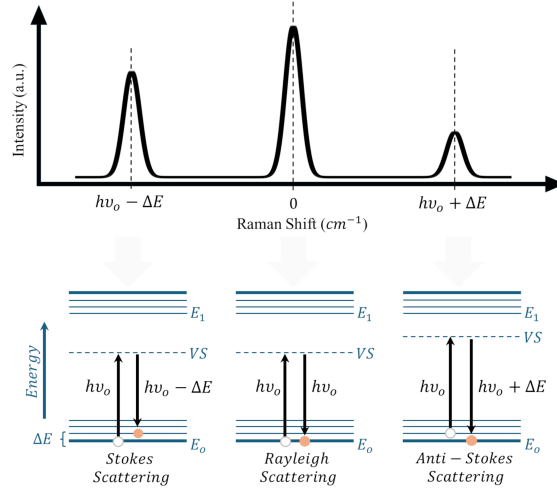


Figure 1.8: Overview of Rayleigh, Stokes and Anti-stokes scattering with their respective Jablonski diagrams.

If incident photons gain energy from the vibrating molecules of the medium and subsequently scatter with increased energy, this phenomenon is referred to as anti-Stokes Raman. Conversely, if the scattered photons lose energy, the process is known as Stokes Raman.

The probability of Raman scattering is exceedingly small compared to Rayleigh scattering, occurring only about one out of a million incident photons [42]. This low probability necessitates the use of sensitive detection methods for observation and subsequent analysis. Despite this limitation, Raman scattering remains a powerful tool for analysis. Fig. 1.8 illustrates Jablonski diagram for Rayleigh and Raman scattering.

⁵Named after Sir Chandrasekara Venkata Raman, the scientist who discovered it.

1.2 Light propagation in Tissues

Biological tissues present a highly complex heterogeneous environment. When light enters tissue, it is rapidly scattered due to variations in refractive index of the cellular and subcellular structures present in these layers [43, 44]. In light tissue interactions, although both Rayleigh and Mie scattering occurs but Mie scattering events are predominant [45]. In most biological tissues, g typically ranges from 0.8 to 0.95, indicating that photons are largely scattered in forward direction [45]. Simultaneously, absorption occurs due to endogenous chromophores such as hemoglobin, melanin, lipids, water, collagen and many more, each with distinct absorption spectra. Absorption results in attenuation of light and hence directly influences the penetration depth and energy distribution within the tissue. Additionally, biological tissues also contain several fluorophores, each of which has a distinct peak absorption and emission wavelength. Some common endogenous fluorophores include NADH, flavins, collagen, elastin and lipofuscin. Overall, light transport in biological tissues arises from the combined effects of these absorption, scattering and fluorescence events.

The interaction of light with biological tissues is strongly wavelength dependent. Wavelengths from $\sim 700\text{ nm}$ to 1100 nm define the therapeutic (also called optical) window [46]. In this spectral range, absorption by both hemoglobin and water is relatively low, allowing light to penetrate deeper into tissues. The resulting higher numbers of scattering events as compared to absorption in this window leads to diffuse spread of light in tissues [47].

1.2.1 Potential and Challenges

The interaction between light and biological tissue is fundamentally determined by the anatomical and physiological properties of the tissue. This relationship is bidirectional. Incident light is modulated through various optical interactions in tissue and conversely tissue can get influenced by light as well. In the context of biomedical applications the former offers significant potential for non-invasive screening, diagnostics and physiological monitoring. While the latter enables applications in intraoperative guidance and therapeutic interventions.

Extracting information about tissue from the way light is absorbed, scattered and re-emitted by biological tissues has enabled the development of a wide array of optical sensing techniques. The ability to measure blood oxygenation has led to applications like pulse oximetry, neonatal cerebral oxygenation monitoring and detection of angiogenesis⁶. Raman shifted light in tissues is another area which has a number of applications for metabolic monitoring [48]. These screening and monitoring capabilities hold potential for developing into point of care platforms. Technological developments have led to their integration into wearable monitoring platforms, further enhancing their potential for continuous and personalized healthcare solutions.

Energy transfer happens as a result of inelastic scattering or due to absorption. This is utilized in fluorescence guided surgery where exogenous fluorophores such as fluorescein, indocyanine green (ICG), 5-Aminolevulinic Acid (5-ALA) etc are administered, once activated enhance recognition of structures like tumors, blood vessels, or lymphatic pathways. The energy transfer in tissues can also result in

⁶Increase in vascularization

localized heating of tissue. This forms the basis of several clinically established and emerging modalities such as photothermal therapy and laser ablation. Another important application of light sensitive molecules getting activated is photodynamic therapy (PDT) where photosensitizing agents once activated leads to generation of reactive oxygen species that selectively induce cell death in diseased tissues. These light based therapeutic interventions offer reduced invasiveness, localized treatment and the potential for repeatability.

However, despite immense potential there are several challenges that limit the widespread adoption of light based modalities. The highly scattering and heterogeneous nature of biological tissues complicates light propagation, resolution and penetration depth. This creates difficulty in localization of the place where change to light happened. Moreover the overlapping absorption spectra of biological chromophores can hinder quantitative interpretation of the collected light. These limitations may be addressed by developing robust models of light transport and integrating multimodal approaches.

Despite these challenges, the possibility of interacting with tissues in a minimally invasive way that preserves tissue integrity make optical technologies highly promising tools for biomedical applications.

1.3 Optical Sensing Techniques

Optical sensing techniques build upon the unique potential of light-tissue interactions to extract relevant information. By probing absorption, scattering, fluorescence and Raman signatures, these techniques can translate optical responses from illuminated tissue into meaningful physiological and diagnostic insights. They offer relatively cost effective, non-toxic and minimally invasive rapid solutions for clinical applications. Past few decades have seen rapid multidisciplinary developments to improve sensitivity, specificity, portability to enhance their clinical acceptance [49–51]. The following sections explore some of the common optical spectroscopic modalities in greater detail, highlighting their principles, capabilities and biomedical applications.

1.3.1 Continuous wave diffuse reflectance spectroscopy

Continuous Wave (CW) systems represent the simplest and most widely used optical systems. These systems employ a constant-intensity light source for sample illumination and detect the emerging diffusely reflected light. The detected signal carries information about the sample's internal structure and biochemical composition.

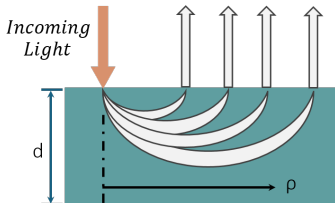


Figure 1.9: Schematic representation of probing depth (d) as a function of varying SD separation (ρ) in CW systems.

The penetration depth of CW light is primarily governed by the distance between the light source and the detector commonly referred to as source-detector (SD) separation. A greater separation enables deeper tissue interrogation but also increases the likelihood of absorption and scattering events resulting in weaker signal-to-noise ratio (SNR). Effect of SD separation on probing depth is illustrated in Fig. 1.9.

CW systems are widely used due to their simplicity, low cost and portability, making them well-suited for point-of-care diagnostics and real-time physiological monitoring [52, 53]. They offer rapid, non-invasive insights into tissue characteristics and are commonly applied in clinical and research settings [54–59]. However, a key limitation of CW systems is their inability to independently quantify the absorption and scattering

coefficients of tissue. Instead, the detected diffuse spectrum represents a combined attenuation effect of both [60]. Despite this, CW systems remain valuable, particularly in applications where qualitative assessment, real-time feedback and device portability is required.

1.3.2 Photon Time-of-flight spectroscopy

Photon Time-of-Flight (pToF) spectroscopy is a well-established optical technique for probing internal structure and optical properties of a sample. The fundamental principle involves delivering a short laser pulse and detecting the temporally broadened pulse at a set distance from the source as shown in Fig. 1.10. The temporal profile of the detected pulse is influenced by the optical properties of the sample being investigated.

A key advantage of pToF over CW systems, 1.3.1, is its ability to decouple μ_a and μ'_s simultaneously rather than yielding a single absolute intensity measurement [61]. This is possible because absorption and scattering affects different parts of the detected pulse. μ'_s affects the temporal shift of the detected pulse peak, whereas μ_a influences the slope and amplitude of the temporal profile [61]. As light propagates in the medium undergoes multiple scattering events, it takes longer for the photon to travel to detector leading to peak shift and pulse broadening. On the other hand when light undergoes absorption events it reduces the detected peak intensity. The effect of varying μ_a and μ'_s on the shape of the peak is illustrated in Fig. 1.11 below:

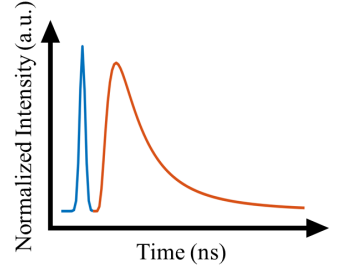


Figure 1.10: Representative photon Time of flight (pToF) curve with the blue being the input pulse while the orange is the change in the shape of the pulse at detector.

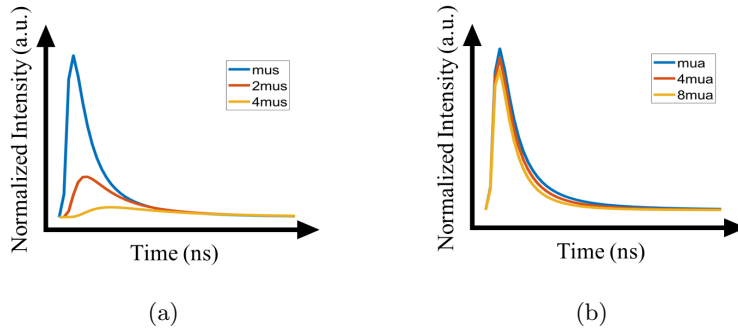


Figure 1.11: (a) Effect of variation of μ_s on pulse broadening. (b) Effect of variation of μ_a on pulse broadening.

Early implementations of pToF systems utilized streak cameras coupled with spectrometers, which converted the temporal distribution of photons into spatial information for continuous detection [62]. However, operational complexity and low SNR limited their widespread use in biomedical applications. Advances in detection technology, particularly the development of Time-Correlated Single Photon Counting (TCSPC), has significantly overcome these challenges. TCSPC achieves high temporal resolution by time-stamping individual photon arrivals relative to a reference pulse, enabling the construction of precise photon arrival time histograms [63]. The excellent sensitivity, compact form factors and improved portability offered, makes modern pToF systems more suitable for clinical and research applications. Optical mammography, diffuse optical tomography, monitoring of blood oxygenation and hemoglobin concentration are some of the interesting biomedical applications of pToF systems [63, 64].

1.3.3 Raman spectroscopy

Raman spectroscopy (RS) is another widely used optical sensing technique. Rooted in the principle of inelastic scattering, it's a powerful analytical tool for probing molecular structure and composition of samples under investigation. It features minimum sample preparation, non-destructive measurements and insensitivity to water [65]. These benefits position RS as an important technique for medical diagnostics among other applications.

For a vibrational transition to be Raman active, a change in the polarizability⁷ of the molecule must occur [66]. In contrast, infrared (IR) spectroscopy, another vibrational spectroscopic technique, requires that the vibration involve a change in dipole moment⁸ for it to be IR-active. This makes IR more sensitive to polar functional groups (e.g. O-H, C-O) while RS more sensitive to symmetric non-polar bonds (e.g. C-C, S-S) [66]. As a consequence, water being a polar molecule is a weak Raman scatterer. This makes RS well-suited for studying biological specimens in their native aqueous environments.

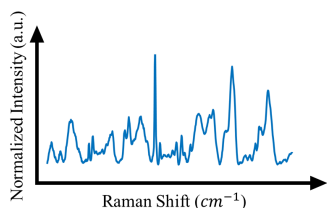


Figure 1.12: Representative normalized Raman spectrum of blood plasma.

Raman spectrum is essentially a graphical representation of specific molecular vibrations in the form of peaks typically plotted with Raman shift on x-axis in cm^{-1} and intensity on y-axis. A representative RS signal is illustrated in Fig. 1.12. The position of the peak along the x-axis provides information about the type of chemical bonds and functional groups present, while the intensity of the peak is related to the concentration of the scattering species [66]. This pattern of the peaks is the spectral fingerprint of the substance being probed. It enables identification and analysis of the substance. Several variables can influence SNR of Raman spectra all the way from laser wavelength, laser power, integration time, optical alignment, temperature, background fluorescence and detector efficiency. These will be discussed in detail in 4.2.

Advancements in technology have enhanced RS capabilities leading to techniques like Surface-Enhanced Raman Scattering (SERS), Resonance Raman Spectroscopy, Confocal Raman Microscopy, Coherent Anti-Stokes Raman Scattering (CARS), Tip-Enhanced Raman Spectroscopy (TERS), Spatially offset Raman spectroscopy (SORS) and other variants [67–69].

1.4 Summary

This chapter has laid the fundamentals behind interaction of light with biological tissues, all essential in this thesis. It discusses how light propagation in tissues is influenced by multiple optical interactions, including scattering, absorption and to a lesser extent fluorescence. Spectroscopic techniques utilize these interactions to extract insights about tissue composition, molecular structure and physiological states. A lot of research effort is being directed towards their seamless integration within the existing clinical pathways. The rest of the thesis builds up on the background concepts presented herein.

⁷Polarizability refers to the ease with which the molecule's electron cloud can be distorted by an external electric field, such as that from incident light.

⁸A dipole moment refers to the separation of positive and negative electrical charges within a molecule.

LIGHT PROPAGATION MODELS

This chapter provides a comprehensive overview of the theoretical frameworks used to model light transport in biological tissues. As discussed in chapter 1, biological tissues present a complex scattering and absorbing environment. Understanding how light propagates within is essential to utilize the immense potential of light-tissue interactions for biomedical applications. The focus of this chapter is on Radiative Transfer Equation (RTE) and its widely used approximations that are relevant to this work. The strengths and limitations of these models along with underlying assumptions are discussed for their application in rest of the thesis.

2.1 Theoretical Models of Light Propagation in Biological Tissues

Light matter interactions, discussed in 1.1, start to become more and more complex as the number of interacting atoms or molecules increases. Various models have been proposed to understand the complexity of the interactions between light and matter. The well-known Maxwell's equations provide a comprehensive theoretical structure for analysis of electromagnetic wave propagation [70]. However, their mathematical complexity increases substantially when applied to highly scattering or diffusive media such as biological tissues [71]. Modeling of diffuse light propagation becomes a lot more practical when considering particle nature of light.

2.1.1 Radiative Transport Equation

Radiative transport equation (RTE) effectively approximates the complex wave interactions by focusing on the transport of energy rather than the electromagnetic field [72]. It offers a macroscopic approach for modeling light propagation in biological tissues.

RTE makes a number of key assumptions regarding the medium [72]. The diffusive media is treated as having randomly distributed absorption and scattering centers. Absorption events result in termination of light propagation. Scattering events are assumed to be elastic only, so just deflection of light from the original direction occurs while preserving photon energy. Additionally, it is assumed that the medium is static i.e. optical properties such as μ_s, μ_a, g, n are time invariant but can vary spatially [72].

RTE is derived by balancing the incoming and outgoing energy in an infinitesimal volume, dA , in the medium. The mathematical description

of light propagation takes spatial and temporal distribution of radiance¹, $L(\vec{r}, \hat{s}, t)$, at a point. It then balances the various mechanisms of radiance change within dA . RTE can be written as:

$$\begin{aligned} \frac{\partial L(\vec{r}, \hat{s}, t)}{\partial t} \cdot \frac{1}{v} = & - \underbrace{\mu_t L(\vec{r}, \hat{s}, t)}_1 \pm \underbrace{\nabla L(\vec{r}, \hat{s}, t) \cdot \hat{s}}_2 \\ & + \underbrace{\mu_s \int_{4\pi} \Phi(\hat{s}', \hat{s}) L(\vec{r}, \hat{s}', t) d\Omega'}_3 + \underbrace{S(\vec{r}, \hat{s}, t)}_4 \end{aligned} \quad (2.1)$$

where

$L(\vec{r}, \hat{s}, t)$ is radiance at position $\vec{r}$, in direction $\hat{s}$ and at time t within an infinite volume element dA , v is the speed of light in diffusive media, $\Phi(\hat{s}', \hat{s})$ represents scattering phase function and Ω is solid phase angle.

The four terms on the right-hand side of Eq. (2.1) individually describe various factors that might affect light propagation in the medium:

- **Term 1:** It describes loss of radiance in the direction $\hat{s}$ due to combined effect of absorption and scattering referred to as total attenuation coefficient, μ_t
- **Term 2:** It represents how the radiance changes as it moves through space along direction $\hat{s}$.
- **Term 3:** The scattering phase function denoting probability of light propagating in direction $\hat{s}'$ being scattered in direction $\hat{s}$ within solid angle $d\Omega$. This integral term accounts for radiance scattered into direction $\hat{s}$ from all other directions $\hat{s}'$ over the unit sphere 4π .
- **Term 4:** This represents any external or internal sources of radiance in the medium in direction of $\hat{s}$.

RTE is a powerful model that bridges the gap between fundamental wave optics and practicality. However, the presence of an integral on right side of Eq. (2.1) complicates obtaining an analytical solutions to RTE. This necessitates use of numerical² or stochastic³ approaches like finite element methods and Monte Carlo simulations to approximate solutions. These approaches often require considerable computational power. To overcome this limitation, RTE can be further simplified using certain assumptions.

2.1.2 Diffusion Approximation

P_N approximation is the simplification of RTE by expanding the angular dependence of the radiance in terms of spherical harmonics up to order N . The increasing hierarchy of N provides more accuracy but at the cost of computational complexity. The special case of $N = 1$ results in the widely used Diffusion Approximation (DA) to RTE for modeling diffusive media [73]. DA reduces the number of variables in Eq. (2.1) by making following assumptions:

¹The light intensity per unit solid angle.

²Deterministic algorithms to solve problem.

³Random probability distribution.

- **Isotropic radiance:** Predominantly scattering nature of the medium over absorption allows radiance $L(\vec{r}, \hat{s}, t)$ to be assumed nearly isotropic. This implies that the angular variations in radiance are so small that it can be expressed simply in terms of fluence rate ⁴, $\Phi(\vec{r}, t)$, and the flux vector ⁵, $\vec{J}(\vec{r}, t)$, as:

$$L(\vec{r}, \hat{s}, t) \approx \frac{1}{4\pi} \phi(\vec{r}, t) + \frac{3}{4\pi} \hat{s} \cdot \vec{J}(\vec{r}, t) \quad (2.2)$$

- **Negligible flux variation:** The flux varies smoothly in time at distances larger than the mean free path. The mean free path being the mean distance a photon travel before its direction is randomized due to scattering to the point that the initial direction is completely lost. It is mathematically quantified as $l' = (1/\mu'_s)$.
- **Isotropic light source:** The initial emission of light is isotropic.

The relation between fluence and flux is known as Flick's law and is given by:

$$\vec{J}(\vec{r}, t) = -D \nabla \phi(\vec{r}, t) \quad (2.3)$$

where D [m] is the diffusion coefficient defined as

$$D = \frac{1}{3(\mu_a + \mu'_s)} \quad (2.4)$$

In diffusion regime i.e under the assumption $\mu'_s \gg \mu_a$, Eq. (2.4) can be modified to

$$D = \frac{1}{3(\mu'_s)} \quad (2.5)$$

Implementing the above mentioned assumptions in RTE, Eq. (2.1), yields the time dependent diffusion approximation:

$$\frac{1}{v} \frac{\partial \Phi(\vec{r}, t)}{\partial t} + \mu_a \Phi(\vec{r}, t) - \nabla \cdot (D \nabla \Phi(\vec{r}, t)) = S(\vec{r}, t) \quad (2.6)$$

DA provides a computationally efficient model for light transport in tissue while providing reasonable accuracy. However, it should be applied in the context where the underlying assumptions remain valid. Any deviation from the fundamental assumption of $\mu'_s \gg \mu_a$ would invalidate DA to RTE [72]. Further, an anisotropic light source can only be regarded as almost isotropic after traveling a distance greater than mean free path length. This implies that a sufficiently large source detector (SD) separation provides better validity. For SDs smaller than mean free path length the DA no longer holds [72].

2.1.3 Modified Beer Lambert Law

The modified Beer-Lambert law (MBLL) is another simplification of RTE but in contrast to DA, it is valid when scattering and absorption both are significant. MBLL is in fact an extension of classic Beer-Lambert Law, Eq. (1.1), which assumed light attenuation to be dominated by absorption. However, when scattering within the medium is significant as well, the light attenuation is affected by a combined effect of absorption and scattering. In such scenarios path taken by

⁴The power incident on an infinitesimal sphere divided by the cross-sectional area of that sphere.

⁵The power transferred per unit area.

light to reach detector exceeds the simple linear distance between source and detector. MBLL accounts for this change in travel distance by introducing a differential path length factor (DPF). MBLL is given as [74]:

$$I(\lambda) = I_0(\lambda)e^{-\mu_a(\lambda) dDPF(\lambda)+G} \quad (2.7)$$

where I is the measured diffuse reflected light intensity, I_0 the injected light into the sample, d is the SD separation, DPF the differential path length factor and G is a medium and geometry dependent constant.

The inclusion of DPF in Eq. (2.7) is crucial for accurate quantification of highly scattering mediums such as biological tissues. DPF generally ranges from 3 to 6 in biological tissues [37] and can be given as [74]:

$$DPF(\lambda) = \frac{1}{2} \sqrt{\frac{3\mu'_s(\lambda)}{\mu_a(\lambda)}} \quad (2.8)$$

MBLL has been applied extensively in the field of spectroscopy due to its simplicity and fairly accurate estimation.

2.1.4 Measurement of Tissue Optical Properties

For extracting optical properties, the models discussed in the previous sections are applied to model response of the medium to light. A solution to RTE can be achieved analytically by applying inversion algorithm or through numerical methods such as Monte Carlo simulations or finite element analysis. While this is very detailed modeling of light transport, where assumptions hold value DA provides a less complex solution. Extracting optical properties through DA is often an inversion problem where measured light is compared with the model prediction and then the optical properties are fitted to minimize the difference between the observed and theoretical data. Methods like least squares fitting, Levenberg-Marquardt are some of the common minimization algorithms used. The latter being considered an optimum minimization tool for non-linear problems [75].

2.1.5 Potential and Challenges

Biological tissues contain randomly distributed heterogeneous constituents that both, scatter and absorb light. This intrinsic complexity presents significant challenges for optical modeling. Tissue's biochemical composition and microstructure governs light-tissue interactions by determining the optical properties namely μ_a and μ'_s . Variations in these parameters reflect underlying physiological or pathological changes, thereby enabling non-invasive probing of tissues.

Accurate modeling of light propagation in tissue is essential for extracting these optical properties. RTE provides a comprehensive theoretical framework for describing light transport in scattering and absorbing media. Two primary methodologies are commonly employed to estimate tissue optical properties [76]. The first relies on forward modeling, where the optical parameters are derived by minimizing the difference between experimentally measured spectra and those predicted by a theoretical light transport model such as the diffusion approximation (DA) or Monte Carlo (MC) simulation. The second approach in contrast to forward simulations employs inverse modeling techniques that directly regresses the optical properties from the measured spectra.

The determination of optical properties through non-invasive probing and by extension the biochemical composition, holds significant promise for biomedical applications. Light based tools can serve as diagnostic tools, differentiate tumorous tissues from their healthy counterparts and monitor treatment efficacy.

2.2 Summary

This chapter provides an overview of light propagation models in biological tissues, beginning with the Radiative Transport Equation (RTE), which offers a rigorous framework for characterizing light-tissue interactions but often requires computationally intensive numerical or stochastic solutions. To overcome this challenge, a simplified diffusion approximation (DA) is introduced, offering practical analytical solutions under conditions of high scattering and low absorption. The chapter further examines the Modified Beer–Lambert Law (MBLL) as a practical approach for relating optical measurements to tissue chromophore concentrations. These models are the foundation of the work discussed in this thesis. In essence, light transport modeling is a powerful tool for non-invasive assessment of tissue physiology and pathology. At the same time, the very complexity that makes these optical methods so informative also creates challenges. Achieving a balance between accuracy and computational efficiency remains a critical objective for advancing these models toward practical clinical and research applications.

TISSUE MIMICKING PHANTOMS

Optical tissue mimicking phantoms are artificial constructs designed to replicate the interaction of light with biological tissues. They provide a controlled, reproducible and ethically favorable alternative to biological samples [77]. By accurately simulating optical properties such as absorption and scattering, phantoms enable system characterization, calibration and inter-laboratory standardization [77]. Their role extends from preclinical development to clinical validation of diagnostic and therapeutic devices, highlighting their significance in advancing biomedical technologies. This chapter provides a comprehensive discussion on phantom design considerations, fabrication methodologies and flexibility of mimicking heterogeneous structures.

3.1 Design Considerations for Optical Phantoms

Biological tissues are highly complex exhibiting varying optical, mechanical, thermal and acoustic properties. Ideally a phantom material should replicate these properties simultaneously. However, despite the availability of a large number of tissue making materials it is very challenging to find a single material that encompasses all these properties simultaneously [78]. As a result, phantoms are realized for specific applications. Optical phantoms are typically engineered to mimic only the optical properties of tissue, using appropriate concentrations of absorbers and scatterers dispersed within a supportive bulk material [78]. Based on the choice of this bulk material phantoms can be broadly classified into liquid and solid types, each with distinct advantages and limitations. Ultimately the choice between liquid and solid phantoms needs to be guided by experimental requirements.

3.1.1 Liquid Phantoms

Liquid phantoms are easy to prepare and cost-effective. They are typically composed of water-based solutions containing absorbers and scatterers [79]. The dynamic nature of liquid phantoms allows for rapid adjustments in optical properties as per the evolving need of the experiments [79]. However, the inability of liquid phantoms to retain a defined shape is a major limitation. They also suffer from a shorter shelf life due to evaporation, microbial growth and sedimentation of scattering particles [80, 81]. These compromise consistency of optical properties overtime hence limiting their long term use.

3.1.2 Solid Phantoms

Solid phantoms on the other hand, offer superior mechanical stability and long-term reproducibility. The base material for solid phantom includes water or oil and synthetic polymer based materials [82]. Gelatin, agar and polyacrylamide are some of the commonly used water-based materials [82]. They are biologically similar to human tissues, low cost, easy to source, have a relatively simple manufacturing process and offer ease of manipulation and modification. However, their mechanical robustness is limited. They are also prone to degradation from microbial contamination and dehydration, which restricts their shelf life and utility in long-term applications. In contrast, oil and synthetic polymer based materials such as styrene-ethylene/butylene-styrene (SEBS), gel, epoxy resin and silicone overcome this limitation hence offering longevity [82]. These materials provide improved longevity and are suitable for multi-session use. However, their fabrication tends to be more complex, time-consuming and less flexible to post-fabrication modification of optical properties.

3.1.3 Tuning Optical Properties

Fabrication of optical phantoms benefits from a diverse array of materials, each tailored for specific application. The appropriate quantity of absorber and scattering media are incorporated in the bulk material to replicate desired tissue optical properties.

Scattering coefficients can be tuned using intralipid, titanium dioxide (TiO_2) or aluminum oxide (Al_2O_3), microspheres or other scattering agents [83]. Intralipid is in fact generally the mostly used scattering agent [84]. It is basically a lipid emulsion suspension that is extensively used as an intravenous nutritional support in medical settings. This makes intralipid a low cost and easily accessible material. Absorption coefficient can be tuned using india ink, dyes such as methylene blue, Indocyanine green (ICG) etc or biologically relevant chromophores like haemoglobin etc. Other additives can be added based on the desired features of the phantom including fluorophores for monitoring or antimicrobial agents to extend shelf life.

To enhance homogeneity, the mixture is stirred vigorously or sonicated to ensure uniform dispersion of particles. The choice of solid versus liquid bulk material further influences fabrication protocols and the ultimate usability of the phantom.

3.2 Mimicking Tissue Heterogeneity

Tissues exhibit substantial heterogeneity in both structure and function [85–88]. This influences light-tissue interactions. Understanding this heterogeneity and incorporating it within optical phantoms enhances their predictive accuracy. It is however very challenging given the variations across patients, organs and even within organ [89, 90]. Fabricating a multi-layered phantom with each layer a homogenous representation is one way to bring optical phantoms closer to biological tissues [91–94]. However, the complexity of human anatomy necessitates more sophisticated models. Incorporating anatomical landmarks and geometries derived from medical imaging modalities such as medical resonance imaging (MRI), computed tomography (CT) or ultrasound scans facilitate the creation of anthropomorphic phantoms that more accurately reflect the structural complexity [95]. Such anatomically accurate models are

able to capture spatial variation in optical properties improving phantom utility for clinical validation studies.

3.3 3D Printing Anthropomorphic Phantoms

High fidelity optical anthropomorphic phantoms are invaluable simulation tools providing detailed insights. Advances in 3D printing technologies have boosted the realization of anthropomorphic phantoms [96]. The technology's high level of customization allows easy adaptation to specific requirements. A wide range of printing materials can be used to 3D print the phantoms [96]. However, directly printing an optical anthropomorphic tissue phantom is not a possibility due to the need of incorporating various optical properties into one single phantom. Different structures representing organs would need to be printed separately using filaments corresponding to desired properties which is a challenge in itself [97]. Then afterwards assembling all structures together for the final anthropomorphic phantom still leads to deviation from targeted optical properties. An alternate possibility to realize anthropomorphic phantoms using 3D technology is to print negative plastic molds and then pour inside a suitable tissue mimicking material to fabricate the anthropomorphic phantom such as illustrated in Fig. 3.1. This gives more control over the optical properties of the phantoms being fabricated. Numerous realization of such phantoms have been reported [98–101].

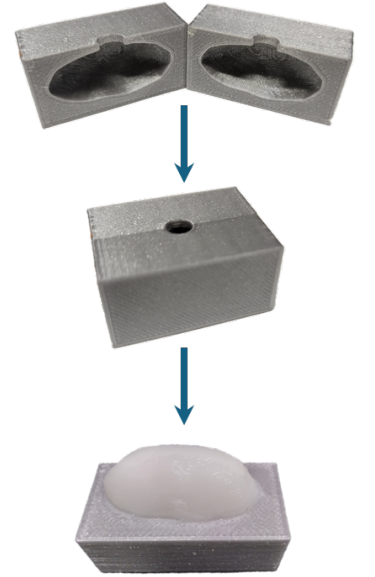


Figure 3.1: Fabricating phantoms from negative molds.

Anatomical details can be extracted from MRI, CT and ultrasound [97]. These would describe the surface structure and associated geometries. Surface smoothing may be required to reduce irregular boundaries introduced by the resolution of the imaging modality. The files can be modified with the help of any CAD software to develop negative molds. The next stage would be to describe the 3D model as a stereolithography (STL) file. These files basically are a collection of interconnected triangles to realize the surface geometry of the 3D model [102]. Triangles are commonly used because they always form a planar surface regardless of how their three vertices are connected [102]. So they can be used to represent any surface e.g a quadrilateral can be realized by using two triangles. The density of these triangular meshes determines the quality of the print [103]. STL files are not 3D printable on their own, a software is used to slice STL model into layers for the printer to understand [103]. This final file containing the slicing info in G-code format is loaded in the printer to print.

3.4 Solid-Liquid Phantoms for realizing Anthropomorphic Features

Clinically relevant fluorophore are mostly water soluble for allowing intravenous administration. Liquid phantoms support the use of intravenous agents, but lack structural integrity to create complex structures or inclusions. For applications requiring precise placement of mechanical instruments such as optical fibers external support is needed. Fig. 3.2 illustrates one such liquid phantom arrangement where an external post is providing support for insertion of optical fibers and the set of beakers is providing the possibility of inclusion.

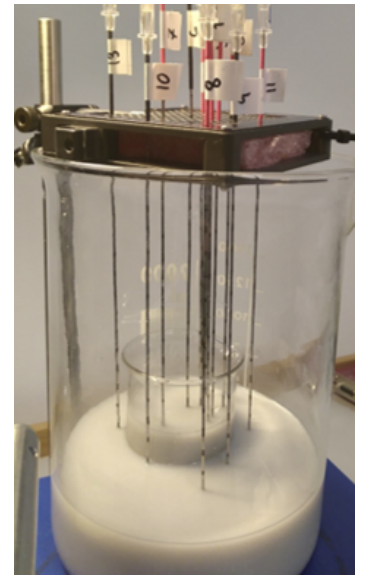


Figure 3.2: Liquid phantom with fibers mounted at specific locations. The beaker inside represents an inclusion with a different set of optical properties than the liquid phantom outside.

Solid phantoms on the other hand are not compatible with intravenous agents but they provide mechanical robustness and long-term stability. This makes them ideal for repeated use and precise placement of optical fibers. Fig. 3.3 depicts a solid phantom for prostate anatomy

with a number of fibers precisely inserted at specific coordinates.

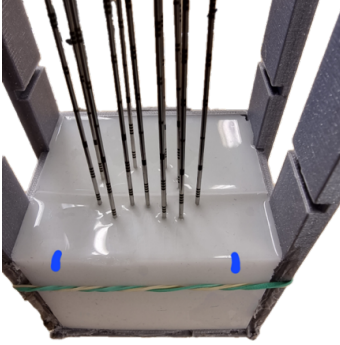
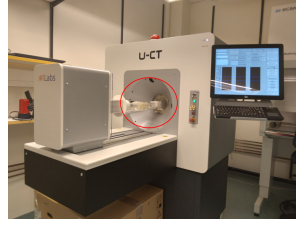
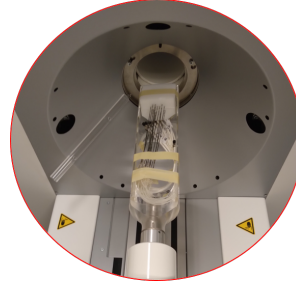


Figure 3.3: A solid phantom with fibers mounted at specific locations still inside negative mold.



(a)



(b)

Figure 3.4: (a) Micro CT machine with phantom inside ready for scan. (b) Close up of phantom placement from red circle in (a).

A completely solid anthropomorphic phantom however, may not be suitable for applications requiring mechanical insertion of fibers as it carries the risk of movement of fibers during transportation as well during the course of experimentation. This becomes particularly crucial for applications involving anatomically complex regions such as during prostate photodynamic therapy (PDT), where surrounding structures, tissue boundaries and fiber placement precision can significantly impact the therapeutic outcome. To investigate this further a micro-CT was performed on such a phantom, as shown in Fig. 3.4. It did reveal air gaps confirming this suspicion as illustrated in Fig. 3.5.

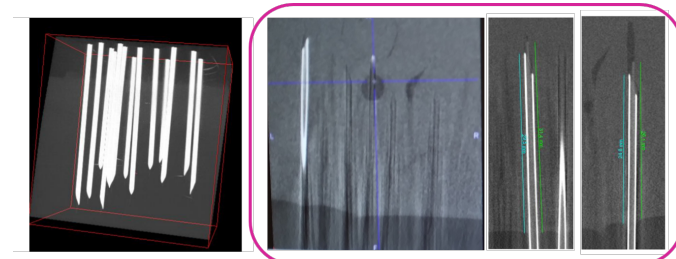


Figure 3.5: Results of CT scan revealing air gaps in the solid silicone phantom.



Figure 3.6: A solid phantom with a inclusion in the middle for a liquid phantom.

Given these trade-offs of solid and liquid phantom, a hybrid solid-liquid phantom approach that combines the strengths of both solid and the liquid phantoms could offer mechanical stability and anatomical fidelity of solid phantoms along with use of intravenous fluorophores. The use of solid-liquid interface also addresses the possible issue of air gaps resulting from translational or rotational movement of fibers. Fig. 3.6 depicts one such phantom where a solid silicone shell houses an inclusion in the middle for a liquid phantom.

3.5 Diffuse Fluorescence Tomography

Diffuse fluorescence tomography (DFT) is a powerful technique that enables assessment of spatial distribution of fluorophores. In phantom studies, DFT is frequently employed to evaluate system performance, optimize reconstruction algorithms treatment planning and monitoring. Tissue-mimicking phantoms for DFT typically incorporate fluorescent dyes such as indocyanine green (ICG), fluorescein, visudyne within a

background medium that replicates the optical scattering and absorption properties of biological tissue [104–106].

3.6 Challenges

One of the primary challenges of phantom fabrication is minimizing inter batch variability, especially in quantitative or longitudinal studies. A major source for variability could arise from the use of scattering agents that are suspended as they carry the risk of sedimentation over time and alter the phantom’s optical properties. Mixing for homogenization, degassing and ensuring no sedimentation prior to curing are all critical steps. Additionally for heat sensitive materials such as gelatin or agar, a tighter temperature control during preparation is critical to avoid premature solidification or degradation. It is important to maintain consistent manufacturing conditions so as to keep manufacturing variability to a minimum.

Proper storage is another important challenge. Once manufactured phantoms should be stored properly. Evaporation in liquid phantoms can change concentrations of scatterers/absorbers, thus altering optical coefficients. Degradation due to environmental exposure e.g. light, oxygen, humidity etc can lead to degradation of dyes, photobleaching or even emergence of background fluorescence over time [107–109].

The capabilities to create patient specific models has significant potential in personalized medicine. However high anatomical detail of anthropomorphic phantom adds complexity to fabrication procedures. This limits their widespread adaptability to applications that would benefit from optical and tissue architecture information such as therapeutic interventions. From a standardization point of view, replicating complex contours and structures with high reproducibility is a challenge in different settings due to variation in printing resolution of 3D printers. This creates a challenge for multi center trials which can be mitigated by fabricating phantoms at one center only.

3.7 Summary

In summary, this chapter presents a comprehensive discussion on various types, fabrication procedures and challenges for realizing optical phantoms. The primary design considerations involve optimizing the combination of bulk, absorber and scattering media to attain the desired properties. It also discusses anthropomorphic structures to improve the tissue simulation capabilities of optical phantoms by bringing them closer to the anatomical realities. Diffuse fluorescence tomographic reconstruction is also discussed as a means of assessing spatial distribution of fluorophore inside the phantom. This chapter lays the groundwork for a novel hybrid anthropomorphic phantom discussed in Paper **III**. It presents a novel approach for combining solid and liquid phantoms for incorporating tissue heterogeneities. This hybrid phantom holds potential in improving reconstruction algorithms for accurate dosimetry estimations as demonstrated in Paper **IV** which presents a novel two-stage tomographic reconstruction algorithm for evaluation of spatial distribution of the fluorophores. The realized hybrid phantom has also been demonstrated with bioresorbable fibers in Paper **V**.

INSTRUMENTATION AND EXPERIMENTAL SETUP

The focus of this chapter is on the design and implementation of the experimental setup used in subsequent chapters. It outlines design principles, major components and best practices. The chapter also discusses the decision factors that affect key parameters such as SNR, repeatability and system stability. The experimental systems covered in this chapter have been utilized in Paper **I**, Paper **II**, **VI** and **VII**.

4.1 Hybrid CW ToF Instrumentation

This section provides an overview of key instruments used for implementing continuous wave diffuse reflectance spectroscopy (CW) and photon time-of-flight (pToF) spectroscopy techniques. It describes the design of the fiber-optic probe and the instrumentation associated with each technique.

4.1.1 Probe Design

The fiber-optic probe is a critical component that acts as the interface between the optical system and the sample under investigation. Its function is to deliver light to the tissue and collect the diffusely reflected light. Typically, the probe consists of a bundle of optical fibers arranged in source-detector (SD) configuration. In this setup the source fibers emit light into the specimen while the detector fibers capture the reflected or transmitted light. As discussed in 1.3.1, the SD separation directly influences the photon path length and probing depth. The optimum SD should be chosen based on adequate SNR, desired probing depth and minimal cross talk between adjacent pairs.

The probe must be designed to ensure mechanical stability and flexibility during use. Any movement or misalignment of fibers during the course of measurements could compromise accuracy and lead to errors in reproducibility. The area of interest should be uniformly illuminated by the probe. To improve coupling efficiency between the probe and the specimen, index-matching pads, gels or films can be employed [110]. These materials reduce the signal reflection by creating a continuous optical path with a refractive index that matches the surrounding area. Improvement in 3D-printed materials, has allowed for rapid prototyping of various probe geometries. For clinical applications the materials used to house the fibers must be optically inert and biocompatible [111].

4.1.2 Continuous Wave Systems

CW systems as already discussed in 1.3.1 are designed to perform diffuse reflectance measurements from a medium over a broad spectral range.

Core Components

The key components of such systems include a light source, probe and a detector. An appropriate light source should be stable and provide continuous illumination. Typical sources include super-continuum lasers, halogen lamps and xenon lamps. The geometry of probe is another design parameter, which has already been covered in detail in Section 4.1.1. The final core component is a detector. A highly sensitive detector is essential to effectively capture the diffusely reflected light. To enable broadband spectral analysis, a spectrometer is typically employed for high-resolution measurements across the entire wavelength range.

The collected diffusely reflected light is influenced by the refractive indices of the material, as well as the shape and size of structures within the specimen. Therefore it is essential to ensure that the extracted spectral characteristics reflect only these intrinsic properties, rather than artifacts introduced by instrumental noise or environmental fluctuations. Calibration is typically performed by comparing the system's response to a standard with known reflectance properties [112]. The standard calibration procedure used to correct recorded spectra in a single experiment is presented in Eq. 4.1 [112]:

$$R_{\text{normalized}} = \frac{R_{\text{measured}} - R_{\text{Bkg}}}{R_{\text{standard}} - R_{\text{Bkg}}} [au] \quad (4.1)$$

Experimental Challenges

Obtaining reproducible measurements in continuous wave diffuse reflectance spectroscopy requires careful control of the experimental setup and consistent procedures. The probe must be placed firmly on the sample surface to maintain stable contact and minimize angular variation, which can strongly influence the measured reflectance.

Measurements should only be started once the light source has become stable. Any fluctuation in intensity can lead to errors in reproducibility. A sufficient warm-up time must be allowed for the light source, the duration of which is typically mentioned in data sheet otherwise can easily be empirically determined by continuously monitoring the output intensity over time. The source is considered stable when the variation in intensity falls below 2% of the mean value, indicating minimal drift [113].

Optimum selection of acquisition time is also critical to achieve good SNR. The integration time should be adjusted to ensure that the measured reflectance signal is above the system's dark noise at shorter durations. While at longer durations, it should not lead to detector saturation. SNR can also be enhanced by performing multiple measurements at the same sampling location and then averaging the resulting spectra.

4.1.3 Photon Time of Flight Systems

pToF systems are designed to measure photon temporal characteristics of light within a medium. By measuring the temporal distribution of photons pToF technique enables the estimation of key parameters such as the absorption coefficient (μ_a) and the reduced scattering coefficient (μ'_s).

Core Components

The setup configuration for a typical pToF can be divided into three sections; source arm, detection arm and reference arm.

Source Arm: The source arm typically comprises of a supercontinuum laser source for broadband pulses and a Pellin Broca prism for wavelength tuning. The source is then split into two arms in ratio of 90 : 10. The former is directed onto the sample under investigation using probe as discussed in 4.1.1, while the latter is used as reference arm. To avoid detector saturation attenuation filters can be used in either of these arms.

Detection Arm: The diffusely reflected light is collected through the fiber optic probes and directed to the detectors. Single-photon avalanche diodes (SPADs), Silicone photomultiplier (SiPM) and InGaAs photomultiplier (PMT) are commonly used detectors. The signal from detector is processed by time-correlated single photon counting (TCSPC) boards which produce a temporal point spread function or commonly called a histogram.

Reference Arm: The reference arm directs a small portion of light, i.e., 10%, onto the detector. This serves to compensate for any distortions or drifts affecting the signal.

Estimating optical properties

As discussed in chapter 2, to obtain optical properties inversion algorithms as well as numerical and stochastic approaches such as Monte Carlo simulations are commonly employed to approximate solutions.

For inversion algorithm approach, measured data is compared to analytical solution of DA and the optical parameters are iteratively adjusted to minimize discrepancies. In practice, the assumption of isotropic source is not entirely true so the analytical curve is convolved with Instrumental Response Function (IRF) to account for temporal width of the light source, response time of the detector and the signal dispersion in optical fibers. The fitting range is set so as to exclude data points on the rising edge of the time-resolved curve where the photon counts fall below 80% of the peak intensity, as DA does not accurately account for early-arriving photons. Similarly, the trailing portion of the curve (the tail) is truncated at 1% of the peak value to prevent distortions from low-count statistics. For minimizing the difference between the observed and theoretical data methods like least squares fitting, Levenberg-Marquardt etc are some of the common algorithms used [114]. The latter being considered an optimum minimization tool particularly for non-linear optimization problems [75].

On the other hand, when DA assumption doesn't hold numerical methods to retrieve the optical properties are employed. A library of Monte Carlo simulations can be created at different scattering values. This serves as the base set of simulations to obtain results without absorption effects. Any particular reduced scattering value can be interpolated from this lookup table and then a simple multiplication by a factor $\exp(\mu_a vt)$, where v is the speed of light accounts for the absorption effect. Then very similar to the case of inversion algorithm, a convolution is performed with IRF followed by minimization.

Experimental Challenges

One of the key challenges for pTOF systems is photon pile-up effect. It happens when a photon arrives during dead time of the device i.e. the time in which TCSPC board has detected an event and is getting ready for the next event detection. This pile-up effect can lead to having a combined effect of incoming photons leading to inaccurate temporal distributions. It could become a significant source of uncertainty. To prevent pile-up, the average time between incoming particles should be larger than the detector's dead time. For example, a laser operating at a repetition rate of 40 MHz has a time interval between consecutive pulses:

$$T = \frac{1}{f} = \frac{1}{40 \text{ MHz}} = 25 \text{ ns} \quad (4.2)$$

So if the detector cannot reset in $< 25 \text{ ns}$ it would lead to pile-up effect. Various techniques have been proposed in literature to prevent or reject pile-up effects including high-speed electronics can overcome this slow readout or using algorithms [115, 116]. As a rule of thumb, the detected photon rate should be kept below $< 10\%$ of the excitation pulse rate to avoid pile-up effects [117].

4.2 Raman Spectroscopy Instrumentation

This section provides an overview of the essential aspects for implementing a Raman spectroscopic investigation. It discusses factors that influence Raman spectral quality such as choice of excitation source, component configuration, sample preparation protocols, criteria for substrate selection and methods for data acquisition. Common experimental challenges are also discussed to highlight factors that can impact spectral quality.

4.2.1 Core Components

A Raman system essentially comprises of a laser light source, associated optics to guide laser to the sample and collect the scattered light and a spectrometer equipped with a charge-coupled device (CCD) detector.

Laser Source

The source wavelength is an important design consideration. Common wavelengths include 488 nm, 512 nm, 532 nm, 633 nm, 785 nm, 830 nm and 1064 nm [118, 119]. As discussed in 1.1.4, Raman scattering efficiency scales according to λ^{-4} signifying that shorter wavelengths yield a stronger signal. However, for biological specimens shorter wavelengths owing to their ability to excite electronic transitions in many chromophores also induce high fluorescence which can mask the Raman spectrum [120]. On the other hand, NIR region owing to their less energy are less likely to be absorbed and hence offer reduced fluorescence [121]. This makes longer wavelengths the preferred choice for fluorescent or biological samples but this comes at a cost of fewer scattering photons i.e. a weaker Raman signal. Generally, 785 nm balances the tradeoff between strength of Raman signal and sample fluorescence [119].

Laser power is another key parameter. For accurate and reliable spectral acquisition, it is important to preserve the physical and chemical properties of the sample being investigated. Although higher laser power densities can improve scattering efficiency, they also pose an increased risk of photothermal damage to the sample [122]. In biological

samples, excessive heating can cause irreversible changes in cellular fluids and proteins [122]. Optimizing the laser power at the sample is therefore essential to achieve a balance between signal strength and sample integrity.

Optical Delivery and Collection System

The design of optical delivery and collection system is fundamental in determining the performance of a Raman spectroscopic setup. A series of mirrors and lenses are used in the light path to efficiently deliver light onto the sample and subsequently collect the scattered light. As discussed in 1.1.4, the probability of Raman scattering is low so each component must be carefully selected and optimally aligned.

On the light delivery path, first a narrow band pass filter is used to clean the incoming laser from any light outside excitation bandwidth. This removes any spectral impurity that might have been introduced such as from a fiber between laser and the microscope. The resulting monochromatic excitation improves spectral resolution [123]. The cleaned laser input is directed to the sample. Typically, Raman systems are designed in back scattering configuration i.e. light delivery and collection are done through the same objective, as shown Fig. 4.1, providing compact system design. A dichroic mirror plays a crucial role in this configuration. It directs the laser light through a microscope objective to the sample.

Use of microscope objective is standard practice in Raman spectroscopy. It allows precise focusing of the laser onto the sample. The resolving power of the microscope can be given by [123]:

$$R_0 = \frac{0.61\lambda}{NA} \quad (4.3)$$

where NA is the numerical aperture which essentially determines the range of angles over which the system can accept or emit light [124], as shown in Fig. 4.2. It's given as [123]:

$$NA = n \sin \theta \quad (4.4)$$

where n is the refractive index of the medium between the objective lens and the sample, while θ is half the angle of the light cone formed from a focus point on the sample to the objective. Greater numerical aperture allows for more scattered light collection and finer spatial resolution [123].

The scattered light through the objective reaches the dichroic which allows the longer wavelength scattered light from the sample to pass through. Subsequently the scattered light passes through long pass edge filter to ensure removal of Rayleigh (input) laser line and only let Raman scattered light reach the spectrometer.

Spectrometer

The weak Raman spectral signal places considerable demands on the performance of the spectrometer. Optimizing the performance of a Raman spectrometer involves balancing resolution, sensitivity, spectral range and physical constraints to better suit the intended application. Key components in a spectrometer include a dispersive element, typically diffraction grating and a detector, most commonly a CCD [120]. The use of diffraction gratings allows efficient dispersion of light into its wavelength components [125]. While the use of CCDs offers high

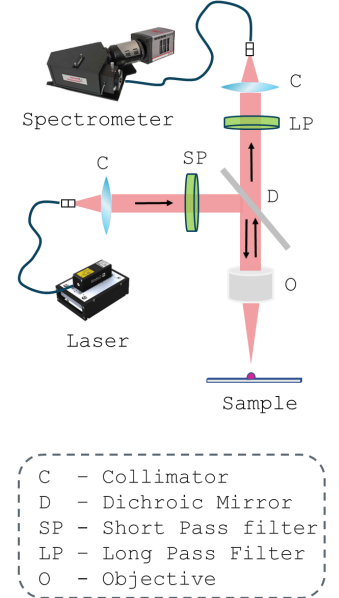


Figure 4.1: A representative Raman light delivery and collection system.

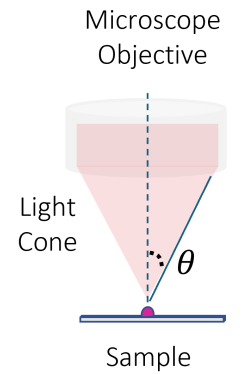


Figure 4.2: Light cone formed from microscope objective to the sample. Here θ represents half the acceptance angle for light rays from a specimen.

Table 4.1: Summary of optimization of key parameters for improving spectral resolution and their tradeoffs

Optimization	Tradeoff
↑ Grating Groove Density (<i>lines/mm</i>)	Narrower spectral range as signal is more spread out
↓ Entrance Slit Width (μm)	Reduced SNR as less light throughput
↑ Focal Length (<i>mm</i>)	Larger instrument footprint
↑ CCD Pixel Density ($\mu\text{m}/\text{mm}$)	Reduced sensitivity due to smaller pixel size

sensitivity that is beneficial for detecting subtle Raman peaks [126].

The scattered light from the sample travels through the optical delivery and collection system and enters the spectrometer via the notch filter ¹. Next comes the diffraction grating, the component that determines the spectral resolution ² of the spectrometer. The angular separation introduced by the grating is mapped to spatial separation at the image plane, a relationship quantified by the linear dispersion ³, l . The reciprocal of linear dispersion can be given as [127]:

$$\frac{d\lambda}{dl} = \frac{d \cos \theta}{mf} \quad (4.5)$$

where d is the spacing between grating lines, θ is the diffraction angle with respect to the grating surface normal, m is the diffraction order and f is the focal length of the focusing mirror. Table 4.1 summarizes parameters for improving spectral resolution.

On operational end, data collection through a spectrometer requires setting up an acquisition time. It is another key instrumental parameter that can influence spectral data. Longer exposure time ⁴ allows more Raman scattered photons to be collected hence enhancing spectral quality. As already discussed in 4.2.1 high laser power can cause photothermal damage. A key point to remember is that even at low powers, long exposure times of the spectrometer can still cause heating and induce thermal effects in the sample due to deposition of more energy ($E = P * t$). Lowest possible settings that yields adequate signal should be the starting point of the optimization.

4.2.2 Sample Preparation

Appropriate sample preparation is essential for reliable and reproducible Raman spectroscopic results. Biological samples should be handled with gloves and stored in clean containers prior to analysis. Care should be taken to preserve the native state of the specimen. It is crucial to minimize exposure to ambient light and environmental contaminants. For longitudinal experiments to ensure consistency and reliability same experimental protocol should be followed.

¹A notch filter is employed to block out Rayleigh scattering, allowing only the inelastically scattered light to pass through.

²The smallest difference that a spectrometer can distinguish two closely spaced peaks from each other.

³A measure of how far apart two wavelengths are separated on the detector in *mm*

⁴Where exposure time is the duration of a single detector frame or scan

4.2.3 Substrate Selection

The choice of Raman substrate can significantly impact SNR of the collected spectra [128]. There exists high degree of variability between substrates depending on the operating wavelengths and the Raman spectroscopy variant [129]. Ideal substrates are the ones which are chemically inert and lead to minimal Raman background to prevent interference with the analyte's spectrum.

For conventional Raman, literature documents the use of several Raman-grade substrates such as calcium fluoride, quartz, magnesium fluoride, glass, fused silica steel, aluminium etc [129]. Glass slides are among the cheapest substrates available. Glass slides can fluoresce under certain wavelengths especially with ultraviolet-visible (UV-Vis) excitation. On the other hand use of aluminum substrates not only offers background suppression but also provides a consistent Raman spectra regardless of excitation wavelength [130]. Generally, aluminum foil wrapped around a glass slide is a popular choice for substrate as it offers a flat surface ideal for placing drop-cast or dried samples, minimizes substrate interference and is cost effective.

4.2.4 Experimental Challenges

Primary goal of any data acquisition is to achieve adequate SNR. Raman spectroscopy is no different. As already discussed, key factors governing successful experimentation include careful optimization of laser wavelength laser power, optical alignment and exposure time.

For longitudinal studies maintaining identical settings is important to minimize instrumental variations and ensure consistency. However, this necessity presents a significant challenge in some cases such as varying fluorescence intensity among the same specimens. This could stem from a number of reasons including variations in sample thickness, unevenness of the substrate etc. These variations pose challenges in identifying an integration time applicable to all samples. Same acquisition settings may cause spectra from certain samples to exceed the detector's dynamic range, leading to signal saturation and distortion of the spectral profiles. On the other hand for some specimens the fixed parameters can result in weak Raman scattering leading to poor SNR. A good rule of thumb is to utilize some of the initial samples as range finding to figure out empirically the parameters that would work for majority of the specimens. While some post-processing techniques can ensure weaker SNR is still comparable with rest of spectra but information lost as a result of saturated spectra cannot be reliably corrected. These post-processing techniques will be further discussed in detail in chapter 5.

Precise laser focusing is critical to ensure adequate SNR. However, finding an optimum focus point can sometimes be challenging due to unevenness of surface, tilt or other inhomogeneities of the sample. A poorly focused laser can lead to compromised spectral quality. This issue can be minimized by doing multiple spectral acquisition at several random spots on a sample and then averaging them. This minimizes the impact of focusing errors. To be consistent, the number of random spots to be measured should be fixed for all samples throughout the duration of the study.

4.3 Summary

This chapter explores the design principles behind typical CW, pToF and Raman experiments. A comprehensive understanding of design principles is essential for setting up a successful experiment and obtaining reliable results. The application of the instrumentation discussed herein are carried on in chapter 5 and 6 which serve as the groundwork for Paper **II** and Paper **VII**.

BIOFLUID ANALYSIS

Biofluids such as blood, urine, cerebrospinal fluid etc are widely used in biomedical research and clinical diagnostics. They provide minimally invasive access to molecular and cellular information. Raman spectroscopy (RS) has emerged as a promising analytical technique for probing biofluids, offering label-free, non-destructive and highly specific molecular characterization. Building upon the discussion of experimental design considerations presented in previous chapter, 4, this chapter focuses on the data treatment and interpretation of Raman spectra obtained from biofluids, with particular emphasis on blood. Ongoing challenges related to standardization and clinical challenges are also discussed.

5.1 Blood and its Biomedical Relevance

Blood enables various functional processes within the human body. Its role ranges from serving as a medium for transport of nutrients, hormones and waste products while also facilitating cellular communication and regulating immune response [131]. It is a rich source of molecules including proteins, nucleic acids, metabolites, lipids and extracellular vesicles [132]. These molecular constituents can serve as biomarkers that reflect the physiological and pathological state of the human body. This makes blood an invaluable resource for biomedical applications.

Blood analysis is typically performed using well-established analytical techniques including flow cytometry, mass spectrometry, immunoassays, high-performance liquid chromatography (HPLC) and polymerase chain reaction (PCR) [133–135]. However, the targeted assessment of specific biomarkers is time intensive and may overlook other indicators, particularly in complex, multifactorial conditions such as inflammatory bowel diseases. Omics-based platforms offer a more detailed understanding of the molecular mechanisms facilitating more comprehensive biological profiling [136, 137]. However, the integration of these advanced approaches into routine clinical practice remains limited. Key barriers include the need for micro-sampling and the implementation of complex data analysis workflows. Thus, techniques that can reliably identify multiple biomarkers, require minimal sample preparation and are not resource intensive could benefit clinical decision making.

5.2 Potential of Raman Spectroscopy for Blood Analysis

Raman spectroscopy holds considerable promise as a powerful analytical technique for biochemical characterization of complex biofluids such as

blood. The resulting biochemical fingerprints reflects the molecular composition and structural features of these fluids. Minimal sample preparation, rapid results and the capability to detect multiple biomarkers simultaneously enhances its suitability for blood based diagnostics. Its integration into blood analysis holds great potential for improving early disease detection, monitoring treatment efficacy and advancing personalized medicine approaches.

5.3 Blood Preparation for Raman Acquisition

Slide preparation for Raman acquisition of blood and related products involves depositing a drop on the substrate of choice to acquire spectra. Typically the drop is dried before spectral acquisition to enhance signal strength and avoid effects of evaporation while acquiring the spectrum. Drying often leads to heterogeneous deposition due to faster drying on the edges which drives capillary action from center to edges to replenish the lost liquid on the edges [138]. This stronger deposition at the periphery of the drop leads to the well-known coffee ring shape [139]. This heterogeneous deposition can be taken into account by averaging multiple measurements during data acquisition. Use of vacuum pump increases the likelihood of uniform deposition as compared to open air drying. Another approach is to use a layering approach, where one layer is dried first and then a new layer is added on top. Stacking multiple layers like this can help produce clearer features.

5.4 Raman Signal Interpretation

Raw Raman spectrum is a mixture of sample composition and numerous randomly distributed parasitic effects caused by the sample itself, measurement procedure, instrumental and environmental factors [140]. These artifacts can lead to incorrect conclusions when comparing across instruments or even the same instrument over time. To overcome this various data pretreatment methods have to be employed before interpreting the results.

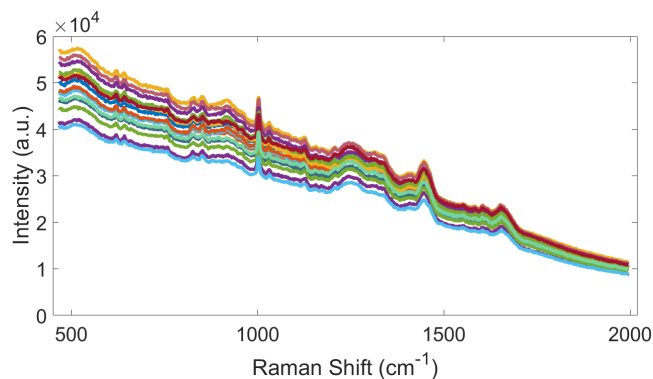


Figure 5.1: Representative raw spectra from multiple measurements at random spots.

5.4.1 Data Treatment

Spectral calibration using standard reference materials must be performed on both the wavelength and intensity axes to ensure the reliability and accuracy of Raman measurements. Once the system is properly calibrated, it is ready for the acquisition of spectral data. The recorded spectra then undergoes various data treatment steps. These typically

include spike removal, axis calibration, background subtraction, baseline correction, spectral smoothing and normalization. Such procedures can be implemented using MATLAB®, Python, or other suitable software platforms.

Cosmic Ray Removal

Sometimes cosmic spikes might appear in the spectrum. Their origin has been attributed to random hitting of high energy cosmic particles on spectrometer camera pixels during signal acquisition [141]. They appear as narrow and high intensity features anywhere along the wavenumber axis [141]. This makes them quite distinguishable from spectral features of the specimen under measurement. Fig. 5.2 illustrates a spike, notice the contrast in shape from rest of spectral features. Typically, long acquisition times of the biological specimen increases the likelihood of these rays being present.

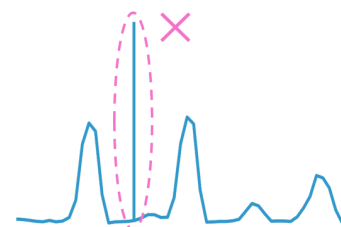


Figure 5.2: Spike represented by dotted lines.

Spike detection can be achieved by finding abnormally high intensity changes along the wavenumber axis within a spectrum. This can be achieved in an automated way by setting a certain intensity threshold for the scan or by comparing the intensities of the neighboring pixels. A second approach in practice is to record multiple spectrums and then compare them. Owing to the random nature of spike origin they will unlikely appear at the same wavenumber in successive measurements of the same specimen. Hence outliers/artifacts can easily be identified as spikes. This method carries minimal risk of affecting the actual features as compared to the first method, however it necessitates multiple acquisitions which might not always be possible.

Once identified, spikes can be removed by replacing with an interpolated value between the two points on each side of the spike. For Raman data presented in this thesis spikes were identified visually to avoid ambiguity with narrow peaks and ultimately removed by taking the average of the points on either side.

Background Removal

Background measurements account for signal contributions from the surrounding. They should be recorded with the same experimental settings as the acquisition for the specimens but without the excitation source. This recorded background is directly subtracted from the specimen spectrum before proceeding with rest of the data treatment. It is therefore crucial to record background free from cosmic rays to avoid introducing a downward signal in the specimen signal.

X-axis Calibration

The Raman shift (X-axis) represents the energy difference between the incident and scattered light. To standardize the Raman shift, reference samples are measured to identify prominent spectral peaks. These peaks are then mapped to their known positions in a pre-recorded standard spectrum and a curve fitting, typically a quadratic function, is done. This calibrated axis is subsequently applied to the Raman spectra of unknown samples.

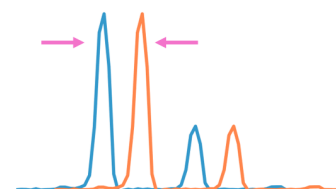


Figure 5.3: X-Axis Calibration.

Common calibration standards include acetaminophen, naphthalene, nylon and neon-argon lamps [142]. Where appropriate, these standards can be placed in custom 3D-printed holders to improve measurement reproducibility. Visualization of the wavenumber calibration process can be

achieved by overlaying the recorded and reference spectra and adjusting one along the wavenumber axis, as illustrated in Fig. 5.3.

Y-axis Calibration

Similar to X-axis calibration, Y-axis calibration or intensity calibration corrects the recorded signal intensity by measuring a known standard broad-band light source. The measured signal is first calibrated using the X-axis calibration and then a Y-axis correction factor is calculated by comparing this measured spectrum with the reference spectrum of the light source as depicted in Fig. 5.4. All the recorded sample spectrum in a particular acquisition sequence can then be calibrated by multiplying with this correction factor.

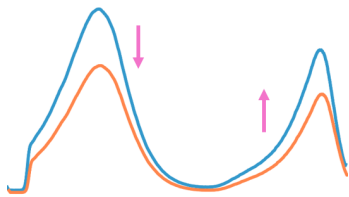


Figure 5.4: Y-Axis Calibration.

Baseline Removal

The actual Raman signal is superimposed on a varying background signal. Baseline removal step isolates the Raman signal by setting an arbitrary boundary line between both and then removing the background contribution. This is a critical step as even the introduction of small artifacts can lead to overshadowing of the Raman signal [143]. The background varies from sample to sample so several baseline correction methods have been proposed [144]. Manual methods of baseline estimation are time consuming and subjective. A number of semi-automated and automated baseline correction algorithms have also been proposed. Common methods include differentiation, polynomial fitting, Fourier transformation, Rolling Ball, Wavelet Transform and Asymmetric Least Squares (ALS) [144–146]. Each has its own benefits and pitfalls. Differentiation can distort Raman features, Fourier transform requires intervention to define upper and lower limits. Rolling Ball method conceptually simulates a ball rolling under the spectrum which is great for curved and complex baselines. Wavelet transform assumes a well separated background which is a rare possibility in biological samples. The effectiveness of these algorithms in determining true baseline is dependent on selection of the most appropriate method for a specific data and the choice of parameters which in turn is dependent on subjective judgment. Recently deep learning based methods that train neural networks for optimizing baseline have also emerged [147]. However, training these models requires a lot amount of data and is computationally intensive as well. Although the baseline corrected spectrum is free from all major artifacts but before it can be compared with others, normalization needs to be carried out.

Normalization

Normalization is the critical step in data treatment routine aimed at enhancing comparability across multiple spectrum of the same sample. Experimental conditions mainly focusing offsets, sample inhomogeneities and fluctuation of excitation intensity can lead to variability in absolute intensities between successive measurements [148]. Normalization reduces the effect of these variations by adjusting the spectrum to a common scale. Min-max, area, mean intensity, vector and standard normal variate (SNV) are some of the commonly used normalization approaches [148, 149]. Each of these has its own strengths, ultimately the choice depends on the kind of recorded data and the use case. Min-max normalization scales the intensity values of the Raman spectrum, so they fit within a fixed range, usually between 0 and 1. Area normalization divides all intensity values by the total area under the spectrum. Normally used for quantitative comparison. Mean intensity normalization normalizes all intensity values relative to the mean of all the intensities. Vector

normalization treats the spectrum as a vector and scales it to unit length i.e each intensity value is divided by Euclidian norm ¹ of the spectrum. SNV normalizes individual spectrum by centering it to zero mean and scaling it to unit variance.

Noise Filtering

Noise filtering is an optional preprocessing technique that improves clarity of spectral features by reducing noise. Spectra can become noisy due to environmental interference, low signal intensity or short acquisition times [150]. Noise filtering can improve SNR for such data hence making peaks easier to detect. Common noise filtering methods include moving average, Gaussian and Savitzky-Golay filtering [151]. These techniques minimizing abrupt variations by fitting data points within a defined window. Moving average simply replaces each data point in the spectrum with the average of its neighboring points. Gaussian smoothing acts as a weighting average filter where the weights follow a Gaussian distribution centered on the point of interest, so that points near the center of the window contribute more heavily than those at the edges. Savitzky-Golay filtering by contrast, fits a low-order polynomial to the data within a moving window and replaces the central value with the corresponding fitted value. Each of these methodologies has its own trade-offs, the best choice depends on the recorded data and the noise level. The single most important parameter for these methods is the selection of window size. Smoothing can be applied in conjunction with baseline removal and normalization steps. Although smoothing is not always essential, it is often beneficial. But it should be applied cautiously as over smoothing can distort subtle spectral features [152].

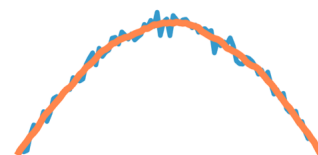


Figure 5.5: Orange line depicts smoothing using Savitzky-Golay filter.

5.4.2 Data Analysis

The treated Raman spectra is ready for qualitative and quantitative interpretation. To analyze Raman spectroscopy data, it is essential to distinguish subtle differences between samples and identify significant spectral features. In this context, statistical methods are employed to rigorously assess the significance [153]. Kruskal-Wallis test is one such non-parametric ² statistical tool that compares groups. This test is particularly useful in RS data analysis where its underlying assumptions are met including independent observations, non-normal distribution and comparable group variances [154]. For all pairwise comparisons in a group, the result is in the form of a p-value between 0 and 1 [155]. A value less than 0.05 is considered indicative of statistical significance. Box plots are commonly used to visualize intensity distribution of Raman peaks. These plots provide a clear representation of the median, interquartile range and potential outliers, aiding in the interpretation of differences between groups [154].

To assess the strength of the relationships between two groups, correlation can be computed for describing the degree and direction of the relationship. Among the various measures of association, the Pearson's correlation is widely used for continuous variables [156]. For RS data this fundamental statistical concept can offers insights into the degree of similarity between spectral features of different sample types. By examining these correlations it is possible to determine whether specific Raman peaks exhibit consistent patterns across two groups¹.

¹The length of a vector expressed as the square root of the sum of the squares of its components.

²Statistical methods that make minimal assumptions about the underlying distribution of the data

This enables the investigation of potential associations between them. Similar to Kruskal-Wallis, a p value less than 0.05 indicates that the correlation coefficient is statistically significant [157].

Together, these statistical approaches, non-parametric statistical testing (Kruskal-Wallis) and correlation analysis (Pearson's Correlation), offer a robust approach for analyzing RS data. They enable researchers to uncover meaningful relationships from spectral features, distinguish between different sample types and draw reliable conclusions despite data distribution and variability challenges of RS data.

5.5 RS Biofluid Experimental Challenges

RS analysis of biofluids like blood plasma offers a minimally invasive diagnostic approach but faces significant reproducibility challenges across populations, instruments and healthcare settings. A primary limitation is the inherently weak Raman scattering signal, often obscured by strong fluorescence backgrounds, complicating the detection of low-abundance biomarkers. Advancements like surface-enhanced Raman spectroscopy (SERS) can improve sensitivity. However, the dynamic and heterogeneous nature of biofluids introduces variability due to inter-individual differences like diet, medication, hormonal fluctuations and underlying health conditions [158, 159]. Pre-analytical factors such as sample collection, storage duration, transport conditions and freeze-thaw cycles also critically influence spectral quality and metabolic stability [160]. In fact, an estimated 70 % of laboratory errors occur in this phase [161]. Proper handling including storage at 4 °C for short-term and -80 °C for long-term preservation, aliquot division to avoid repeated thawing and careful temperature stabilization is essential to maintain molecular integrity. Creating and following standard protocols throughout the course of a study is essential for distinguishing biological variability from these methodological artifacts.

5.6 Summary

Biofluid analysis represents an important avenue for advancing biomedical diagnostics and personalized medicine. Owing to their rich biochemical composition and accessibility, biofluids offer immense potential for minimally invasive disease monitoring. This chapter explores the application of Raman spectroscopy for biofluid analysis, particularly blood, covering key aspects including sample preparation, signal acquisition, spectral treatment and interpretation. This chapter lays the groundwork for more in-depth exploration presented in Paper **VII** which explores how variations in Raman spectra of blood can be correlated with the spectra from tissues.

TISSUE CHARACTERIZATION

Biological media is highly scattering in the optical window ($\sim 700 \text{ nm}$ to 1100 nm) resulting in highly diffuse photon propagation. Extracting useful information in this window opens doors for non-invasive optical diagnostics and therapeutic monitoring. The key optical parameters can provide quantitative information about chromophore concentrations and tissue composition. The need to assess optical properties of tissues arises from attempts to solve the forward or the inverse problem of predicting diffuse light distribution in tissues (chapter 2). This chapter discusses continuous wave (CW), time domain (TD) and frequency domain (FD) techniques for the extraction of optical properties.

6.1 Diffuse Optics

Tissue characterization is fundamental to diagnostics, therapeutic and biomedical research. Optical measurements provide a non-invasive means to characterize tissue giving valuable insights. Biological tissues represent an environment of cells and organelles of various shapes, sizes and optical properties. These structures serve as absorbing and scattering centers for incoming light.

Absorption is predominantly due to tissue chromophores including hemoglobin, melanin, lipids, water, collagen etc. Prior knowledge of these chromophores for various wavelengths gives the possibility of estimating these tissue chromophores using Eq. (1.2). Similarly as discussed in 1.1.4, reduced scattering has a contribution of Mie and Rayleigh scattering. The scattering wavelength dependence for a broad range of scattering particle sizes can be described by the following empirical relation [162]:

$$\mu'_s(\lambda) = a_{Mie} \left(\frac{\lambda}{\lambda_0} \right)^{-b} + a_{Rayleigh} \left(\frac{\lambda}{\lambda_0} \right)^{-4} \quad (6.1)$$

where λ_0 is the reference wavelength, a_{Mie} and $a_{Rayleigh}$ is the Mie and Rayleigh scattering amplitude respectively, reflecting density of scatterers in the medium while b is the scattering power depending upon the size of the scatterers. Eq. (6.1) describes how reduced scattering coefficient decreases with increasing wavelength. Although both types of scattering are present in tissues, the Rayleigh component is mostly overshadowed by the predominant Mie scattering [162].

6.2 Methods for Extracting Optical Properties

Optical properties can be derived by injecting light into a medium and then studying the spatial and temporal profile of the detected light. Mul-

multiple wavelengths can be employed for this purpose. Primary modalities of diffuse optics are Continuous Wave (CW), Frequency Domain (FD) and Time Domain (TD) each having its unique strengths and limitations.

6.2.1 Continuous Wave (CW)

CW systems utilize a CW light source to measure the intensity attenuation of light after it propagates through tissue. It's cost effective, simple and capable of broadband measurements. However, the system yields a single intensity value per wavelength that has a combined effect of μ_a and μ'_s termed as effective attenuation, μ_{eff} . This single measurement has two unknowns hence insufficient to independently evaluate absorption and scattering properties.

Spatially resolved CW measurements address this limitation by acquiring diffuse reflectance at multiple SD separations d . Although CW systems cannot completely disentangle μ_a and μ'_s but it still provides reasonable estimation that is valuable for tissue characterization and monitoring physiological changes. This approach is based on DA, discussed in chapter 2, which predicts decay in diffusion intensity with increasing distance from source. The detected diffuse intensity $I(d)$ at SD separation d can be approximated by the following equation [74]:

$$\ln[I(d)d^2] = k - d\mu_{\text{eff}} \quad (6.2)$$

Where k is a constant that depends on μ_{eff} which in turn can be given as:

$$\mu_{\text{eff}} = \sqrt{3\mu_a(\mu_a + \mu'_s)} \quad (6.3)$$

Under diffusion regime scattering is predominant, i.e $\mu'_s \gg \mu_a$, allowing effective attenuation in Eq. 6.3 to be approximated as:

$$\mu_{\text{eff}} \approx \sqrt{3\mu_a\mu'_s} \quad (6.4)$$

The slope of the resulting graph of various spatial measurements, as illustrated in 6.1 yields μ_{eff} . Then using power law dependence in eq 6.1, fitting algorithms such as Levenberg-Marquardt can be used to fit $a, b, \mu_a(\lambda)$. Hence utilizing the distance-dependent reflectance profile intensity, it becomes possible to estimate μ_a and μ'_s . This way spatially resolved CW approaches provide a practical approach to extract optical parameters. An important thing to note is that the reliability of the results is dependent on how strongly DA assumption holds.

6.2.2 Time Domain (TD)

TD approaches makes use of pulsed lasers and sensitive detectors. The time gating capability inherently provides the benefit of depth sensitivity of media under test as late arriving photons have probed greater depth [61]. As discussed in 1.3.2, since absorption and scattering affect different parts of the detected pulse TD approaches can detangle absorption and scattering contributions simultaneously. An inherent benefit is that since temporal shift is being calculated the systems are less sensitive to losses in absolute amplitude such as coupling losses. However, on the other hand these systems are susceptible to drift and calibration challenges, require bulky instrumentation and broadband measurements are time intensive.

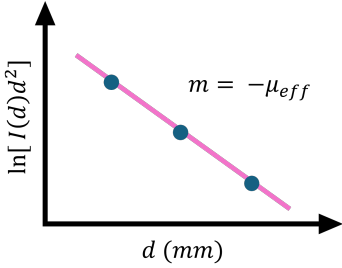


Figure 6.1: Spatially resolved CW diffuse reflectance.

6.2.3 Frequency Domain (FD)

FD systems introduce amplitude modulated light into the sample and detect it after propagating through tissue. The phase shifts and amplitude changes relative to a source or some other reference signal gives information about absorption and scattering coefficient of the sample [163]. Similar to pToF systems, it can decouple absorption and scattering parameters. The ability of fast sampling adds to their attractiveness especially for clinical applications. It requires less sophisticated instrumentation as compared to pToF but on the other hand, non-availability of broadband sources is a major challenge that limits FD systems to only a few wavelengths [164, 165].

6.3 System Characterization and Performance Assessment

Phantoms serve as a means for characterizing system performance. MEDPHOT protocol is a well-established standard for assessing instrument performance in retrieving absorption and scattering coefficients. It contains a set of 32 optical phantoms with varying optical properties for studying accuracy, linearity, noise, stability and reproducibility of the instrument [166]. The benchmarking capabilities offered supports reproducibility and reliability of measurements. Generally a coefficient of variation (CV) of less than 3% for three measurements is acceptable level of variation of the system [167].

6.4 Potential and Challenges

Broadband measurements offer a significant advantage by enabling detection of unknown or disregarded absorbers such as low concentration biomolecules in tissues. Each of the techniques discussed in 6.2 has its own pros and cons. CW systems are broadband and simple to deploy in a clinical setting. However, they only reveal a single intensity attenuation that has a combined effect of scattering and absorption. Employing spatially resolved measurements can partially overcome this limitation and partially decouple optical parameters. However, a main disadvantage is that the short SD separation results in only superficial probing hence compromises quantitative accuracy [61].

FD approaches, on the other hand permit disentangling of optical parameters but they are typically restricted to a few modulation frequencies only. Theoretically, TD approaches are equivalent to FD approaches but in practice owing to this limitation that's not always a reality. This leads to a large number of SD channels making the system bulky. To address the challenge of limited wavelength coverage of FD approaches, several studies have reported the development of hybrid CW–FD systems, where broadband CW measurements are combined with FD measurements at discrete wavelengths to estimate optical parameters [164, 168–170]. These take greater foothold in clinical space owing to much more decreased hardware foot print. However, while these systems can quantify absolute optical properties and offer better depth sensitivity than CW alone, TD systems are able to provide depth discrimination and absolute quantification of properties [171]. This is of relevance when investigating biological media.

6.5 Summary

This chapter describes the techniques for measurement of tissue optical properties (absorption and scattering) from the spatial or temporal profile of the light detected after propagation in tissues. The information can either be in response to a broadband light, a laser pulse or a modulated wave representing CW, TD and FD approaches. The tradeoffs of each must be considered in context of intended application. This chapter lays the foundation for Paper **II** which presents the design, development, characterization and experimental validation of a prototype system based on a hybrid analytical model combining CW and TD approaches.

CONCLUSIONS

Tissue characterization is of significant clinical importance for diagnosis, treatment planning and ongoing monitoring. Optical technologies provide a non-invasive means for probing tissues. Through the analysis of detected light using appropriate light propagation models, optical signals can be correlated with underlying biochemical changes within tissue. This work has sought to develop a framework for translating optical techniques into clinical practice by evaluating their potential for quantitatively assessing the physiological and pathological state of tissues. The thesis addresses this goal through the development of analytical modelling, design of anatomically realistic tissue-mimicking phantoms and investigation of biofluid-based spectroscopy as indirect indicator of tissue pathology. Looking ahead, the integration of these approaches into clinical workflows presents significant opportunities for advancing diagnostic and therapeutic practices. The key contributions and future work corresponding to each research objective are outlined below.

7.1 Retrieval of Tissue Optical properties

Complementing the overarching aim of this thesis, Paper **I** demonstrated the direct clinical utility of continuous wave diffuse reflectance spectroscopy (CW-DRS) for early detection of oral cancer. This study established that spectral signature modulated as a result of underlying biochemical changes can act as a diagnostic clinical tool for discriminating healthy tissue from diseased. These findings offered a strong motivation for advancing optical techniques towards more quantitative tissue characterization. Building on this evidence of clinical feasibility, the analytical model developed in Paper **II** addresses the broader methodological challenges of accurate broadband optical property extraction. It integrates continuous wave and photon time of flight (pToF) modalities into a hybrid algorithm, enabling estimation of absorption and scattering coefficients under clinically realistic conditions. The broadband characterization enhances the ability to detect low-concentration chromophores as well as improving potential for early disease detection. This combined approach leverages the broad spectral coverage and simplicity of CW systems alongside the depth sensitivity and temporal resolution of pToF techniques. Compared to the individual modalities, this hybrid approach offers a balanced solution prioritizing accuracy, speed and clinical feasibility for extracting physiologically meaningful optical parameters. By improving sensitivity to low concentration chromophores and enhancing depth resolved characterization, the hybrid model expands the diagnostic potential demonstrated by the CW study. The CW-DRS and the hybrid analytical model represent both the theoretical and applied dimensions

of optical diagnostics. One validating the diagnostic potential of optical spectroscopy in a real clinical context while the other advancing the methodological tools for tissue characterization. Together they reinforces the central hypothesis of this thesis i.e optical modalities hold potential to non-invasively provide physiologically relevant diagnostic information for informed clinical decision making. Future investigations should validate the hybrid analytical model in heterogeneous patient cohorts for exploring its translation into specific clinical applications.

7.2 Tissue Mimicking Phantoms

A second objective was to determine whether optical phantoms could be engineered to incorporate both the structural and optical complexity of human anatomy. Paper **III** using a hybrid solid-liquid interface demonstrates an anatomically correct optical tissue-mimicking phantom for therapeutic planning. The ability to provide realistic conditions for incoming light improves its usefulness for interstitial photodynamic therapy (iPDT) planning. The developed phantom represents an advancement beyond conventional phantoms, which typically prioritize either anatomical or optical fidelity but rarely achieve both. This improved realism allowed for further advances, including a two-stage dosimetry reconstruction algorithm for iPDT application introduced in Paper **IV**. The complex hybrid solid-liquid phantom concept was also explored using bioresorbable fibers in Paper **V**. Collectively, these developments highlight how anatomically and optically faithful phantoms can accelerate translation of optical therapies to the clinic. While the current phantom provides an anatomically accurate yet static platform, future work should focus on transforming it into a dynamic phantom. Inclusion of finer structures such as blood vessels to account for blood perfusions, mimicking the pulsatile nature of vessel are some of the potential avenues to explore. Such developments would substantially strengthen system validation and treatment planning.

7.3 Biofluid Spectral Analysis

The final component of this work explored whether biofluid-based optical spectroscopy can serve as a minimally invasive means of assessing pathological and physiological state of tissue. The rich molecular content of biofluids changes in response to disease activity making them suitable for monitoring and therapeutic assessments. The ease of accessibility and inexpensive extraction adds to their attractiveness. The study presented in Paper **VII** demonstrate that Raman spectroscopy of blood can identify spectral signatures associated with Crohn's disease, an inflammatory disorder of the gastrointestinal tract. By correlating Raman spectral features with paired biopsy samples, the study accounts for inherent inter-patient and intra-patient variability in biofluid composition. This approach positions biofluid spectroscopy as a complementary diagnostic and monitoring tool that can extend the reach of optical techniques beyond direct tissue interrogation in a clinically relevant disease model. Compared with standard biochemical assays Raman analysis offers the advantages of simultaneous determination of various metabolites making it potentially suitable for longitudinal monitoring. This work opens doors for providing alternate monitoring and assessment tools. The patient-specific insights with increased comfort holds potential in broadening accessibility and acceptance of optical technologies in routine care. Efforts should be directed towards increasing cohort size and investigating effect of various medications on blood spectral profile for a comprehensive patient centered future.

7.4 Integrated Perspective

This thesis explored optical techniques for quantitative assessment of physiological and pathological state of tissues for clinically significant applications. It has presented diagnostics, analytical modelling, anatomically realistic tissue-mimicking phantoms and biofluid based spectroscopy to examine both direct and indirect optical interrogation of tissues.

Each research objective addresses a distinct challenge inherent to optical biophotonics, yet their combined contribution provides a unified framework. The CW-DRS study validates the diagnostic potential of optical spectroscopy in a clinical context. Moving towards more quantitative tissue characterization the analytical modelling component establishes a rigorous basis for interpreting light–tissue interactions. This enables the extraction of physiologically relevant optical biomarkers from measurable signals. By linking observable optical responses to underlying tissue properties, this work advances the reliability and interpretability of optical measurements.

Complementing the diagnostic aspect, the development of anatomically realistic hybrid tissue phantoms provides a controlled yet physiologically relevant platform for experimental validation. These phantoms bridge the gap between in vivo tissues and simplified optical phantom models by incorporating structural complexity as well. This additional capability makes them valuable tools for optimizing patient specific therapeutic strategies. This integration ensures that theoretical predictions can be meaningfully refined in controlled but clinically realistic scenarios.

The investigation of biofluid-based spectroscopy extends the scope of optical assessment to situations where direct tissue access may be limited or impractical. This approach broadens the applicability of optical techniques by demonstrating that optical signatures in biofluid can reflect underlying tissue pathology. This opens pathway for longitudinal monitoring or minimally invasive diagnostics and reinforces the adaptability of biophotonic methods across diverse clinical constraints.

The integration of the research objectives collectively illustrates how optical methods can support diagnostic, therapeutic and monitoring objectives within a single conceptual framework. The analytical model provides quantitative insight, the anatomically realistic phantom enables robust validation and optimization while biofluid spectroscopy enhances monitoring accessibility. Together, these contributions affirm the central hypothesis of this work that optical techniques can capture clinically meaningful physiological and pathological information. This highlights the potential of optical biophotonics to evolve into a unified and patient-centered healthcare ecosystem.

7.5 Summary

This thesis investigated the potential of optical techniques to address challenges in tissue identification and characterization through diagnostic, analytical modelling, phantom development and biofluid spectral analysis. The findings demonstrate the potential of optical diagnostics in a clinical setting, show that broadband analytical models can extract meaningful optical parameters, indicate that anatomically informed hybrid phantoms can enhance system validation and treatment planning, and reveal that spectroscopy of biofluids can provide minimally invasive

indicators of tissue pathology. This work advances tissue identification towards a more patient-centered future while opening promising avenues for further exploration in the field of biophotonics.

COMMENTS ON THE PAPERS

I Diagnostic potential of diffuse reflectance spectroscopy and feature selection in identifying oral potentially malignant disorders

This study combines continuous wave diffuse reflectance spectroscopy with machine learning for detecting early pathological changes leading to oral potentially malignant disorders (OPMD). Feature selection using LASSO identified key diagnostically relevant wavelengths linked to haemoglobin, absorption, oxygenation and tissue scattering. All indicative of underlying biochemical changes in tissues. The approach demonstrated potential of continuous wave diffuse reflectance spectroscopy for real-time, non-invasive oral cancer screening and biopsy guidance. I was responsible for building of real time interface for data acquisition, system validation, clinical data collection as well as assisted in the writing of the manuscript. This manuscript is still in progress.

II Hybrid multi-distance continuous wave and discrete-wavelength time-domain analytical model for broadband optical characterization

Diffuse optical spectroscopy is a powerful tool to noninvasively measure optical properties of biological tissues. This can provide valuable insights into tissue composition, blood oxygenation and other physiological parameters. Continuous-wave (CW) diffuse reflectance systems are simple and cost effective but they are incapable of independently resolving absorption and scattering coefficients. Frequency Domain (FD) and Time-Domain (TD) methods overcome this limitation however FD systems are limited by non-availability of broadband sources while TD systems are limited by complex instrumentation and time consuming measurements. This work presents an analytical model by combining pToF and CW approaches for broadband extraction of optical properties. The proposed model offers accuracy and depth sensitivity of pToF systems, while minimizing the system complexity and physical footprint as well as reducing time intensive nature of full broadband time-resolved systems. I was responsible for data collection, analysis, algorithm development and manuscript preparation. This manuscript is still in progress.

III Hybrid heterogeneous phantoms for biomedical applications: a demonstration to dosimetry validation

This paper presents a novel phantom design for realizing anatomically realistic prostate phantoms anatomy for photodynamic therapy (PDT) application. Combining the strengths of solid and liquid phantoms, it features a photosensitizer, Visudyne, infused gelatin lesion representing tumor within an intralipid filled prostate shape, enclosed in a silicone outer shell representing surrounding tissue. Plastic molds were created for the various anatomical structures based on patient specific ultrasound data and ultimately put together to realize this hybrid phantom. PDT like light delivery was achieved using 13 optical fibers placed at different positions as determined by SpectraCure's IDOSE® software. The same software was used for tomographic evaluation. The proposed heterogeneous phantom holds potential to facilitate further development and optimization of tomographic reconstruction algorithms. I contributed significantly to all aspects including the manufacturing, assembly, testing, and validation of phantoms. Additionally, I was involved in conducting the final measurements using the SpectraCure P18 system. I was also responsible for writing the manuscript.

IV Two-stage diffuse fluorescence tomography for monitoring of drug distribution in photodynamic therapy of tumors

Determining spatial distribution of photosensitizing drug in PDT is crucial for dosimetry decisions. This work introduces a two-stage diffuse optical tomography algorithm for real-time, interstitial fluorescence measurements to accurately map drug distribution. The first stage uses standard tomographic reconstruction methods and then feeds its results into the second stage. Capabilities and limitations of the proposed algorithm were analyzed using computer simulations. Experimental validation was carried on prostate mimicking tissue phantoms, realized using the process proposed in Paper **III**. Light delivery and data collection was done using SpectraCure's IDOSE® software. The proposed algorithm can be used for monitoring temporal evolution of the photosensitizing drug concentration to adjust the light dose in real time during therapy session. I manufactured the phantoms of various concentrations utilized for experimental verification of the proposed algorithm, involved in final measurements using the SpectraCure P18 system as well as assisted in the writing of the manuscript.

V Resorbable optical fibers for interstitial photodynamic therapy — assessment of photosensitizer spatial distribution in tumors

This work investigates the potential of bioabsorbable optical fibers for light delivery and monitoring the spatial distribution of photosensitizing drugs in interstitial PDT. Tumor-mimicking inclusions containing Verteporfin, a PS routinely used in PDT, were realized by utilizing the process proposed in Paper **III**. Performance evaluation was done in comparison to conventional silica fibers using SpectraCure's IDOSE® software. Use of bioabsorbable fibers features capability to be dissolved in physiological media over a clinically relevant time. This bypasses the need for fiber extraction after a PDT session, a benefit that holds potential for extended and real-time monitoring of relevant tissue characteristics, or biochemical information. I fabricated the phantoms for demonstration of the fibers as well as assisted in the writing of the manuscript.

VI Mobile multi-configuration clinical translational Raman system for oral cancer application

This study reports the design and development of a versatile, cost-effective Raman spectroscopy system capable of operating in three configurations designed specifically for analysis of biological specimens, both in vivo and ex vivo. The system includes a customized Raman microscope for ex vivo specimen analysis, a novel photonic crystal fiber (PCF) stage to measure analyte in native state, and a multimodal fiber optic probe for in vivo tissue analysis. The modular system offers flexibility in terms of samples that can be analyzed hence holding immense potential for clinical applications. I was involved in ex vivo specimen collection as well as in vivo spectroscopic data collection that was used for testing and verification of the system.

VII Biomolecular Fingerprint of Crohn's Disease Using Raman Spectroscopy

This work explores utilization of Raman spectroscopy of blood plasma samples as a minimally invasive tool for monitoring Crohn's disease activity. The need for monitoring arises from the relapsing and remitting nature of the disease. As compared to tissue biopsies, the ease of blood collection contributes significantly to patient comfort. To this purpose investigation was done into underlying biochemical changes in blood spectra of disease, inflammatory status and the treatment regime. Histopathological outcome from paired biopsies served as the gold standard. The results hold potential for personalized disease management such as treat2target approaches. I was responsible for preparation of supporting documents for ethical approval of this study, setting up of specimen collection protocols, handling biospecimen logistics from clinic, processing and measuring the specimens and data treatment of acquired spectra. I was also responsible for writing the manuscript.

REFLECTION ON MY DOCTORAL TRAINING

Pursuing a doctorate has been an enriching and transformative experience. One that has not only expanded my technical understanding but also significantly shaped who I am as a professional. I find it fitting to reflect on the wealth of experience gained.

Each research project brings about its own set of challenges. My journey was no different. Whether they be instrument issues, things not going as per initial plan, balancing deadlines, need for protocol refinement or sorting documentary formalities. These challenges in many ways helped improve my adaptability by developing skills for troubleshooting setbacks and staying focused under pressure. I believe this didn't just prepare me for a research career. It also gave me transferable skills I rely on every day, from project management and problem-solving to a mindset of continuous learning

A major part of my work revolved around clinical exploration. This has undoubtedly been one of the most rewarding segment of my doctoral journey. I was involved in various aspects all the way from drafting supporting documents for ethical approval, looking into safety and risk management when dealing with biological specimens, developing data management plans in line with FAIR principles and GDPR guidelines and managing day-to-day operations. Working alongside clinical and technical staff has helped in developing a mindset for identifying a clinical need and developing a solution for addressing it. The perspective developed will no doubt enhance the practical adaptability of my future work.

I feel truly fortunate to have been part of the Marie Curie PHAST-ETN. Its intersectoral secondments and EU-wide training events broadened my perspective in ways I hadn't anticipated. Moving between partner institutes exposed me to diverse approaches, while workshops and summer schools enriched my skills on both technical and transferable levels. These experiences played a meaningful role in shaping my professional development. In particular, the grant writing training proved instrumental because of how it contributed to three successful funding applications during the time of my PhD: BEHealSy program under the EIT Health label and three Erasmus+ training awards. I look forward to building on the connections formed as part of these projects and continuing to work together in the years ahead to help translate novel medical technologies into commercial reality.

During my doctoral training I had the opportunity to work within a diverse research team, bringing together professionals from various cultural and academic backgrounds. This strengthened my ability

to navigate cross-cultural dynamics and foster collaborations where diverse perspectives are respected. Acting as the corresponding author on two manuscripts taught me how to coordinate with editors and respond constructively to reviewer feedback. I also got an opportunity for reviewing a manuscript myself, allowing me to see the process from the other side sharpening my critical perspective and judgment. Additionally, in line with my institution's policy, I actively participated in at least two education and public engagement activities each year to promote STEM. These activities helped me develop the ability to present complex scientific content to a broad and varied audience.

In many ways, this journey was a test of persistence and adaptability, but also one of discovery and growth. The learning continues...!

ACKNOWLEDGEMENTS

Undertaking a PhD has been one of the most challenging yet extremely rewarding endeavor. I would like to acknowledge and extend my heart-felt gratitude to the following who have made the completion of this milestone possible.

First, I wish to express my sincere gratitude to my supervisors Prof. Stefan Andersson-Engels, Dr. Sanathana Konugolu-Venkata-Sekar and Mr. Ray Burke for their stimulating discussions, generous approachability and constant encouragement throughout the development of this thesis. I owe a great deal of thanks to my research advisor Dr. Rekha Gautam as well. I truly appreciate the efforts that they all put into teaching and mentoring. Without their precise and appropriate guidance, I could not have come this far.

I would like to extend my sincere thanks to all collaborators and coauthors who have supported this work. I am especially grateful to SpectraCure AB (Sweden), Politecnico di Milano (Italy) and BioPixS (Ireland) for providing access to their facilities and resources, which played a crucial role in advancing the experimental aspects of this thesis. I also wish to thank the clinical collaborators that I had the privilege to work with at APC Microbiome (Ireland), Cork University Hospital (Ireland) and Mercy University Hospital (Ireland). Their ongoing support and clinical insight have been invaluable in shaping the direction of the projects and ensuring relevance to real-world medical needs.

A heartfelt thank you to the PHAST-ETN for the outstanding training opportunities that have played a pivotal role in equipping me with skills helpful for this thesis as well as shaping my future career. Special thanks to the PHAST coordination office for making all of this possible through their exceptional administrative support.

I would like to thank Tyndall National Institute for providing me with an environment that fosters multidisciplinary research and collaborations. Moreover, I am grateful to all my colleagues, past and present, in the Biophotonics@Tyndall particularly Siddra, Dario, Hui, Hasti. Thank you for all the valuable discussions and fun filled memories. I also highly appreciate Sarah's support with various administrative matters.

Finally, I express my deepest gratitude to my family for their constant love and encouragement. I feel truly fortunate to have such wonderful siblings whose constant support and best wishes have always been with me. Words cannot fully capture my thankfulness to my parents without their sacrifices, support and prayers, this milestone would not have been possible. I wholeheartedly dedicate this thesis to them.

FUNDING INFO

The research activity presented in this thesis has been kindly supported by the following organizations:

- European Union’s Horizon 2020 research and innovation programme “Photonics for Healthcare: multiscale cancer diagnosis and therapy” (PHAST) under the Marie Skłodowska-Curie grant agreement No 860185.
- Science Foundation Ireland (SFI) under grant agreement number SFI/15/RP/2828 & SFI-12/RC/2276 P2 & SFI-22/RP-2TF/10293.

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PAPERS

Diagnostic potential of diffuse reflectance spectroscopy and feature selection in identifying oral potentially malignant disorders

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XYZ 00(0), 000-000 (xxxx).

Diagnostic potential of diffuse reflectance spectroscopy and feature selection in identifying oral potentially malignant disorders

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Abstract.

Significance

Early detection of oral potentially malignant disorders (OPMD) is essential to prevent progression to oral cancer. This requires objective, non-invasive tools capable of identifying subtle, subclinical physiological alterations in oral tissue associated with disease progression.

Aim

This study investigated the diagnostic potential of diffuse reflectance spectroscopy (DRS) combined with machine learning-based feature selection for identifying spectral bands of importance and differentiating OPMD from healthy oral mucosa across multiple anatomical sites.

Approach

In vivo broadband DRS (377 - 1075 nm) spectra were acquired from 82 participants including 22 healthy controls and 60 OPMD patients. Least Absolute Shrinkage and Selection Operator (LASSO) was used to select diagnostically relevant wavelengths, which were subsequently used for tissue classification using Linear Discriminant Analysis (LDA) with leave-one-out cross-validation.

Results

Distinct spectral differences were observed between OPMD and healthy tissues, corresponding to variations in haemoglobin absorption, oxygenation and tissue scattering. LASSO identified 14 key wavelengths primarily within 380-600 nm and 900-1075 nm spectral regions. Classification accuracy improved when using selected features compared to full-spectrum analysis, achieving 87% accuracy for cheek mucosa and 89% for ventral surface of tongue. Contralateral normal-appearing sites exhibited intermediate spectral behaviour, suggesting early field effects.

Conclusions

DRS combined with LASSO-based feature selection enables accurate and objective discrimination of OPMD lesions suitable for real-time implementation. The method provides physiological insights into early optical changes and supports the development of compact, multispectral devices for non-invasive oral cancer screening and biopsy guidance.

Keywords: Diffuse reflectance spectroscopy, oral potentially malignant disorders, oral cancer, feature selection, LASSO, non-invasive diagnostics.

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1 Introduction

Oral cancer is the 16th most common cancer worldwide, with a survival rate strongly associated with the stage of the disease at the time of diagnosis.^{1,2} More than 45% of the cancer cases have metastasised to the lymph nodes when first diagnosed.^{2,3} Early-stage diagnosis remains challenging due to the lack of obvious clinical signs and the limited sensitivity of visual examination or commercially available oral screening methods. More than 90% of oral cancers constitute squamous cell carcinomas (OSCC), which originate from genetic mutations in epithelial cells⁴ that trigger uncontrolled cell proliferation. In some cases, the body's defence mechanisms are unable to repair DNA damage or eliminate these abnormal cells through apoptosis, leading to neoplastic transformation.⁵ A critical stage in oral carcinogenesis is the development of oral potentially malignant disorders (OPMD), a group of precancerous conditions that increase the risk of progression to OSCC.⁶ Identifying these early-stage abnormalities can help facilitate timely intervention and improve patient outcomes.

OPMD often presents as persistent white or red patches, or as easily bleeding sores on the lips or within the oral cavity, such as leukoplakia and lichen planus. As the disease progresses, these lesions can develop into non-healing ulcers, frequently accompanied by numbness, tenderness, pain and difficulty in swallowing. Figure 1 illustrates the typical progression of these lesions within oral tissue. The diagnostic procedure for OPMD involves reviewing the patient's medical history and conducting a visual examination. However, a definitive diagnosis relies on histopathological evaluation of the biopsy sample. Despite its high accuracy, biopsy is invasive, time-consuming, impractical for multifocal lesions and sometimes prone to variability in sampling and interpretation.⁷ Traditional imaging techniques such as computed tomography (CT) and magnetic resonance

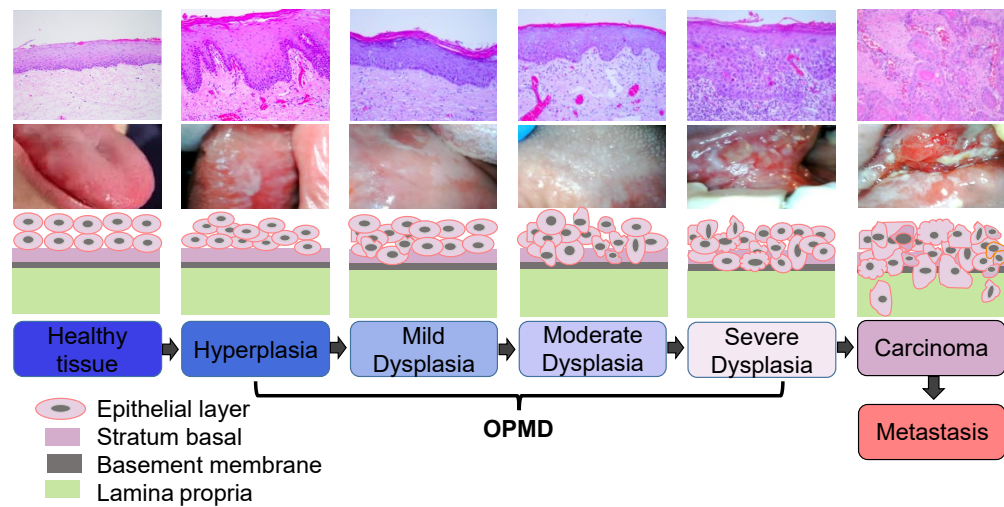


Fig 1 Progressive morphological changes in oral mucosa, from healthy tissue to hyperplasia and oral potentially malignant disorders (OPMDs), progressing through mild, moderate, and severe dysplasia to carcinoma and metastasis. Key anatomical layers, including the epithelial layer, stratum basale, basement membrane, and lamina propria, are indicated.

60 imaging (MRI) can help to assess the extent and progression of the lesions, but are expensive and
 61 unsuitable for screening and routine monitoring. Given these limitations, non-invasive diagnostic
 62 approaches are being widely explored.

63 Several studies have explored exhaled breath analysis to detect volatile organic compounds
 64 that exhibit distinct concentration profiles associated with disease progression.^{8,9} Other research
 65 focuses on blood- and saliva-based biomarkers, such as circulating tumor cells, DNA and RNA
 66 fragments;¹⁰⁻¹² specific proteins¹³ including CD44,¹⁴ Cyfra 21,¹⁵ CA-125;¹⁶ inflammatory markers
 67 such as C-reactive proteins.¹⁷ These biomarkers are typically analysed using complicated labora-
 68 tory techniques like enzyme-linked immunosorbent assays (ELISA),¹⁷ next generation sequenc-
 69 ing¹⁸ or polymerase chain reaction (PCR).¹⁹ Salivary IL-1 β and IL-8 have also been reported as
 70 promising biomarkers for diagnosing oral cancer.²⁰ Moreover, saliva-based home test kits, such
 71 as “CancerDetect Test for Oral and Throat Cancer”^{21,22} have been introduced. However, most of

these methods lack FDA approval or widespread clinical acceptance and often struggle to detect biomarkers at ultra-low concentrations during the OPMD stage.^{23,24} Therefore, there remains an urgent need for a cost-effective, non-invasive, real-time screening tool that can be easily integrated into clinical workflows.

Among various emerging technologies, optical spectroscopy offers a promising, label-free approach to tissue characterisation, providing non-invasive insights into biochemical and morphological changes associated with disease progression. Techniques such as fluorescence, Raman spectroscopy and diffuse reflectance spectroscopy (DRS) have demonstrated potential in differentiating between normal and abnormal oral tissues.¹³ Fluorescence-based adjunctive optical screening devices, such as Vizilite and VELscope, have been developed to enhance the visualisation of oral mucosa abnormalities. While offering improved lesion contrast, their diagnostic accuracy remains limited.²⁵ On the other hand, DRS encodes information related to tissue's biochemical and structural properties including haemoglobin concentration, oxygenation levels and scattering changes due to alterations in epithelial thickness and cellular morphology, typically indicative of OPMD progression.²⁶ Some works have explored the combination of fluorescence with DRS.²⁷⁻²⁹ For example, a commercially available device, Identafi®, combines autofluorescence from epithelial and stromal layers and reflectance in 500-600 nm region to visualise vasculature with only moderate sensitivity and specificity in differentiating oral lesions.³⁰ Previous studies focusing solely on DRS have reported effective discrimination between normal, OPMD and malignant tissues.^{31,32} Many of the studies have concentrated on buccal mucosa and tongue lesions - the most frequent OPMD sites - reporting in-vivo accuracy values in a wide range from 75% up to 98%.³²⁻³⁴ Other studies explored the potential of DRS as a tool for the evaluation of resection margins³⁵ and biopsy guidance³⁶ with encouraging results. While a majority of the works analyse the full DRS spectrum

(typically 400 - 900 nm), others focus on ratio-metric changes related to haemoglobin absorption peaks.^{37,38} However, DRS data are inherently complex and high-dimensional, often containing redundant and correlated spectral features. These characteristics pose challenges for direct classification, often leading to model over-fitting or reduced diagnostic accuracy. Therefore, identifying the most informative spectral features that contribute to tissue differentiation is essential for developing robust and interpretable diagnostic models.

Unlike prior DRS-based studies that primarily analyse entire spectra, this work systematically identifies and validates the most diagnostically informative spectral regions using feature selection. In this study, we investigate DRS as a potential tool for the regular screening of OPMD and as an adjunct tool for biopsy guidance. Using broadband (377 - 1075 nm) DRS measurements acquired *in vivo* from 82 participants, combined with feature selection and machine learning (ML) algorithms, we identify the most discriminative spectral regions for tissue characterisation. This analysis reveals spectral biomarkers linked to early pathological changes, facilitating objective and interpretable approach to OPMD diagnosis. The primary objectives of this study are: (1) to acquire and analyze broadband DRS spectra from normal and OPMD oral tissues; (2) to identify the most relevant spectral features contributing to tissue differentiation; and (3) to evaluate classification performance using both the entire broadband spectra and the optimised feature set. Overall, this work aims to establish a non-invasive, real-time, and cost-effective diagnostic framework to improve early detection and clinical management of OPMD.

2 Materials and methods

2.1 Clinical study design

This *in vivo* study involved 82 participants - 22 healthy controls and 60 OPMD patients with lesions present in different anatomical locations in the oral cavity. Two of the OPMD participants had multifocal lesions. Ethical approval was granted by the Cork Research Ethics Committee (CREC) of University College Cork (UCC) according to ref.: ECM 4 (y) 13/4/2021 & ECM 3 (kkkkk) 09/05/2023. The research was conducted according to the CREC ethical guidelines and 1964 Helsinki Declaration and its subsequent amendments. Written informed consent, clinical and demographic information, including diagnosis, medical history, smoking and alcohol status were obtained from all participants. Participants were advised to refrain from food or drinks (except water) at least an hour before data collection. They also all rinsed their mouths with water for one minute before the examination. A dental surgeon confirmed the general condition of the oral cavity, including the identification of lesions and healthy tissue. Histopathology results were considered for confirming the disease status.

2.2 Measurements workflow

For data collection, the clinician gently dried the tissue with sterilized gauze and carefully positioned the probe on the tissue. Data was collected from six tissue types, including the cheek mucosa (CM), ventral and dorsal surface of the tongue (VST and DST, respectively), lips, gingiva (Gum) and anterior floor of mouth (AFM) from the control (H) and OPMD groups. The tissues in OPMD group were further subdivided into lesion (OPMD) and contralateral site (OPMDH - confirmed by the clinician). Initially, to optimize the procedure and keep the overall measurement time to a minimum, 9 spectra were recorded from each tissue for the first 17 participants. For the

Table 1 Total number of DRS spectra from each tissue type.

Tissue	CM	VST	DST	Lip	Gum	AFM
Total number of spectra	1386	1376	1215	1233	1125	1074
Total number of lesions	28	19	7	6	2	0
Number of OPMD spectra	375	258	102	78	33	0
Number of OPMDH spectra	777	798	789	831	768	750
Number of H spectra	324	321	324	324	324	324

136 remaining patients, we aimed to collect 15 spectra from each tissue, with the actual number de-
 137 pending on the condition of the oral cavity, the availability of contralateral tissue, and the comfort
 138 of the participant. In total, 7410 spectra were collected during the study from six locations/tissue
 139 types. The total number of DRS measurements per tissue type is summarized in Table 1.

140 For classification purposes, data collected from all tissues were labelled into the following
 141 three categories: H – all measurements from the control group, OPMD – all lesions from the
 142 OPMD group, OPMDH – all healthy tissues from the OPMD group, including the contralateral
 143 site. For this preliminary study, we focused only on CM and VST tissues, as the number of lesions
 144 in the other anatomical locations was insufficient for comprehensive analysis, but will be included
 145 in future studies. Moreover, OPMDH spectra were collected from all healthy-appearing tissues in
 146 the OPMD group, only the contralateral site corresponding to the lesion location (e.g., the opposite
 147 side of CM or VST) was considered for the analysis here to ensure anatomical comparability. A
 148 total of 1056 spectra from CM (324 H, 375 OPMD and 357 OPMDH) and 837 spectra from VST
 149 (321 H, 258 OPMD and 258 OPMDH) were analysed.

150 2.3 Diffuse reflectance spectroscopic system and probe design

151 DRS system (Figure 2) used for this *in vivo* study is based on a highly sensitive spectrometer (QE
 152 Pro, Ocean Insight) ranging from 355 nm to 1100 nm with a 1024 x 58 element CCD array. The
 153 tissue was illuminated by a tungsten-halogen broadband light source (HL-2000-HP, FHSA Ocean

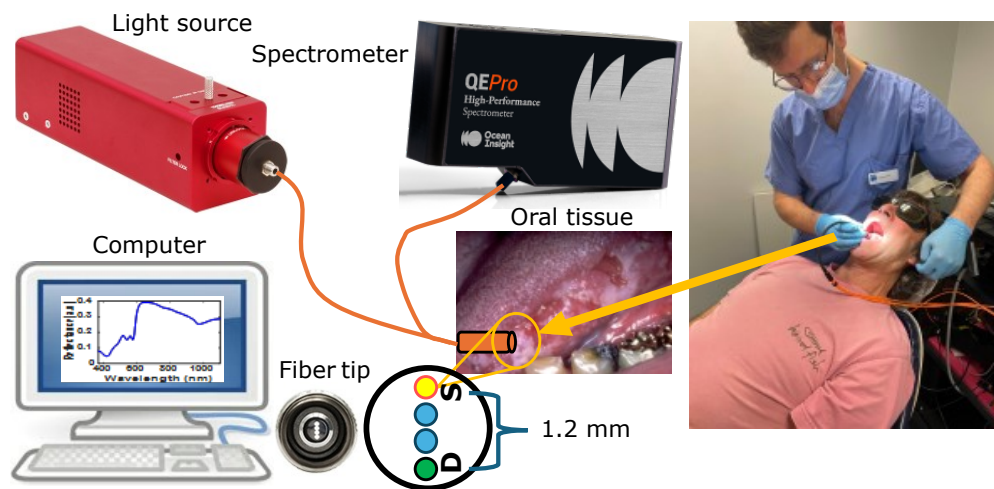


Fig 2 Schematic image of the DRS system and probe configuration.

Insight), with an emission wavelength range from 360 nm to 2400 nm. A low OH 1-to-4 Fan-Out fibre bundle with 400 μm core multimode fibre with linear common end (BFL44LS01- Thorlabs) delivered and collected light from the tissue. Although the probe contained three detector fibres, only the fibre with a 1.2 mm source–detector distance (SDD) was employed for this study. This distance was selected as it provides information regarding the epithelial layer (typically between 400 and 250 μm for CM and VST^{29,39,40}) and superficial stroma, which are the most relevant for distinguishing healthy tissue from OPMD. The shorter SDD fibres were not utilised as in the case of a thicker epithelial layer, they would only sample superficial volume and would not provide information on the stromal layer, which was found to be beneficial in previous studies.^{35,41}

2.4 DRS data collection and preprocessing

Each DRS measurement was recorded for 50 ms and averaged over 20 repetitions. Data was calibrated using a Spectralon reflectance standard (WS-1-SL Diffuse Reflectance Standard, Ocean Insight) and corrected for dark counts (which was measured by recording spectra of reflectance

standard with the light source switched off) by using standard reflectance formula given in Eq.1 :

$$R = \frac{I_{tissue} - I_{background}}{I_{standard} - I_{background}}, \quad (1)$$

where R is the calibrated reflectance, I_{tissue} is the intensity of the reflectance collected from tissue, $I_{background}$ is the intensity of the background with the illumination source off, $I_{standard}$ is the reflectance intensity collected using the standard. The data was truncated to 377 - 1075 nm to avoid the influence of a poor signal-to-noise ratio related to low input light intensity and detector sensitivity. Subsequently, a second-degree polynomial Savitzky-Golay filter was applied with window size 5 and spectra were binned over 5 pixels to filter out high-frequency noise and approximate the resolution of the instrument; and finally the data was normalized to zero mean and unit variance. The resultant DRS data was saved in the form of an $n \times m$ matrix, where n represents the number of DRS spectra (observations) and m represents the number of variables (wavelengths). To check the statistical difference between groups Kruskal-Wallis test was used in MATLAB with a significance level of $p < 0.05$.

2.5 DRS feature selection

After data pre-processing, each DRS spectrum consisted of $m = 150$ wavelengths (features). To reduce dimensionality and identify the most relevant features for classification purposes, the Least Absolute Shrinkage and Selection Operator (LASSO) feature selection model was applied. LASSO minimizes the sum of squared residuals along with an L1 penalty on the coefficients. The L1 penalty shrinks some of the coefficient estimates towards zero, thereby allowing feature selection.⁴² The β_j for each predictor variable is estimated by minimizing the following cost function:

$$\text{Cost function} = \sum_{i=1}^n (y_{real}^{(i)} - y_{pred}^{(i)})^2 + \alpha \sum_{j=1}^m |\beta_j| \quad (2)$$

where, n is the number of DRS spectra, m is the number of features (wavelength), $(y_{real}^{(i)})$ and $(y_{pred}^{(i)})$ are the true and predicted values of the i^{th} spectrum. The term $\alpha \sum_{j=1}^m |\beta_j|$ is L1 penalty, β_j is the regression coefficient of j^{th} feature, α is a regularization parameter controlling the strength of the penalty term L1. A larger value of α leads to more pronounced regularization, driving more coefficients close to zero, resulting in the development of a sparser model. Conversely, smaller values of α reduce the amount of regularization, allowing more wavelengths to retain non-zero coefficients and thus increasing the number of features contributing to the model. The α parameter was selected using cross-validation to balance between prediction error and over- or under-fitting. LASSO regression was applied on the scaled dataset and only variables with non-zero coefficients were retained for inclusion in the predictive model. The contribution of each wavelength is quantified through LASSO weights, which guides the development of a classification model.⁴³

2.6 Classification models

Following feature selection, both tissues H and OPMD were classified by Linear Discriminate Analysis (LDA), which maximizes the separation between the two groups based on the selected features. In addition, LDA is particularly effective when the two groups have distinct but overlapping properties, as observed in spectral data from healthy and OPMD tissues. The model was validated using the leave-one-out cross-validation (LOOCV) method to ensure robustness and minimize bias. The performance of the LASSO-LDA model was confirmed in the form of a confusion matrix and receiver operator curves (ROC). Data processing, feature selection and classifica-

tion tasks were performed using Python (version 3.12) with standard scientific libraries including NumPy, Pandas, sciPy, scikit-learn, Seaborn and Matplotlib for data handling, analysis, modelling and visualization.

3 Results and discussion

DRS spectra are sensitive to changes in tissue composition and microstructure in OPMD lesions caused by alterations in light scattering and absorption properties within epithelial and stromal layers.^{26,33,41} Figure 3 illustrates the average DRS spectra for CM and VST tissues in three groups: healthy (H), participants with OPMD and contralateral site of the lesion (OPMDH). Although both CM and VST are considered nonkeratinized sites, they were analyzed separately throughout the study due to anatomical differences in epithelial thickness and underlying vascularity.⁴⁴ The presented spectra were normalised using the reflectance standard, preserving physically meaningful intensity differences. The standard deviation (shown as shaded areas in the graph) of each group demonstrates high intra- and inter-patient variation related to tissue heterogeneity, typically observed in *in vivo* DRS measurements. All three groups exhibit very similar spectral shapes with small variations in intensities across different wavelength ranges. In general, CM tissue exhibits a higher reflectance than VST, indicating a higher attenuation of light in the latter, which can be a result of a thinner epithelial layer and stronger absorption in the underlying stroma. Intensity-wise, the mean DRS spectrum of the contralateral side (OPMDH) resembles more healthy tissue in the case of CM, and that of OPMD tissue in the case of VST.

The DRS spectrum below 600 nm is dominated by haemoglobin absorption, which is an important biomarker for OPMD progression due to frequent neo-angiogenesis and changes in oxygen consumption and delivery that occur within the lesion.^{26,37} For CM, healthy tissue in this spectral

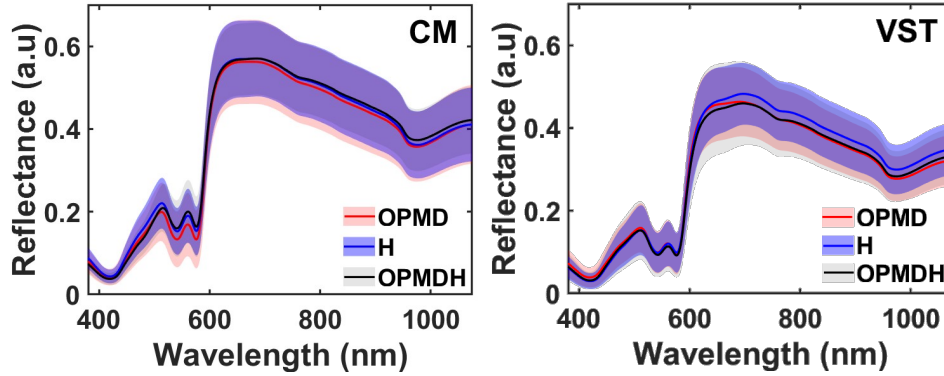


Fig 3 Mean DRS spectra of CM and VST. Blue, red, and black curves correspond to healthy (H), OPMD, and contralateral site (OPMDH), respectively. Shaded areas represent standard deviation across all measurements.

region consistently exhibits a higher reflectance, while the OPMD group typically has the lowest reflectance, indicating increased blood content and vascularization in the lesion. In contrast, for VST OPMD group exhibits the highest reflectance in the 400–500 nm region compared to H and OPMDH, while in the region of 500–600 nm all three groups overlap. This relative change in OPMD reflectance likely exhibits site-specific differences in scattering and absorption, potentially linked to altered epithelial morphology in tongue lesions. To quantify these differences, the ratio of reflectance values at 540 and 575 nm ($R_{540/575}$), representative of relative haemoglobin absorption, was calculated for both CM and VST as shown in figure 4. Although both peaks are related to oxyhaemoglobin, their relative intensities change depending on the balance between oxy- and deoxyhaemoglobin, making them a sensitive indicator of tissue oxygenation status. Changes in this ratio can therefore highlight vascular and metabolic alterations associated with the progression of OPMD. In CM, OPMD lesions showed the highest $R_{540/575}$ compared to H and OPMDH, indicating increased oxygenation and blood volume within the lesion, which is consistent with previous studies.^{32,37,45} A similar but more pronounced trend was also observed in VST, with OPMD ex-

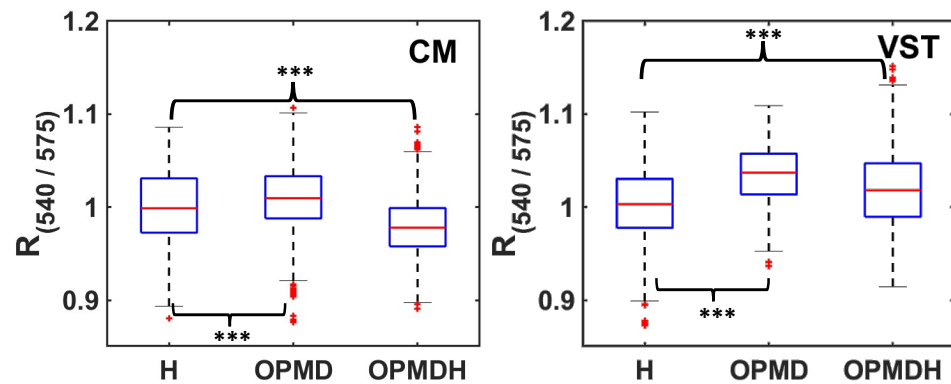


Fig 4 Boxplots of the reflectance ratio $R_{540/575}$ for CM (left) and VST (right) tissues. OPMD lesions show higher ratios compared to healthy and contralateral sites, indicating increased oxy-haemoglobin absorption and suggesting vascular and metabolic alterations linked to disease progression. Both OPMD and OPMDH groups are significantly different from H. *** shows that p-value ≤ 0.001

hibiting the highest ratio. OPMDH exhibits an intermediate behaviour that suggests subtle changes in haemoglobin absorption and oxygenation in the clinically normal-appearing contralateral site. This could be a sign of early vascular changes in OPMDH and aligns with the concept of field cancerization, where molecular and histopathological changes in the normal-appearing mucosa adjacent to oral lesions may predispose these areas to the development of future lesions.^{46–48} For instance, Siar et al. reported that patients with OPMD are at greater risk of developing a second lesion, most probably in the contralateral anatomical site.⁴⁶ Another study reported that half of the patients with OPMD were more vulnerable to field change and had an increased risk of developing second or multiple lesions.⁴⁹ Collectively, these results indicate that subtle vascular and oxygenation changes in the contralateral site may reflect early alterations associated with lesion formation. These findings highlight the potential of DRS to detect early, spatially-resolved tissue alterations that are not visually apparent.^{27,32}

Beyond the oxyhemoglobin absorption peaks, distinct site- and tissue-specific changes were

observed in the near-infrared (NIR) from 600 to 1075 nm. In this spectral region, absorption is relatively reduced with contributions from hemoglobin at shorter wavelengths and due to water absorption beyond 900 nm,^{50,51} making reflectance more sensitive to scattering changes and allowing deeper photon penetration.⁵² Although OPMDH spectra in CM resembled healthy tissue, they consistently exhibited higher reflectance than both OPMD and H, suggesting underlying structural and compositional changes in the contralateral site - even in the absence of visible lesions. Across the same region, OPMD showed lower reflectance than healthy tissue. The thicker epithelial layer in CM can partially mask subtle scattering-related differences, yielding similar reflectance profiles for OPMD and H. Nonetheless, this reduction likely reflects changes in stromal composition and microvascular density, consistent with previous reports of alterations in premalignant oral tissues.^{34,41} In VST, group-specific optical changes were more pronounced in the NIR region. Healthy tissue exhibited the highest reflectance above 620 nm, while OPMDH showed a trend similar to OPMD, again indicating subtle alterations in the contralateral site and supporting the field cancerization phenomena reported in oral premalignant lesions.^{46,49} The spectral changes in OPMD tissue are likely driven by localized variations in haemoglobin concentration, microvascularization and structural remodelling in the epithelium and stroma.²⁶ The consistent subtle dip around 760 nm - associated with deoxy-haemoglobin absorption - was present in all tissue types.⁵³ Its flattening was observed in CM OPMD tissue compared to healthy tissue, indicating altered deoxyhaemoglobin absorption and increased oxygenation of the OPMD lesions.

These spectral differences and the $R_{540/575}$ ratio highlight key DRS spectral regions capable of differentiating OPMD from healthy tissue. However, moving beyond visual interpretation requires a more objective and scalable approach to identify diagnostically meaningful spectral features. To achieve this, we applied LASSO-based spectral feature selection, enabling us to systemati-

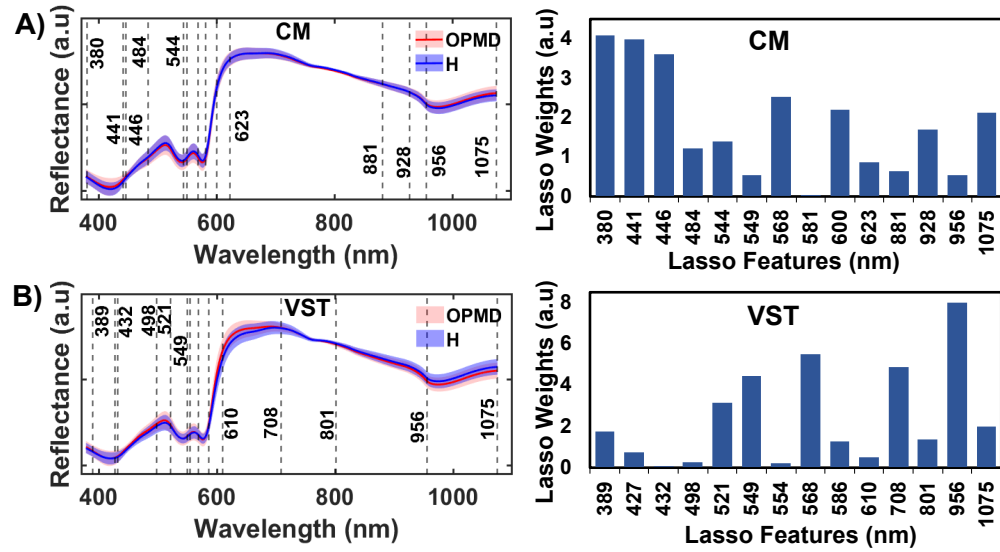


Fig 5 LASSO selected features projected on scaled average DRS spectra of H (blue) and OPMD (red) of A) CM and B) VST with their respective LASSO weights shown as a bar plot on the right.

278 cally identify wavelengths that contribute the most significantly to distinguishing spectra between
 279 OPMD and healthy tissue. The algorithm selected 14 most important features for the classification
 280 between healthy and OPMD in CM and VST locations. Figure 5 represents the LASSO-selected
 281 features overlaid on the averaged DRS spectra for H and OPMD tissues. Their respective LASSO
 282 weights, indicative of their importance to the classification result, are shown as a bar plot on the
 283 right. These features were selected using regularization parameter alpha ($\alpha = 0.0005$) for both CM
 284 and VST. In both tissues, LASSO-selected features correspond predominantly to spectral regions
 285 associated with haemoglobin absorption, oxygenation status and scattering properties. In partic-
 286 ular, in CM the highest weights were achieved for wavelengths in the short wavelength region
 287 (377 - 500 nm), showcasing the dominant role of haemoglobin absorption. For VST, on the other
 288 hand, bands related to oxy-hemoglobin in the 500-600 nm range and water absorption peak appear
 289 more prominent. Several of the features, with varying weights, were selected for both tissue types,

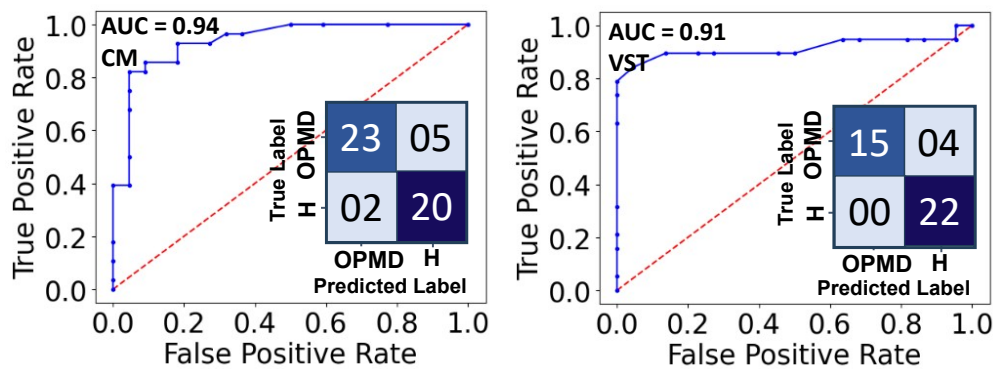


Fig 6 Confusion matrix and ROC curve for CM (left) and VST (right) for LASSO selected features.

such as 549 nm, 568 nm, 956 nm and 1075 nm. The selection of these wavelengths reinforces the hypothesis that vascular changes and oxygen metabolism are key to distinguishing OPMD from healthy tissue in non-keratinized tissue types. Additionally, the features selected between 700 – 1100 nm suggest changes in the scattering properties of the deeper tissue layers, potentially indicating malignant progression.^{32,45,50,52} The ability of LASSO to identify and prioritize wavelengths provides biological interpretability and guidance for designing compact optical devices for clinical use. For example, a portable multispectral imaging system with discrete bands instead of the full broadband spectrum can be designed for rapid screening procedures.⁵⁴ It is worth noting that some of the selected wavelengths are closely spaced (e.g. 441 and 446 nm for CM), making them even more suitable as a choice for narrow-band approach.

Following the LASSO feature selection, LDA was applied to classify healthy and OPMD tissue using a leave-one-patient-out approach achieving an accuracy of 87% and 89% for CM and VST, respectively. The corresponding confusion matrices and receiver operator characteristic (ROC) curves shown in Figure 6 demonstrate the performance of the model. The ROC curve indicates strong classification ability with area under the curve (AUC) of 0.87 and 0.86 for CM and VST,

Table 2 Comparison of the classification results for CM and VST tissues.

Tissue	Classification input	Sensitivity by patient	Specificity by patient	Accuracy by spectra	Balanced accuracy by patient
CM	LASSO features	82.2 %	90.9 %	79.0 %	87.0 %
	All wavelengths	89.3 %	72.7 %	80.8 %	81.0 %
VST	LASSO features	79.0 %	100 %	81.2 %	89.0 %
	All wavelengths	68.4 %	86.4 %	75.9 %	77.0 %

respectively, underscoring the potential of DRS for the screening and diagnosis of OPMD. Table 2 details the classification performance using sensitivity, specificity and accuracy at both patient and spectrum level for CM and VST. For both tissue types LASSO selected features outperformed full-spectrum analysis. In CM, accuracy level of 87% was achieved on selected features, compared to 81% for all wavelengths. Similarly in VST, the accuracy reached 89% compared to 77% for the entire wavelength range. These results indicate that full spectrum analysis leads to over-fitting of the model and poor generalization capability, while classification on LASSO-selected features of importance yields more robust results.

To further understand the physical aspect of the LASSO-selected features and their behaviour in the three studied groups, box plots were generated for H, OPMD, and OPMDH (figure 7) for all 14 features. These plots illustrate the distribution of measured DRS signals and their differences between the lesion, healthy, and contralateral sites for CM and VST. While the model was trained on H and OPMD data, examining OPMDH spectra at the selected wavelengths allows for assessment of field effects in normal-appearing contralateral site and provides insight into early optical changes associated with OPMD. Many of the selected features demonstrate significant differences between classes. In the case of CM tissue, all peaks selected between 377 - 600 nm show significant differences between H and OPMD, with some of them also demonstrating significant differences between H and OPMDH. In the case of VST, only wavelengths up to 435 nm and

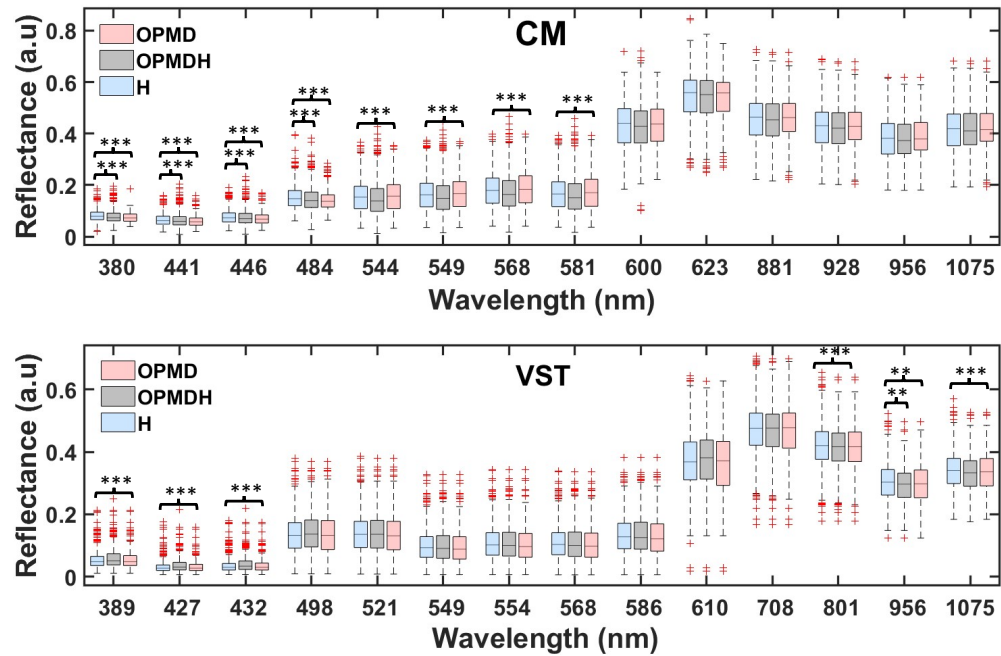


Fig 7 Box plots of LASSO-selected spectral features for CM (top) and VST (bottom). Each box represents the interquartile range, the horizontal line indicates the median, whiskers show the minimum and maximum values, while '+' sign represents outliers. Statistically significant pairs were highlighted with '*' sign (** - $p < 0.01$, *** - $p < 0.001$)

above 800 nm were statistically significant. Notably, some features with high LASSO weights did not show large absolute differences between H and OPMD, and in contrast, some of the features with low LASSO scores turned out to be significant. This indicates that LASSO captures subtle multivariate contributions rather than relying solely on univariate significance. The behaviour of OPMDH group doesn't follow a clear pattern as discussed previously and shown in figure 3; it trends closer to H in some spectral ranges, while in others aligns more with OPMD. This variability underscores the heterogeneous nature of contralateral tissue,⁴⁹ which may carry some optical signatures of field changes without a visible lesion.^{55,56}

Although most diagnostic signatures lie within the visible spectrum, DRS captures subtle optical

variations in tissue that are imperceptible to the human eye. Early dysplastic transformations begin with microstructural and biochemical alterations - such as early changes in stromal oxygenation, neovascularization, nuclear enlargement and increased cellular density - that modify tissue absorption and scattering. These changes can precede the clinically visible morphological alterations, allowing DRS to detect early dysplastic transformations even in clinically normal-appearing tissue. Unlike visual examination, DRS provides quantitative and reproducible measurements that can support consistent interpretation across users and clinical settings. This objectivity is particularly valuable in improving diagnostic yield by guiding biopsy toward optically abnormal sub-regions within heterogeneous lesions.

4 Conclusion

This study demonstrates that DRS, when combined with LASSO-based feature selection, can objectively distinguish OPMD lesions from healthy oral mucosa with accuracies above 85%. Key spectral biomarkers identified within the visible and near-infrared regions correspond to physiological alterations in hemoglobin absorption, tissue oxygenation, and scattering — all indicative of early neoplastic transformations. The application of feature selection improved model robustness and interpretability compared to full-spectrum analysis, highlighting the feasibility of a compact multispectral diagnostic approach.

Importantly, subtle spectral deviations observed in clinically normal contralateral sites (OPMDH) support the concept of field cancerization and suggest that DRS could detect preclinical biochemical alterations invisible to the naked eye. This finding underscores its value as a complementary, non-invasive tool for early OPMD detection and biopsy guidance.

While the present study validates DRS performance across two major non-keratinized oral

sites, future work will extend to a larger cohort encompassing diverse anatomical regions and dysplasia grades. Incorporating quantitative optical modelling, histopathological correlation, and advanced machine learning frameworks will further enhance diagnostic precision and generalizability. Ultimately, the integration of DRS into routine clinical workflows — potentially combined with Raman or fluorescence modalities — may enable real-time, label-free optical biopsy for improved early detection and management of patients with OPMD.

Acknowledgements

This publication has emanated from research conducted with the financial support of Taighde Éireann – Research Ireland under Grant number SFI/15/RP/2828 and SFI/22/RP-2TF/10293 and EUH2020 MSCA-ITN project ‘Photonics for healthcare: multiscale cancer diagnosis and therapy’ (PHAST-ETN) (Grant No. 860185). For the purpose of Open Access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

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507 List of Figures

- 508 1 Progressive morphological changes in oral mucosa, from healthy tissue to hy-
509 perplasia and oral potentially malignant disorders (OPMDs), progressing through
510 mild, moderate, and severe dysplasia to carcinoma and metastasis. Key anatomi-
511 cal layers, including the epithelial layer, stratum basale, basement membrane, and
512 lamina propria, are indicated.
- 513 2 Schematic image of the DRS system and probe configuration.
- 514 3 Mean DRS spectra of CM and VST. Blue, red, and black curves correspond to
515 healthy (H), OPMD, and contralateral site (OPMDH), respectively. Shaded areas
516 represent standard deviation across all measurements.
- 517 4 Boxplots of the reflectance ratio $R_{540/575}$ for CM (left) and VST (right) tissues.
518 OPMD lesions show higher ratios compared to healthy and contralateral sites, indi-
519 cating increased oxy-haemoglobin absorption and suggesting vascular and metabolic
520 alterations linked to disease progression. Both OPMD and OPMDH groups are sig-
521 nificantly different from H. *** shows that p-value ≤ 0.001
- 522 5 LASSO selected features projected on scaled average DRS spectra of H (blue) and
523 OPMD (red) of A) CM and B) VST with their respective LASSO weights shown
524 as a bar plot on the right.

- 525 6 Confusion matrix and ROC curve for CM (left) and VST (right) for LASSO se-
- 526 lected features.
- 527 7 Box plots of LASSO-selected spectral features for CM (top) and VST (bottom).
- 528 Each box represents the interquartile range, the horizontal line indicates the me-
- 529 dian, whiskers show the minimum and maximum values, while '+' sign represents
- 530 outliers. Statistically significant pairs were highlighted with '*' sign (** - $p <$
- 531 0.001, ** - $p < 0.01$)

532 **List of Tables**

- 533 1 Total number of DRS spectra from each tissue type.
- 534 2 Comparison of the classification results for CM and VST tissues.

Hybrid multi-distance continuous wave and discrete-wavelength time-domain analytical model for broadband optical characterization

M. D. Ghauri, S. K. Kothuri, P. Lanka, M. S. Nogueira , S. Andersson-Engels and S. Konugolu.

XYZ 00(0), 000-000 (xxxx).

Hybrid multi-distance continuous wave and discrete-wavelength time-domain analytical model for broadband optical characterization

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Abstract

Significance: Diffuse optical spectroscopy provides noninvasive access to tissue absorption and scattering properties, but existing methodologies face trade-offs between simplicity, measurement time and the ability to independently resolve optical parameters across broad wavelength ranges.

Aim: This study aims to develop and evaluate a hybrid analytical model that combines multi-distance continuous-wave (CW) reflectance with photon time-of-flight (pToF) measurements to enable broadband extraction of tissue absorption and scattering coefficients using a simplified instrumentation approach.

Approach: The model incorporates CW diffuse reflectance measurements with pToF-derived reduced scattering coefficients at four discrete wavelengths (690, 720, 780, and 910 nm). Scattering amplitude and power are estimated using well known wavelength-dependent power-law relationship and then extrapolated for broadband μ_s' . It is then combined with CW-derived effective attenuation to analytically recover broadband μ_a .

Results: The hybrid approach maintains the depth sensitivity and accuracy characteristic of pToF systems while reducing the measurement time and system complexity associated with full broadband time-domain instrumentation. Preliminary validation using optical tissue phantoms demonstrates accurate recovery of broadband optical properties and confirms the model's feasibility.

Conclusions: The proposed model offers a compact and efficient alternative for broadband optical property extraction, with the potential to enable real-time tissue diagnostics using substantially simplified hardware compared with conventional time-resolved systems.

Keywords: broadband optical characterization, photon time-of-flight (pToF), continuous-wave diffuse reflectance, diffuse optical spectroscopy

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1 Introduction

Biological tissues exhibit complex optical behavior arising from their heterogeneous structural and biochemical composition. Diffuse optical spectroscopic techniques provide powerful, noninvasive tools for probing these tissues by quantifying key optical properties, including the absorption coefficient (μ_a) and the reduced scattering coefficient (μ_s'). These techniques rely on analytical,

stochastic, or numerical models to interpret light–tissue interactions. Broadband measurements, typically spanning 600–1000 nm, offer a distinct advantage by enabling the detection of unknown or low-concentration biomolecules and chromophores within tissue ¹. The extracted optical parameters are essential for recovering physiologically relevant information, such as hemoglobin concentration, blood oxygenation, water and lipid content and other structural or functional tissue constituents ^{2,3}.

Continuous-wave (CW) diffuse reflectance remains the most commonly used modality due to its simplicity, portability and high signal-to-noise ratio (SNR) ⁴. The standard measurement scheme includes injecting light into the tissue and detecting it at a certain known distance. However, CW measurements alone suffer from a fundamental limitation. They cannot independently recover absorption and reduced scattering coefficients. Instead they give a single intensity values with a combined attenuation effect of both. Spatially resolved CW measurements is one way to overcome this limitation but it still reveals effective attenuation, μ_{eff} , only ⁵. Additional information must be incorporated beyond multi-distance CW intensity measurements to extract both absorption and scattering independently.

FD approaches on the other hand do decouple absorption and scattering parameters and also offer faster sampling making them suitable for clinical applications. However, due to absence of broadband sources and detectors they are typically restricted to a few modulation frequencies only ^{6,7}. On the other hand, Time-resolved techniques such as photon time-of-flight (pToF) spectroscopy overcome this limitation and at the same time are able to provide information regarding absorption and scattering. An added advantage of these systems is that they can also provide depth segmentation information as late arriving photons have probed greater depth ⁸. Despite these advantages, pToF systems are primarily limited by complex instrumentation, low

64 throughput and longer acquisition times⁹. Clinical applications often impose limitations on overall
65 instrument foot print and measurement time. Therefore there is need for tissue optical
66 characterization that combines compact instrumentation and fast acquisition with reliable
67 depth-resolved measurements.

68 In this work, we present a hybrid analytical model that combines multi-distance CW diffuse
69 reflectance with pToF measurements at four discrete wavelengths for broadband extraction of
70 optical properties. The pToF data provides reliable μ_a and μ'_s at four specific wavelengths. By
71 utilizing the well-established wavelength dependence of reduced scattering in tissue broadband μ'_s
72 can be extracted from these four wavelengths. These extrapolated μ'_s are then used as an input to
73 the model along with μ_{eff} values derived from the slope of the spatial CW measurements for
74 analytical extraction of μ_a across the broadband range. The combined strengths of CW and pToF
75 systems offers a more practical and efficient method to characterize biological tissues in clinical
76 settings without the need for complex, time-consuming measurements and bulky instrumentation.
77 A preliminary evaluation of the model's performance was conducted using optical phantoms.

78

79 2 Material and Methods

80 2.1 Analytical Framework

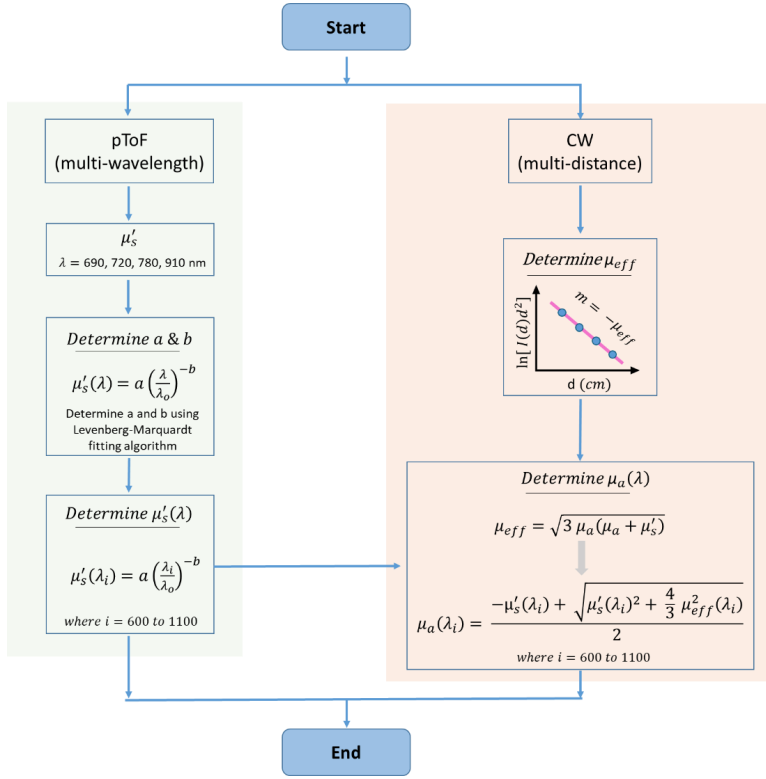


Fig. 1: Hybrid analytical model algorithm flow chart.

This analytical model uses reduced scattering values measured at multiple wavelengths by photon time-of-flight (pTOF) systems to estimate the scattering amplitude, a , and scattering power, b . These parameters are obtained by fitting the data to the well-known power-law relationship using the Levenberg–Marquardt minimization algorithm¹⁰:

$$\mu'_s(\lambda) = a \left(\frac{\lambda}{\lambda_0} \right)^{-b} \quad \text{Eq. (1)}$$

where λ_0 is a reference wavelength. Once both a and b are estimated the reduced scattering spectrum $\mu'_s(\lambda)$ across the full wavelength range can be predicted.

On the other hand, in spatially resolved CW diffuse reflectance spectroscopy (DRS) measurements relationship between intensity, I , and the distance, d , is given by the following equation ¹¹:

$$\ln(I(\lambda) * \rho^2) = k - \rho * \mu_{eff}(\lambda) \quad Eq. (2)$$

where $\mu_{eff}(\lambda)$ is the wavelength dependent effective attenuation coefficient.

The relationship between the absorption, scattering and effective attenuation coefficients is typically expressed using following equation:

$$\mu_{eff} = \sqrt{3\mu_a(\mu_a + \mu'_s)} \quad Eq. (3)$$

The $\mu'_s(\lambda)$ and $\mu_{eff}(\lambda)$ estimations from eq (1) and eq (2), eq (3) can provide broadband absorption, $\mu_a(\lambda)$, properties. The process flow is illustrated in **Fig. 1**.

2.2 Probe Design

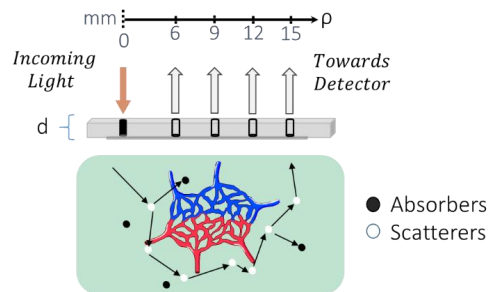


Fig. 2: 3d printed probe holder in reflectance geometry showing fixed source-detector (ρ) separation of 6, 9, 12, 15 mm.

A multi-distance probe holder was printed using a standard 3D printer (Original Prusa i3 MK3S+, PRUSA Research). The design included five linearly arranged cylindrical holes for optical fibers to be inserted. After printing, the base of the probe is lined with a black masking tape. Five 1000 μm core 0.39 NA low -OH -Silica patch cables (M35L01 Fiber Patch Cable, Thorlabs, Munich, Germany) were manually inserted and leveled with the bottom surface of the masking tape. The first fiber acted as the source, while the rest acted as detection fibers at fixed source-detector separations, ρ , of 6, 9, 12 and 15 mm from the source fiber as shown in **Fig. 2**.

2.4 Photon Time of Flight Instrumentation

The system employed a pulsed supercontinuum laser source ((80 MHz repetition rate, 500–2500 nm; FIU-15, Fianium, UK). Wavelength tuning was achieved with a Pellin–Broca prism and then the selected wavelength was delivered through a 50 μm multimode source fiber to the sample. For detection, a 200 μm multimode fiber guides the collected light to a SPAD detector (SPAD, PDM-100 μm active area, MPD, Italy). Time gating was achieved using a time to digital converter (TDC, Picoharp 300, Picoquant, Germany) for generating pToF histograms. All measurements were conducted in reflectance mode with a 15 mm separation between the source and detector fibers. More about the system could be read in ^{12,13}.

2.5 Continuous Wave Diffuse Reflectance Instrumentation

The system comprises of a broadband light source (HL -2000, Ocean Optics, Edinburgh, United Kingdom) as the excitation source and a visible wavelength spectrometer (QE-Pro, Ocean Optics, Edinburgh, United Kingdom) for spectrally resolving the measured signal. The source fiber was fixed while the detection fibers were kept changing for different SD configurations. OceanView, the standard software from Ocean optics was used for collecting data from the spectrometer.

129 2.6 Measurement Protocol

130 Prior to data acquisition the probe was cleaned using standard IPA wipes and then positioned
131 perpendicularly on the sample surface with gentle but firm contact. Ambient illumination was
132 minimized to create a controlled dark room environment. For pToF measurements first an
133 instrument response function (IRF) was recorded and then a reference phantom measurement was
134 done. Then data was acquired for the four selected wavelengths 690, 720, 780 and 910 nm for the
135 sample under test. Following pToF measurements, CW diffuse reflectance measurements were
136 initiated. The acquisition sequence begins with collection of dark spectra and followed by
137 reflectance standard measurements. This was done sequentially for all SD pairs i.e 6, 9, 12 and 15
138 mm. Then the measurements of the sample under test can be carried out. For all data recordings
139 integration time was adjusted iteratively to achieve approximately 90% of the saturation limit of
140 the spectrometer.

141 3 Results and Discussion

142 In this work, we introduced a hybrid analytical model that combines multi-distance continuous-
143 wave (CW) diffuse reflectance with photon time-of-flight (pToF) measurements at four discrete
144 wavelengths, 690, 720, 780 and 910 nm, to extract broadband optical properties of tissue.

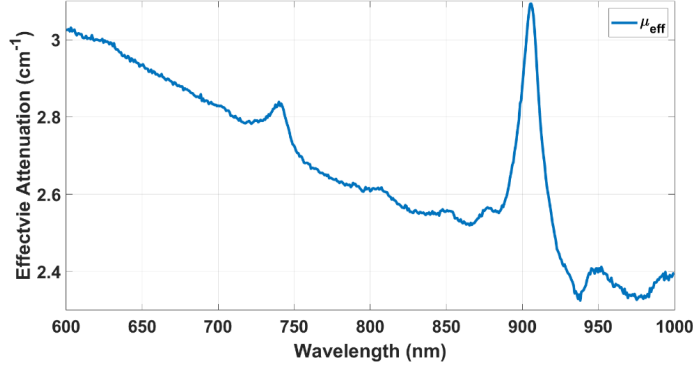


Fig. 3: Effective attenuation, μ_{eff} , spectrum (600-1000 nm).

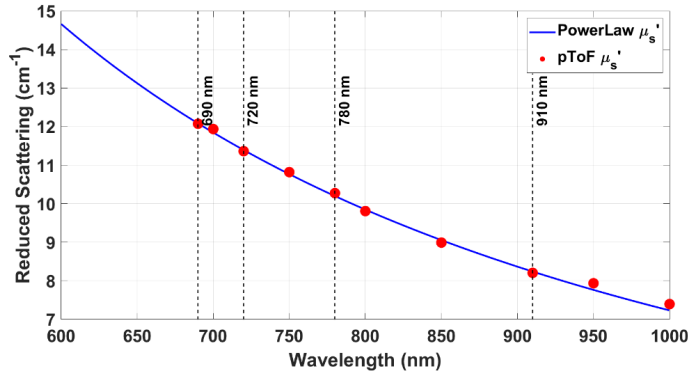
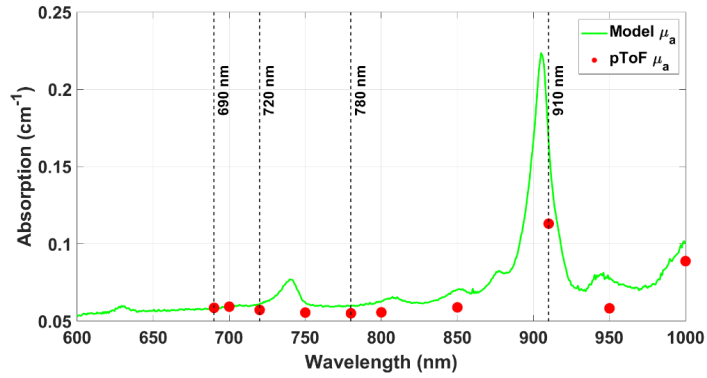


Fig. 4: Broadband (600-1000 nm) reduced scattering, μ'_s , spectrum obtained using power law.

The dotted lines represent the four data points used as input to the power law while red dots represent the reference experimental pToF measurements.



151

152 **Fig. 5:** Broadband (600-1000 nm) absorption, μ_a , spectrum obtained from eq (3). The dotted
 153 lines represent the four data points used as input to the analytical model while red dots represent
 154 the reference experimental values from pToF measurements.

155

The combination of CW and pToF measurements significantly enhances the extraction of
 156 optical properties in a practical and clinically relevant manner. The primary advantage of our
 157 hybrid model is its ability to extract both absorption (μ_a) and reduced scattering (μ_s') independently,
 158 which has long been a challenge for CW-based systems. The introduction of pToF measurements,
 159 even at a limited number of wavelengths, enables the independent determination of μ_a and μ_s' at
 160 those wavelengths. This choice not only ensures that we can probe essential tissue properties but
 161 it also strikes a practical balance between spectral coverage and measurement efficiency. This is
 162 particularly valuable given the well-established wavelength dependence of reduced scattering in
 163 biological tissues allows for the extrapolation of broadband scattering information from these few
 164 available data points. By combining this with the spatially resolved CW data, we can accurately
 165 estimate μ_a across a broad wavelength range without the need for full broadband time-resolved
 166 instrumentation. This hybrid approach is advantageous not only in its ability to extract more

precise optical properties, but also in its practicality, as it reduces measurement time and equipment complexity compared to existing methods that rely on broadband pToF systems.

The choice of four discrete wavelengths used in our study, 690, 720, 780 and 910 nm, is based on the ability to probe key tissue chromophores like hemoglobin, water and lipids as well as their commercial availability as pulsed optical sources ¹⁴. The wavelengths 690 nm and 720 nm fall within the hemoglobin-dominated absorption region of biological tissue, where oxyhemoglobin and deoxyhemoglobin are the primary chromophores ². These wavelengths are therefore widely used in NIRS systems to monitor blood oxygenation, tissue perfusion and cerebral hemodynamics ^{15,16}. The overlap of hemoglobin absorption features across these wavelengths enables spectroscopic differentiation of oxygenated versus deoxygenated tissue, thereby supporting a range of physiological and diagnostic applications, including the detection of tissue abnormalities that alter local oxygenation.

In contrast, the wavelengths 780 nm and 910 nm probe deeper into the near-infrared (NIR) window. The 780 nm wavelength marks the beginning of the NIR spectral region, where hemoglobin absorption persists but gradually diminishes. At 910 nm, water and lipid absorption becomes increasingly significant ¹⁷. Water absorption increases toward its well-known overtone band near 960 nm, while lipids exhibit measurable features around ~890 nm, making 910 nm particularly useful for assessing tissue hydration and lipid composition. Dysregulation of adipose tissue could lead to a wide range of metabolic disorders including insulin resistance, cardiovascular diseases, fatty liver disease and chronic inflammation highlighting the diagnostic value of incorporating lipid-sensitive wavelengths ^{18,19}. Elastin, a major structural protein found in connective tissues such as arteries, lungs, skin and ligaments also exhibits measurable absorption across 550–1350 nm ²⁰. One of its absorption peak is at 910 nm ²⁰. As it provides the essential

elasticity it is valuable for studies of tissue aging, vascular remodeling, and pathological changes in the extracellular matrix ^{21,22}. These longer wavelengths therefore complement the hemoglobin-sensitive bands by enabling access to additional biochemical information, especially metrics related to tissue water content and compositional changes. By using these four wavelengths and extrapolating the optical coefficients, our measurement approach enables joint assessment of tissue oxygenation, hydration and structural composition, providing a simpler and more clinically feasible characterization of biological tissue.

Despite the clear advantages, there are several limitations and challenges to consider as well. First, the model assumes that the wavelength dependence of μ_s' is known and well-characterized, which is generally true for typical tissue types. But deviations may occur in case of complex and abnormal heterogeneous tissues. In such cases a lookup table from Monte Carlo simulations may be needed to further refine the output of analytical model and improve accuracy. Another limitation is that, the four discrete wavelengths for pToF measurements were chosen based on probing chromophores like hemoglobin, water and lipids. If four wavelengths can be chosen to spread more evenly over the broadband range, depending on availability of pulsed laser diodes, it will improve the accuracy of the algorithm. Finally, while the model reduces the complexity of broadband time-resolved measurements, it still requires careful calibration CW and pToF systems to ensure reliable estimation of the optical properties.

4 Conclusion

We present a hybrid analytical model that integrates multi-distance CW diffuse reflectance with discrete wavelength pToF data to enable practical and efficient broadband optical property extraction. By leveraging the portability and low-power requirements of CW instrumentation together with the depth sensitivity of time-resolved measurements, the model overcomes the bulky

hardware and long acquisition times typically required for broadband pToF systems. Using only a small number of single-distance pToF measurements, it achieves accurate broadband estimation without extensive time-resolved data. This approach offers a balanced solution that reduces system complexity, measurement time and device footprint making it well suited for clinical translation. The simplified hardware supports compact, low-power and potentially wearable implementations. The combination of CW simplicity and pToF sensitivity positions this method as a promising tool for noninvasive broadband tissue characterization in applications such as bedside monitoring, intraoperative diagnostics and point-of-care devices. While further refinement and validation are needed, preliminary results indicate strong potential for advancing diffuse optical spectroscopy toward practical clinical deployment.

Disclosures

The authors declare that there are no financial interests, commercial affiliations, or other potential conflicts of interest that could have influenced the objectivity of this research or the writing of this paper.

Code, Data, and Materials

Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

Acknowledgments

This work is funded by European Union's Horizon 2020, under the Marie Skłodowska-Curie grant agreement No 860185 and Research Ireland, Grant numbers SFI/15/RP/2828; SFI-22/RP-2TF/10293. For the purpose of Open Access, the author has applied a CC BY public copyright licence to any author accepted manuscript version arising from this submission.

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Hybrid heterogeneous phantoms for biomedical applications: a demonstration to dosimetry validation

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Opt Express. **15(2)**, 863-874 (2024).



Hybrid heterogeneous phantoms for biomedical applications: a demonstration to dosimetry validation

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Abstract: Phantoms simultaneously mimicking anatomical and optical properties of real tissues can play a pivotal role for improving dosimetry algorithms. The aim of the paper is to design and develop a hybrid phantom model that builds up on the strengths of solid and liquid phantoms for mimicking various anatomical structures for prostate cancer photodynamic therapy (PDT) dosimetry validation. The model comprises of a photosensitizer-embedded gelatin lesion within a liquid Intralipid prostate shape that is surrounded by a solid silicone outer shell. The hybrid phantom was well characterized for optical properties. The final assembled phantom was also evaluated for fluorescence tomographic reconstruction in conjunction with SpectraCure's IDOSE software. The developed model can lead to advancements in dosimetric evaluations. This would improve PDT outlook as a clinical treatment modality and boost phantom based standardization of biophotonic devices globally.

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1. Introduction

Photodynamic therapy (PDT) is a light-based therapeutic modality for cancerous as well as a number of noncancerous health conditions [1]. Dosimetry of PDT is a multi-disciplinary field relevant to planning, monitoring and evaluation of PDT clinical output [2]. It is focused on finding an optimum combination of tissue oxygenation, fluence rate, spatial distribution of photosensitizer (PS) and other factors to determine PDT outcome [3]. Depending on the targeted area, preparation for PDT involves oral, intravenous or transdermal administration of PS [1]. This administered PS is excited at an appropriate wavelength to systematically cause cytotoxicity [1]. The type and concentration of PS used depends on the specificity of the treatment area and drug resistance status of the patient [4]. An optimal PS would exhibit low dark toxicity, selectivity to target tissue, water solubility, high quantum yield in an aqueous environment and short lived photosensitivity [5]. Technological advancements in light delivery mechanisms and exploration of novel PS have increased the general acceptance of PDT as a promising modality. However, a

#514994

Journal © 2024

<https://doi.org/10.1364/BOE.514994>

Received 4 Dec 2023; revised 12 Jan 2024; accepted 12 Jan 2024; published 18 Jan 2024

better understanding of PDT dosimetry from cellular level to tissue response is essential for its wide clinical adaptation.

Tissue heterogeneity is inherent to many if not all treatment sites in the body. These anatomical features create challenges for accurate PS dosage estimations and fiber placements for light delivery. This can lead to adverse outcomes such as retention in non-target tissues or tumor relapse due to incomplete light delivery or insufficient accumulation of PS at treatment site [6]. This limits the clinical effectiveness of PDT [4]. Tissue phantoms that consider tissue heterogeneity will be helpful in providing realistic scenarios of PS distribution and thereby indirectly help in understanding the PS diffusion once applied in real tissue. Phantoms are already widely used in developing biomedical technological solutions [7,8]. In the case of PDT along with the understanding of spatial distribution, incorporating PS into these phantoms could potentially provide insights into precision, predictability and reliability of real-time dosimetric evaluations [9,10]. 3D printing technologies have been reported in the literature to create anthropomorphic phantoms of various anatomical sites [11–14]. However, despite realistic representation, they fail to incorporate diverse well calibrated optical properties [15].

Anthropomorphic optical phantoms having specific optical properties have been documented in the literature [15,16]. However, replicating optical properties and anthropomorphic features into a phantom for PDT application despite technological advancements still presents various degrees of challenges. Polymer-based solid phantoms with specific optical properties lack the flexibility to change PS concentration and fiber positions [17]. Moreover, inserting additional fibers or movement of already present fibers during the course of study may introduce air pockets which can lead to erroneous results. Additionally, widely used PS are water soluble and not soluble in curing materials like silicone or epoxy [18]. On the other hand, liquid phantoms address these limitations but lead to difficulties in creating tissue heterogeneities and also suffer from a relatively short shelf life [19]. For interstitial PDT applications using liquid phantoms necessitate additional support structures for mounting fibers at specific positions. A phantom model that could address the above-mentioned challenges can enable optimization and bring innovation in PDT dosimetry.

In this study, we present a hybrid heterogeneous prostate PDT phantom combining the strengths of both solid and liquid phantoms. A gelatin-based solid tumorous region inside a liquid Intralipid “prostate” surrounded by a solid silicone outer shell was designed and fabricated. The water-based gelatin tumor phantom allows utilization of widely used PS such as Visudyne and offers the possibility of changing PS concentration. The use of an Intralipid liquid prostate avoids the introduction of air bubbles at the interface of tumor and surrounding phantom. A solid silicone outer shell enables the mounting of fibers at specific locations. The ease of manufacturing, tunability of tumor geometry and PS concentration enhance its utility for realistic heterogeneous tissue phantom studies for PDT dosimetry. The trend of PS concentration in phantoms was evaluated using fluorescence capabilities. The optical properties of the phantom recipe were characterized using time-domain diffuse optical spectroscopy. A preliminary evaluation of the tomographic reconstruction of the hybrid phantom is demonstrated.

2. Material and methods

2.1. Structure of hybrid phantom

Prostate tissue phantoms were created based on real patient ultrasound data [20]. Segmentation of various tissue structures from the data is presented in Fig. 1(a). Figure 1(b) illustrates their respective computer-aided design (CAD) files.

To reduce the fabrication challenges urethra and rectum were no longer considered the tissues of interest. The final hybrid phantom realized has three distinctive regions: tumor, prostate, and surrounding tissue. The gelatin-based tumor is inside an Intralipid-filled prostate enclosed by solid silicone surrounding tissue. This assembling of the three distinctive phantoms is in

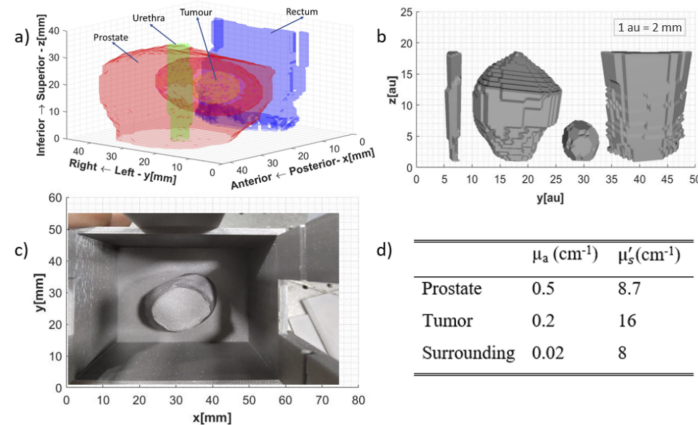


Fig. 1. (a) Tissue 3D structural layout based on the ultrasound data (b) CAD files for the anatomical features identified (Left to Right: Urethra, Prostate, Tumor, Rectum,) (c) Outer mold for the silicone phantom showing the placement of prostate inside (d) Optical properties at 690 nm [21,22].

accordance with the geometry identified in Fig. 1(a). The respective molds of these three structures for phantom manufacturing were printed using a standard 3D printer (Original Prusa i3 MK3S+, PRUSA Research). The optical properties of the three distinctive regions were taken from the literature [21,22] and are tabulated in Fig. 1 (d).

2.2. Phantom recipe & fabrication

Chemicals: Gelatin (G2500, Sigma-Aldrich USA), Intralipid 20% w/v (Fresenius Kabi, Ltd., Germany), India ink (Higgins, 44201 Chartpak Inc, USA), Carbon Black [18] (QL3261, Polycraft Black Silicone Pigment, UK), Titanium dioxide [18] (248576, Sigma-Aldrich, USA), Visudyne (Cheplapharm Arzneimittel GmbH, Germany). All solutions were prepared using deionized water.

Fabrication – Solid Silicone Phantom: The solid silicone outer shell was fabricated by following the process documented by Konugolu Venkata Sekar *et al* [18]. Briefly, silicone was polymerized using carbon black and titanium oxide as absorber and scatterer respectively. The steps including stirring, ultrasound, vacuum and others were closely followed [18]. Figure 1 (c) shows the final mold and placement of the prostate for realizing the desired silicone phantom. In addition, a bulk phantom (cylinder – diameter 8.3 cm and thickness 4 cm) was fabricated from the same mixture for time domain optical properties characterization.

Fabrication - Liquid Intralipid Phantom: Liquid Intralipid prostate tissue phantom was realized by first diluting India ink in water to achieve 0.76% dilution. This served as the stock for realizing liquid phantom. Subsequently, 2.0 g from this stock and 4.4 g Intralipid were mixed with water to achieve a final volume of 200 g for attaining optical properties of prostate stated in Fig. 1 (d).

Fabrication – Gelatin Phantom: Gelatin tumors were prepared by modifying the development procedure in literature to suit our application [23]. A temperature of 37 °C was monitored using a digital thermometer and maintained continuously throughout the phantom making process. The synthesis process followed to achieve optical properties (as mentioned in Fig. 1 (d)) is illustrated

in Fig. 2. Initially, 0.49% India ink stock solution was prepared in water. Then 287.4 g of water was transferred in an adequate glass beaker and placed on a hot plate to raise temperature to 37 °C and continuously stirred at 300 rpm. Once the desired temperature was achieved 5 more minutes were allowed to achieve homogenous temperature conditions. Afterwards, 51 g of Intralipid and 3.3 g of India ink from the stock solution were added. After ~5 minutes 39.6 g of gelatin powder was slowly added to the beaker to avoid cluster formation. The mixture was continuously stirred for 20 more minutes to ensure a uniform blend. This point marks the completion of Phase 1 of making gelatin phantoms.

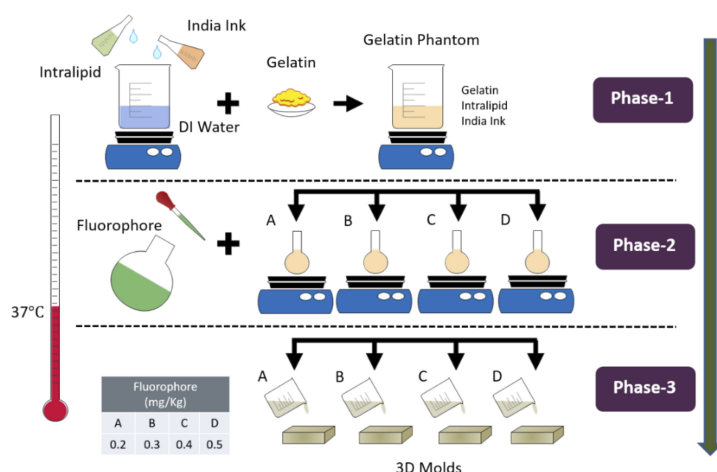


Fig. 2. Synthesis process of gelatin phantoms.

In Phase 2, a Visudyne solution was initially prepared to achieve 0.01 mg/ml concentration of verteporfin (an active substance in Visudyne) as fluorophore stock solution. From the final blended solution realized in Phase 1, 28 g was transferred into each of four separate 50 ml glass beakers (A, B, C, D) kept on a hot plate under constant stirring. Fluorophore from stock was added in the amounts of 0.60, 0.90, 1.20 and 1.50 g to beaker A, B, C, D respectively to achieve dosage of 0.20, 0.30, 0.40, 0.50 mg/kg (fluorophore concentration in mg / phantom mass in kg). Deionized water was added in each beaker to make up the final mass of 30 g. The remaining solution in the main beaker was used for making two phantoms as control groups. The first control group phantom was created by transferring 28 g of the blended solution in Phase 1 to another beaker and adding 2.0 g water. Whereas for the second control group 230 g of the blended solution and 16 g of water was added to create bulk phantom for optical properties characterization. For each of these solutions, series A to D and the control group phantoms, 15 minutes were allowed to ensure adequate mixing. Afterwards, the phantoms were sonicated for 1 minute to remove air bubbles.

In Phase 3, the realized phantoms were poured into the respective molds. After ~5 minutes these molds were placed in the freezer at -20 °C for 1 hour to solidify. The phantoms were then stored in the refrigerator at 4 °C in the air and light tight mold until further analysis and use.

2.3. Instrumentation

After the curing process, the optical properties of the three realized bulk phantoms were characterized at 690 nm using a time domain diffuse optical spectroscopy system [24,25]. The system consists of pulsed supercontinuum laser (20 MHz rep. rate, 400 to 1750 nm, SC450, Fianium, United Kingdom), the wavelength selection was achieved using Pellin Broca prism and the selected wavelength is coupled into a 50 μm multimode source fiber. The detection system consists of a 200 μm multimode detection fiber coupled to a SPAD (SPAD, PDM-100 μm active area, MPD, Italy) detector. The photon counts from detector were histogrammed using time to digital converter (TDC, PicoHarp 300, Picoquant, Germany) to generate photon time of flight (pTOF) curves. The measurements were performed in reflectance geometry at 2 cm source detector distance.

Next, the three distinctive regions were assembled together such that the gelatin-based tumor is inside an Intralipid filled prostate enclosed by solid silicone surrounding tissue similar to as shown in Fig. 3. This phantom geometry is in line with the various tissue structures already presented in Fig. 1(a). Then, the realized gelatin phantoms representing tumors were subjected to interstitial optical measurements to characterize the diffuse fiber to fiber fluorescence signal as illustrated in Fig. 3. Optical fibers, mimicking transperineal insertion, were placed into the hybrid model. A 5.0 mm source-detector (SD) distance was maintained between the two fibers. Figure 3 illustrates the configuration of the optical system for characterizing the fluorescence capabilities of the phantom. It comprises an illumination source, associated filters and a spectrometer. Illumination was achieved by a 690 nm laser diode, a short-pass 700 nm excitation filter (FESH0700, Thorlabs), a long-pass 750 nm emission filter (FELH0750, Thorlabs), at 750 nm and a UV-Vis spectrometer (QEPro, Ocean Optics).

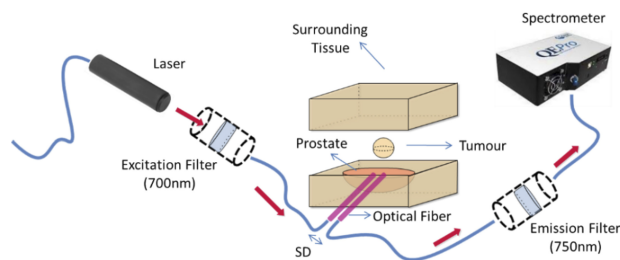


Fig. 3. Schematic of optical arrangement used for characterizing gelatin phantoms.

2.4. Tomographic reconstruction for PDT

SpectraCure's P18 system for interstitial PDT along with IDOSE software was used for determining fiber placement positions and data collection as well [26,27]. For light delivery and monitoring for this particular prostate and tumor geometry the algorithm identified a minimum of 13 bare-end optical fibers for treatment (point source illumination and light collection). Hence, the final assembled phantom was subjected to interstitial light delivery using 13 fibers. The fiber positions identified are illustrated in Fig. 4.

Needle piercings were made at these locations in the silicone phantom for inserting optical fibers inside. The urethra and the rectum are just shown for orientation purposes, as already stated they are not present in the final realized phantom.

The diffuse fluorescence tomographic reconstruction methods were developed in Matlab using NIRFAST package [28,29] and previous work by Axelsson *et al* [30]. In the current

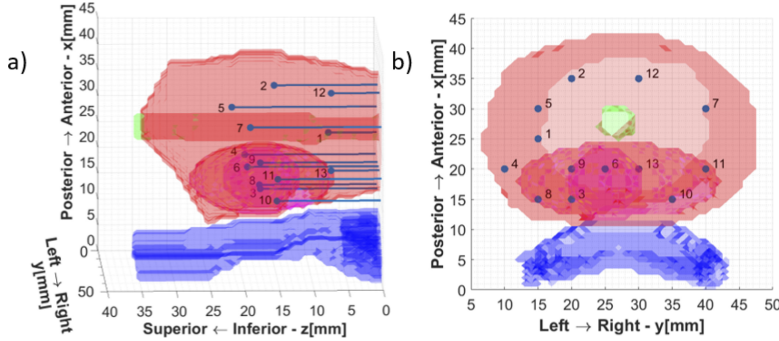


Fig. 4. Fiber positions for the tumor geometry (a) Lateral View (b) Top View.

implementation, the space is represented by a finite element mesh with around 26,000 nodes. The distribution of nodes was such that more nodes were placed in regions around the fibers, along all three axes, compared to the periphery of the medium. It is required to solve for ~26,000 unknowns (fluorescent absorption coefficients) with only 156 data points (obtained by 13 fibers acting as both source and detector – all possible source-detector pairs). Every data point is a ratio between the fluorescent signal compared to the excitation signal, known as normalized Born ratio [31]. The excitation and fluorescent signals are collected by the same fiber, when one of the other 12 fibers is emitting light.

A vector of measurements $\mathbf{M}$ contains all 156 data points and is compared to the forward model $\mathbf{F}(\boldsymbol{\eta})$ for given values of fluorophore yields $\boldsymbol{\eta}$ in all mesh nodes. Fluorophore yield in each node is a product of the fluorophore absorption coefficient in that node μ_{af} , and fluorescence quantum yield γ : $\eta = \mu_{af}\gamma$. Forward model is calculated from the diffusion equation, as described in Ref. [30]

$$F_{s,d}(\boldsymbol{\eta}) = \frac{1}{U_x(\vec{r}_s, \vec{r}_d)} \sum_{n=1}^{N_n} U_m(\vec{r}_d, \vec{r}_n) U_x(\vec{r}_s, \vec{r}_n) \Delta V \eta_n \quad (1)$$

where $U_{x,m}(\vec{r}_s, \vec{r}_d)$ is the forward solution for excitation light (x) or fluorescent emission light (m), at the position of the source $\vec{r}_s$, detector $\vec{r}_d$ or node $\vec{r}_n$, ΔV is the element volume, η_n is the fluorophore yield in node n , and the number of nodes is N_n .

The inverse problem consists of finding the vector of fluorophore yields, or fluorophore absorption coefficients in all nodes such that the difference between the measurements $\mathbf{M}$ and the forward model $\mathbf{F}(\boldsymbol{\eta})$ is minimized. Since the problem is ill-posed, Tikhonov regularization is applied, such that the objective function to minimize becomes [30]

$$\Omega = \|\mathbf{M} - \mathbf{F}(\boldsymbol{\eta})\|^2 + \lambda \|\mathbf{L}(\boldsymbol{\eta} - \boldsymbol{\eta}_0)\|^2 \quad (2)$$

In this implementation, regularization matrix $\mathbf{L}$ was set to identity matrix, i.e., no geometrical prioring was done, since the emphasis was to demonstrate the usefulness of the fluorescent signal obtained from hybrid phantoms, not to geometrically bias the solution to have a more accurate quantitative reconstruction. Initial guess for the fluorophore yields in all nodes was set to 0, therefore, $\boldsymbol{\eta}_0 = 0$. A variant of Levenberg-Marquardt algorithm was applied for numerical convergence to a solution in iterative steps. Regularization parameter λ is set to the maximum of the diagonal of the Hessian matrix $\mathbf{H} = \mathbf{J}^T \mathbf{J}$, with $\mathbf{J} = \frac{\partial \mathbf{F}(\boldsymbol{\eta})}{\partial \boldsymbol{\eta}}$ being Jacobian matrix, calculated at the beginning of each iteration. In each iteration, λ is scaled according to new Hessian matrix

values, and additionally divided by a factor $\sqrt[4]{10}$. After each iteration i , fluorophore yields vector takes the updated value [30]

$$\eta_i = \eta_{i-1} + (\mathbf{J}^T \mathbf{J} + \lambda \mathbf{I})^{-1} \mathbf{J}^T (\mathbf{M} - \mathbf{F}(\eta_{i-1})) \quad (3)$$

The iterative method stops when the decrease of the square of the 2-norm of the error $\|\mathbf{M} - \mathbf{F}\|^2$ is less than 2%, or maximum number of 20 iterations is reached.

More general reconstruction methods, compared and analyzed in simulations, and finally validated on a series of standardized phantoms described in this work, will be a subject of future publications.

3. Results & discussion

This study presents the design and development of a hybrid heterogeneous prostate phantom combining the strength of both the solid as well as liquid phantom for PDT application.

3.1. Phantom characterization

The three distinctive bulk phantoms were characterized in collaboration with BioPixS using the state-of-the-art time domain diffuse optical spectroscopy system [24]. The characterization results are tabulated in Table 1 along with the uncertainty of optical properties (absorption, reduced scattering) measurements. The properties can be followed up using the trace code: BioPixS0014.

Table 1. Optical properties of the final realized phantoms

Wavelength	Tissue Type	Absorption	Scattering	Absorption Coefficient of Variation	Scattering Coefficient of Variation
nm		cm^{-1}	cm^{-1}	%	%
690	Surrounding	0.03	9.1	2	1
720	Surrounding	0.03	8.6	1	1
780	Surrounding	0.03	8.2	1	1
690	Tumor	0.20	15.7	0.2	1.2
720	Tumor	0.20	15.0	0.0	1.0
780	Tumor	0.20	13.7	3.1	3.0
690	Prostate	0.51	7.3	1.0	0.7
720	Prostate	0.51	7.1	0.6	0.4
780	Prostate	0.48	6.7	3.1	3.1

The diffuse fluorescence signal from the phantom was studied at 690 nm. The fluorescence response of the phantom to the changing concentrations of the photosensitizer after background correction is depicted in Fig. 5 (a) and (b). The coefficient of variation (CV) for three repetitions was found to be less than 3% which is the expected CV of the system [18]. The gelatin tumor with 0.4 mg/kg concentration of verteporfin was subjected to PDT-like light delivery at 80 mW for one hour. The photobleaching effect was observed as depicted in Fig. 5 (c).

Wavelength selection for PDT is limited to the therapeutic window however wavelengths higher than 800 nm are generally insufficient to create the photodynamic effect on its own [3]. Most clinical PS are therefore illuminated within 600 nm to 800 nm range [3]. The selection of 690 nm as excitation source was made to match the excitation wavelength for verteporfin. For a photosensitizer with a different excitation wavelength, the same hybrid approach can be followed for phantom manufacturing with changes in concentrations of scatterer and absorber respectively

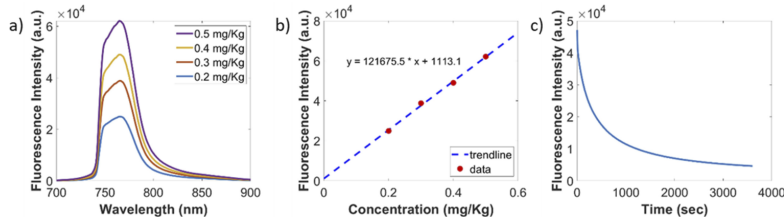


Fig. 5. Background Corrected: (a) Fluorescence emission results (b) Proportionality plot between fluorescence intensity and PS concentration (c) Photobleaching effect in gelatin phantom.

to match the optical properties at that particular wavelength. Research effort targeted at exploring innovative materials and recipes has been reported in the literature for the development of multi-wavelength tissue phantoms [32]. However, despite their appeal the manufacturing process of such phantoms is not easy. In the interest of a simple fabrication process the proposed phantom has been limited to one wavelength only i.e., 690 nm.

3.2. Tomographic reconstruction: preliminary results

The final assembled phantom was subjected to PDT-like light delivery using 13 fibers as shown in Fig. 6 (a). The experimental studies performed resemble a simplified but anatomically realistic model of light propagation inside the human prostate. The preliminary reconstruction result is shown in Fig. 6 (b) for one selected slice corresponding to the center of the fluorophore sphere ($z = 18$ mm). A homogeneous quantum efficiency of 10% for fluorescence from verteporfin was assumed to reconstruct the fluorophore absorption coefficient [33,34]. The white contour shows the interface between the prostate and the surrounding tissue, or in the case of the phantom, between the liquid part and the solid silicone outer shell. The reconstructed fluorophore spatial distribution in the chosen plane matches the corresponding cross section of the gelatin tumor sphere in the hybrid phantom.

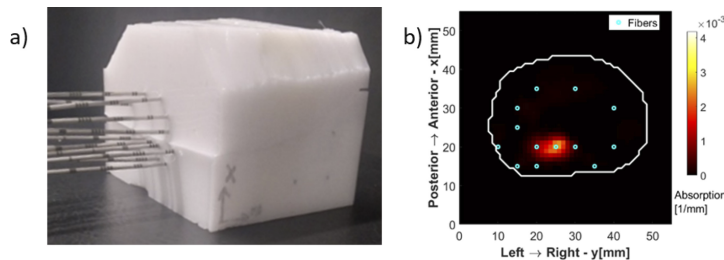


Fig. 6. (a) Final assembled hybrid phantom ready for measurement (b) Tomographic Reconstruction.

3.3. Manufactured phantom & validation

The hybrid phantom constitutes a gelatin-based solid tumorous region inside a liquid prostate surrounded by a solid silicone outer shell. The phantom recipe for each of the distinctive organs

assumes homogenous optical properties throughout. The photosensitizer selected was verteporfin because it is currently being investigated for use in prostate cancer PDT [35,36].

Phantom development was an iterative process with each iteration further refining the recipe as well as minimizing occurrences of human error. The gelatin tumor phantoms with different concentrations of verteporfin along with the control group and the surrounding silicone phantom are depicted in Fig. 7 (a).

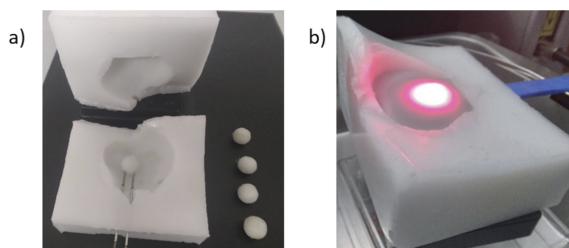


Fig. 7. (a) Tumors for varying concentrations of verteporfin along with outer shell (b) Light delivery in gelatin tumor surrounded by liquid prostate within the lower half of the silicone outer shell.

The final assembly of the phantom involves tumor placement inside the prostate region and the insertion of optical fibers. While inserting optical fibers mimicking transperineal insertion inside the gelatin tumor it might change orientation or get deformed due to external pressure. A similar effect is also exhibited by the prostate during actual PDT sessions [37]. The surgeons rely on ultrasound or magnetic resonance imaging (MRI) for transperineal insertion of optical fibers to ensure that fibers are placed correctly and at the same time minimize damage to the surrounding organs such as urethra, rectum etc. [37]. In our case use of liquid phantom as prostate overcomes the deformation and displacement issue while inserting fibers. On the other hand, the gelatin phantom does suffer from this issue. However, since the outer silicone shell is divided into two halves the operator can simply remove the upper half of the outer silicone shell, insert the fiber and then place the gelatin phantom in the fiber at a specific location. No slippage of gelatin tumors from the fiber was observed even without liquid prostate. Once satisfied by the position of the gelatin tumor the operator can fill it with liquid prostate as shown in Fig. 7 (b). Then, the upper half of the silicone outer shell needs to be placed back. Afterwards, the rest of the liquid phantom was injected into the prostate cavity using a syringe through one of the needle piercings in the outer silicone shell. Slight leakages were observed from the phantom when the syringe was taken out and fiber was placed in that needle piercing as well. During and after the course of measurements no change in position of gelatin phantom was observed.

The solid silicone outer shell keeps the inner phantoms and the position of fibers at the right location without the need of any additional support. The benefit of having a liquid phantom inside the solid silicone outer shell is that the translational or rotational movement of fibers during the course of the study does not introduce air gaps. In comparison to a fully solid heterogeneous phantom, this hybrid approach offers a way to ensure that the optical properties do not get modified during the study leading to erroneous results. The use of liquid prostate also offers the possibility to accommodate changes in tumor geometry as well as the possibility to tailor tumor placement anywhere in the prostate. The selection of gelatin for mimicking the tumorous part was done because it is a widely available gelling material. Gelatin phantoms are biocompatible, resemble human tissues closely and allow the incorporation of water soluble photosensitizer [38]. Gelatin also offers a simpler manufacturing process that can be pursued even in a low resource setting. If needed a change in tumor geometry can be achieved by using a different custom-made

mold during the phantom making process. Although verteporfin was used as the photosensitizer for the demonstrated phantom, the same development and quantification process can be followed for testing the efficiency of other photosensitizers. Both of these scenarios allow for reusing other parts of the phantom. All these factors add to the attractiveness of using gelatin to mimic a tumor for PDT application.

3.4. Limitations

During PDT-like light delivery measurements using 13 fibers, instances of leakages of the liquid phantom were observed from the region where the distance between the prostate boundary and the outer boundary of the silicone outer shell was small. A container similar to Fig. 1 (c) was printed using the same 3D printer to submerge the final assembled phantom in the liquid phantom to ensure that no instances of leakages interfere with measurements. The phantom design of the silicone outer shell will be further evolved to resolve this issue.

The ideal shelf life of gelatin phantoms was observed to be almost twenty days under optimum storage conditions after which they start to dry out or start microbial growth. Almost the same shelf life has been reported by Gautam *et al* [23]. Improved storage mechanisms or the addition of preservatives could potentially further enhance shelf life. In the case of the latter, care must be taken to ensure that the optical properties of the phantom are not affected. Also, following up on the hybrid phantom design further studies can be done with the incorporation of more anatomic sites such as urethra and rectum. Although the CAD files were generated for these structures as well, these were omitted from this proof-of-concept study to keep complexity to a minimum. The approach to incorporate urethra and rectum will be to make both urethra and rectum as solid phantoms. The prostate surrounding phantom will be modified to provide space for the urethra and rectum solid structures which will be assembled together, and prostate liquid phantom will be filled in the end. Additionally, the outer silicone shell can be reshaped to reproduce the entire pelvic geometry. The mechanical properties of the outer phantom can be tuned to be similar to the perineum so that the same resistance is felt when inserting optical fibers for prostate PDT. Such a phantom has the potential to serve as a training platform for clinicians/surgeons for practicing or pedagogical purposes. This would also assist in improving robotic interventions.

4. Conclusions

The design, manufacturing and characterization of a hybrid solid-liquid prostate tissue phantom has been reported in this study. The hybrid approach combines the benefits of both types of phantoms to present an anatomically realistic patient specific treatment site model. The proposed phantom contains a gelatin tumor inside a liquid prostate enclosed in a silicone outer shell representing surrounding tissue. Use of gelatin for making tumors offers adjustable photosensitizer concentration, customizable tumor geometry and easy needle placement for PDT application. Liquid prostate ensures that any fiber movements during measurements do not lead to changes in optical properties due to air gaps. While the silicone based surrounding tissue helps the mounting of the fibers at specific locations. Gelatin tumor characterization demonstrated a directly proportional trend between fluorescence signal and PS concentrations as well as photobleaching effect. Preliminary fluorescence tomographic reconstruction results using SpectraCure IDOSE software are promising. Although the hybrid phantom has been demonstrated herein for prostate cancer, it has potential to be applied for evaluating other treatment sites in the body as well. Reliable dosimetry assessment is central to positive treatment outcome. It is anticipated that the use of the proposed heterogeneous phantom would facilitate further development and optimization of reconstruction algorithms for improved dosimetry evaluations.

Funding. European Commission (101016087, 101017113, 860185); Science Foundation Ireland (SFI/15/RP/2828, SFI-12/RC/2276_P2); Irish Research eLibrary.

Acknowledgements. This study is funded by EU H2020 MSCA-ITN project ‘Photonics for healthcare: multiscale cancer diagnosis and therapy’ (PHAST-ETN) (Grant No. 860185), Science Foundation Ireland (Grant No. SFI/15/RP/2828 & SFI-12/RC/2276_P2), European projects VASCOVID (Grant No. 101016087), and TinyBrains (Grant No. 101017113). Open access funding provided by Irish Research eLibrary.

Disclosures. SKVS and SAE are shareholders of BioPixS Ltd with an interest in tissue optical phantoms. SAE and JSw are minority shareholders of SpectraCure AB with an interest in Prostate cancer IPDT.

Data availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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PAPER IV

Two-stage diffuse fluorescence tomography for monitoring of drug distribution in photodynamic therapy of tumors

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J Biomed Opt. **30(1)**, 015003 (2025).

Two-stage diffuse fluorescence tomography for monitoring of drug distribution in photodynamic therapy of tumors

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ABSTRACT. **Significance:** The spatial distribution of the photosensitizing drug concentration is an important parameter for predicting the photodynamic therapy (PDT) outcome. Current diffuse fluorescence tomography methods lack accuracy in quantifying drug concentration. The development of accurate methods for monitoring the temporal evolution of the drug distribution in tissue can advance the real-time light dosimetry in PDT of tumors, leading to better treatment outcomes.

Aim: We develop diffuse optical tomography methods based on interstitial fluorescence measurements to accurately reconstruct the spatial distribution of fluorescent photosensitizing drugs in real-time.

Approach: A two-stage reconstruction algorithm is proposed. The capabilities and limitations of this method are studied in various simulated scenarios. For the first time, experimental validation is conducted using the clinical system for interstitial PDT of prostate cancer on prostate tissue-mimicking phantoms with the photosensitizer verteporfin.

Results: The average relative error of the reconstructed fluorophore absorption was less than 10%, whereas the fluorescent inclusion reconstructed volume relative error was less than 35%.

Conclusions: The proposed method can be used to monitor the temporal evolution of the photosensitizing drug concentration in tumor tissue during photodynamic therapy. This is an important step forward in the development of the next generation of real-time light dosimetry algorithms for photodynamic therapy.

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Keywords: diffuse optical tomography; fluorescence; photodynamic therapy; photosensitizing drug; inverse problem; tissue phantoms

Paper 240308GR received Oct. 23, 2024; revised Jan. 6, 2025; accepted Jan. 7, 2025; published Jan. 30, 2025.

1 Introduction

Photodynamic therapy (PDT) is a promising approach in cancer treatment.¹ It relies on photodynamic action, a dynamic interaction between light, a photosensitizing agent, and oxygen,

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resulting in tissue destruction.² None of these three components is individually toxic, but together they initiate a photochemical reaction whose product is a highly reactive singlet oxygen. Its significant toxicity leads to direct tumor cell death via apoptosis or necrosis, damage to tumor vasculature, and activation of innate and adaptive responses against tumors.^{3,4} PDT's minimal invasiveness and selectivity in killing malignant cells while sparing surrounding tissue make it effective in treating early-stage tumors and a good choice for inoperable tumors.³ PDT has many assets that make it suitable for cancer treatment, either as a first option or combined with other methods (e.g., chemotherapy, radiotherapy, surgery). The light is non-ionizing, and no cumulative toxicity allows repeated treatments of recurrent malignancies.^{3,5} The short penetration of this light in tissue has led to the evolution of PDT in two directions: one is the development of photosensitizers (PSs) activated at wavelengths where light is less attenuated,⁶ and the other is the interstitial placement of optical fibers for treating solid tumors deeply embedded into the body.⁷⁻⁹ It is essential to have access to light dosimetry algorithms to get a favorable response to PDT.¹⁰

The destruction of malignant tissue in PDT depends on the type and dose of PSs used, the time between their administration and light delivery, total light dose and its fluence rate, and tumor oxygen concentration.³ Therefore, to improve the light dosimetry during PDT treatments, information on PS distribution, light fluence rate, and oxygen concentration should be available in real-time.¹¹⁻¹⁵ Some PSs have the important property to accumulate to a higher degree in tumors, rather than in healthy tissue.⁶ Most PSs are fluorescent in nature.⁷ These properties of PS enable better tumor localization for dose planning, as well as assessment of treatment progression.

Real-time dosimetry during interstitial PDT treatments has already been employed in clinical applications.¹⁶⁻¹⁹ In prostate cancer treatment, Johansson et al.¹⁸ have shown that real-time dosimetry allows for the delivery of a specific dose of light to the target tissue while sparing the organs at risk. However, the distribution of the PS drug within the patient tissue should have been considered during the light dose planning, and the lack of reliable methods for its estimation is the reason why it still has not been considered. To solve this, the present work aims to develop methods for real-time monitoring of the PS spatial distribution during the PDT treatment. This could be beneficial in the future to improve PDT dosimetry algorithms considering additional factors, such as the fluorescent properties of PS.²⁰ PS distribution and concentration in the tumor are a major determinant of photochemical oxygen depletion, and as such, knowledge of it is beneficial for accurately predicting the treatment outcome.²¹

In this work, we develop diffuse optical tomography (DOT) models and methods for the real-time reconstruction of the spatial distribution of the PS drug by utilizing its fluorescent properties, which we will refer to as diffuse fluorescence tomography (DFT). The reconstruction algorithm we propose here consists of two stages. The first stage (S1) relies on standard tomographic reconstruction methods²²⁻²⁴ without structural a priori information. The second stage (S2), presented here for the first time, uses the results from S1 as inputs and significantly reduces the discrepancy between the reconstructed fluorophore absorption and its ground-truth (GT), compared to S1. The reconstruction methods are implemented in computer software, then tested and quantitatively evaluated in numerical simulations, and their limitations and potential improvements are discussed. Prostate tissue-mimicking phantoms with verteporfin, a PS used in PDT (excited by light around 690 nm),²⁵ were prepared following the work by Ghauri et al.,²⁶ to provide realistic experimental validation of the developed methods. The measurements are performed with SpectraCure's P18 system for interstitial PDT of prostate cancer.^{8,16} This is, to the best of our knowledge, the first time a quantitative tomographic reconstruction of the concentration of the PS verteporfin has been demonstrated on a clinical system for interstitial PDT of prostate cancer.

2 Model

2.1 Diffusion Equation for Fluorescence

We assume a highly scattering medium, with the absorption coefficient μ_a , the reduced scattering coefficient μ'_s , satisfying $\mu_a \ll \mu'_s$, and all the conditions of the diffusion approximation.²⁷ The diffusion coefficient is defined by $D = \frac{1}{3\mu'_s}$ and the effective attenuation coefficient $\mu_{\text{eff}} = \sqrt{\frac{\mu_a}{D}}$.

We consider not necessarily a homogeneous medium, so the aforementioned coefficients will be in general dependent on the spatial coordinates: $\mu_a(\vec{r})$, $\mu_s'(\vec{r})$, and $D(\vec{r})$. We distinguish the optical properties and quantities at excitation from those at fluorescence emission wavelengths, by adding a letter in the subscript— x for the excitation and m for the fluorescence emission. If the fluorophore absorption coefficient μ_{af} is defined as the probability of absorption of a photon at the excitation wavelength, per unit pathlength it has covered, and if the fraction of such absorbed photons which result in the fluorescent re-emission is γ , known as the fluorescence quantum yield,²⁸ then the fluorescent yield η can be defined as the product of the two,²²

$$\eta(\vec{r}) = \gamma\mu_{af}(\vec{r}), \quad (1)$$

by assuming γ is independent of the fluorophore spatial distribution. Considering a continuous-wave (CW) light source $q_0(\vec{r})$, the steady-state diffusion equation for the fluence rate at excitation wavelength Φ_x is²⁷

$$(-\nabla D_x(\vec{r})\nabla + \mu_{ax}(\vec{r}))\Phi_x(\vec{r}) = q_0(\vec{r}), \quad (2)$$

Where we assumed $\mu_{af} \ll \mu_{ax}$, i.e., the equation is unaffected by the presence of fluorophores. The source of fluorescence emission $q_m(\vec{r})$ is defined where the fluorophores exist in space, and its strength is proportional to the excitation fluence and the fluorescent yield,

$$q_m(\vec{r}) = \eta(\vec{r})\Phi_x(\vec{r}). \quad (3)$$

The fluence rate at fluorescence emission wavelength Φ_m is found from the following diffusion equation^{7,29}

$$(-\nabla D_m(\vec{r})\nabla + \mu_{am}(\vec{r}))\Phi_m(\vec{r}) = \eta(\vec{r})\Phi_x(\vec{r}). \quad (4)$$

Assuming $\mu_{af} \ll \mu_{am}$, the emission light fluence from Eq. (4) can be obtained by

$$\Phi_m(\vec{r}) = \int_V \Phi_x(\vec{r}')\eta(\vec{r}')G_m(\vec{r}, \vec{r}')d\vec{r}', \quad (5)$$

where $G_m(\vec{r}, \vec{r}_s)$ is Green's function—solution of Eq. (4) when a source term is replaced by a unitary source $\delta(\vec{r} - \vec{r}_s)$. In a special case of an infinite, homogeneous medium, the analytical expression for Green's function is²⁷

$$G(\vec{r}, \vec{r}_s) = \frac{\exp(-\mu_{\text{eff}}|\vec{r} - \vec{r}_s|)}{4\pi D|\vec{r} - \vec{r}_s|}. \quad (6)$$

One possible way of modeling the spatial dependence of the optical properties is the implementation of the finite element mesh, where each element is a region of small volume and its specific optical properties. This method is known as the finite element method (FEM), see Sec. 2.2.

2.2 Calculation of Fluence by Finite Element Method

The whole space (V) considered is divided into a finite number (N_e) of elements of finite volume. Every element is defined by its vertices—nodes of the finite element mesh. The elements are usually tetrahedrons or cubes, in this work, we will use tetrahedrons; therefore, every element will have four nodes. Adjacent elements share one triangular face with three common nodes. However, it is also possible that two elements have one shared segment (two common nodes) or even only one shared vertex (one common node). Elements can not have other intersections, apart from a trivial empty set. The fluence Φ in any point ($\vec{r}$) of the medium is approximated by its finite element representation $\Phi^h(\vec{r})$, as a weighted sum of fluences in mesh nodes Φ_j ^{30–32}

$$\Phi(\vec{r}) \approx \Phi^h(\vec{r}) = \sum_{j=1}^{N_n} u_j(\vec{r})\Phi_j, \quad (7)$$

where N_n is the number of nodes. The weights $u_j(\vec{r})$ depend on the geometrical relations between $\vec{r}$ and node coordinates $\vec{r}_j$, and they are basis functions which span the whole space V . A function $u_j(\vec{r})$ corresponding to node j should be chosen in such a way that it is equal to one in that node,

i.e., $u_j(\vec{r}_j) = 1$, and equal to zero in all other mesh nodes, $u_j(\vec{r}_i) = 0$ for $i \neq j$. A set of coordinates $\vec{r}$ where a basis function $u_j(\vec{r})$ is different from zero is limited (it is said that these basis functions have limited support).

To solve for the finite element representation of the fluence Φ^h , we start from the diffusion equation [Eq. (2)], omitting the subscripts for the wavelength

$$(-\nabla D(\vec{r})\nabla + \mu_a(\vec{r}))\Phi^h(\vec{r}) = q_0(\vec{r}). \quad (8)$$

Multiplying both sides by some function $v(\vec{r})$ (Galerkin method³³) and integrating over the whole space volume V gives

$$\int_V v(\vec{r})[-\nabla D(\vec{r})\nabla + \mu_a(\vec{r})]\Phi^h(\vec{r})dV = \int_V v(\vec{r})q_0(\vec{r})dV. \quad (9)$$

After exploiting mathematical identities and theorems to transform the derivative of the product, volume integral into a surface integral and applying the Robin boundary condition,^{27,34} Eq. (9) (when $\Phi^h(\vec{r})$ is expanded), for specific $v(\vec{r}) = u_i(\vec{r})$ becomes

$$\begin{aligned} \sum_{j=1}^N \left\{ \int_V D(\vec{r})\nabla u_i(\vec{r})\nabla u_j(\vec{r})dV + \int_V \mu_a(\vec{r})u_i(\vec{r})u_j(\vec{r})dV + \frac{1}{2A} \oint_S u_i(\vec{r})u_j(\vec{r})dS \right\} \Phi_j = \\ = \int_V u_i(\vec{r})q_0(\vec{r})dV, \end{aligned} \quad (10)$$

where the closed surface S is defined by the boundaries of volume V and A is the effective Fresnel coefficient.^{27,34} Writing analogous equations for all basis functions $u_i(\vec{r})$, $i = 1, \dots, N_n$, the system of equations is obtained³²

$$\left(\mathbf{K} + \mathbf{C} + \frac{1}{2A}\mathbf{B} \right) \cdot \Phi = \mathbf{A} \cdot \Phi = \mathbf{Q}, \quad (11)$$

where $\mathbf{K} = [K_{ij}]$, $\mathbf{C} = [C_{ij}]$, $\mathbf{B} = [B_{ij}]$, and $\mathbf{A} = [A_{ij}]$ are $N_n \times N_n$ matrices with entries defined by

$$\begin{aligned} K_{ij} &= \int_V D(\vec{r})\nabla u_i(\vec{r})\nabla u_j(\vec{r})dV; \\ C_{ij} &= \int_V \mu_a(\vec{r})u_i(\vec{r})u_j(\vec{r})dV; \\ B_{ij} &= \oint_S u_i(\vec{r})u_j(\vec{r})dS; \\ A_{ij} &= K_{ij} + C_{ij} + \frac{1}{2A}B_{ij}; \end{aligned} \quad (12)$$

while

$$\Phi = [\Phi_1 \quad \Phi_2 \quad \dots \quad \Phi_{N_n}]^T, \quad (13)$$

and

$$\mathbf{Q} = \left[\int_V u_1(\vec{r})q_0(\vec{r})dV \quad \int_V u_2(\vec{r})q_0(\vec{r})dV \quad \dots \quad \int_V u_{N_n}(\vec{r})q_0(\vec{r})dV \right]^T \quad (14)$$

are $N_n \times 1$ column vectors.

Numerical computation of the integrals in Eqs. (12) and (14) is performed within element volumes, exploiting the limited support of basis functions, which results in non-zero integrands only within certain elements. Therefore, all matrices $\mathbf{K}$, $\mathbf{C}$, $\mathbf{B}$ and the resulting system matrix $\mathbf{A}$ are sparse. For calculating the source vector, depending on the coordinate of the point source $\vec{r}_s$, the element which contains that source is found (here we consider only point sources; in FEM in general, sources can be distributed as well). The barycentric coordinates of $\vec{r}_s$ are found with respect to the tetrahedron nodes, and finally, the corresponding integrals are calculated. The fluence vector Φ is computed using fast numerical methods for the inversion of sparse matrices.⁷

To calculate the fluence of the fluorescent light, Eq. (5) is used, where first the fluence Φ_x is computed. The Green's function $G_m(\vec{r}, \vec{r}')$ is computed similarly, just finding $G_m(\vec{r}', \vec{r})$ instead, which is computationally faster, because the source is fixed at $\vec{r}$, and the result is the same because of the reciprocity theorem.^{35–37}

Now, we have the methods to solve the forward problem—calculating light fluence at excitation and fluorescent wavelengths at arbitrary positions in the medium, knowing the optical properties.

2.3 Inverse Model

We assume to know the optical properties of the background medium—absorption coefficients $\mu_a(\vec{r})$ and reduced scattering coefficients $\mu_s'(\vec{r})$. Our goal is to reconstruct the fluorescent yield $\eta(\vec{r})$ everywhere in the medium, starting from the fluorescent signal measurements. The measurement data is collected by n interstitially placed optical fibers, and the light is delivered through the same fibers. When light is emitted from one fiber to the medium, the other $n - 1$ are collecting, and that is repeated for every fiber delivering light. In this way, all $n(n - 1)$ source-detector pairs are covered, and the light is detected at both—the excitation and the fluorescent wavelengths. To cancel out the experimental uncertainties, such as the source power, or collection efficiency of a fiber, we express the measurements in the form of the normalized Born ratio³⁸—the ratio between the signals detected at the fluorescent and the excitation wavelengths, respectively. Therefore, we have $N_m = n(n - 1)$ measurement points, defined as Born ratios, every measurement point corresponding to one source-detector pair. The forward model, expressed in terms of a vector of finite element mesh nodal values of fluorescent yields, $\boldsymbol{\eta} = (\eta_1, \eta_2, \dots, \eta_{N_m})$, gives the expression for the measurements from the source with index s and the position $\vec{r}_s$, and the detector with index d , at the position $\vec{r}_d$ ^{22,26}

$$F_{s,d}(\boldsymbol{\eta}) = \frac{1}{G_x(\vec{r}_d, \vec{r}_s)} \sum_{i=1}^{N_n} G_x(\vec{r}_i, \vec{r}_s) G_m(\vec{r}_i, \vec{r}_d) \eta_i \Delta V, \quad (15)$$

where η_i is the fluorescent yield in node i , with a spatial coordinate $\vec{r}_i$, ΔV is the element volume, and G_x and G_m are Green's functions solutions at the excitation and fluorescent emission wavelengths, respectively, as in Sec. 2.1. These solutions $G_{x,m}(\vec{r}_d, \vec{r}_s)$ are obtained by computing the corresponding fluence at $\vec{r}_d$ when the unitary source is placed at $\vec{r}_s$. Note that this Eq. (15) follows from Eq. (5) when the normalization to the excitation signal is done and after numerical implementation of the integration over all the elements of the finite element mesh.

The arranged pairs (s, d) of source and detector indices are mapped to a set of integer numbers from 1 to N_m . The mapping function is the following: $f(s, d) = d - 1 + (n - 1)(s - 1) + p(s, d)$, where $p(s, d) = 0$ if $s < d$ and $p(s, d) = 1$ if $s > d$. The forward model vector of the estimated Born ratios is then obtained

$$\mathbf{F}(\boldsymbol{\eta}) = (F_1(\boldsymbol{\eta}), \dots, F_i(\boldsymbol{\eta}), \dots, F_{N_m}(\boldsymbol{\eta})), \quad \text{where } i = f(s, d). \quad (16)$$

The same mapping is done on measurement points, creating the measurement vector $\mathbf{M}$, which contains all N_m Born ratios of the detected fluorescent and excitation light signals. The discrepancy between the measurements and the forward model is the error vector $\boldsymbol{\delta}(\boldsymbol{\eta}) = \mathbf{M} - \mathbf{F}(\boldsymbol{\eta})$.

The inverse problem can be formulated as estimating the vector of fluorescent yields $\boldsymbol{\eta}$ such that the error vector $\boldsymbol{\delta}(\boldsymbol{\eta})$ is minimal in some metrics. A standard metrics is the Euclidean or 2-norm, which we denote as $\|\boldsymbol{\delta}\|$. The number of unknowns N_n (components of vector $\boldsymbol{\eta}$) is usually much greater than the number of measurements N_m ($N_n \sim 10^4 \gg N_m \sim 10^2$), so the problem is ill-posed, and a regularization should be added to its formulation. A common approach is Tikhonov regularization,^{32,39} where a quadratic term with the 2-norm of the vector of unknowns is included in the objective function. The goal is to minimize the following^{22,26}

$$\Omega(\boldsymbol{\eta}) = \|\mathbf{M} - \mathbf{F}(\boldsymbol{\eta})\|^2 + \lambda \|\mathbf{L}(\boldsymbol{\eta} - \boldsymbol{\eta}_0)\|^2, \quad (17)$$

where λ is the regularization parameter, $\mathbf{L}$ is the regularization matrix (dimensions $N_n \times N_n$), and $\boldsymbol{\eta}_0$ is the initial estimate (guess) for the vector of unknowns $\boldsymbol{\eta}$.

If $\boldsymbol{\eta}$ is the parameter that minimizes the cost function $\Omega(\boldsymbol{\eta})$, then the first derivative $\frac{\partial \Omega(\boldsymbol{\eta})}{\partial \boldsymbol{\eta}}$ is equal to zero, which implies

$$-2 \frac{\partial \mathbf{F}(\boldsymbol{\eta})^T}{\partial \boldsymbol{\eta}} (\mathbf{M} - \mathbf{F}(\boldsymbol{\eta})) + 2\lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta} - \boldsymbol{\eta}_0) = 0. \quad (18)$$

Defining the Jacobian as $N_m \times N_n$ matrix $\mathbf{J} = \frac{\partial \mathbf{F}(\boldsymbol{\eta})}{\partial \boldsymbol{\eta}}$, we can write

$$\mathbf{J}^T (\mathbf{M} - \mathbf{F}(\boldsymbol{\eta})) = \lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta} - \boldsymbol{\eta}_0). \quad (19)$$

If $\boldsymbol{\eta}_i$ and $\mathbf{J}_i$ are the estimates vector and the Jacobian of iteration i , then, the forward model calculated for the iteration $i + 1$ can be, using the Taylor expansion, approximated as

$$\mathbf{F}(\boldsymbol{\eta}_{i+1}) \approx \mathbf{F}(\boldsymbol{\eta}_i) + \mathbf{J}_i (\boldsymbol{\eta}_{i+1} - \boldsymbol{\eta}_i), \quad (20)$$

where it is assumed that the estimates vectors of successive iterations $\boldsymbol{\eta}_{i+1}$ and $\boldsymbol{\eta}_i$ are close enough to have a good approximation by keeping just the first order term. Rewriting Eq. (19) for the next iteration $\boldsymbol{\eta}_{i+1}$ while exploiting the forward model linearization around $\boldsymbol{\eta}_i$ in Eq. (20) gives

$$\mathbf{J}_i^T (\mathbf{M} - \mathbf{F}(\boldsymbol{\eta}_i) - \mathbf{J}_i (\boldsymbol{\eta}_{i+1} - \boldsymbol{\eta}_i)) = \lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta}_{i+1} - \boldsymbol{\eta}_0), \quad (21)$$

which can be rewritten as

$$\mathbf{J}_i^T \boldsymbol{\delta}(\boldsymbol{\eta}_i) - \mathbf{J}_i^T \mathbf{J}_i (\boldsymbol{\eta}_{i+1} - \boldsymbol{\eta}_i) = \lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta}_{i+1} - \boldsymbol{\eta}_i) + \lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta}_i - \boldsymbol{\eta}_0). \quad (22)$$

Finally, the update equation for the fluorescent yield estimates vector is²²

$$\boldsymbol{\eta}_{i+1} = \boldsymbol{\eta}_i + (\mathbf{J}_i^T \mathbf{J}_i + \lambda \mathbf{L}^T \mathbf{L})^{-1} (\mathbf{J}_i^T \boldsymbol{\delta}(\boldsymbol{\eta}_i) - \lambda \mathbf{L}^T \mathbf{L} (\boldsymbol{\eta}_i - \boldsymbol{\eta}_0)). \quad (23)$$

Equation (23) is general, and in this work, we apply a modified Levenberg-Marquardt algorithm (similar to Axelsson et al.²² and Dehghani et al.³²) where the update equation is

$$\boldsymbol{\eta}_{i+1} = \boldsymbol{\eta}_i + (\mathbf{J}_i^T \mathbf{J}_i + \lambda_i \mathbf{L}^T \mathbf{L})^{-1} \mathbf{J}_i^T \boldsymbol{\delta}(\boldsymbol{\eta}_i), \quad (24)$$

with λ being initialized to the maximum of the diagonal of the Hessian matrix: $\lambda_0 = \max\{\text{diag}(\mathbf{J}_0^T \mathbf{J}_0)\}$ and updated in every iteration to $\lambda_i = \max\{\text{diag}(\mathbf{J}_i^T \mathbf{J}_i)\} \cdot 10^{-i/4}$. A more detailed discussion about the regularization follows in Sec. 2.4.

The iterative procedure of updating $\boldsymbol{\eta}$ stops when the relative decrease of the norm of the error vector $\|\boldsymbol{\delta}\|$ is $<2\%$ or the predefined maximal number of iterations (around 20) is reached.³²

2.4 Regularizations

The solution obtained from the inverse model is regularized and does not have the minimum norm of the error vector. However, it has some properties that are imposed by the choice of the regularization matrix—which can make it “smoother” (less abrupt spatial variations of the reconstructed fluorescent yield) or have similar (very close) values in predefined regions or even very sharp transitions between the regions.

The default regularization applied in this paper is without prior information about the geometry, setting the regularization matrix of the Tikhonov regularization to an identity matrix $\mathbf{L} = \mathbf{I}$. This adds some “inertia” on the main diagonal of the Hessian matrix $\mathbf{J}^T \mathbf{J}$, which provides numerical stability when computing inverses of large-dimensional matrices. As a consequence, the solution appears “smooth” in space.

It is possible to include a priori knowledge about the geometry of the problem, by assuming there are regions in space (for example different tissue types) that should have similar levels of PS drug accumulation—for example, inside the tumor the highest, around the tumor lower, background the lowest. If we are not reducing the number of unknowns of the original inverse problem, thus permitting different values of reconstructed fluorophore absorption coefficients within the regions, the geometrical biasing of the solution is called soft priority, and the entries of the regularization matrix $\mathbf{L} = [L_{ij}]$ are defined as^{22,40}

$$L_{ij} = \begin{cases} 1, & \text{if } i = j; \\ \frac{1}{N_{R(i)}}, & \text{else if nodes } i \text{ and } j \text{ belong to the same region } R(i) \equiv R(j) \text{ with } N_{R(i)} \text{ nodes;} \\ 0, & \text{otherwise.} \end{cases} \quad (25)$$

Suppose we restrict to only a few possible values for the reconstructed fluorophore absorption coefficients, i.e., divide the whole geometry of the medium into N_r non-overlapping regions, where all nodes within a particular region have the same reconstructed value. In that case, the number of unknowns is reduced to N_r , and the geometrical biasing of the solution is called hard prioring. When it is reasonable to assume homogeneous properties within the regions of the tissue, this regularization results in quantitatively more accurate reconstruction, as reported by Dehghani et al.⁴¹ and Srinivasan et al.⁴² The main idea underlying this method will be applied in S2, where the results obtained without any geometrical biasing in S1 will be used to define the regions in S2 (see Sec. 2.5).

It is, of course, possible to apply any regularization different from Tikhonov, and some examples are given in the work by Jagannath and Yalavarthy⁴³ and in the review by Okawa and Hoshi.⁴⁴

2.5 Second Stage of the Reconstruction (S2)

The inverse model provides the solution for the fluorescent yields in all nodes, using regularization and the Levenberg-Marquardt iterative algorithm, and we refer to this method as S1. The solution obtained at this stage is usually smoother than it should be (obtained fluorescent yields do not show abrupt spatial variations, because of the regularization) and therefore has a quantitatively larger error. This is more evident in homogeneous regions, where almost the same fluorophore absorption coefficient is expected over the whole volume of the region. This is the key assumption for S2. Assuming the entire medium can be divided into N_r non-overlapping regions (sets of finite element mesh nodes) of homogeneous fluorophore absorption coefficients, the inverse problem to solve becomes more straightforward—the number of unknowns is reduced to N_r , while still having N_m measurement points. Usually, $N_r \ll N_m$.

The regions are defined after S1, by looking at the obtained spatial distribution of fluorescent yields, i.e., fluorophore absorption coefficients. The values of fluorophore absorptions in the mesh nodes ($\mu_{af1}, \mu_{af2}, \dots, \mu_{afN_n}$) are compared with thresholds. For the two finite element mesh nodes belonging to the same element (tetrahedron), we say they are adjacent or neighboring. Starting from the mesh node m_1 with the highest fluorophore absorption coefficient in the medium, the first region is initialized as the set containing only that node. The average fluorophore absorption coefficient of this node and all its neighboring nodes is $\mu_{af}^{\max,1,\text{avg}}$. In the next iteration, this region can be expanded by including all the neighboring nodes of the node m_1 , which have fluorophore absorption greater than or equal to the threshold $t_p \mu_{af}^{\max,1,\text{avg}}$, where $0 < t_p < 1$ is the given fraction that determines the peak threshold. This procedure is repeated as long as there are neighboring nodes (k) of any of the nodes from the first region, not yet included in the first region, which satisfy the condition $\mu_{afk} \geq t_p \mu_{af}^{\max,1,\text{avg}}$. After the iteration in which all the adjacent nodes, not yet included in the first region, had fluorophore absorption below the threshold $t_p \mu_{af}^{\max,1,\text{avg}}$, the first region is concluded. Then, in case there is a node m_2 with the highest fluorophore absorption coefficient among all nodes outside of the first region, satisfying the condition $\mu_{afm_2} \geq t_b \mu_{af}^{\max,1,\text{avg}}$, for the given background threshold fraction $0 < t_b < 1$, the second region is initialized as the set containing only that node (m_2). The average fluorophore absorption coefficient of this node and all its neighboring nodes is $\mu_{af}^{\max,2,\text{avg}}$. The iterative procedure for including neighboring nodes continues, now with the threshold $t_p \mu_{af}^{\max,2,\text{avg}}$, defining the second region. In case there is a node (m_3) in the finite element mesh which is not included in the first two regions, and still satisfies the condition $\mu_{afm_3} \geq t_b \mu_{af}^{\max,1,\text{avg}}$, the next region is defined, and so on. The first time the highest value of μ_{af} outside of the regions defined so far is below the threshold $t_b \mu_{af}^{\max,1,\text{avg}}$, the number of regions is concluded by ascribing all the remaining nodes to the background region. The flowchart diagram of this algorithm is shown in Fig. 1.

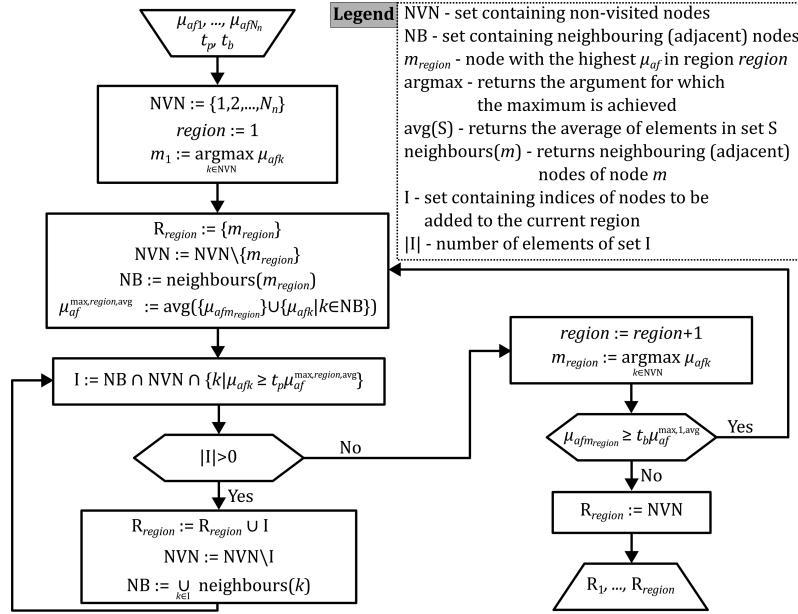


Fig. 1 Algorithm for defining the regions of homogeneous fluorophore absorption $R_1, \dots, R_{N_r}$ based on inputs from S1—reconstructed fluorophore absorptions in all nodes $\mu_{af1}, \dots, \mu_{afN_n}$, for chosen thresholds t_p, t_b .

The transition matrix $\mathbf{T}$ (also called a priori matrix⁴¹), with dimensions $N_n \times N_r$, provides the mapping between the original vector $\boldsymbol{\eta}^{(1)}$ of N_n fluorescent yields, as a result of S1, and the vector of unknowns $\boldsymbol{\eta}^{(2)}$ in S2, which has only N_r components. A matrix entry T_{ij} is equal to 1 if the node i belongs to region j ; otherwise, it is equal to 0. The relations between $\boldsymbol{\eta}^{(1)}$ and $\boldsymbol{\eta}^{(2)}$, as well as between the reduced (S2) Jacobian $\mathbf{J}^{(2)}$ and the full (S1) Jacobian $\mathbf{J}^{(1)}$ are

$$\boldsymbol{\eta}^{(1)} = \mathbf{T}\boldsymbol{\eta}^{(2)}, \quad \mathbf{J}^{(2)} = \mathbf{J}^{(1)}\mathbf{T}. \quad (26)$$

The inverse problem is now solved without regularization, by applying the Gauss-Newton^{45,46} iterative method with the updated equation for $\boldsymbol{\eta}_i^{(2)}$

$$\boldsymbol{\eta}_{i+1}^{(2)} - \boldsymbol{\eta}_i^{(2)} = ((\mathbf{J}^{(2)})_i^T \mathbf{J}_i^{(2)})^{-1} (\mathbf{J}^{(2)})_i^T \boldsymbol{\delta}(\boldsymbol{\eta}_i^{(1)}). \quad (27)$$

The initial guess for the vector $\boldsymbol{\eta}_0^{(2)}$ is the vector of averages of the fluorescent yields in regions, obtained from S1, thus not expecting to be too far from the “solution” (value of $\boldsymbol{\eta}$ for which $\|\boldsymbol{\delta}(\boldsymbol{\eta})\|$ is minimal). Because the system of equations is now overdetermined, the forward model in this stage cannot match all the measurement points but provides a smaller error $\|\boldsymbol{\delta}\|$ than in S1. This error is evaluated for different divisions of the medium into regions, by changing the threshold fractions t_p and t_b . A pair of threshold fractions (t_p, t_b) is given as input to the regionalization algorithm defined in Fig. 1. The output of the regionalization algorithm is the array of regions $R_1, R_2, \dots, R_{N_r}$. The regionalization algorithm is executed for different threshold pairs (t_p, t_b) , resulting in different divisions of the medium into regions, i.e., different arrays $R_1, R_2, \dots, R_{N_r}$. The regionalization (division) for which the error $\|\boldsymbol{\delta}(\boldsymbol{\eta}^{(2)})\|$ is the smallest, and the corresponding vector $\boldsymbol{\eta}^{(2)}$, are the final results of S2.

3 Materials and Methods

3.1 Computer Implementation

A finite element mesh is defined over the considered inhomogeneous medium, and the FEM is used to compute the forward model fluences and normalized Born ratios in Eq. (15). The mesh elements are tetrahedrons, and the mesh creation and all FEM computations are done in MATLAB (The MathWorks Inc., 2022) using the open-source NIRFAST package.^{32,47} Mesh nodes are placed more densely around the fiber tip positions to capture more dynamic spatial variations of fluence near the light sources and detectors. The resulting meshes had from 20,000 to 70,000 nodes, and between 90,000 and 320,000 elements, with an average size of around $(5 \times 5 \times 5) \text{ cm}^3$. The mesh, used to calculate the forward model, is called the forward mesh. Details about the forward meshes used in this work are given in Table 1. The reconstruction mesh, used to solve the inverse problem, has a lower resolution (for example 6480 nodes) to reduce the computational cost and improve the numerical stability of the Hessian matrix inversion. The interpolation from the forward mesh onto the reconstruction mesh and vice versa is done in every iteration of the reconstruction procedure: the forward model and the corresponding Jacobian are first computed on the forward mesh, then the dimension of the problem is reduced by interpolating nodal values from higher resolution forward to lower resolution reconstruction mesh, on which the update equation [Eq. (24)] is solved, and the obtained nodal values are extrapolated back from the reconstruction to the forward mesh. Mesh resolution will affect the quality of the reconstruction and the time needed for the convergence. One should find a balance between the number of measurement points, the number of unknowns (size of the forward mesh), the computational power, the regularization, the accuracy of the forward model, and finally, the solution.

For S2, adaptive thresholds were implemented—the threshold fractions t_p and t_b from Sec. 2.5 are varied, resulting in the one providing the lowest final $\|\delta\|$. This takes more computational time but is more robust than the approach with fixed thresholds. In the current implementation, it is assumed $t_p = t_b$, and nine equidistant values for the threshold fractions are taken from the interval (0.33, 0.87).

The graphical representation of the reconstructed fluorophore absorption relies on the mapping from the forward mesh nodal values to the values in arbitrary positions in space, where every point in space is within an element, and its barycentric coordinate within the element determines the contribution of each of the element nodes. The graphical representation is a 2-dimensional (2D) color map of fluorophore absorption coefficients in a single xy -plane (the chosen slice perpendicular to the z -axis, see Figs. 4, 5, 7, 8, 9, and 11). Note that even the homogeneous GT fluorophore distributions will appear in different colors, because of this mapping between the FEM mesh and 2D graphics mesh. Blue circles denote fiber tip projections in xy -plane. After S2, the space is divided into a few regions of homogeneous fluorophore absorption coefficients with sharp borders. It is possible to provide a 3-dimensional (3D) representation of the regions using semi-transparent boundary surfaces [see Figs. 2 and 10(e)]. This way, the estimates of the reconstructed inclusion (region) volumes are also given.

Table 1 Finite element meshes used in this work, with the details about the size, numbers of nodes, and elements.

Mesh	Size (mm × mm × mm)	Number of nodes	Number of elements
1	52 × 57 × 40	28,296	130,124
2	52 × 57 × 40	64,325	312,676
3	40 × 50 × 34	49,921	237,041
4	40 × 50 × 34	20,745	92,705
5	50 × 50 × 40	49,900	240,333
6	50 × 50 × 40	22,972	104,406

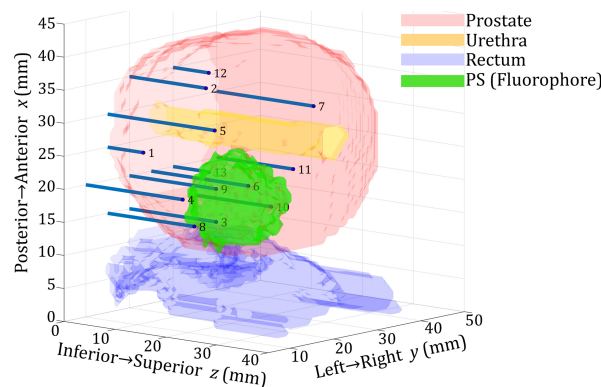


Fig. 2 3D model of prostate tissue (semi-transparent red) with the urethra (semi-transparent blue), and fluorescent PS (green). Blue lines and blue circles represent fibers and their tips (labeled with numbers), respectively. PS distribution shown is a result of the reconstruction from the simulation with default parameters (see Sec. 3.3) and without noise. The same Mesh 1 (see Table 1) was used for data generation and inverse problem solving. A coarser 6480-node mesh was used for matrix inversion. 3D models of the prostate, urethra, and rectum obtained from clinical ultrasound data. Labels on axes x , y , and z correspond to the sides and directions of the patient's body.

Table 2 Fiber coordinates used in simulations.

Fiber number	1	2	3	4	5	6	7	8	9	10	11	12	13
x (mm)	25	35	15	20	30	20	30	15	20	15	20	35	20
y (mm)	15	20	20	10	15	25	40	15	20	35	40	30	30
z (mm)	7	15	17	19	21	19	19	17	17	15	15	7	7

3.2 Prostate Anatomy and Interstitial Fiber Placement

In Fig. 2, we give a 3D model of relevant tissues, fiber positions, and the reconstructed fluorophore distribution in space. The reconstruction was performed on simulated data, following the default scenario described in Sec. 3.3, without noise. The same finite element mesh was used for data generation and reconstruction. This graphical representation should help the reader to understand the geometry of the interstitial fiber placement. Fiber coordinates (see Table 2) and 3D models of the prostate, urethra, and rectum are obtained from clinical ultrasound data, as in our previous work.²⁶ The fiber configuration in a particular PDT treatment is the result of Cimmino's optimization method, where the minimum threshold light dose is delivered to the target volume while minimizing the surrounding tissue exposure.^{8,48}

3.3 Numerical Simulations

Computer codes for numerical simulations, including data creation, reconstruction, and graphical representation, were implemented in MATLAB. Simulated measurement data were created by applying the forward model FEM, described in Sec. 3.1.

In all simulated scenarios, a single-spherical inclusion was considered. Unless other specified, the following parameters were used as default. GT background fluorophore absorption $\mu_{afbg,gt} = 0.01 \text{ cm}^{-1}$, GT inclusion fluorophore absorption $\mu_{afi,gt} = 0.10 \text{ cm}^{-1}$, reduced scattering coefficient $\mu'_s = 8.7 \text{ cm}^{-1}$, and absorption coefficient of the background $\mu_a = 0.50 \text{ cm}^{-1}$. The inclusion was centered at $(x_C, y_C, z_C) = (19, 25, 18) \text{ mm}$, with radius $R = 6.7 \text{ mm}$. A total of 13 fibers were placed²⁶ at the positions given in Table 2 and shown in Fig. 2.

3.4 Evaluation of the Reconstruction

To quantitatively evaluate the performance of the reconstruction, here, we introduce the following relative errors:

- reconstructed volume of the inclusion, relative error δV_i ;
- reconstructed fluorophore absorption coefficient of the inclusion, relative error $\delta\mu_{afi}$;
- reconstructed fluorophore absorption coefficient of the background, relative error $\delta\mu_{afbg}$;
- average absolute value of the relative error of the reconstructed fluorophore absorption coefficient $\langle |\delta\mu_{af}(\vec{r})| \rangle$ in the whole medium (shorter $\langle |e| \rangle$).

Note that it only makes sense to define relative errors δV_i , $\delta\mu_{afi}$, and $\delta\mu_{afbg}$ for the results of the second stage of the reconstruction, because these parameters refer to the regions defined only in that stage. On the other hand, the absolute value of the relative error $|e|$ can be defined for every node, and we will compare the average in the whole medium $\langle |e| \rangle$ after S1 and after S2.

3.5 Phantoms

Tissue-mimicking phantoms were realized to validate the proposed model. A hybrid approach of combining solid and liquid phantoms similar to the work by Ghauri et al.²⁶ was followed. The liquid solution used for background optical properties of the tissue phantom is a water solution of Intralipid (200 mg/ml, Fresenius Kabi, Ltd., Germany) and India ink (a - Rotring, Germany; and b - Higgins, 44201 Chartpak Inc., USA). India ink (a) was first diluted in water to 1%, then 4.1 ml of this stock solution was added to a solution containing 35.4 ml of intralipid (20%w/v) and 960 ml of water, mixed with a magnetic stirrer to achieve homogeneous properties: absorption coefficient $\mu_{ab} = 0.24 \text{ cm}^{-1}$ and the reduced scattering coefficient $\mu'_{sb} = 8.3 \text{ cm}^{-1}$ (both at 690 nm). The optical properties were chosen to mimic those of a human prostate.⁴⁹ The solid inclusions were realized as spheres of radius $R = 5.5 \text{ mm}$. The material used was water-based gelatin with the addition of proper amounts of Intralipid and India ink (b) to achieve the absorption coefficient $\mu_a = 0.20 \text{ cm}^{-1}$ and $\mu'_s = 15.7 \text{ cm}^{-1}$. The same protocol as in our previous work²⁶ was applied to make these phantoms. The PS used was verteporfin, an active substance of the drug Visudyne (Cheplapharm Arzneimittel GmbH, Germany). This PS is excited at around 690 nm and re-emits fluorescent light at wavelengths longer than 700 nm. Solid inclusions were realized with different concentrations of verteporfin, ranging from 0.3 to 1.8 mg/kg. Optical characterization of bulk properties of both solid and liquid phantom components was done using a time-domain diffuse optical spectroscopy system.⁵⁰

3.6 Experimental Setup

The measurements were performed using SpectraCure's P18 system.^{8,18} The system consists of a light delivery unit with 18 photonics modules. Each photonics module (PM) has a laser diode (690 nm), and two detectors—the first detecting excitation light (wavelengths below 700 nm) and the other detecting fluorescence or infrared light (wavelengths above 700 nm). Each PM is SC-connected to a 45-cm-long patch fiber with a core diameter of 400 μm and $\text{NA} = 0.37$. Each patch fiber is further SMA-connected to a 200-cm-long silica fiber with the same core diameter and $\text{NA} = 0.22$. This fiber, inserted through a brachytherapy needle, has its bare end (tip) free in the tissue phantom (around 2 mm beyond the needle tip) to deliver and collect light, although not simultaneously. PMs are controlled by SpectraCure's Interactive Dosimetry by Sequential Evaluation (IDOSE) software⁸ for how long the light will be sent or collected. We used $n = 12$ PMs, and therefore, 12 fibers were placed in the tissue phantom, through brachytherapy needles, which were supported at pre-determined positions (holes) in the brachytherapy template (horizontal plane) and specific pre-determined depths (along the vertical axis). The fiber arrangement has been decided based on different simulations of real measurements (see Figs. 8 and 9), such that the chosen configuration, reported in Table 3, results in enough signals above the cut-off threshold (Sec. 4.1), for all tested fluorophore concentrations.

By controlling all 12 PMs, tomographic data acquisition is possible: while one fiber is delivering light, the other 11 are collecting—both excitation (690 nm) and infrared fluorescence emission—and this is repeated such that all 12 fibers deliver light, one at a time (monitoring sequence). There are usually several monitoring sequences (3 to 5), during the session, one after

Table 3 Another fiber configuration used in simulations and in experiments.

Fiber number	1	2	3	4	5	6	7	8	9	10	11	12
x (mm)	15	20	15	20	25	30	20	25	30	35	30	35
y (mm)	30	35	20	25	30	35	15	20	25	30	15	20
z (mm)	25	15	15	20	20	25	25	15	25	15	15	25

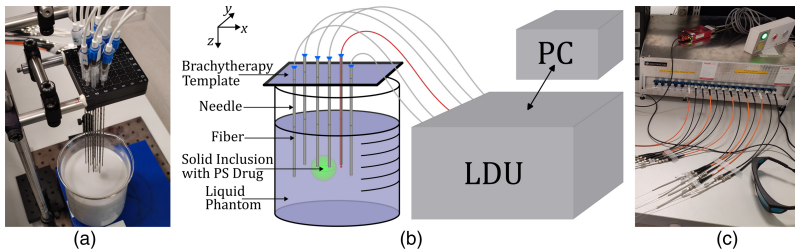


Fig. 3 (a) Gelatin sphere attached to two brachytherapy needles (corresponding to fibers 4 and 5) before immersing into a liquid background. (b) Schematics of the laboratory setup: LDU—light delivery unit, and PC—personal computer, with the software sending instructions to and receiving the data from LDU. (c) SpectraCure's P18 LDU with optical fibers connected to its ports.

another, to follow the PS drug concentration evolution over time (which is of interest in a dynamic environment, such as in vivo during PDT). After each monitoring sequence, a tomographic reconstruction of the fluorophore absorption can be performed. The tissue phantom (Sec. 3.5) is a liquid solution, with the addition of solid fluorophore inclusions suspended by brachytherapy needles. All the fibers and needles are vertical and immersed in the liquid background, only two of the fiber tips are positioned inside the solid fluorophore inclusions. The experimental setup is shown in Fig. 3.

4 Results and Discussion

4.1 Simulation Study on the Effect of Different Parameters

In simulations, we first examine how the size of the inclusion affects the reconstruction of relative errors. The parameter varied here is the radius of the spherical inclusion (R). The reconstructions after S1 and after S2, are compared with the GT fluorophore absorption distribution, as shown in Fig. 4. Data were generated on the mesh different from the one used for the reconstruction (see the caption of Fig. 4).

For all the considered sphere sizes, relative errors of the reconstructed fluorophore absorption coefficients of the inclusion and the background are always at similar levels (of the order of (1 to 10)%). The absolute value of the relative error of the reconstructed volume is always <20%, which corresponds to about 6% error in linear dimensions (radius or diameter). This can be ascribed to the finite element mesh limitations and differences between the mesh used for data creation and the mesh used for the reconstructions (see the caption of Fig. 4). Considering the colors in Fig. 4, it is possible to observe that the spatial sensitivity is dependent on the fiber positions—S1 reconstruction resulted in some detectable fluorophore absorption (dark red blurs) around the fibers, whereas the rest of the background resulted in values close to 0 (black). S2 of the reconstruction compensates for these artefacts taking advantage of the homogeneous regions assumption. The reconstructed background has a maximum error of <10%. A clear evidence of the benefits of the two-stage reconstruction approach is the following: an average absolute value of the relative error of the reconstruction $\langle|e|\rangle$ after S1 was always around 90%, whereas this average is drastically reduced after adding S2, resulting in $\langle|e|\rangle$ in range (1 to 8)%.

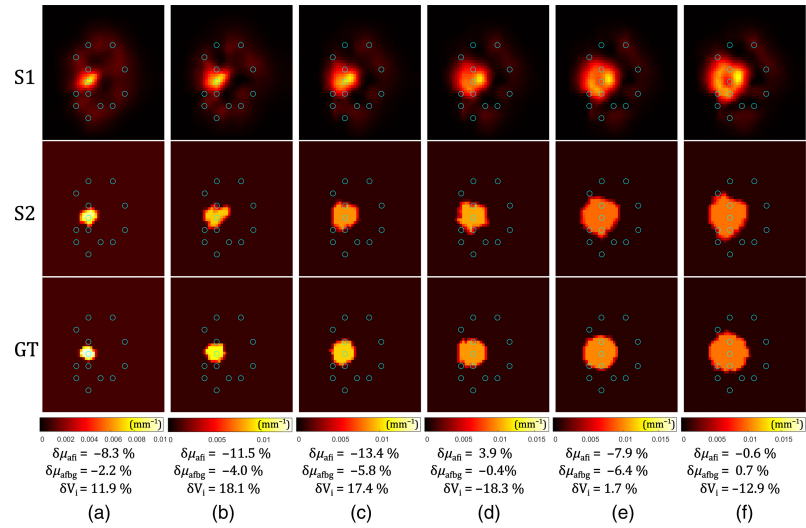


Fig. 4 Reconstruction of spherical fluorophore inclusions of different simulated sizes (R): (a) 3 mm, (b) 4 mm, (c) 5 mm, (d) 6 mm, (e) 7 mm, and (f) 8 mm. The results after the S1 reconstruction are in the first row, followed by the S2 reconstruction in the second row, and finally compared with the GT in the third row. The plane cut is perpendicular to the z -axis, through the center of the inclusion sphere. Fluorophore absorption coefficients are shown with different colors (color bars below the GT images). Blue circles represent fiber positions. Relative errors for the reconstructed fluorophore absorption coefficients of the inclusions $\delta\mu_{afi}$ and the background $\delta\mu_{abg}$, and the volumes of the inclusions δV_i , are given below the images. Data were generated using the denser Mesh 2, whereas the inverse problem was solved using Mesh 1 (see Table 1), with a coarse 6480-node mesh used for matrix inversion.

It is interesting to note that the introduction of S2 in the reconstruction algorithm has a similar effect on the background artifact removal and the spatial confinement of the reconstructed inclusion as the quadratic source term in the diffusion equation for fluorescence in the case of upconverting nanoparticles.⁵¹

The second varied parameter is the fluorophore absorption coefficient of the inclusion (μ_{afi}), which is directly proportional to the concentration of the fluorophore. Figure 5 shows the reconstruction results for different ratios of μ_{afi}/μ_{abg} , where the background fluorophore absorption was kept constant, $\mu_{abg} = 0.010 \text{ cm}^{-1}$. Data were generated on the mesh different from the one used for the reconstruction (see the caption of Fig. 5).

We see that as the inclusion's fluorophore absorption coefficient increases, the reconstructed volume of the inclusion is more accurate. When the volume is underestimated, the absorption coefficient is overestimated and vice versa. Relative errors of the reconstructed fluorophore absorption coefficients of the inclusion and the background are always at similar levels (of the order of (1 to 10)%). When the fluorophore absorption of the inclusion is similar to the background [Fig. 5(a), $\mu_{afi}/\mu_{abg} = 2.5$], the reconstruction S2 resulted in three regions, and therefore, a larger error for the reconstructed background, as the region between the background and the inclusion was a "transitional" region with the reconstructed fluorophore absorption coefficient 32% higher than the GT background. In this particular case, the average absolute value of the relative error $\langle |e| \rangle$ after S2 was 34%, whereas after S1, it was 89%. In all other cases, the initial $\langle |e| \rangle$ of around 90% after S1 was reduced to a value in the interval (2 to 10)% by adding S2.

Next, we incorporate noise in the analysis to see how the reconstruction performs in the face of uncertainty in input data. Noise in signals is a general term and can stem from the physical limitations of the detection system. The ratio of this noise compared with the useful signal can usually be reduced by increasing the excitation power or extending the measuring time.

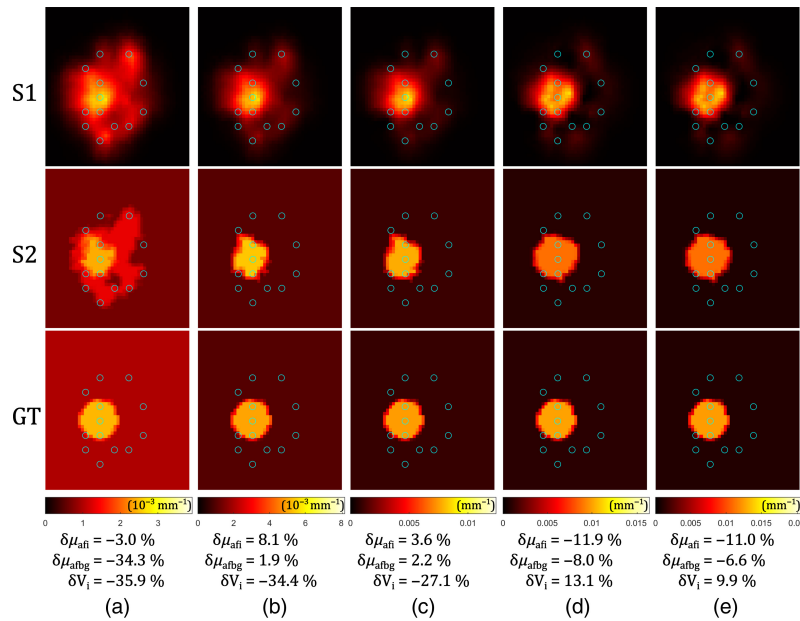


Fig. 5 Reconstruction of spherical ($R = 6.7$ mm) inclusions of different simulated fluorophore absorption coefficients (μ_{afl}): (a) 0.025 cm^{-1} , (b) 0.050 cm^{-1} , (c) 0.075 cm^{-1} , (d) 0.100 cm^{-1} , and (e) 0.125 cm^{-1} . The results after the S1 reconstruction are in the first row, followed by the S2 reconstruction in the second row, and finally compared with the GT in the third row. The plane cut is perpendicular to the z-axis, through the center of the inclusion sphere. Fluorophore absorption coefficients are shown with different colors (color bars below the GT images). Blue circles represent fiber positions. Relative errors for the reconstructed fluorophore absorption coefficients of the inclusions $\delta\mu_{afl}$ and the background $\delta\mu_{abg}$, and the volumes of the inclusions δV_i , are given below the images. Data was generated using the denser Mesh 2, whereas the inverse problem was solved using Mesh 1 (see Table 1), with a coarse 4590-node mesh used for matrix inversion.

Other sources of noise come from our limited knowledge about the physical system, and from the model, which is likely very simplified compared with reality. Random variables representing the noise are added here to the theoretically computed signals (obtained by FEM). Every random variable (for every measurement point and both the excitation and the fluorescence emission wavelengths) is taken from a Gaussian distribution with a mean value of 0 and a standard deviation equal to the corresponding theoretically computed signal, multiplied with an amplitude factor, varied here from 0 to 7%. The Gaussian distribution for noise is chosen to model not only the statistics of photon detection (shot noise) but also any other uncertainties. For the comparison, in our CW detection system, a Gaussian noise with an amplitude factor of 1% corresponds to the Poisson noise for detected power of $4.5 \cdot 10^{-10}$ W, whereas 3% of amplitude factor for Gaussian noise corresponds to the detected power of $4.5 \cdot 10^{-11}$ W for Poisson noise. The noise modeled by a Gaussian distribution with noise amplitude factors of 3% or more is a good way to model uncertainties even beyond the standard deviations of photon detection for relevant signal levels (see Sec. 4.2 for the infrared signal cut-off threshold). The reconstruction was performed on the same finite element mesh used for data generation to eliminate uncertainties due to FEM resolution limitations. Figure 6 was obtained by repeating the same method of generating noisy data 5x to find the averaged absolute values of the reconstruction relative errors.

The average relative errors for the reconstructed volume, fluorophore absorption coefficients of the inclusion, background, and every node in the mesh are plotted in Fig. 6. For the noise amplitude factor less or equal to 2%, all the relative errors from S2 reconstruction are less than

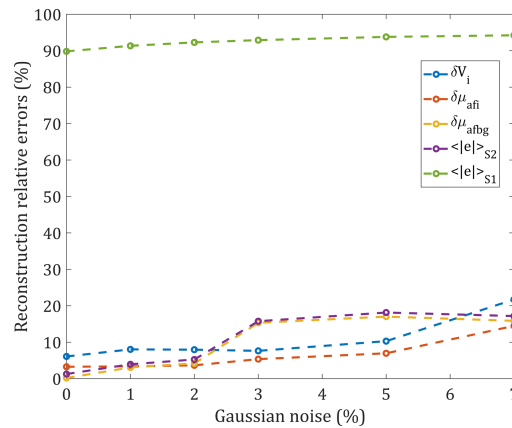


Fig. 6 Reconstruction S2 relative errors (δV_i , $\delta \mu_{afj}$, $\delta \mu_{afbg}$, $\langle |e| \rangle_{S2}$) and S1 relative error ($\langle |e| \rangle_{S1}$) for different simulated noise levels (calculated at points where the Gaussian-profile noise amplitude factor was 0, 1%, 2%, 3%, 5%, and 7%). The same mesh was used for data generation and reconstruction (Mesh 1 from Table 1), whereas the matrix inversion was performed on a coarser 6480-node mesh.

10% (all of them but volume with less than 6% relative error). It was observed that the reconstruction quality decreases when the noise amplitude factor is 3% or more. A noise amplitude of 10% already results in distorted reconstruction images, usually having more than the two expected regions, which have even higher errors (not shown in the graph, because the classification of the two expected regions is not relevant). It is possible to observe that the relative error of the volume reconstruction and the fluorophore absorption of the inclusion is still acceptable (<10%) as long as the noise amplitude factor is not >5%. The benefits of using S2 can be seen by comparing the average absolute values of the relative errors for S2 $\langle |e| \rangle_{S2}$ and S1 $\langle |e| \rangle_{S1}$, in Fig. 6. Although the S1 relative error is always around 90%, the S2 relative error is less than 6% for the noise amplitude factor less or equal to 2% or less than 20% for the noise amplitude factor between 3% and 7%.

For the parameters varied so far (fluorescent inclusion size, fluorophore concentration, and noise level), the S1 reconstruction average absolute value of the relative error was around 90%, and similar values have been reported in the literature.^{24,52} The necessity of using structural a priori information to obtain quantitatively accurate reconstructions was stated by Lin et al.²⁴ Here, we demonstrated that it is possible to obtain quantitatively more accurate results if the second stage (see Sec. 2.5) is added to the reconstruction algorithm, even without any structural (geometrical) prior in the regularization (see Sec. 2.4). Similar approach to our proposed method, without any structural a priori information, was applied by Kwong et al.,⁵² using the technique called “temperature-modulated fluorescence tomography.” However, the important difference is that while there a functional a priori information was obtained by exploiting the thermal properties of specifically designed fluorophores, our proposed method uses only the results of S1 as an input for S2 and therefore more general fluorophores can be used. Both approaches resulted in comparable S2 reconstruction relative errors (up to around 15% for the fluorophore absorption coefficient and 35% for the volume).

Optical properties of the medium around the fibers are given as inputs to the reconstruction algorithm. If the forwarded (given) parameters do not match the actual optical properties of the medium, the reconstruction is affected. Table 4 shows to what extent. Although the absolute value of the relative error for the reconstructed volume is <26% in all but one of the cases considered, most of it naturally comes from the finite element mesh discretization of space, and the limited resolution for the volume calculation. More interesting is the error for the reconstructed fluorophore absorption coefficient of the inclusion. The fluorophore absorption is close to the GT value, within about 20%, for the optical properties closer to the default absorption of 0.50 cm^{-1}

Table 4 Simulated actual and given background absorption ($\mu_{a,actual}$, $\mu_{a,given}$), reduced scattering coefficients ($\mu'_{s,actual}$, $\mu'_{s,given}$), and the resulting relative errors of the reconstructed volume (δV_i) and fluorophore absorption coefficient of the inclusion ($\delta\mu_{afi}$). The same Mesh 2 (Table 1) was used for data generation and reconstruction.

$\mu_{a,actual}$ (cm ⁻¹)	$\mu_{a,given}$ (cm ⁻¹)	$\mu'_{s,actual}$ (cm ⁻¹)	$\mu'_{s,given}$ (cm ⁻¹)	δV_i (%)	$\delta\mu_{afi}$ (%)
0.30	0.30	8.7	8.7	-12.0	4.7
0.30	0.50	8.7	8.7	-23.0	20.1
0.50	0.30	8.7	8.7	0.6	-10.2
0.50	0.50	8.7	8.7	-11.1	3.2
0.50	0.50	7.3	7.3	-11.7	3.8
0.50	0.50	7.3	8.7	-15.2	-6.8
0.50	0.50	8.7	7.3	-7.4	15.0
0.50	0.50	15.0	15.0	-5.5	0.3
0.50	0.50	15.0	8.7	-1.1	46.3
0.50	0.50	8.7	15.0	-18.0	-29.7
0.20	0.20	8.7	8.7	-12.8	5.7
0.20	0.50	8.7	8.7	-44.8	52.7
0.50	0.20	8.7	8.7	25.5	-21.5

and reduced scattering of 8.7 cm⁻¹ (the first seven rows of Table 4). Larger deviations, which can also occur in clinical reality, are reported in the last six rows of Table 4. The reconstructed fluorophore absorption coefficient is overestimated in the cases where the background absorption is overestimated or the background reduced scattering is underestimated, and vice versa.

The fluorescent inclusions considered so far were different from the background only in terms of the fluorophore absorption coefficient, or the concentration of the fluorophores. Now we take into account the possibility that the inhomogeneity (spherical inclusion) added to the background may have different optical properties—absorption and scattering (inclusion: $\mu_a = 0.20$ cm⁻¹ and $\mu'_s = 15.7$ cm⁻¹, background: $\mu_{ab} = 0.50$ cm⁻¹ and $\mu'_{sb} = 7.3$ cm⁻¹). Figure 7 shows how the reconstruction is affected if the absorption and the reduced scattering coefficient of the medium are treated as homogeneous (c) in the whole medium (without specifying optical properties around each of the fibers), compared with the case where the correct input is given (after taking into account where the fibers are in respect to the spherical inclusion) (b). Note that this “correct” input is still imperfect in describing the medium optical properties. However, it is realistic because the IDOSE^{8,18} algorithm can estimate the properties around each fiber. The limiting factor is the mapping between the fiber coordinates and FEM node coordinates, i.e., mesh resolution. However, the reconstruction with more realistic optical properties is significantly better than that with plain, homogeneous optical properties (see Fig. 7). Similar conclusions about how the absorbing and scattering inhomogeneities affect the reconstructed fluorophore absorption have been reported by Abascal et al.⁵³ and Soubret et al.⁵⁴

Experimental data always contain a certain level of noise. For the measurement points with a low signal level (for example, when the detector fiber is far from the source fiber, or absorption in between the fibers is high), the signal-to-noise ratio can become so low that these measurement points become unreliable. It is possible that a fiber is partly blocked or acquires low signals. As a consequence, even though the fluorescent signal is low, the excitation wavelength signal is also low, and the Born ratio can become high because the “detected fluorescent signal” is dominated by background noise with a level comparable to or even a few orders of magnitude greater than the actual fluorescent signal. Such measurement points must not be forwarded to the

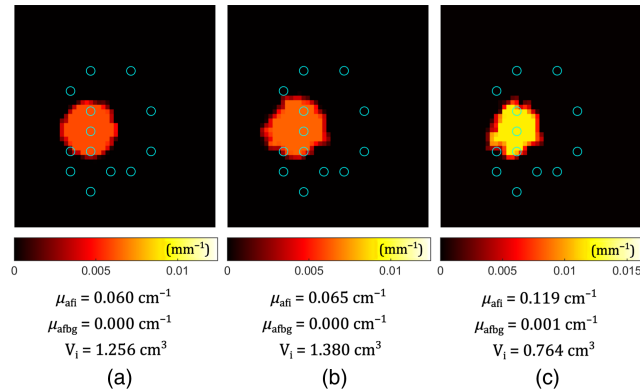


Fig. 7 Simulation of the scenario where the absorption and the reduced scattering coefficients of the inclusion (μ_a , μ'_s) differ from those of the background (μ_{ab} , μ'_{sb}). Slice through the spherical inclusion center. Fluorophore absorption coefficients are shown with different colors. Blue circles represent fiber positions. Below the images, values are given for the fluorophore absorption of the inclusion μ_{afi} , fluorophore absorption of the background μ_{atbg} , and the volume of the inclusion V_i for (a) GT case, (b) reconstruction with correct optical properties around the fibers, and (c) reconstruction with just homogeneous optical properties in the whole medium.

reconstruction algorithm. Because the inverse problem is ill-posed, every unreliable data point can amplify the reconstruction error, resulting in a reconstructed image very different from the truth.

Here, we simulate the data processing of real measurements. All measured fluorescent signals below a certain threshold (P_{cut}) are discarded, leaving $N'_m \leq N_m$ values. This means that the reduced measurement vector has N'_m rows, as well as the reduced Jacobian matrix ($N'_m \times N_n$). Reconstruction was performed on four different scenarios—the same spherical inclusion size and position ($R = 5.5$ mm, center at $(x_C, y_C, z_C) = (20, 22, 18)$ mm), but different μ_{afi} , equidistantly covering the interval from 0.015 to 0.030 cm^{-1} . The optical properties of the inclusions were the same as for solid inclusions used in phantoms, and the optical properties of the background were the same as those used for liquid phantom preparation, see Sec. 3.5. The optical properties around the fibers were set accordingly. Reconstruction results are shown in Fig. 8. The GT volume is $V_{\text{gt}} = 0.697$ cm^3 . It can be visually observed that in the xy -plane through the center of the sphere ($z = 18$ mm), the fluorophore spatial extent is not fully reconstructed. This is a consequence of the data cut-off. The same is true in the perpendicular, z -direction. As a result, the whole volume of the inclusion is underestimated, leading to an overestimation of the fluorophore absorption coefficients. For the cases shown in Fig. 8, the reconstructed volume is (72 to 84)% of the GT volume, whereas the fluorophore absorption coefficient is overestimated by (33 to 42)%. The threshold for simulated measurement data cut-off was $P_{\text{cut}} = 4.5 \cdot 10^{-11}$ W, resulting in $N'_m = 50$ (out of $N_m = 156$) above-threshold measurement points in the case of the lowest concentration (a) and $N'_m = 61$ in the case of the highest concentration (d).

The effect of the instrument cut-off and the reconstructed volume underestimation described above can be diminished if the fibers are placed more favorably for solving the problem of DFT. The fiber configuration (see Table 2) considered in simulations is from a realistic clinical scenario. The priority in clinical PDT is to cause the photodynamic effect in tumorous tissue and only there. Neglecting this condition, we suggest another fiber configuration, with 12 fibers instead of 13, but having better spatial sensitivity around the spherical inclusion. The sphere's center was at $(x_C, y_C, z_C) = (22.5; 27.5; 20.0)$ mm, and the fiber coordinates are defined in Table 3. The same optical properties of the background and the inclusion, the same size of the inclusion, as used to obtain the results in Fig. 8, were applied in six reconstructions shown in Fig. 9. GT values for μ_{afi} varied from 0.015 to 0.090 cm^{-1} in equal steps. The cut-off threshold was kept $P_{\text{cut}} = 4.5 \cdot 10^{-11}$ W, resulting in $N'_m = 54$ (out of $N_m = 132$) above-threshold

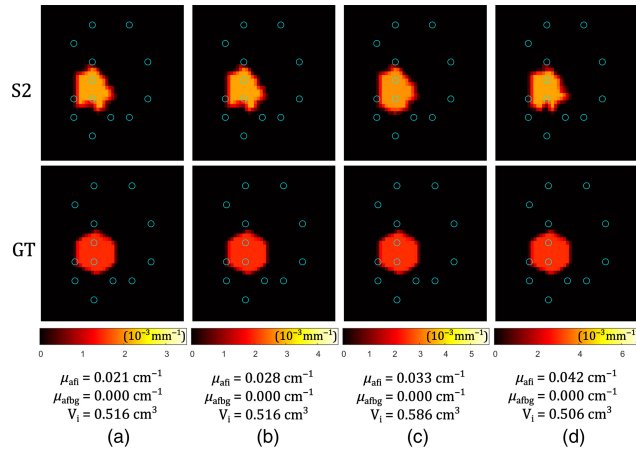


Fig. 8 Simulation of instrument cut-off effect in a configuration with 13 fibers (Table 2). Blue circles represent fiber positions. Slice through the spherical inclusion center—comparison between the S2 reconstructed image and the GT. Below the images, reconstructed values are given for the fluorophore absorption of the inclusion μ_{afi} , fluorophore absorption of the background μ_{afbg} , and the volume of the inclusion V_i for different GT fluorophore absorptions of the inclusion ($\mu_{afi,gt}$): (a) 0.015 cm^{-1} , (b) 0.020 cm^{-1} , (c) 0.025 cm^{-1} , and (d) 0.030 cm^{-1} . GT fluorophore absorption of the background was $\mu_{afbg,gt} = 0$. Data were created using Mesh 3, and the reconstruction was performed using Mesh 4 from Table 1.

measurement points in the case of the lowest concentration (a), and $N'_m = 98$ in the case of the highest concentration (f). The reconstructed volume is (77 to 82)% of the GT volume, whereas the fluorophore absorption coefficient is overestimated by (21 to 27)%.

It is interesting to see how the reconstruction algorithm performs when the information on the actual fiber positions is uncertain. This uncertainty has one of the following two roots. First, in clinical practice, ultrasound imaging of needle and fiber tips has inherent measurement

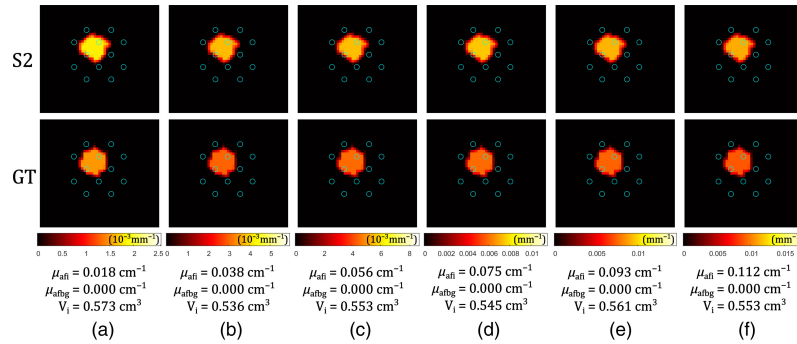


Fig. 9 Simulation of instrument cut-off effect in a configuration with 12 fibers (Table 3). Blue circles represent fiber positions. Slice through the spherical inclusion center—comparison between the S2 reconstructed image and the GT. Below the images, reconstructed values are given for the fluorophore absorption of the inclusion μ_{afi} , fluorophore absorption of the background μ_{afbg} , and the volume of the inclusion V_i for different GT fluorophore absorptions ($\mu_{afi,gt}$): (a) 0.015 cm^{-1} , (b) 0.030 cm^{-1} , (c) 0.045 cm^{-1} , (d) 0.060 cm^{-1} , (e) 0.075 cm^{-1} , and (f) 0.090 cm^{-1} . GT fluorophore absorption of the background was $\mu_{afbg,gt} = 0$. Data were created using Mesh 5, and the reconstruction was performed using Mesh 6 from Table 1.

Table 5 Simulated errors (δx_i , δy_i , δz_i) of the estimation of actual fiber coordinates, random variables uniformly distributed over the defined intervals, with the resulting average relative errors for the reconstructed volume (δV_i) and fluorophore absorption coefficients of the inclusion ($\delta \mu_{\text{af}}$) and the background ($\delta \mu_{\text{abg}}$), for seven scenarios (averaged over the number of scenarios resulting in the specific number of regions). The same Mesh 2 (Table 1) was used for data generation and reconstruction.

δx_i (mm)	δy_i (mm)	δz_i (mm)	Regions	Scenarios	δV_i (%)	$\delta \mu_{\text{af}}$ (%)	$\delta \mu_{\text{abg}}$ (%)
[-0.5; 0.5]	[-0.5; 0.5]	[-1; 1]	2	5	-8.8	5.5	2.0
[-0.5; 0.5]	[-0.5; 0.5]	[-1; 1]	3	2	6.2	-6.2	-16.0
[-1; 1]	[-1; 1]	[-2; 2]	2	5	-23.6	16.8	3.6
[-1; 1]	[-1; 1]	[-2; 2]	3	2	-41.9	33.4	-15.0

uncertainties, due to the needle bending or different lengths of fibers beyond the needle tips. Second, if the ultrasound is not used, as in our measurements on phantoms (see Sec. 4.2), there is no feedback on the actual fiber positions. The desired (aimed, planned) fiber coordinates are those chosen as optimal for the particular PDT treatment (see Sec. 3.2). The desired coordinates can be corrected by estimates of actual fiber positions based on ultrasound images, after fiber insertion into the patient's body. These coordinates, denoted as $x_{i,\text{given}}$, $y_{i,\text{given}}$, and $z_{i,\text{given}}$, for $i = 1, \dots, 13$, and defined in Table 2, are then given as inputs to the tomographic reconstruction algorithm. On the other hand, $x_{i,\text{actual}}$, $y_{i,\text{actual}}$, and $z_{i,\text{actual}}$ are actual positions of inserted fibers, which can be more or less different from those given to the reconstruction algorithm. For this simulation, actual fiber coordinates were obtained according to: $x_{i,\text{actual}} = x_{i,\text{given}} + \delta x_i$, $y_{i,\text{actual}} = y_{i,\text{given}} + \delta y_i$, and $z_{i,\text{actual}} = z_{i,\text{given}} + \delta z_i$, where δx_i , δy_i and δz_i are random variables taken from uniform distributions on intervals defined in Table 5. In this simulation, two realistic levels of error amplitudes were considered, each with seven random realizations (scenarios) of actual fiber positions. Table 5 shows the average relative errors for the reconstructed volume and fluorophore absorption coefficients of the inclusion and the background. Bigger coordinate uncertainties lead to larger reconstruction errors, with some scenarios resulting in more than two regions. It can be concluded that the reconstruction is sensitive to the correct knowledge of the fiber positions, and to have accurate reconstruction, only limited errors in the fiber position estimation can be accepted.

Finally, instead of a homogeneous fluorophore concentration inside the sphere and in the background, with a step-like transition between the two, we could consider a continuous transition from the highest concentration at the sphere center, to the constant background level concentration outside of the sphere of radius $R = 12$ mm. The continuous transition has the Gaussian profile [solid line in Fig. 10(d)]. From Fig. 10(d), it can be seen that the background fluorophore absorption is accurately reconstructed in S2, which is an improvement compared with S1. Although it is evident that a few homogeneous regions are not enough to accurately approximate the Gaussian profile, reconstructed discrete values for μ_{af} are not far from GT values. The number and size of regions depend on adaptive thresholds. Specifically in this simulation, two degrees of freedom for the peak (t_p) and the background threshold (t_b) were allowed, i.e., t_p and t_b can have mutually different values under the condition that $t_b \leq t_p$, and both t_p and t_b are from the defined set of discrete values (see Sec. 3.1). Figure 10(e) shows reconstructed regions with their volumes and fluorophore absorption coefficients in 3D.

4.2 Phantom Validation

The optical and geometrical properties of the realized phantom are described in Sec. 3.5. The gelatin-based spherical inclusions of seven different verteporfin concentrations were tested: $c_0 = 0$, $c_1 = 0.3$ mg/kg, $c_2 = 0.6$ mg/kg, $c_3 = 0.9$ mg/kg, $c_4 = 1.2$ mg/kg, $c_5 = 1.5$ mg/kg, and $c_6 = 1.8$ mg/kg. The experimental procedure is described in Sec. 3.6. The fiber configuration used is defined in Table 2. The center of the spherical inclusion was placed at $(x_C, y_C, z_C) = (22.5, 27.5, 20.0)$ mm. For each spherical inclusion, the measurement had five

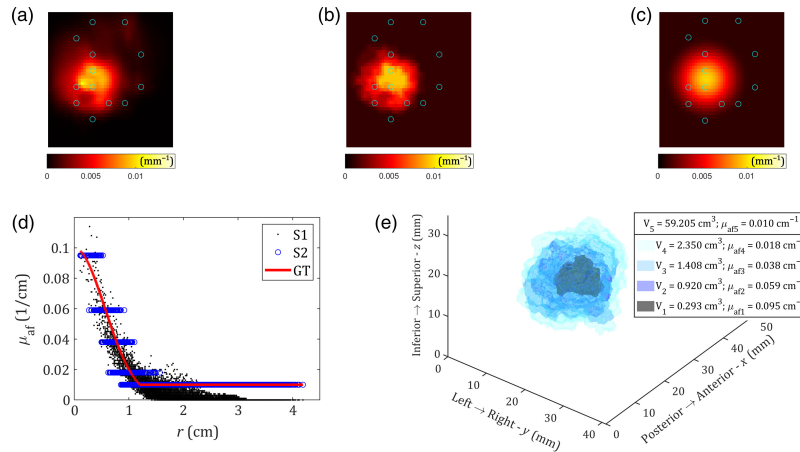


Fig. 10 Simulation of the scenario where the fluorophore absorption coefficient of the spherical ($R = 12$ mm) inclusion follows a Gaussian profile, with the peak $\mu_{afi} = 0.10$ cm⁻¹ at the sphere center $(x_C, y_C, z_C) = (20, 22, 18)$ mm and background outside of the sphere $\mu_{afbg} = 0.01$ cm⁻¹. The plane cuts perpendicular to the z -axis, at $z = 18$ mm, are shown as color maps for (a) S1 reconstruction results, (b) S2 reconstruction results, and (c) GT. Blue circles represent fiber positions. (d) $\mu_{af}(r)$ dependence calculated at mesh nodes, where r is the distance of the node from the sphere center, for S1 reconstruction (black dots), S2 reconstruction (blue circles), and GT Gaussian $\mu_{af}(r) = \mu_{afi} \exp(-\ln(\mu_{afi}/\mu_{afbg})r^2/R^2)$ (red solid line). (e) 3D representation of S2 results. Data were generated using the denser Mesh 3, and the inverse problem was solved using Mesh 4 (see Table 1), whereas the matrix inversion was performed on a coarser 7140-node mesh.

sequences, and the average was taken to increase the signal-to-noise ratio. Before forwarding the data to the reconstruction algorithm, raw measurement data were processed such that every infrared detected value below $4.5 \cdot 10^{-11}$ W was discarded, resulting in the reduced vector of measurements having $N'_m = 46$ elements (out of $N_m = 132$) in the case of concentration c_1 , and $N'_m = 75$ in the case of concentration c_6 . This cutting-off is done to make the reconstruction

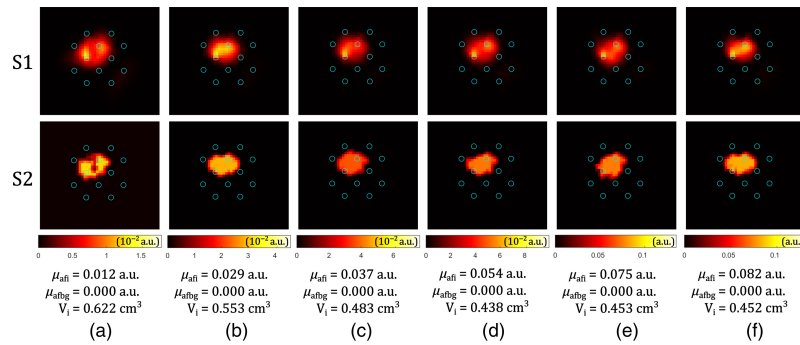


Fig. 11 Tomographic reconstruction of the fluorophore absorption, cut plane $z = 20$ mm, for different verteporfin concentrations applied in the gelatin spheres (c): (a) 0.3 mg/kg, (b) 0.6 mg/kg, (c) 0.9 mg/kg, (d) 1.2 mg/kg, (e) 1.5 mg/kg, and (f) 1.8 mg/kg. Results of the two stages of the reconstruction are shown (S1 and S2) in different rows, the values for the reconstructed fluorophore absorption coefficient of the inclusion μ_{afi} , background μ_{afbg} , and the estimated volume of the inclusion V_i are given below. Blue circles represent fiber positions. Columns (a)–(f) have different color bars.

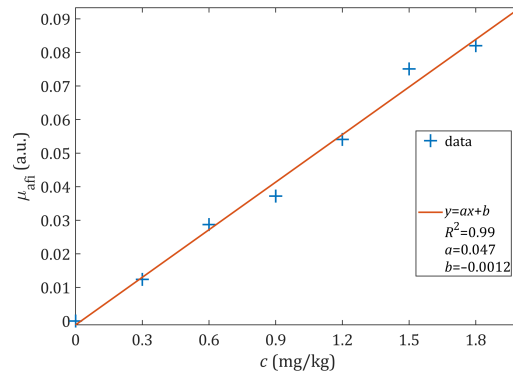


Fig. 12 Reconstructed fluorophore absorption coefficient (μ_{afl}) of solid spherical inclusions for different applied concentrations of verteporfin (c). Experimental data marked with +, parameters of the linear fit (red line) shown in the legend.

more robust by avoiding amplification of measurement points with low levels of detected infrared power, as explained in Sec. 4.1.

The tomographic reconstruction of fluorophore absorption was performed on Mesh 6 from Table 1, and a lower resolution 6137-node mesh was used for matrix inversion. Reconstruction results for six nonzero verteporfin concentrations are shown in Fig. 11, with the plane cut through the sphere center ($z = 20$ mm). The reconstructed volume of the inclusion is underestimated as expected, (63 to 89)% of the ideal sphere volume (Sec. 4.1). The reconstructed fluorophore absorption coefficient is expressed in arbitrary units for two reasons. First, the reconstructed volume is not reliable, and when the volume is underestimated, the fluorophore absorption coefficient is overestimated. However, all the reconstructed volumes are close to each other, and the arbitrary unit can be used for relative comparison of the reconstructed PS drug concentrations. Second, the fluorescence quantum yield, defined in Sec. 2.1, was assumed to be $\gamma = 10\%$.^{55,56} If this value is wrong, all the results obtained from experimental measurements cannot be expressed in a specific SI unit, but in a scaled unit which can be used for a relative comparison.

The relative comparison of the reconstructed fluorophore absorption coefficients for seven verteporfin concentrations (including zero) applied in phantoms is graphically shown in Fig. 12. A linear ($y = ax + b$) least-squares fitting was performed through seven data points, resulting in the coefficient of determination $R^2 = 0.99$, and the free term $b = -0.0012$, suggesting a direct proportionality between the fluorophore absorption and the fluorophore concentration. The obtained linearity parameters are comparable with those from other fluorescence tomography quantification methods.^{57,58} Our tomographic reconstruction algorithm validated on verteporfin phantoms shows the potential for capturing quantitative differences in the PS drug concentration by using SpectraCure's P18 clinical system.

4.3 Limitations and Potential Improvements

The main limitations of the suggested DFT reconstruction methods are determined by the mathematical nature of the problem and the physical limitations of the measurement process. There is also a computational factor that plays an important role in potential real-time clinical applications. To have a better spatial resolution, more nodes should be added to the finite element mesh, and this requires more measurement points, i.e., source-detector pairs. Physically, because the prostate volume is already small and the tumor region is even smaller, placing many brachytherapy needles of ~ 1 mm diameter is not a simple task for a clinician. Moreover, the high spatial density of metal needles inside a relatively small tissue volume affects the light propagation and the model derived from the diffusion equation. SpectraCure's P18 system used in experiments has 18 photonics modules; therefore, slots for up to 18 optical fibers, which is more than enough for most prostate cancer PDT applications, but imposes a theoretical limit of a maximum $18 \cdot 17 = 306$ measurement points for DFT.

The time required to solve the inverse problem in two stages, having as inputs the measurements expressed as Born ratios and the optical properties estimated around the fibers, is around 4 to 5 min on CPU Intel i9, 10th generation, 3.7 GHz, with 32 GB RAM. The computations can be faster or slower, depending on the finite element mesh resolution. The choice of the finite element mesh is important and it has to take into account the dimensions of the medium, the number of fibers, and time constraints (in the case of real-time clinical applications). For a clinically realistic measurement with a set of 12 to 13 fibers interstitially placed inside a volume of around $(5 \times 5 \times 5) \text{ cm}^3$, finite element meshes of around 20,000 to 30,000 nodes were used.

Data acquisition by this system can still be improved. Increasing the numerical aperture of the fibers is desired, and already in the latest system, $\text{NA} = 0.37$ is used, which is better than $\text{NA} = 0.22$ used in this work (see Sec. 3.6). Leakage of 690 nm light through filters to the infrared detectors should be rejected as much as possible or precisely estimated and compensated for. To make the reconstruction algorithm work robustly, regardless of noise and expected errors in detection, a certain threshold below which all the infrared signals are to be discarded, should be carefully determined. It would be useful to predict in simulations how much the reconstructed volume is underestimated, as a consequence of this cutting-off, and take it into account as a correction for the reconstruction from real measurement data.

Optical properties around the fibers are estimated according to already developed methods.^{8,18} This is a good input to the fluorescence reconstruction algorithm but could be improved in terms of spatial resolution and decoupling of absorption from the scattering. However, that would result in more complex problems and a potential need for time-domain systems. On the other hand, if it is of interest to reduce the computational complexity and achieve faster reconstructions, simpler models can be developed by assuming a homogeneous background medium, and analytical expressions are given in Sec. 2.1, removing the need for FEM calculations.

Note that in the current model, the effect of metal needles and the optical properties of silica fibers is not considered. We believe that this will not affect the reconstruction significantly, because the effective optical properties around the fibers are estimated as mentioned above and the Born ratio is used to express the measured fluorescence. Light propagation at fluorescent wavelengths is not expected to differ significantly from light propagation at the excitation wavelength. As the Born ratio of the detected fluorescent and excitation wavelength signals is forwarded to the reconstruction algorithm, all uncertainties including the inhomogeneous or different optical properties in the medium and in small volumes occupied by the fibers and needles, are expected to be canceled or negligible. However, it is suggested to model the presence of optical fibers and brachytherapy needles in the medium, but the diffusion equation approach would become more complex. Another possible approach is to compare the effects on the Born ratio in Monte Carlo simulations in the two cases—when the fibers and needles are modeled and when they are not.

A precise knowledge of the fiber positions is important, as it is an input to the forward model used in reconstructions. The feedback information about the fiber positions is available in the clinical setting, obtained from the ultrasound probe. This was not available in phantom experiments, and we used recommended (aimed, desired) fiber positions as the actual fiber positions (see Secs. 3.2 and 4.1). In the reality of the experiments with phantoms, because the gelatin sphere was attached to the fibers, precise positioning of all the fibers and the sphere relative to them was not possible. More precise information about the actual fiber positions would improve the accuracy of the forward model, which is expected to result in better reconstruction of the PS drug concentration.

Moreover, it would be beneficial to separately characterize fluorophore inclusions, before the tomographic reconstruction in a liquid background, to verify that the concentration of fluorophores (PS drug) is uniformly distributed over the inclusion volume. The phantom used in this paper had a simplified geometry, unlike the hybrid phantom from our previous work²⁶ whose prostate boundaries were defined using the 3D model obtained from the clinical ultrasound data. The effect of such geometry could be explored and considered in the future.

Finally, the algorithm for determining the regions as inputs to S2 could be implemented in many different ways. One direction for further exploration is to extend the allowed set for adaptive threshold fractions t_p and t_b , for example by not imposing $t_p = t_b$, including more than nine

different values, or extending the empirically determined interval (0.33, 0.87) (Sec. 3.1). Other suggested directions include application of clustering algorithms based on statistical pattern recognition or artificial neural networks. Moreover, it is expected that the structural a priori information in S1 (which can be obtained from ultrasound during the treatment) would improve the accuracy of the inputs for S2 and therefore the final results of S2.

5 Conclusion

We have proposed a novel two-stage approach for estimating the spatial distribution of the fluorescent PS drug starting from CW measurement data at excitation and fluorescence emission wavelengths, acquired by interstitially placed optical fibers. S1 of the proposed algorithm relies on standard DOT reconstruction methods, without any geometrical prior. The results from S1 are then used as inputs for S2, presented here for the first time. The reconstruction methods were implemented in MATLAB, using the NIRFAST package. Numerical simulations in various scenarios were performed to test the newly developed methods and find their limitations. As found in simulations with a single homogeneous fluorescent spherical inclusion, it is essential to increase the signal-to-noise ratio to have a reliable reconstruction of the volume of the inclusion and its fluorophore absorption coefficient. Various inclusion sizes and fluorophore concentrations were investigated. More accurate results are obtained if the optical properties around the fibers are correctly taken into account, compared with the case where a mismatch between the actual optical properties and those forwarded to the reconstruction algorithm exists. The novel two-stage approach resulted in the average absolute value of the reconstruction relative error of the order of (1 to 10)%, compared with around 90% in a standard single-stage approach. The tomographic reconstruction methods were validated on prostate tissue-mimicking phantoms to estimate the spatial distribution and concentration of fluorescent PS verteporfin used in the PDT of prostate cancer. The reconstructed fluorophore absorption coefficients had a good direct proportionality with the applied concentrations of verteporfin, whereas the reconstructed volume of the inclusion had a relative error (10 to 35)%. The results from experimental measurements show the potential for further development of the proposed DFT methods and their eventual clinical in vivo application for monitoring the PS drug concentration in real-time during the PDT of tumors.

Before transferring to clinics, these methods should be validated on the next generations of phantoms of different shapes, sizes, PS concentrations, and with various fiber configurations. In parallel, the reconstruction methods can be used offline to find the correlation between the prostate cancer treatment outcome and the amount of the PS and its spatial and temporal evolution in already finished treatments.

Disclosures

JS and SAE are minority shareholders of SpectraCure AB and are interested in prostate cancer interstitial photodynamic therapy. SKVS and SAE are shareholders of BioPixS Ltd. with an interest in tissue optical phantoms.

Code and Data Availability

The data presented in this article are publicly available in Zenodo at <https://doi.org/10.5281/zenodo.14713507>. The software code may be available upon a reasonable request to the corresponding author.

Acknowledgments

This project is funded by EU H2020 MSCA-ITN project “Photonics for Healthcare: multiscale cancer diagnosis and therapy” (PHAST-ETN) (Grant No. 860185), Science Foundation Ireland (Grant No. SFI/15/RP/2828, SFI-12/RC/2276_P2, and SFI-22/RP-2TF/10293).

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**Resorbable optical fibers for interstitial
photodynamic therapy — assessment of
photosensitizer spatial distribution in tumors**

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J Biomed Opt. **30(5)**, 058001 (2025).

Resorbable optical fibers for interstitial photodynamic therapy—assessment of photosensitizer spatial distribution in tumors

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ABSTRACT. **Significance:** Optical-quality bioresorbable implants, which gradually dissolve within the body, are gaining increasing interest due to their potential to eliminate the need for revision surgeries. These implants show significant promise in treating deep-seated tumors in high-risk areas, such as the brain, and offer extended capabilities for monitoring interstitial physiological parameters or pharmacokinetics through photonic technologies.

Aim: A proof-of-principle validation has been conducted on calcium phosphate glass (CPG)-based bioresorbable optical fibers to assess their capability to monitor the spatial distribution of photosensitizing (PS) drugs in tumors—an essential parameter to optimize for enhanced treatment outcomes in photodynamic therapy (PDT).

Approach: *Ex vivo* validation was performed on liquid phantoms with solid tumor-mimicking inclusions containing the fluorescent PS drug. In-house developed bioresorbable fibers, with optical characteristics similar to silica fibers used in current PDT systems, were utilized. For the first time, these fibers were used for the interstitial acquisition of fluorescent signals, followed by the tomographic reconstruction of the drug distribution in the phantom. The results were compared with those obtained from a standard clinical system equipped with silica fibers.

Results: The reconstructed drug distribution with bioresorbable fibers agreed with that obtained using the same system with standard silica fibers.

Conclusions: We reveal the potential of further exploring CPG bioresorbable optical fibers for interstitial PDT.

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Keywords: photodynamic therapy; bioresorbable photonics; photosensitizer distribution; optical fibers; diffuse optical tomography; fluorescence

Paper 240385GR received Dec. 19, 2024; revised Mar. 8, 2025; accepted Apr. 15, 2025; published May 14, 2025.

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1 Introduction

Photodynamic therapy (PDT) is a promising cancer treatment modality that involves administering a photosensitizing (PS) drug either topically or intravenously, followed by irradiation of the light at a wavelength that aligns with the PS's absorbance band. In the presence of oxygen, this initiates a cascade of events leading to direct tumor cell death, damage to the tumor's microvasculature, and the activation of a localized inflammatory response.^{1–3} The effectiveness of PDT depends on a combination of parameters^{2,4} including the type and dose of the PS, the timing between its administration and light exposure, knowledge of the distribution of PS within the tumor, tumor oxygen levels, total light dose, and fluence rate and PDT-induced blood flow changes.^{5,6} There is a growing interest in developing integrated systems that allow real-time interstitial monitoring of these parameters during PDT.^{7–9} These systems are designed to enable real-time fluence modulation, delivering a more uniform dose to the tumor and improving treatment precision and outcomes. Such advancements are expected to enhance the performance of current clinical interstitial PDT systems, which remain underutilized in clinical practice despite being the first Food and Drug Administration (FDA)-approved drug-device combination in the 1990s.¹⁰

Fiber optic technology is widely used for endoscopic and interstitial light delivery in PDT, enabling the treatment of deep-seated tumors and internal organs.¹¹ In clinical practice, silica-based fiber-optic endoscopy is a well-established method for guiding light. Nevertheless, these endoscopes are inserted only during the procedure and are not suitable for extended monitoring.¹² A major limitation of traditional silica-based fibers is their non-degradable nature, requiring surgical removal after treatment. This not only increases the risk of infection, fibrosis, and procedural complications but also poses a significant challenge in delicate, high-risk environments such as the brain.^{13–15} In multi-session PDT, the repeated insertion and removal of fibers exacerbate patient discomfort, procedural risks, and clinical workload.¹⁴

The motivation for this study stems from the clinical need for precise, minimally invasive, and self-dissolving light delivery and real-time monitoring systems for interstitial PDT, particularly for the treatment of deep-seated and surgically inoperable tumors such as glioblastoma, head and neck, or intra-abdominal tumors.^{16,17} An ideal interstitial PDT tool for such scenarios should efficiently deliver light to deep tumor tissues while naturally degrading post-treatment, eliminating the need for removal, enabling real-time dosimetry to ensure uniform and effective light exposure, and allowing minimally invasive monitoring of drug distribution to optimize therapeutic outcomes. With the development of novel biocompatible and degradable optical fibers for interstitial PDT applications, new therapeutic tools are becoming possible.^{18,19} Relevantly, bioresorbable optical fibers based on calcium phosphate glass (CPG) meet these criteria by combining bioresorbability, optical efficiency, and functional integration.²⁰ Their composition mimics bone minerals, ensuring biocompatibility. They dissolve in physiological media over a clinically relevant time, bypassing the necessity of extraction procedures.^{21,22} Their optical properties can be tailored based on the composition. To the best of our knowledge, CPG fibers possess the lowest optical loss in the category of biocompatible and degradable optical fibers reported in the literature.²³ Along with light delivery, they possess the potential for extended and real-time monitoring of relevant physiological signals, tissue characteristics, or biochemical information.²⁴

CPG-based low-loss optical fibers were first developed using a fiber drawing technique in 2016 by Ceci-Ginistrelli et al.²⁰ Since then, they have been extensively studied for their *in vitro* solubility,²⁵ biocompatibility, and mechanical properties.²⁶ Systematic investigations into their degradation behavior have demonstrated complete dissolution within 1 month, with less than 10% variation in optical power transmission during the first 2 weeks of fiber dissolution.²⁵ In a study by Podrazký et al.,²⁷ mice subcutaneously implanted with CPG fibers showed no clinical signs of adverse effects for up to 5 weeks. CPG fibers possess good thermomechanical properties and have also been recently validated for diffuse optical techniques such as diffuse optical spectroscopy (DOS)²⁸ and diffuse correlation spectroscopy (DCS)²⁹ for monitoring complementary physiological parameters, paving the way for interstitial continuous monitoring of tissue oxygenation, hemodynamics, and metabolism. Despite their benefits, CPG fibers have not been explored for interstitial PDT application.

This study investigates the potential of CPG fibers for light delivery and monitoring in interstitial PDT. Specifically, our objective was to assess whether these fibers could be used

to accurately map the spatial distribution of PS drugs within tumor-mimicking solid phantom inclusions using diffuse fluorescence tomography (DFT). We utilized custom-fabricated multi-mode (MM) CPG fibers along with a well-tested solid-liquid phantom approach.³⁰ The performance of CPG fibers was evaluated against standard silica fibers in terms of their ability to map the PS distribution within the phantoms. This novel approach represents a step toward a minimally invasive therapeutic device that would reduce patient burden and improve the treatment efficacy of deep-seated tumors.

2 Materials and Methods

2.1 CPG Fiber Fabrication

MM bioresorbable CPG optical fiber employed in this study was fabricated through a rod-in-tube technique using an in-house developed drawing tower.²⁰ The glasses for the fiber were made by conventional melt quenching method using high-purity biocompatible chemicals ($\text{P}_2\text{O}_5 \cdot \text{CaO} \cdot \text{Na}_2\text{O} \cdot \text{SiO}_2 \cdot \text{MgO}$). The wavelength-dependent refractive index $n(\lambda)$ of the core (n_{core}) and cladding (n_{cladd}) glasses were then measured at different wavelengths (633, 825, 1061, 1312, and 1533 nm) using a Metricon 2010 prism coupler (Metricon Corporation, United States), with an estimated error of ± 0.0005 . The refractive index values at the wavelength 690 nm (i.e., the wavelength used in DFT experiments reported here, see Sec. 2.2) were then obtained through interpolation using Cauchy's equation. These values were $n_{\text{core}} = 1.5323$ and $n_{\text{cladd}} = 1.5135$. The refractive index difference in core and cladding glass was attained through increasing MgO molar content in the core glass as a substitute for CaO, as detailed in the work by Ceci-Ginistrelli et al.²⁰ The numerical aperture (NA) of the CPG fiber was calculated as $\text{NA} = \sqrt{n_{\text{core}}^2 - n_{\text{cladd}}^2} = 0.24$. This value of NA for CPG fibers is close to the $\text{NA} = 0.22$ of standard silica fibers (Spc Sterile Bare Fiber UVH 400 N SC, LightGuideOptics International Ltd., Germany) employed in clinical PDT systems.

The preform of the fiber consists of a core rod obtained by casting the melt into a 12-mm-diameter cylindrical mold, and a cladding tube with a 10.7-mm outer diameter, realized through an in-house extrusion facility.³¹ The core rod was then stretched to 5.4-mm diameter to fit into the cladding tube. The stretched rod was inserted in the cladding tube and the rod-in-tube preform was further stretched into several 1-m fiber sections using the fiber drawing tower. Optical microscopy (Nikon ECLIPSE E 50i, Nikon Instruments Inc., United States) equipped with the image analysis software ToupView (ToupTek Photonics, China) was used to measure the fiber diameter at the fiber's cross-section. The measured core diameter of the CPG fiber closely matches the diameter of the standard silica fiber (400 μm). The details about the fabrication can be found in previous publications.^{20,31}

For practical experimental convenience, the fabricated fibers were cleaved (CT-106, Fujikura, Japan) into 11 sections of 25 cm each (see Sec. 2.3), which were SMA connectorized at one end by following the protocol for the connectorization of optical fibers.³²

2.2 Phantom Preparation

Tissue-mimicking phantoms with tumorous inclusions were realized. A well-tested hybrid solid-liquid phantom approach based on our previously reported work was followed.³⁰ Briefly, the gelatin-based solid inclusion phantom representing the tumor was submerged in a liquid intralipid-based phantom. Two inclusions, one spherical (volume of 0.6 cm^3) and one ellipsoidal (volume of 3.2 cm^3), were made and stored for later use. These inclusions contained fluorescent photosensitizer verteporfin, an active substance of the PS drug Visudyne (Cheplapharm Arzneimittel GmbH, Germany). A 0.67 mg/kg fluorophore concentration (in milligrams) per phantom mass (in kilograms) was maintained in both inclusions. Following the referenced work by Ghauri et al.,³⁰ the optical properties targeted for the solid phantom inclusion (mixture of gelatin, water, intralipid, and India ink) at 690 nm were absorption $\mu_{\text{ai}} = 0.20 \text{ cm}^{-1}$ and reduced scattering coefficient $\mu_{\text{si}}' = 15.7 \text{ cm}^{-1}$, mimicking the optical properties of the tumorous region.

Intralipid-based liquid phantom, mimicking surrounding tissue, was prepared by mixing 4.9 ml of 1% India ink solution (Rotring, Germany) and 35.4 ml of intralipid 20% (Fresenius Kabi, Ltd., Germany) in 960 ml of purified water in a glass beaker, which was placed on a magnetic stirrer for mixing. The recipe was chosen to have the optical properties of absorption

$\mu_{abg} = 0.30 \text{ cm}^{-1}$ and reduced scattering $\mu_{sbg}' = 8.3 \text{ cm}^{-1}$ at 690 nm. Each of the gelatin phantoms stored in the freezer at -18°C was taken out and kept for a sufficient time (around 45 min) at room temperature, to bring it toward a thermodynamic equilibrium with the environment and attain mechanical properties convenient for inserting the fibers within inclusion during the measurement (see Sec. 2.3).

2.3 Experimental Setup and Measurements

The phantoms and fiber assembly is shown in Fig. 1(a). Two sets of 11 optical fibers each, one with CPG, and the other with standard silica fibers, were used for the measurements. All 11 CPG fibers from the first set were inserted through a fixed brachytherapy template and positioned within $2 \text{ cm} \times 2 \text{ cm}$ area of the template [Fig. 1(b)]. This number of fibers was chosen to have enough spatial sensitivity to reconstruct the inclusion and background³³ while not complicating the setup with a dense distribution of fibers.

The glass beaker of 10 cm inner diameter, containing 1 l of the liquid phantom, was placed on a lab jack, under the brachytherapy template. Gelatin-based tumor-mimicking spherical solid inclusion was fixed at the CPG fiber tips [see Fig. 1(c)], in the first measurement configuration. The lab jack was vertically adjusted so that the center of the solid tumor-mimicking inclusion was immersed in the liquid at a depth of 7.5 cm from the surface, and height of 5.5 cm from the bottom [Fig. 1(c)]. In the second measurement configuration, the spherical inclusion was replaced by the ellipsoidal one, within the same liquid phantom. In the case of the spherical inclusion, two fiber tips were within the inclusion, whereas in the case of the ellipsoidal, five fiber tips were within the inclusion [see Fig. 1(b)].

The same experimental procedure was then repeated with the set of silica fibers instead of CPG fibers, inserted at the same 11 positions in the brachytherapy template as before. The same

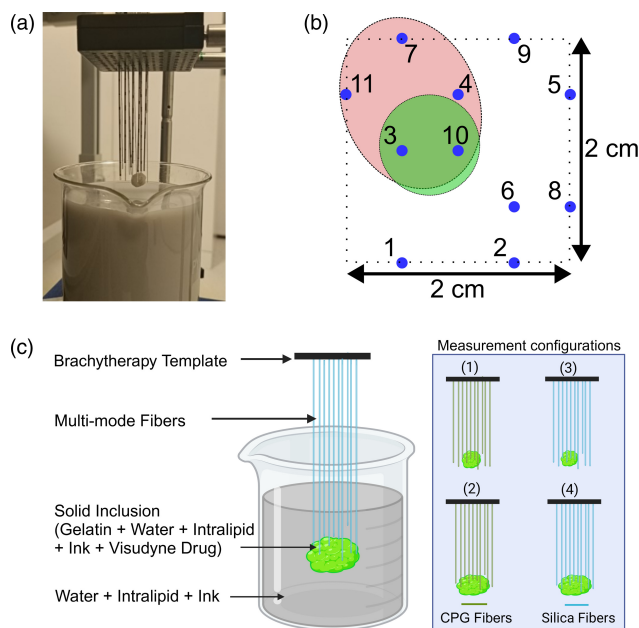


Fig. 1 (a) Experimental setup with CPG fibers, liquid tissue-mimicking phantom, and solid gelatin-based tumor-mimicking fluorescent inclusion, before the measurement. (b) Schematic showing the fiber positioning within the inclusions—spherical (green) and ellipsoidal (red)—where the blue markings represent the 11 fiber centers in the plane (horizontal) parallel to the brachytherapy template. (c) Schematic representing the hybrid phantom and fibers assembly during the measurement (left), along with the simplified schematics of all four measurement configurations (right).

Table 1 Details of the measurements conducted including the types of fibers used, the shape of the inclusion, and the protocol followed during each acquisition cycle.

	Fiber type	Inclusion shape	Protocol in one measurement cycle
1	CPG	Sphere	Light delivery from PM1 through fiber 1 and simultaneous light collection by fibers 2–11 (~3 s).
2		Ellipsoid	
3	Silica	Sphere	Switching the light delivery sequentially from PM1 to PM11, with subsequent light collection by all other fibers before each switch.
4		Ellipsoid	
			Acquisition of $11 \times 10 = 110$ data points.

liquid phantom, and the same solid inclusions as in the first two measurement configurations were used. The spherical one was used in the third configuration and placed at the same position as in the first, whereas the ellipsoidal one was used in the fourth configuration and placed at the same position as in the second [Fig. 1(c)].

During the measurements, PDT-like light delivery was conducted using SpectraCure's P18 system comprising 18 photonics modules (PMs) to which the optical fibers can be connected. Each PM is capable of delivering light at 690 nm (Laser diode LDX-2405-690, LDX Optronics Inc., United States) and detecting (Si photodiode S12915-5K, Hamamatsu Photonics, Japan) both light around that excitation wavelength as well as fluorescent light at longer wavelengths (>700 nm). Two-meter-long silica fibers and 25-cm-long CPG fibers (SMA connectorized) were connected to SC ports in the P18 system via 45-cm SC-SMA patch cables. The choice of this rationale for using shorter sections of CPG fibers is based on the possible future clinical implantation, where long fibers are not required.³⁴ The PMs were calibrated to deliver 170 mW at the output of the SC-SMA patch cable, in accordance with the system specifications.

A complete measurement consists of five cycles. Each measurement cycle involves continuous light delivery from one fiber during a particular time interval while all the other fibers detect this excitation and fluorescence emission light. The cycle is continued by the other fibers sequentially delivering light, one after another (see Table 1). The light delivery from each fiber lasted ~3 s followed by a delay of 0.1 s for shifting the light delivery to the next fiber. The 3 s light delivery per fiber was long enough to reliably measure excitation and fluorescent light without causing photobleaching of the PS due to longer exposure time.³⁰ The measurement data points are obtained by taking the average from all five cycles. Before each measurement, the setup was covered with a dark, light-proof curtain. The summary of all the measurements is given in Table 1.

2.4 Data Processing and Reconstruction

Data collected from tomographic measurements were first processed such that the fluorescent signals below the defined threshold $P_{th} = 5.4 \cdot 10^{-11}$ W were set to zero, and all other fluorescent signals were reduced by the same threshold value P_{th} . This was done to remove the effect from unreliable data points (where the signal is too low compared with the measurement uncertainties), in a way similar to our previous work.³³ The tomographic reconstruction algorithm used here was reported previously.³³ Briefly, 110 data points (all source-detector fiber pairs from 11 fibers), represented as Born ratios between the detected power of fluorescent light (wavelengths >700 nm) and the detected power at the excitation wavelength (690 nm), were given as input to the reconstruction algorithm. The inhomogeneous medium of $(5.0 \times 5.0 \times 3.8)$ cm³ was divided into a finite element mesh of $N_e = 97,906$ tetrahedrons with $N_n = 21,616$ nodes. The computer implementation was done using MATLAB (The Mathworks Inc., 2022) and the NIRFAST package.^{35,36} The reconstruction consists of calculating the forward model for every source-detector pair and solving the inverse problem by iteratively updating the estimates for the fluorescent yields in mesh nodes to minimize the discrepancy between the forward model vector $\mathbf{F}$ and the measurement data vector $\mathbf{M}$. For a given vector of fluorescent yields in all nodes $\boldsymbol{\eta} = (\eta_1, \eta_2, \dots, \eta_{N_n})$ and for a specific source-detector pair (s, d) , the Born ratio from the forward model is^{1,33}

$$F_{s,d}(\eta) = \frac{1}{G_x(\vec{r}_d, \vec{r}_s)} \sum_{i=1}^{N_n} G_x(\vec{r}_i, \vec{r}_s) G_m(\vec{r}_i, \vec{r}_d) \eta_i \Delta V, \quad (1)$$

where ΔV is the element volume, η_i is the fluorescent yield in node i (at the position $\vec{r}_i$), $\vec{r}_s$ and $\vec{r}_d$ are positions of the source and the detector, respectively, and G_x and G_m are Green's functions solutions at the excitation and fluorescent emission wavelengths, respectively.³³

The inverse problem is solved in two stages, referred to as S1 and S2. In S1, because the problem is ill-posed (the number of unknowns (nodes) is much greater than the number of measurement data points), the goal is to minimize the Tikhonov regularization cost function^{1,33}

$$\Omega(\eta) = \|\mathbf{M} - \mathbf{F}(\eta)\|^2 + \lambda \|\mathbf{L}(\eta - \eta_0)\|^2, \quad (2)$$

where λ is the regularization parameter, $\mathbf{L}$ is the regularization matrix, η_0 is the initial estimate for the vector of unknowns η , and $\|x\|$ denotes Euclidean two-norm of vector x . The modification of the Levenberg-Marquardt algorithm was used to calculate the updates for fluorescent yield estimates according to³³

$$\eta_{i+1} = \eta_i + (\mathbf{J}_i^T \mathbf{J}_i + \lambda_i \mathbf{L}^T \mathbf{L})^{-1} \mathbf{J}_i^T \delta(\eta_i), \quad (3)$$

where indices i and $i+1$ denote the ordinal number of the iteration and matrix $\mathbf{J} = \frac{\partial \mathbf{F}(\eta)}{\partial \eta}$ is the Jacobian of the system. The regularization parameter λ is initialized to the maximum of the diagonal of the Hessian matrix: $\lambda_0 = \max\{\text{diag}(\mathbf{J}_0^T \mathbf{J}_0)\}$ and updated in every iteration to $\lambda_i = \max\{\text{diag}(\mathbf{J}_i^T \mathbf{J}_i)\} \cdot 10^{-i/4}$. The regularization matrix $\mathbf{L}$ was set to an identity matrix, so as not to bias the solution with *a priori* information about the geometry. The reconstructed fluorescent yield η is transformed to the fluorophore absorption μ_{af} using the relation $\eta = \gamma \mu_{af}$ and assuming homogeneous quantum efficiency of $\gamma = 10\%$,^{37,38} for fluorescence emission by verteporfin.

S2 of the reconstruction is described in our previous work³³ and assumes the number of unknowns (originally the number of nodes N_n) is reduced to the number of regions (N_r) with homogeneous fluorescent yield. The regions are defined according to the results from S1. The introduction of S2 improves the accuracy of the solution with respect to S1,³³ which is necessary for the quantitative performance comparison between CPG and standard silica fibers. The fluorescent yield estimate updates in S2 are still calculated according to Eq. (3), but without the regularization and with the reduced size of the Jacobian and the vector of unknowns.³³ The iterative method stops when the relative decrease of the two-norm of the difference between the forward model and the measurements vector is $<2\%$, or the maximal number of iterations is reached.³⁶

3 Results

The results of DFT reconstruction from phantom measurements with silica and CPG fibers are shown in Fig. 2. The first two columns compare the performance of silica fibers (a) and CPG fibers (b) in retrieving the PS distribution in the phantom with the spherical tumor-mimicking inclusion. Columns (c) and (d) compare the performance of silica and CPG fibers, respectively, for the measurements with the ellipsoidal tumor-mimicking inclusion. The results shown in the first row (from above) are from S1, whereas the second row corresponds to the results from S2 (Sec. 2.4). The quantified fluorophore absorption and estimated volume of the inclusion obtained in S2 are also indicated in Fig. 2.

Considering the case of the spherical inclusion with the fluorescent PS drug inside, we do not observe any significant difference in the reconstructed fluorophore absorption values obtained using silica fibers and CPG fibers [Figs. 2(a) and 2(b)]. Similar can be said for the case of the ellipsoidal inclusion [Figs. 2(c) and 2(d)]. However, the reconstructed values for the fluorophore absorption coefficient are slightly different for spherical and ellipsoidal inclusions. The reconstructed volume is underestimated in all cases, for spherical and ellipsoidal inclusions. The true volume of the sphere was around 0.6 cm^3 and of the ellipsoid around 3.2 cm^3 . The underestimation of the reconstructed volume leads to overestimating the reconstructed absorption,³³ as discussed in Sec. 4. The reconstructed fluorophore absorption coefficients are

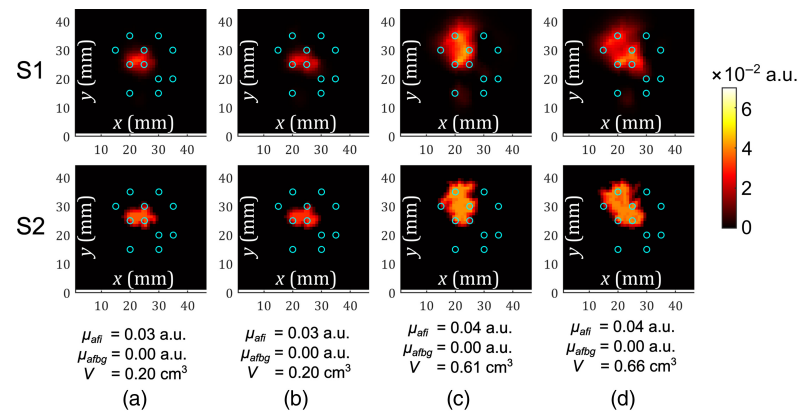


Fig. 2 Tomographic reconstruction results for the measurements with (a) spherical inclusion and silica fibers, (b) spherical inclusion and CPG fibers, (c) ellipsoidal inclusion and silica fibers, and (d) ellipsoidal inclusion and CPG fibers. Slices through the planes cutting the inclusion in halves. Resulting fluorophore absorption (originating from PS) coefficients 2D color map in the first stage (S1) and in the second stage (S2) of the reconstruction shown in different rows. Numerical values for the reconstructed fluorophore absorption coefficients of the inclusion μ_{aff} and the background μ_{abg} , as well as the estimated volume of the inclusion V given below. Blue circles represent fiber projections in the xy -plane.

more overestimated in the case of ellipsoidal inclusion compared with spherical inclusion because the volume of the reconstructed ellipsoid is more underestimated compared with the reconstructed sphere. When the reconstructed volumes are relatively close, as in Figs. 2(a) and 2(b), and Figs. 2(c) and 2(d), the reconstructed fluorophore absorption coefficients can be compared with each other.³³ This implies that CPG and silica fibers performed similarly in DFT reconstructions from our measurements, demonstrating the potential of bioresorbable CPG fibers to monitor the distribution of PS drugs in PDT.

4 Discussion

The aim of this study was to determine the PS spatial distribution monitoring capabilities of CPG fibers within tissue-mimicking phantoms using DFT. The relative comparison between silica and CPG fibers showed good alignment in the reconstructed values for the fluorophore absorption coefficient and the volume of the tumor-mimicking inclusions. The two inclusions with different volumes and shapes were chosen to assess the potential of DFT for monitoring PS distribution regardless of tumor size and morphology. The absolute values for the volume are different from what was expected (the reconstructed volume is underestimated around 3 to 5 times with respect to the ground truth), hence also the absorption.

This volume underestimation was mainly due to the measurement data processing employed here, which is modified from our previous work.³³ The present approach was chosen to trade off the optical power variation among CPG fibers. This power variation could have originated from the process of their in-house connectorization, where the bore size of the available standard SMA connectors (1040 μm) was greater than the diameter of the CPG fiber (870 μm). This might have caused a slight misalignment of the fiber from the central axis of the ferrule. As a consequence, along with CPG material losses,²⁰ the power available at the fiber tip (in the tissue phantom) ranged 75 to 85 mW in different CPG fibers, compared with 150 mW obtained at the silica fiber tips. The variation in optical power among CPG fibers can be reduced by professional connectorization methods.

Because the use of the Born ratio (see Sec. 2.4) helps in canceling out many uncertainties regarding the fiber output and collected powers, there were no significant differences between the reconstructions with silica and CPG fibers. However, for a robust convergence to a solution of

an inverse problem in all the considered cases (both with silica and CPG fibers), a certain level of signal corresponding to the estimated level of noise threshold (Sec. 2.4) had to be subtracted from every measurement data point, causing the volume underestimation.

As stated in our previous work,³³ reconstruction results are sensitive to the true fiber positions, as well as the optical properties of the phantom. In our experiments, the information on the true fiber tip positions was not available once inserted into the phantom. Therefore, it was difficult to achieve exactly the same fiber and inclusion positioning in all four measurement configurations with different fiber types and inclusion shapes. Consequently, small quantitative differences between the reconstructions from the measurements with silica and CPG fibers were expected. It should be noted that the information on the fiber positioning as well as the estimated optical properties of tissue is already available in the clinical setting.^{39,40} Applying similar methods for estimating the implanted CPG fiber positions in future trials could pave the way for more reliable reconstruction.

Overall, in this first test of CPG fibers for DFT in PDT, we observed that there was no significant difference in the reconstruction results obtained from the tomographic measurements with silica and CPG fibers. It should be noted that this test model, comprised of SpectraCure's P18 system for interstitial PDT and phantoms with PS verteporfin, was chosen because it was readily available for a proof-of-principle demonstration. Although the P18 system is currently applied in interstitial PDT of prostate cancer, there is no limitation to follow the same concept and generalize this system for PDT of other internal tumors, such as glioblastoma, where bioresorbable fibers would be highly desirable and better choice than standard non-degradable fibers.

This study establishes the foundation for an interesting perspective in extended interstitial monitoring of PS concentration with implantable bioresorbable fibers and can have positive implications for planning, optimizing, and predicting or monitoring treatment outcomes.⁴¹ Moreover, the concept of implantable optical fibers and implantation procedures has previously been validated with silica MM fibers in mice for long-term monitoring in optogenetic applications.³⁴ Combining DFT with other diffuse optical modalities such as DOS and DCS in the future, for which the bioresorbable fibers have been previously validated,^{28,29} within a single bioresorbable platform could provide information about the complementary physiological and biochemical signals, which will find significant insights in enhancing the interstitial therapeutic outcomes.

5 Conclusion

In this study, we have proposed for the first time the use of bioresorbable optical fibers for DFT in PDT of tumors. By detecting the fluorescent light originating from the PS drug, the spatial distribution of the drug was estimated using the CPG fibers within phantoms with tumor-mimicking inclusions. The results demonstrated good agreement compared with the PDT system equipped with standard silica fibers, in both spherical and ellipsoidal inclusions. The demonstrated concept of monitoring PS distribution in PDT using CPG fibers marks a step forward in the development of an implantable and resorbable multifunctional theranostic platform. Further exploration should focus on extended monitoring with fiber degradation, followed by *in vivo* validation.

Disclosures

JS is a minority shareholder of SpectraCure AB and is interested in prostate cancer interstitial photodynamic therapy. SKVS is a shareholder of BioPixS Ltd with an interest in tissue optical phantoms.

Code and Data Availability

Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

Acknowledgments

This project was funded by the EU H2020 MSCA-ITN project "Photonics for Healthcare: multiscale cancer diagnosis and therapy" (PHAST-ETN) (Grant No. 860185).

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PAPER VI

Mobile multi-configuration clinical translational Raman system for oral cancer application

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Opt Express. **30(10)**, 16572-16584 (2022).

Cite this: *Analyst*, 2023, **148**, 1514

Mobile multi-configuration clinical translational Raman system for oral cancer application

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Early diagnosis of oral cancer is critical to improve the survival rate of patients. Raman spectroscopy, a non-invasive spectroscopic technique, has shown potential in identifying early-stage oral cancer biomarkers in the oral cavity environment. However, inherently weak signals necessitate highly sensitive detectors, which restricts widespread usage due to high setup costs. In this research, the fabrication and assembly of a customised Raman system that can adapt three different configurations for the *in vivo* and *ex vivo* analysis is reported. This novel design will help in reducing the cost required to have multiple Raman instruments specific for a given application. First, we demonstrated the capability of a customized microscope for acquiring Raman signals from a single cell with high signal-to-noise ratio. Generally, when working with liquid samples with low concentration of analytes (such as saliva) under a microscope, excitation light interacts with a small sample volume, which may not be representative of whole sample. To address this issue, we have designed a novel long-path transmission set-up, which was found to be sensitive towards low concentration of analytes in aqueous solution. We further demonstrated that the same Raman system can be incorporated with the multimodal fibre optical probe to collect *in vivo* data from oral tissues. In summary, this flexible, portable, multi-configuration Raman system has the potential to provide a cost-effective solution for complete screening of precancer oral lesions.

Received 23rd November 2022,
Accepted 22nd February 2023

DOI: 10.1039/d2an01921c

rsc.li/analyst

1. Introduction

Oral cancer refers to pathologies affecting the tongue, floor of mouth, upper and lower alveolus, palate, and buccal mucosa.^{1,2} It is described as 16th most common cancer worldwide, often detected in advanced stage, resulting in an overall survival rate of 40–60%.³ The survival rate could be significantly improved to <80%^{4–6} if detected in precancerous stage. Primary care delays in recognizing suspicious symptoms remains an ongoing problem in cancer diagnosis.⁷ To address this, previously various physician-friendly point-of-care optical techniques that can provide easy, inexpensive, rapid and on-site monitoring such as fluorescence, diffuse reflectance and vibrational spectroscopy have been explored.⁸ Among them, Raman spectroscopy has the potential to generate data with detailed molecular information. In addition, Raman spec-

troscopy is preferred for analysing bio-fluids and *in vivo* measurements as water is a weak Raman scatter due to the polar nature of the OH bond, providing minimum interference in the fingerprint region (200–1800 cm⁻¹).^{9,10} Raman spectroscopy is generally a non-invasive approach used to gain chemical and structural information as well as to study molecular interactions.^{11–13} It can be used for quantitative and qualitative analysis of a wide range of samples and materials. It has proven particularly useful in industrial and medical applications having resolved limitations of other spectroscopic techniques.¹⁴ In biomedical optics and clinical applications, Raman spectroscopy in combination with multivariate analysis has proven its potential. The ability of detecting biochemical changes in tissues and cells can lead to a real time clinical diagnostic capabilities. Several studies have highlighted the importance of Raman spectroscopy for tissue differentiation in *ex vivo* and *in vivo* analysis.^{15–17} Typically, a modern Raman spectrometer consist of an excitation source – usually a monochromatic laser, optical components to both transmit laser energy to specimen and collect back scattered signal, a notch filter to reject Rayleigh scattered light, a grating spectrometer and a high-end charged coupled device (CCD) camera to capture a Raman spectrum.

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Analyst

Paper

When the light from a laser interacts with the specimen, a small part of the incident energy is scattered inelastically (1 out of 10^6 photons) to produce a Raman spectrum.¹⁸ In cases where the sample volume is too small or a microscopic area on a large sample needs to be analysed, a Raman spectrometry system coupled with a specially designed microscope would be desirable. Such instruments are commercially available at high cost and are bulky, non-portable, sample volume limited and are not considered suitable for point-of-care application. Additionally, Raman spectrometer coupled with the microscope are generally used as benchtop systems for *ex vivo* analysis owing to its bulkiness and limited flexibility to be used with the fibre optical probe and are therefore not suitable for clinical environment. To facilitate early detection at the primary-care level, as well as in research studies, we envision the use of a highly configurable spectrometry system suitable for seamless integration with the clinical procedure. Here, we describe an affordable, Raman-based instrument capable of both *in vivo* and *ex vivo* analysis employing single platform which can be easily wheeled to provide on-site testing and can be operated by a qualified clinician/lab technician with training and practice. Spectroscopic cell and supernatant analysis would be done to guide if biopsy sampling is necessary. After a positive test result of saliva or cell pellet analysis, *in vivo* measurements can be conducted just before tissue biopsy to guide for a suitable location of biopsy sampling and thereby provide improved sensitivity/specificity of diagnosis. Initially, the data analysis will be done by the experts, to later be automated after collecting sufficient data for machine learning models. To the best of our knowledge, this is the first report describing the design of multi-configurational Raman system

addressing the limitations of the commercial clinical instruments.

The novel Raman system described here is compact, cost effective, portable and easy to assemble and align. It can easily fit into a console for transportation, thereby potentially facilitating point-of-care diagnostics. The design of our instrument is modular, consisting of the following:

Mode (A) Raman microscope – for *ex vivo* saliva cell pellet analysis.

Mode (B) Raman transmission stage – for saliva supernatant analysis using photonic crystal fibre (PCF).

Mode (C) Raman optical fibre probe – for *in vivo* tissue analysis.

This makes it suitable for both *in vivo* and *ex vivo* analysis and can be used in upright and transmission modes for liquid samples. The detailed optical design is given in the Methods section, enabling the easy adaptation by researchers in the field. In this study, we demonstrate the utility of our system in the context of oral cancer screening. The described system is only a prototype. Final system can employ centrifugation microfluidics¹⁹ to separate cell pellet from supernatant, which will minimize human interventions. A typical work-flow of the procedure is shown in Fig. 1.

2. Experimental method

2.1 Instrumentation

Fig. 2 presents the configuration modes of the point-of-care Raman setup developed. This modular instrument consisted of: an illumination source, the sample probing subunit and

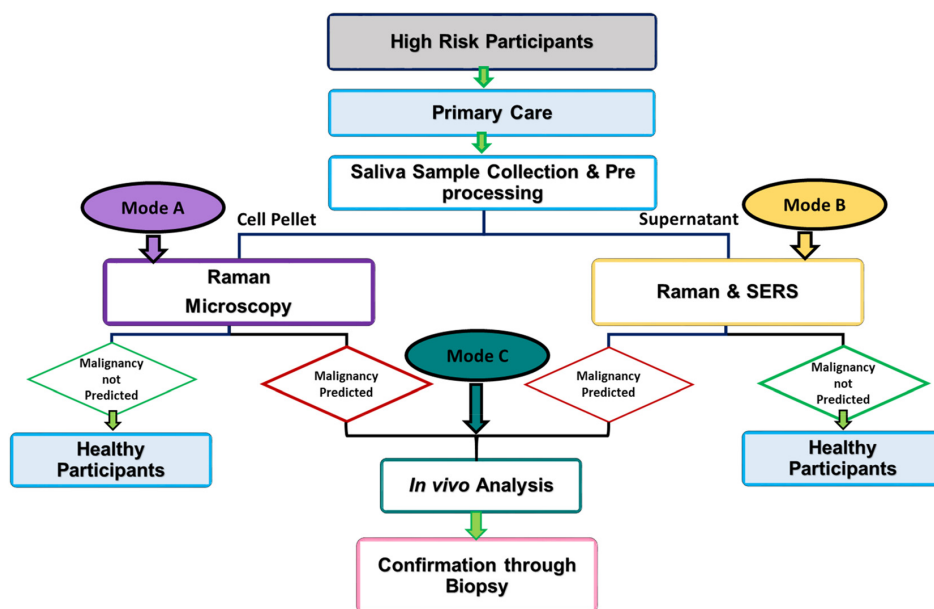


Fig. 1 Flow chart of study protocol with multi-configurational customised Raman system.

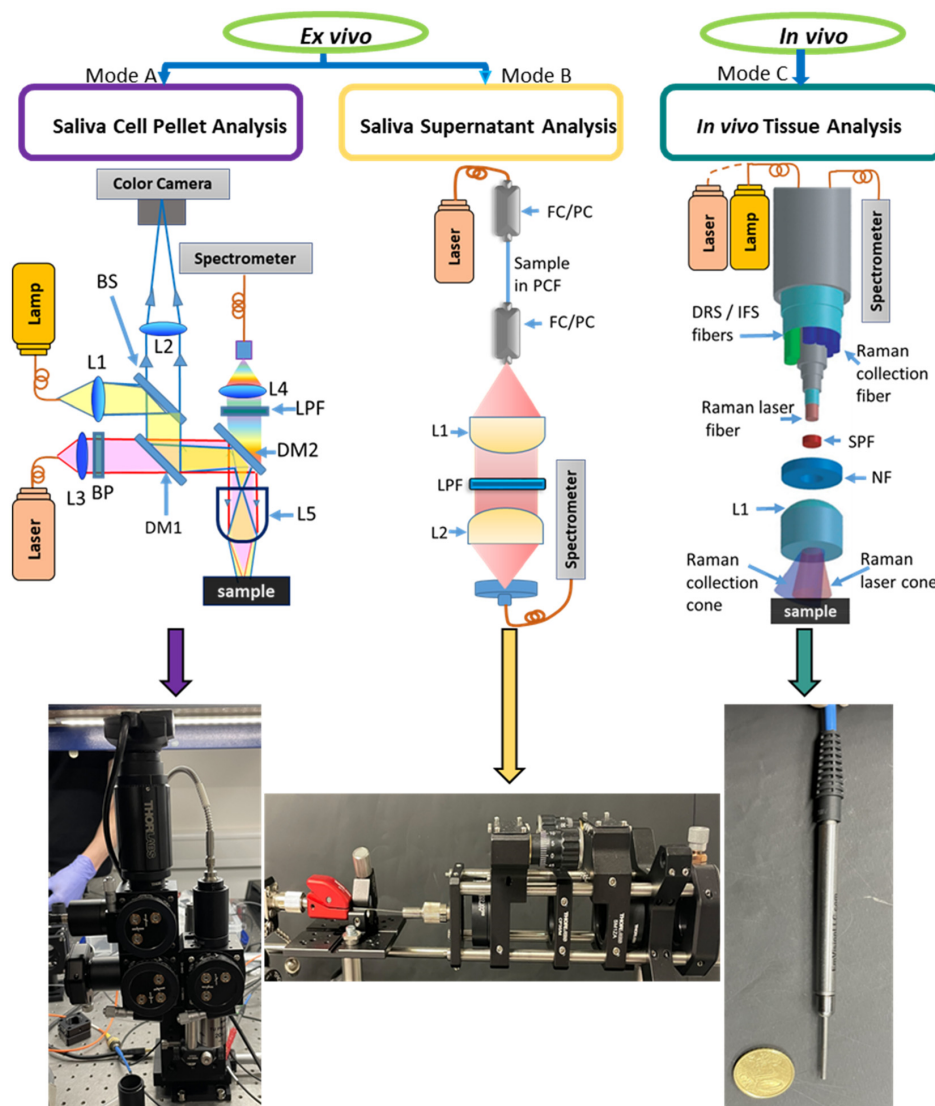


Fig. 2 Three configuration modes of the new customised Raman setup designed to include 3 plug and play modules to be coupled with common light sources and a spectrometer. These modes consist of: Mode (A) customised upright Raman microscope in combination with optical microscopy; Mode (B) transmission mode Raman stage; Mode (C) optical fibre probe for *in vivo* Raman analysis.

the spectrometer. Using the fibre optical connection between the subunits, the sample probing unit was easily switched between the three configuration modes. The illumination was achieved by a 785 nm narrow line width laser with high suppression of side bands (>60 dB suppression at 0.3 nm from the laser peak, Cobolt 08-01 Series, Cobolt, Sweden). The Raman spectrometer was equipped with a diffraction grating with 1200 groves per mm grating density (LS-785, Teledyne Princeton Instruments, USA), a 1340 × 400 pixels back illuminated CCD camera (Blaze: 400HR, Teledyne Princeton Instruments, USA). These subunits can be connected to the

custom-built microscope (Fig. 2, Mode A). This is achieved fibre-optically using a round-to-linear bundle of a 7 × 200 μm core, (Thorlabs BFL200LS02). This Raman system can also utilise the customised transmission stage for the analysis of liquid specimens in transmission mode (Fig. 2, Mode B) and an optical fibre probe for the *in vivo* analysis (Fig. 2, Mode C). Currently, it takes up to five minutes to switch between configurations, mainly fibers. The light sources and calibration are the same for all three configurations. However, the final system will be more automated, which will seamlessly switch between different configurations. This prototype including

Analyst

Paper

microscope and transmission mode stage is designed using off the shelf component in around 10k excluding the spectrometer and detector. The following section describes each of these configurations in detail with the novel optical design.

2.2 Mode (A) Raman microscope for *ex vivo* cell pellet analysis

Fig. 2, Mode A represents the customised Raman microscope which consists of a laser and a visible light source (for sample visualization) connected through optical fibres. Fibre coupled illumination allowed modification of the spot size on the sample plane. Further, this fibre guided light delivery makes the switching of different excitation wavelengths easy. Another important feature of this system is a flexible stage design, which can accommodate different specimen in different dimensions from mm to tens of cm. The specimen was illuminated through an objective lens and Raman scattered photons were collected *via* the same objective lens in backscattering mode and in turn, directed to the spectrometer *via* 7× round-to-linear low OH fibre bundle with 200 µm core (Thorlabs BFL200LS02). Simultaneously, a portion light from sample was patched to a CCD detector. This configuration was used for *ex vivo* analysis of solid and liquid specimens in the reflective mode.

White light imaging optical path. A broadband visible light source (Thorlabs M365LP1, 365 nm, 1350 mW mounted LED) was used to illuminate the specimen for conventional optical microscopy. The light beam entered the microscope *via* lens L1 (Thorlabs LA1951A, $f = 25$ mm) and redirected to the objective lens using a beam splitter BS (Thorlabs, BSW26R, 50:50, 350–1100 nm), dichroic mirror DM1 (Semrock, FF757-Di01) and dichroic mirror DM2 (Semrock LPD02-785RU). The L1 was arranged to provide uniform Kohler illumination of white light on the sample plane. The white light scattered from the sample was reflected by DM2 to DM1 which redirected it to a colour camera through BS and a field lens L2 (Thorlabs LB1437-A, $f = 150$ mm). A high-resolution coloured CMOS camera (Thorlabs DCC1645C) was used to visualize the sample under white light illumination. The field of view (FOV) was controlled using a microscope objective lens L4 (116 × 92 µm for 50×, 290 × 230 µm for 20× and 1160 × 920 µm for 5×). The images were displayed on computer screen using the Thorcam software.

Raman optical path. Monochromatic light (785 nm laser) passed through the collimation lens L3 (Thorlabs A397TM-B, $f = 11$ mm, NA (numerical aperture) = 0.3) and laser cleaning bandpass filter BP (Semrock LD01-785/10-25). Afterwards, the collimated excitation beam passed through the DM1 and was reflected by DM2, which was focused on the sample plane by microscope objective. The spot size of the focused excitation beam was 60 µm with 20× objective lens (Fig. 4). The Raman scattered photons from the sample passed through the objective lens and the excitation light is filtered out using a dichroic mirror (DM2) and long pass filter LPF (Semrock LP02-785RU-25). The filtered Raman light (free of excitation light) was focused on to a low OH circular to linear fibre bundle

(Thorlabs BFL200LS02) using a lens L4 (Thorlabs F810SMA-850, $f = 36.2$ mm). The linear side of fibre was attached to the entrance linear slit of the Raman spectrometer. The Raman spectrometer subunit contained a filter chamber with a long pass filter (Semrock LP02-785RU-25), a high throughput spectrometer (Teledyne Princeton Instruments Acton LS785, $f/2$), and a highly sensitive deep depleted CCD camera (Blaze: 400HR, Teledyne Princeton Instruments, USA). The CCD pixels were binned vertically to combine light from linear slit. The light from the CCD is acquired using the LightField software (Teledyne Princeton Instruments, USA).

2.3 Mode (B) Raman transmission stage for saliva supernatant analysis using PCF

Fig. 2, Mode B and Fig. 3B illustrate a horizontal stage for liquid specimen analysis in transmission mode using PCF. PCF are optical fibers composed of periodic array of air-holes surrounding a solid or hollow-core. In this study, the fiber used is the simplest PCF that is composed of silica core (SC-PCF) at the intersection of three large air holes. Light is guided in the triangular core shape by the total internal reflection mechanism based on the large refractive index difference between the silica and air, and by the reduction of the thickness of the three silica struts enhancing light confinement in the core.^{20,21} To integrate the inherently weak Raman signal from a liquid specimen (saliva supernatant) over a large volume, the SC-PCF with 3.5 µm core diameter, >70 µm air-channel diameter, and NA = 0.5 was filled with aqueous liquid. The external diameter (200 µm) of this SC-PCF is slightly larger than a standard fiber for allowing easier handling. In this SC-PCF, Raman signals are generated by the evanescent wave extended into the holes while excitation light (785 nm) propagates through the solid core.^{20,22,23} The sample holder was used in this way to prolong light-sample interaction length, thereby improving detection sensitivity.²⁴ Another advantage with a longer path length than in a microscope was that the larger volume sampled can be more representative, especially for low concentration analytes. A further advantage was that still a very small volume of analyte was required (about 20–100 nL). A PCF filled with sample solution can be directly placed under the Raman microscope to collect the signal in conventional reflective mode. However, to ease the alignment of input laser beam in to the PCF, we designed a horizontal optical stage enabling the collection of signals in transmission mode. A 785 nm laser beam entered the PCF through an FC/PC connector (ferrule connector/physical contact connector). Raman signal emitted from the other end was collimated by an aspheric lens L1 (Thorlabs S-LAH64, $f = 20$ mm, NA = 0.54, ARC: 650–1050 nm). This lens was selected to match the NA of the PCF enhancing the collection efficiency. The collimated Raman signal, after passing through a long pass filter (LPF) (Semrock LP02-785RU-25), was collected by another lens L2 (Thorlabs N-BK7, $f = 50$ mm, NA = 0.23, ARC: 650–1050 nm) and focused on a low OH circular to linear fibre bundle (Thorlabs BFL200LS02). To transfer the

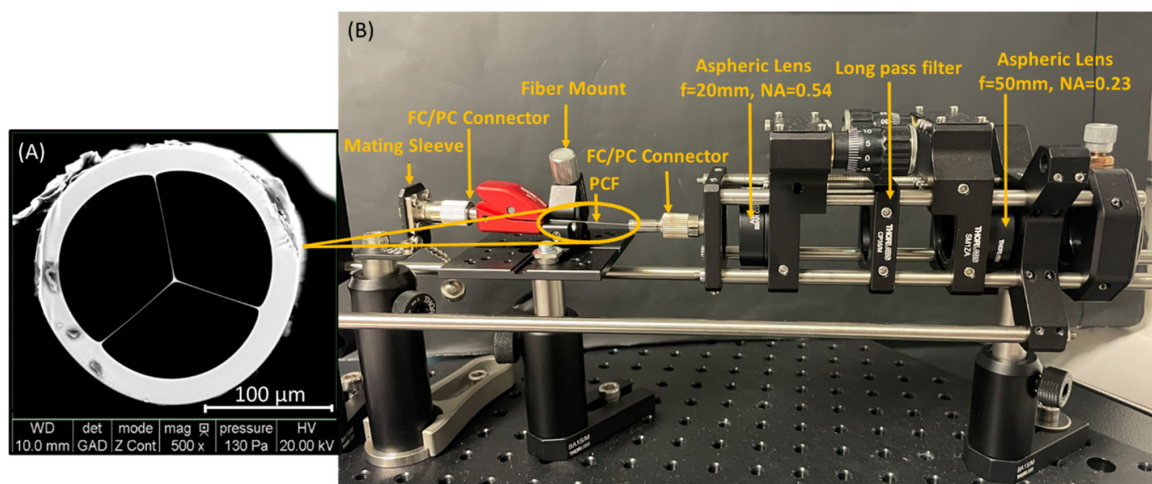


Fig. 3 (A) Cross section of a suspended core PCF designed by the authors (B) transmission mode stage for the analysis of liquid specimen injected into a PCF.

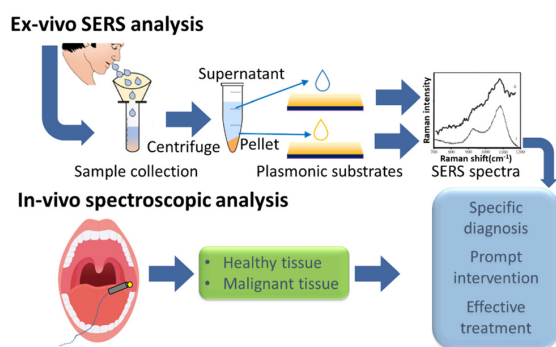


Fig. 4 Sample preparation and methodology for *ex vivo* saliva and *in vivo* tissue analysis.

signal to the spectrometer. The Fig. 3(A) shows the cross section of a typical suspended core PCF used in this study.

2.4 Mode (C) Raman optical fibre probe for *in vivo* tissue analysis

Fiber optics-based Raman spectroscopic system has a tremendous potential for real time *in vivo* tissue analysis in endoscopy or surgical guidance. However, for such applications small and flexible fiber optics probes are mandatory. This requirement has limited the clinical implementation of fiber optics based Raman technology for many years.²⁵ Recent advancement in fiber optics technology and miniaturization of the optical components have improved the *in vivo* implementation.^{26–28} Fig. 2, Mode C shows a multi-spectroscopy fibre-optic probe (EMVision, Loxahatchee, FL, USA) used for the *in vivo* tissue analysis. This customised *in vivo* probe can be used to acquire spectra from multimodal spectroscopic techniques including

Raman, fluorescence (FS) and diffuse reflectance spectroscopy (DRS). The probe consisted of $9 \times 300 \mu\text{m}$ core diameter, 0.22 NA low OH silica fibres arranged in a concentric ring around a $300 \mu\text{m}$ core diameter, 0.22 NA low OH silica fibre used for Raman excitation. Seven out of nine fibres from the circular geometry collected the Raman signal passed through a donut shaped notch filter (NF), which rejected the laser line while transmitting the Raman signals from the probed tissue. The remaining two fibres can be used for DRS and FS. There was a short pass emission filter (SPF) in front of the central Raman illumination fibre to clean input laser line. A two-component customised lens L1 is placed at the probe tip to overlap the focus of excitations light and the area of collection of the Raman light from the tissue. Seven Raman collection fibres were aligned into a single line on the other end of the probe to deliver collected signals to the spectrometer *via* $50 \mu\text{m}$ slit. The accumulated signals from the seven fibres provide an optimum signal-to-noise ratio, needed for biological samples.

2.5 Sample preparation

Patients suffering from oral squamous cell carcinoma and other malignant diseases in the oral cavity going through biopsy and histopathological examination were enrolled in a study approved by the Clinical Research Ethics Committee (CREC) of University College Cork (CREC ref.: ECM 4 (y) 13/4/2021 & ECM 3 (cc) 11/05/2021). Written informed consent was obtained from human participants and study was performed in accordance with the Guidelines of Helsinki.

The performance of the multi-configuration Raman system was demonstrated for different healthy tissues of a single participant and using saliva sample collected from the same participant. A saliva specimen was collected and centrifuged at 10 000 rpm for 10 min at room temperature to separate it in supernatant and cell pellet and stored at -80°C for further

use. For saliva sample analysis with the microscope, 3 drops of 10 μL saliva was placed and dried on a piece of aluminium foil. The cheek and red blood cells from the cell pellet were smeared on the aluminium foil to collect Raman spectra from a single cell layer.

To prepare the PCF sample for saliva analysis in transmission mode stage, a 9–10 cm long PCF with 3.5 μm core diameter and $\text{NA} = 0.5$ was cleaved at both ends. Its polymer jacket was removed from the ends and it was filled with saliva supernatant through capillary action.

In vivo analysis involves measurements on healthy tissues and lesion from the same participants using the fibre-optic probe. For the collection of *in vivo* Raman spectra, the probe was placed to just touch the area of interest.

2.6 Data analysis

Raman spectra were background corrected using the asymmetric least squares method,²⁹ noise smoothing with a Savitzky–Golay filter of polynomial order 2 and a window width of 7, and normalized to their respective mean intensity using MATLAB R2020b software (Mathworks, Inc., Natick, MA, USA).

3. Results and discussion

3.1 Mode (A) Raman microscope for *ex vivo* cell pellet analysis

Analysis of liquid biopsies has recently attracted attention as an emerging field in medical diagnosis. Biological fluids such as blood, saliva, urine and tears are rich in biomarkers that could be indicative of the health status of the sampled

individual.^{30–32} This provides a minimal invasive route for cancer screening and to classify cancer patients based on biomarkers identified.^{33,34} The proposed Raman microscope can be used for the *ex vivo* analysis of any excised tissue or for liquid biopsies.

Fig. 5(A) illustrates Raman spectra from saliva specimen in both dried and liquid phase as well as from the mono layer of red blood cell and cheek cells as shown in Fig. 5B and C respectively. As can be seen, the Raman spectrum of saliva sample is featureless when wet but exhibits distinct peaks when dried. Several peaks characteristic of saliva are discernible.³⁵ For instance, peaks around 1049–1053 cm^{-1} are assignable to C–C stretching vibration of lipids. Furthermore, a well-known Raman bands characteristic of amide-I vibration of salivary proteins at around 1639–1670 cm^{-1} are also discernible. Also, unique to the saliva specimen are two additional peaks at 877 cm^{-1} and 938 cm^{-1} corresponding to a mixture of –C–O–C– and –C–C–, ring and stretching modes.³⁶ The Raman spectrum of red blood cell, on the other hand, was dominated by the haemoglobin signatures clearly differentiating it from cheek cell and saliva. Specifically, prominent peaks at around 1618 cm^{-1} and 1562 cm^{-1} can be assigned to the $\text{C}_\alpha=\text{C}_\beta$ vibration of the vinyl groups, and $\text{C}_\beta-\text{C}_\beta$ stretching modes in heme, respectively. Whereas peaks at 752 and 1395 are associated with pyrrole ring in heme. All the important differentiating Raman bands are presented in Table 1. Previously, Raman has been used to detect structural and biochemical changes in salivary analytes (*e.g.* proteins, amino acids, nucleic acids) accompanied with diseased transformation.¹⁷ However due to generally weak signals and the strong background interference, most of the studies have been done on dried saliva samples to concentrate the analytes or utilized plasmon

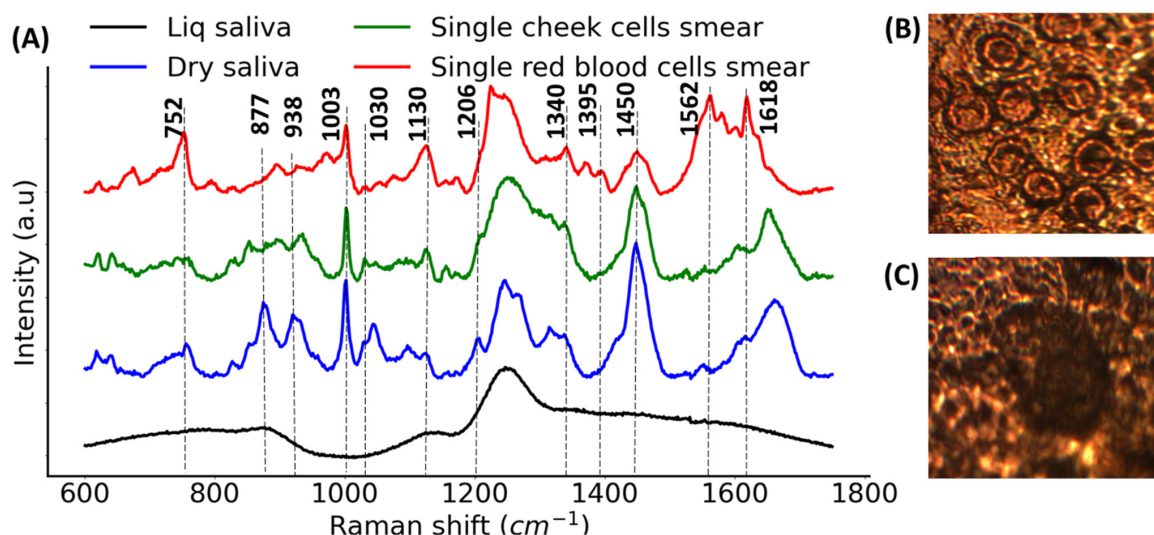


Fig. 5 (A) Pre-processed Raman spectra from saliva specimen in liquid and dry form. Right-optical microscopic view of (B) red blood cells (C) smeared cheek cell on aluminium foil.

Table 1 Spectral assignment of the dominated Raman signatures observed in experimental data^{36,40}

Wavenumber (cm ⁻¹)	Spectral assignments
752	ν (pyrrole breathing) stretch in heme; symmetric ring breathing in tryptophan
877	C–O–C ring (polysaccharides); C–C stretching (hydroxyproline)
938	C–O–C stretching (polysaccharides); C–C stretch α -helix proteins
960	PO ₄ ³⁻ symmetric stretch (hydroxyapatite)
1003	Symmetric ring breathing mode of phenylalanine
1030	C–H in plane bending of phenylalanine
1080–1090	C–C stretch acyl chains (<i>gauche</i>) lipids; PO ₂ ⁻ stretching (DNA); ν (C _β C ₁) asymmetric stretch (hemoglobin)
1130	C–C stretch acyl chains (<i>trans</i>) lipids; C–O stretch polysaccharides; ν (pyrrole half-ring) asymmetric stretch (hemoglobin)
1204–1209	C–C ₆ H ₅ stretch phenylalanine and tryptophan
1220–1320	Amide III-combination of NH in-plane bending and CN stretching
1302	CH ₂ deformation (lipids)
1340	Nucleic acid (adenine, guanine); CH deformation polysaccharides
1395	ν (pyrrole quarter-ring) stretch (hemoglobin)
1440	CH ₂ bending mainly in lipids
1450	CH ₂ bending mainly in proteins
1562	ν (C _β C _β) stretch (hemoglobin)
1618	ν (C _a =C _b) of vinyl groups (hemoglobin)
1656	C=C stretch mainly in lipids
1630–1690	Amide I: mainly C=O stretch in proteins
1740	C=O in lipids

enhancement to improve the weak Raman signals from low concentration analytes.³⁷ The drying process leads to the structural/conformational changes of the analytes (*e.g.* proteins) and SERS struggles with reproducibility. The use of low NA longer focal depth objective lens (*e.g.* 5× or 10×) have poor collection efficiency and a focal length of tens of micron implies interaction of laser light with the sample is limited and thus requires long integration time, typically tens of seconds. These are not suitable for fast detection. Recently, fiber-enhanced Raman spectroscopy (FERS) where hollow core fibers and PCF have been used to confine the excitation light along the fiber (mm) length, thereby enabling the strong and efficient process of light-matter interactions (>50 times of conventional microscopic measurements) leading to enhanced Raman signals.^{38,39} The sample volume probed at the focus of an objective will always be much smaller than the total volume probed over the length of these fibers.³⁸ To implement FERS, we developed the novel transmission mode stage (B) in which signal gets integrated over an extended interaction length with the light.^{23,38,39}

3.2 Mode (B) Raman transmission stage for saliva supernatant analysis using PCF

The interaction of light with the analytes along the PCF length is critical for sensitive detection of low concentration analytes. In such measurements one must consider light attenuation yielding a reduction of transmission signal due to scattering and absorption. It is thus important to characterize the optimum PCF length. For this, the transmission stage was used to optimize the length of PCF using vegetable oil as an analyte because its consistency and refractive index is close to that of saliva (for saliva refractive index $n \approx 1.4$ and for oil $n \approx 1.46$).^{41,42} It also has a distinct Raman signal as discussed in Table 1. Different lengths of PCF ranging from 9 cm to 13 cm were tested. The results are shown in Fig. 6A, highlighting the maximum Raman signal for 9 cm and 10 cm PCF lengths. Subsequent increase in the length >10 cm of the PCF lead to gradual decrease in Raman photons due to absorption/scattering loss. The curves in the Fig. 6B represent Raman signals for different modes of our new Raman system using oil as an

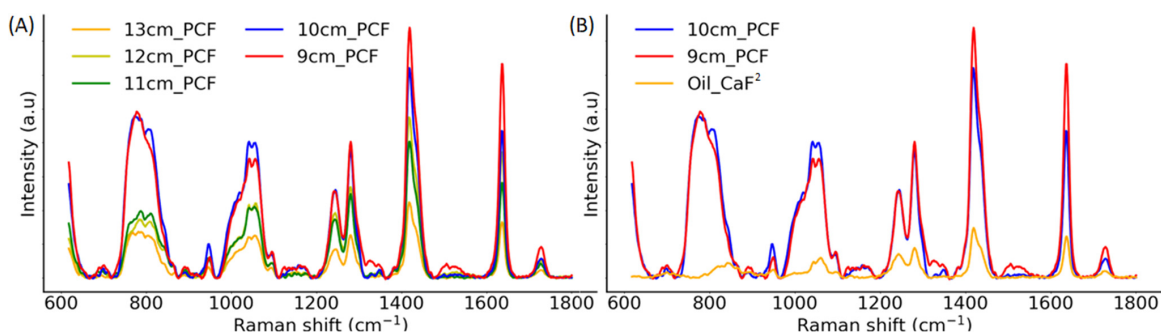


Fig. 6 Comparison of Raman spectra from (A) different length of PCF using vegetable oil as an analyte (B) different instrument configuration modes of the customized system using oil as analyte.

Analyst

Paper

analyte. 9 cm and 10 cm PCF filled with oil provided the highest signals when analysed using transmission mode as compared the oil placed on a 2D CaF_2 substrate and analysed with Microscope in reflection mode. This improvement of signals in PCF compared to 2D substrates would be critical in detecting low concentration oral cancer markers in saliva.

The spectra in Fig. 7 represents Raman signals of 50 μM solution of Rhodamine B (Rhd B) in water filled in the PCF and observed in transmission mode vs. on a CaF_2 substrate and observed under the microscope in reflection mode. Rhd B peaks that were difficult to see on a 2D CaF_2 substrate under the microscope were significantly stronger when analysed through PCF due to increased interaction between the excitation laser and analyte over the length of 9 cm. The observed Rhd B peaks were in line with the previously published literature.⁴³

3.3 Mode (C) Raman optical fibre probe for *in vivo* tissue analysis

Over the time, Raman spectra of various tissue has been observed. Most recognized Raman scatterers are blood, lipid, collagen, tyrosine elastin, myoglobin, carotenoids and nucleic acids *etc.*^{17,44,45} Fig. 8 represents spectra from different tissue types in the oral cavity collected from a healthy participant with the customised fibre optic probe. The probe was gently

placed on the area of interest to collect the Raman data from various tissues. 30 mW laser power was used to collect signal for a time of 4 s, to keep the energy per unit area within the permissible limit of the tissue.

The difference between different tissue types was clearly visible, especially signal from mandible bone was quite prominent at 960 cm^{-1} in the data collected from human gingiva while the lip Raman spectrum was dominated by lipid signals at 1080 , 1302 , 1440 and 1740 cm^{-1} .^{46,47} Other subtle differences in these three different tissue types can be seen in terms of peak height and width at 1003 and 1660 cm^{-1} respectively. The molecular vibrational modes associated with these changes are tabulated in Table 1.

4. Conclusion

Design development and assembly of a versatile customised Raman system is reported in this study. This system can work in three different modes. The first mode was Raman microscope combined with conventional optical microscopy. This microscope has a flexible stage design for sample of different dimensions. We also demonstrated the benefit of the custom built liquid sample transmission stage over microscope in collecting signals from low concentration analytes. This PCF based transmission set-up enabled stronger Raman signals in a relatively small volume. Lastly, the flexible optical fibre based probe is suitable to acquire multiple (Raman, diffuse reflectance and fluorescence) spectroscopic techniques data from the same tissue location *in vivo*. In summary, this flexible Raman system is of great potential for the early diagnosis of oral cancer *via* initial screening using participants saliva followed by Raman spectral signatures guided biopsy of the lesions. The flexible and modular design can be of interest to Raman spectroscopy community and potentially in future primary care.

Author contributions

Siddra Maryam: methodology, system design and development, experiments, data analysis, writing, review and editing; Sanathana Konugolu Venkata Sekar: system design and development, review and editing; Daniyal Ghauri: data collection; Edward Fahy: data acquisition, recruiting participant, manuscript review; Marcelo Saito Nogueira: project planning, manuscript review; Flavien Beffara: PCF design and fabrication; Georges Humbert: PCF design and fabrication; Richeal Ni Riordain: supervision and managing clinical collaboration; Patrick Sheahan: supervision; Ray Burke: supervision and managing clinical collaboration; Kiang Wei Kho: system design and development, experiments, review and editing; Rekha Gautam: system design and development, experiment, data analysis, writing, review and editing; Stefan Andersson-Engels: funding, conceptualization, supervision, review and editing.

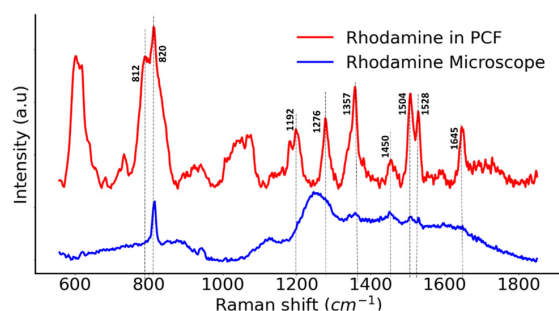


Fig. 7 Raman spectra comparison of Rhd B-filled in the PCF vs. on a 2 dimensional CaF_2 substrate.

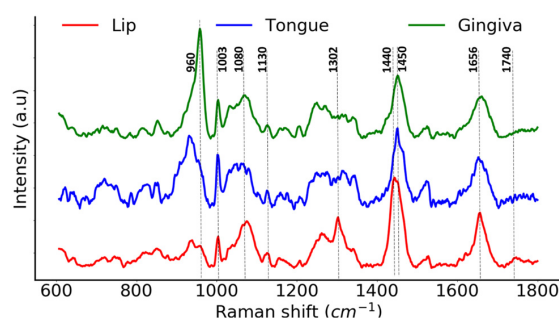


Fig. 8 Spectra from different tissue types in oral cavity collected from the new customised Raman system.

Paper

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Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We greatly acknowledge fruitful discussions with Dr Linda Feeley. The authors will also like to thank Jean Matias for his help in laser beam profile characterization and to Konstantin Grygoryev for his proof reading and improving the English in the manuscript. The authors are grateful for the financial support of Science Foundation Ireland through contract SFI/15/RP/2828.

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PAPER VII

Biomolecular Fingerprint of Crohn's Disease Using Raman Spectroscopy

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J Biophotonics. **19(1)**, e70219 (2026).

RESEARCH ARTICLE OPEN ACCESS

Biomolecular Fingerprint of Crohn's Disease: A Comparative Raman Spectroscopic Study of Blood and Tissue Samples

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Received: 23 October 2025 | **Revised:** 17 December 2025 | **Accepted:** 2 January 2026

ABSTRACT

Crohn's disease (CD) is typically diagnosed through endoscopy and histological analysis of biopsies. Currently, specific noninvasive biomarkers for accurate diagnosis and monitoring of CD remain unavailable. This study investigates Raman spectroscopy (RS) as a noninvasive diagnostic and monitoring tool for CD. Plasma and biopsy samples from 66 participants (55—CD, 11—controls) were analyzed to identify disease-specific molecular alterations. RS revealed significant spectral differences ($p < 0.05$) in amino acids, proteins, carbohydrates, and carotenoids. Decreased valine and arginine levels were consistent with mucosal damage and inflammation, supported by reduced carbohydrate signals indicating barrier impairment. Carotenoid depletion reflected oxidative stress-related inflammation. Correlations between plasma and tissue spectra suggested that plasma RS can reflect local tissue inflammation. Despite the small and heterogeneous cohort, RS detected biochemical shifts toward healthy profiles following biologic therapy. These findings support RS as a minimally invasive approach for detecting inflammation-associated molecular alterations, holding potential to guide personalized disease management.

1 | Introduction

Crohn's disease (CD) is an inflammatory disorder characterized by chronic transmural inflammation along the gastrointestinal tract, most commonly affecting the terminal ileum and colon. Varying incidence and prevalence rates of CD have been reported globally, highlighting its growing public health burden across all ages and ethnic groups [1, 2]. CD typically follows a progressive and destructive course, often leading to complications such as strictures, fistulas, and abscess formation [3]. Patients may remain asymptomatic during the early stages, which necessitates repeated evaluations for adequate decision-making [4]. Overlapping features with other

gastrointestinal disorders further complicate timely and definitive diagnosis, often delaying appropriate management [5]. Such delays may contribute to the development of extra-intestinal manifestations affecting multiple organ systems, including musculoskeletal, ocular, dermatological, neurological, renal, and metabolic complications [6, 7]. Present diagnostic protocols rely on endoscopy combined with histological examination of tissue biopsies [8]. While these are the gold standard for objective disease assessment, the invasiveness of these procedures, along with the need for bowel preparation and associated risks like perforations, prompts the investigation of novel noninvasive tools [9, 10]. Reliable minimally invasive alternatives could offer safer and more patient-friendly

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options for disease monitoring. They can also facilitate implementation of personalized treatment approaches, including Treat-to-Target (T2T) strategies, which emphasize achieving and maintaining tight disease control through inflammation reduction, organ preservation, and improved quality of life for CD patients [11].

Blood-based biomarkers such as C-reactive protein, albumin, and hemoglobin are promising in this context, as they have shown strong correlations with disease activity indicators and are systemically obtainable with ease [12, 13]. Blood analysis relies on established analytical platforms encompassing techniques such as flow cytometry, mass spectrometry, immunoassays, high-performance liquid chromatography, and PCR [14–18]. These methods provide insights into the overall condition of an individual, from nutritional and inflammatory status to immune responses. However, focusing on specific biomarkers can be time-consuming and may overlook other relevant indicators, especially in a multifactorial condition like CD. Emerging platforms, such as omics technologies, enable comprehensive biological profiling, providing deeper insights into molecular mechanisms driving disease progression [19]. Despite their potential, their widespread clinical adoption remains limited due to the need for micro-sampling, advanced instrumentation, and complex data analysis pipelines. These factors pose a substantial challenge, especially in low-resource settings. Thus, developing a rapid, minimally invasive, and accurate diagnostic method is crucial. Identifying reliable biomarkers could streamline diagnosis, reduce dependence on invasive procedures, and improve disease monitoring and treatment decision-making.

Raman spectroscopy (RS) has emerged as a sensitive and versatile analytical technique capable of label-free detection of molecular alterations via inelastic light scattering. It enables the simultaneous analysis of multiple biomolecular targets, providing insights into chemical compositional and structural changes within the sample [20]. These capabilities position RS as a promising modality for patient stratification and disease characterization [21]. Its application in inflammatory bowel disease (IBD) has gained momentum, with recent studies demonstrating its expanding role in both metabolic profiling and biomolecular analysis [22–25]. However, to date, limited research has systematically compared RS profiles from paired tissue biopsies and plasma samples to distinguish between inflamed and non-inflamed (NI) states in CD. This comparison is especially relevant in CD, where treatment is not curative and often necessitates repeated interventions for tight monitoring. Identification of spectral markers in blood plasma could enable minimally invasive, longitudinal monitoring and guide therapy selection.

This study investigates RS-based plasma spectral profiles for stratifying CD patients, aiming to evaluate disease activity relative to healthy controls (HCs) and in relation to inflammatory status determined from histological analysis of intestinal biopsies and treatment regimens. To interpret the results, band assignment is made with reference to pure biomolecules. A comprehensive discussion on biological relevance of key biomarkers is presented based on the existing literature.

2 | Materials and Methods

2.1 | Chemicals

The following biochemicals were used to facilitate accurate peak assignment: linoleic acid (L1376, Sigma-Aldrich, USA), L- α -phosphatidylserine (840032C, Avanti Polar Lipids LLC., USA), L-carnitine (T0846, Targetmol Chemicals Inc., USA), indole (I3408, Sigma-Aldrich), lysine (L5501, Sigma-Aldrich), L-tryptophan (T8941, Sigma-Aldrich), arginine (A5131, Sigma-Aldrich), valine (V0500, Sigma-Aldrich), D-(+)-xylose (X1500, Sigma-Aldrich), L-(–)-fucose (F2252, Sigma-Aldrich), collagen (C7661, Sigma-Aldrich), albumin (A1653, Sigma-Aldrich), and β -carotene (C4582, Sigma-Aldrich).

2.2 | Sample Collection and Processing

This study involved 66 participants (median age 43.5 years old; male/female 35/31), including 55 CD and 11 HCs, enrolled at Mercy University Hospital and Cork University Hospital in Ireland. CD patients underwent colonoscopy for disease assessment or surveillance, while HCs underwent colonoscopy for gastrointestinal symptoms or colorectal cancer surveillance. Biopsy samples were obtained from the most relevant colonic regions, specifically from areas showing the most significant endoscopic inflammation or those deemed most representative of the underlying diagnosis. Table 1 summarizes the demographic characteristics of the participants. Enrollment was conducted in accordance with the protocol approved by the Clinical Research Ethics Committee of University College Cork. Informed written consent was obtained before collection of blood and tissue samples. Blood was drawn into EDTA-coated Vacutainer tubes (BD Biosciences) and transported to laboratory premises on ice to minimize degradation.

TABLE 1 | Cohort demographics.

Characteristics	CD	Healthy
Enrolment (%)	55 (83.3)	11 (16.7)
Median age ^a ($\pm$ SD)	43 ($\pm$ 13.3)	44 ($\pm$ 12.1)
Sex		
Male (%)	31 (56.4)	4 (36.4)
Female (%)	24 (43.6)	7 (63.6)
Therapy		
Advanced therapy (biologics) (%)	34 (61.8)	—
Non-biologics (%)	14 (25.5)	—
Other ^b (%)	7 (12.7)	—
Histological outcome		
Inflamed (NHI > 1) (%)	24 (43.6)	—
Non-inflamed (NHI $\leq$ 1) (%)	26 (47.3)	—
Outcome unknown (%)	5 (9.1)	—

Abbreviation: SD, standard deviation.
^aAge at sample collection.
^bCombination of surgical intervention, biological, or non-biological therapy.

Blood was centrifuged at 10000 rpm for 10 min (Eppendorf 5415D, Germany), and the plasma was stored at -80°C . Biopsy tissues were also stored at -80°C for further analysis.

2.3 | Raman Spectroscopy

2.3.1 | Blood Plasma

Microscope slides were prepared by wrapping glass slides (12383118, Fisher Scientific, USA) with aluminum foil and lightly cleaning the top surface with 70% ethanol (CL02.0539, Chem-Lab, Belgium) soaked wipes (Kimtech Science Kimwipes). Plasma samples were slowly defrosted by transitioning from -80°C to -20°C , then to 4°C , and finally to room temperature. The plasma tube was gently inverted several times to ensure homogeneity. Five $2\mu\text{L}$ drops of blood plasma were pipetted onto the slide, spaced approximately 1 cm apart. The slide was then dried inside a plastic desiccator (17250654, Fisher Scientific) connected to a vacuum pump (12911151, Fisher Scientific). Raman measurements were performed using a custom-built, multi-configuration Raman system [26]. The system was equipped with a 785 nm laser (Cobolt 08-01 Series, Sweden), a spectrometer (LS-785, Teledyne Princeton Instruments, USA), and a back-illuminated CCD camera (Blaze 400HR, Teledyne Princeton Instruments). Spectra were recorded over 30 s using a 50 $\times$ objective lens (Nikon CFI60 TU Plan Epi ELWD, Japan), with a 20 mW laser power at the sample. Three random transverse locations within each drop were analyzed, generating a total of 15 spectra per participant.

Calibration was performed using neon-argon lamps, broadband light from the IntelliCal calibration system (Teledyne), and standard spectra of acetaminophen and naphthalene. The sample collection, processing, and data acquisition workflow is illustrated in Figure 1. For Raman bands assignment and interpretation, pure biomolecules were measured in their original form using the same setup and 20 mW power at 785 nm.

2.3.2 | Tissue

Biopsy tissues were transferred from -80°C to -20°C cryostat (LEICA CM1950, cryostat, Nussloch, Germany), then cryo-sectioned into $30\mu\text{m}$ slices mounted onto CaF_2 slides. Sections were fixed in 70% ethanol for 20 min and air-dried. For each tissue, spectra from 50 spatial points were acquired using a Raman microspectrometer (InVia, Renishaw Inc.) and a motorized xy-sample stage with a 30-s integration time per location. A 785 nm laser ($\sim 20\text{ mW}$) excited the samples through a 50 $\times$ long-working-distance objective lens. System wavelength calibration was performed before measurements using the silicon Raman line at 520.5 cm^{-1} .

2.4 | Inflammation Assessment

Multiple mucosal biopsies were obtained throughout the colon during endoscopic evaluation. Inflammatory activity was assessed histologically using the Nancy histological index (NHI), where a

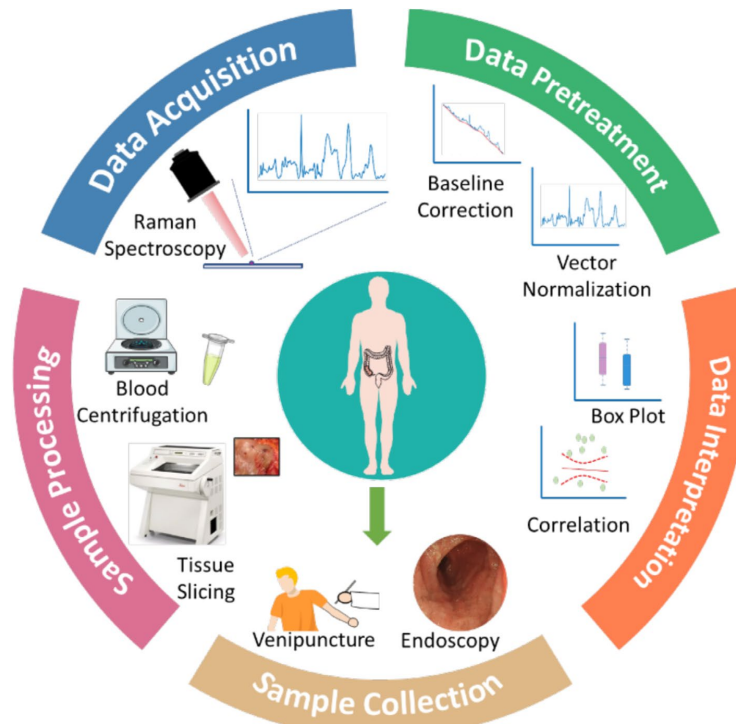


FIGURE 1 | Overview schematic demonstrating the process of sample collection, Raman measurements, and processing.

score of NHI > 1 indicates active (inflamed 'I') disease, and a score of NHI ≤ 1 corresponds to non-inflamed 'NI' (remission) disease [27]. All specimens were reviewed and scored independently by experienced gastrointestinal pathologists blinded to the clinical data.

2.5 | Data Preprocessing and Analysis

Preprocessing of the collected Raman spectra for both qualitative and quantitative analyses was performed in MATLAB following the previously described methodology [28]. Key steps included removal of cosmic rays (if present), background correction, noise smoothing, and normalization, as illustrated in Figure 1. Background correction was conducted using the asymmetric least squares method with parameters $\lambda=60$ and $p=0.0001$ for blood plasma and $\lambda=20000$ and $p=0.0008$ for tissue spectra. Noise smoothing employed a Savitzky-Golay filter with a polynomial order of 2 and a window width of 7. Spectra were then vector normalized. Post-processing, the spectra (15 for plasma and 40–50 for tissue per participant) were averaged to obtain a single representative spectrum for each individual.

Statistical significance of Raman features was assessed using the Kruskal-Wallis test, with a threshold of $p<0.05$, and box plots were generated for significant features. Then, to evaluate the association between tissue and blood plasma spectral parameters, Pearson correlation was conducted.

3 | Results

3.1 | Raman Spectral Comparison

This study employed RS to analyze plasma and intestinal biopsy specimens (colon and ileum) from 55 CD patients and 11 HCs. Inflammation stratification was performed based on histological

assessments by experienced pathologists. Figure 2A,B present the mean Raman spectra ($\pm$ standard error "SE") of plasma and biopsy samples, respectively. Both types of specimens represent a rich source of biomolecules such as proteins, lipids, nucleic acids, metabolites, and signaling molecules [29–32]. However, given their distinct microenvironments, molecular constituents of both specimens differ substantially, leading to visible spectral variations. This can be seen in the differential analysis comparing CD and HCs in Figure 2C,D. This variation between the two specimen types is likely influenced by the predominance of albumin in plasma and the higher collagen and nucleic acid contributions in tissue, reflecting extracellular matrix components and cellularity [33–36].

The relevant Raman bands and their assignments were identified based on previously published literature, as summarized in Table 2. To further support qualitative peak assignments and determine which biomolecules are likely to dominate in regions of overlapping bands, Raman spectra were also acquired from isolated chemicals. For these experiments, commercially obtained samples, including various amino acids, carbohydrates, lipids, proteins, and related metabolites, were directly measured using our customized Raman microscope (Figure 3), given prior evidence of their metabolic involvement in IBD [41–43].

3.2 | Spectral Markers Indicating Molecular Changes

To further explore the biochemical differences, a comprehensive spectral comparison was conducted across three groups: HC versus CD inflamed (I), HC versus CD NI, and CD I versus NI. Out of 55 CD participants, 24 (43.6%) experienced histologically active inflammation 'I', and 26 (47.3%) were categorized as NI or in remission. To capture all sources of variation during spectral acquisition, a representative spectrum for each participant was

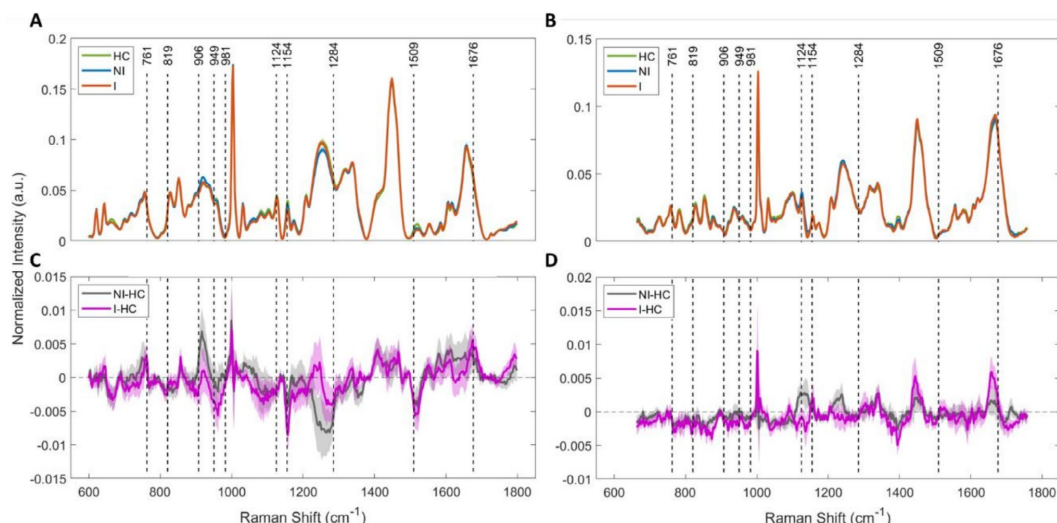


FIGURE 2 | Comparison of averaged Raman spectra of histologically inflamed (I), non-inflamed (NI), and healthy controls (HC) of (A) plasma and (B) tissue from the same participants. The shaded region represents standard error. (C) and (D) represent the corresponding difference spectra.

obtained by averaging data from five plasma drops and all measurement points across the tissue samples, respectively. These averaged spectra were then subjected to statistical analysis ($p < 0.05$). Figure 4 illustrates several spectral markers identified that effectively differentiate active inflammation from HC and

NI cohorts. These markers have been marked in Figure 2 with dotted lines. Overall, RS revealed distinct biochemical alterations in plasma and intestinal tissue from individuals with CD across multiple molecular classes, including amino acids, proteins, carbohydrates, carotenoids, and lipids, as discussed below.

TABLE 2 | Assignment of most relevant Raman bands [22, 23, 26, 37–40].

Raman shift (cm ⁻¹)	Chemical bond assignment	Tentative metabolite association
761	Ring breathing mode, C–N ⁺ (CH ₃) ₃	Indole, tryptophan, carnitine
819	C–C stretch	Fucose (carbohydrates)
879	C–C stretch, C–O–C ring	Amino acids, carbohydrates
906	C–C and C–O stretch	Xylose (carbohydrates)
949	C–C stretch	Valine (amino acids)
970	=C–H out of plane deformation	Lipids
981	C–C and C–N vibration	Arginine
1067	C–C stretch	Lipids
1124	C–O and C–C stretch, COC glycosidic linkage	Carbohydrates
1154	C–C stretch	Carotenoids
1284	Amide III (C–N stretching and N–H bending)	Proteins
1304	CH ₂ twisting/bending	Lipids
1509	C=C stretch	Carotenoids
1676	Amide I (C=O stretch)	Proteins

Significant differences were observed in Raman peaks related to amino acids at 761, 949, and 981 cm⁻¹. The 761 cm⁻¹ band, seen in carnitine and indole ring (Figure 3), showed an increasing trend in CD plasma and a decreasing trend in tissue relative to HCs. The 949 cm⁻¹ (valine) and 981 cm⁻¹ (arginine) bands showed decreasing trends in both plasma and tissue compared to HCs (Figure 4). In addition, protein-related Raman modes also differed between groups, with decreased levels of the amide III band (1284 cm⁻¹) and an increased level of the amide I band (1676 cm⁻¹) in both plasma and tissue of CD participants compared to HCs.

In addition to proteins, significant reductions in carbohydrate-related Raman bands at 819, 906, and 1124 cm⁻¹ were observed in both plasma and tissue of CD participants compared to HCs (Figure 4). These bands also differed significantly between I and NI CD participants. Raman bands at 1154 and 1509 cm⁻¹, associated with carotenoids, were also significantly reduced in plasma as compared to HCs (Figure 4). A similar decreasing trend is also observed in intestinal tissue biopsies at 1509 cm⁻¹ (Figure 4). Lipid-related Raman peaks at 969, 1067, and 1304 cm⁻¹ showed decreasing trends in both plasma and tissue in CD participants (Figure S1). Although differences between CD patients and HCs were not statistically significant in blood plasma, the decreasing trend observed in plasma matched the reductions seen in paired tissue samples.

3.3 | Correlation Between Plasma and Tissue Spectral Markers

To assess whether systemic spectral changes in plasma reflected local intestinal pathology, correlations between plasma and paired tissue Raman intensities were assessed (Figure 5). The data distribution for each of these peaks is shown in Figure S2.

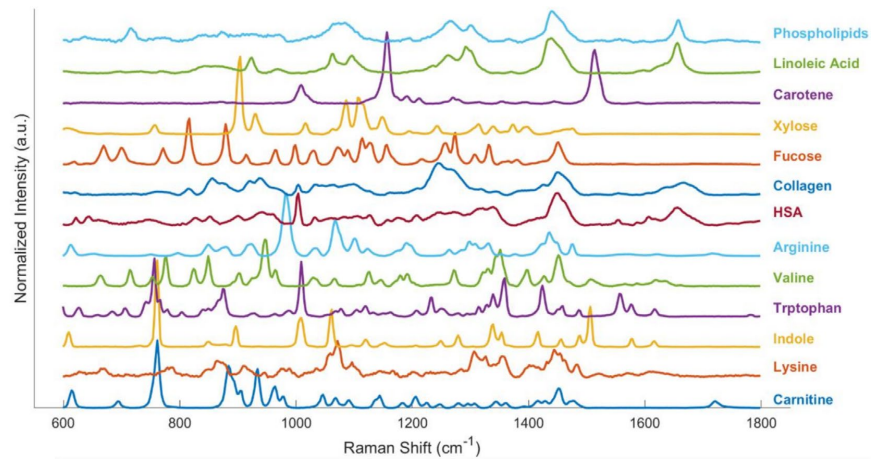


FIGURE 3 | Raman spectra from biomolecules used for the biochemical modeling of plasma and tissue samples of Crohn's disease.

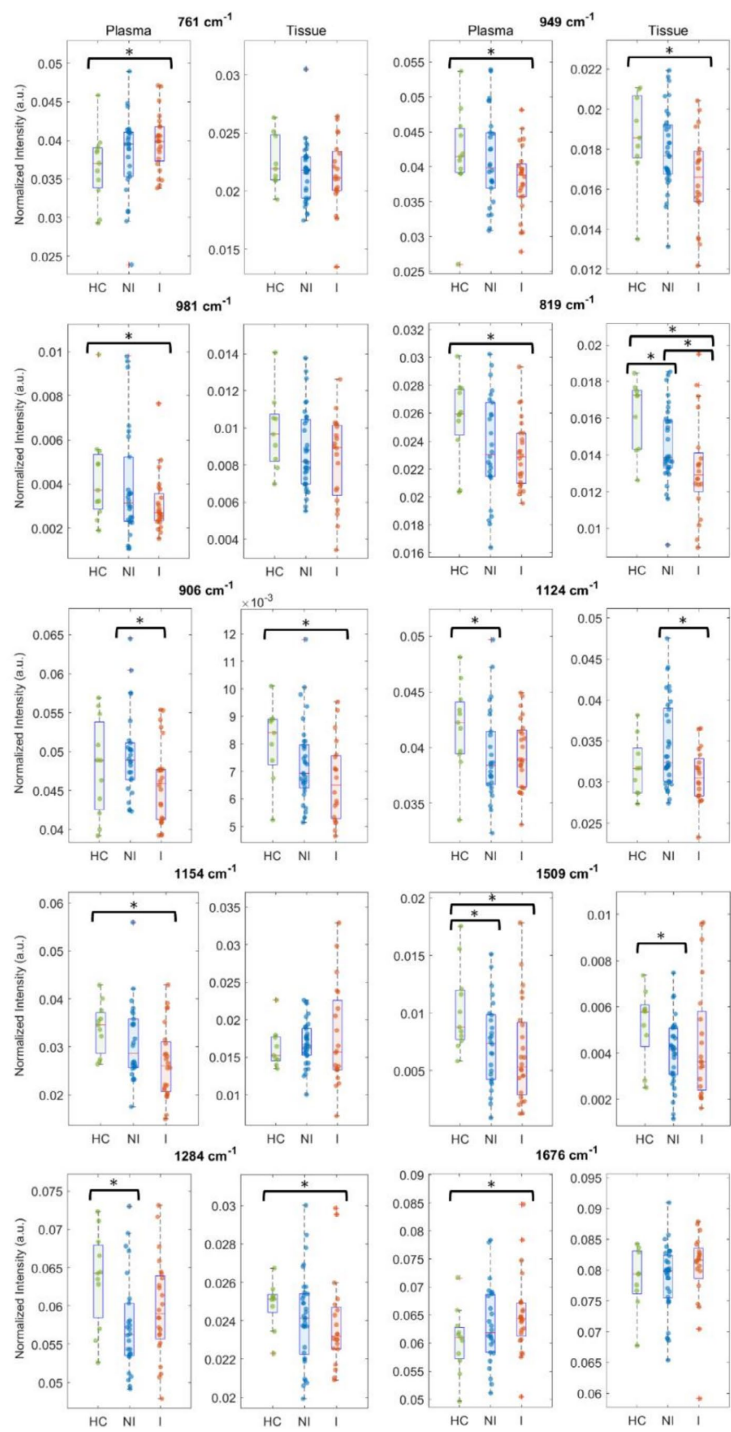


FIGURE 4 | Box plots showing the different Raman intensities of selected peaks with paired samples from both plasma and tissue in healthy control (HC), histologically non-inflamed (NI) and inflamed (I) participants. Significant differences ($p < 0.05$) are marked by an asterisk.

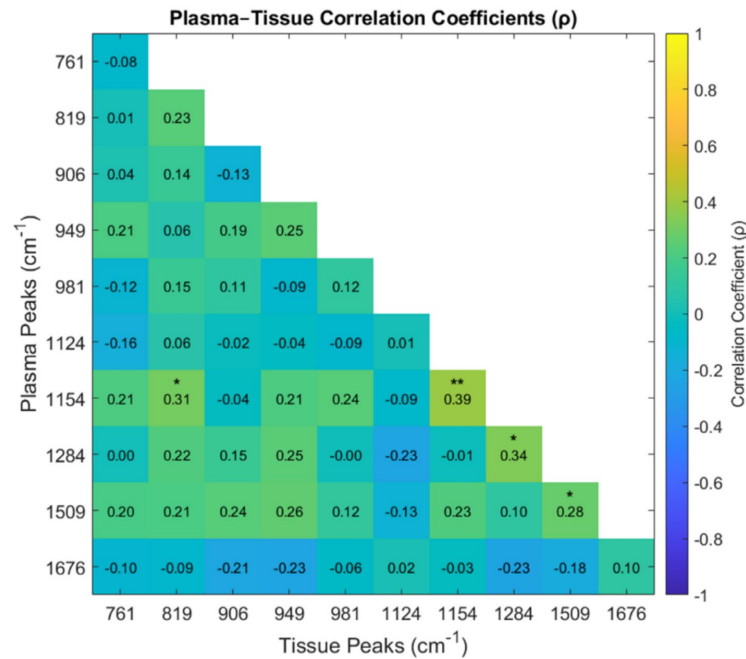


FIGURE 5 | Pearson correlation heatmap showing pairwise associations between plasma and tissue spectral peaks. Only the lower-left triangle is displayed, with significance levels indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$, respectively.

Significant or near-significant correlations ($p < 0.05$) were observed for peaks at 819, 949, 1154, 1509, and 1284 cm^{-1} representing carbohydrates, valine, carotenoids, and protein structure. No significant correlations were observed for peaks at 761, 981, 906, 1124, and 1676 cm^{-1} .

3.4 | Therapy Monitoring

Spectral profiles of patients receiving advanced biologic therapy were compared with those not receiving biologics. Significant differences were identified at 971 cm^{-1} (lipids), 879 cm^{-1} (amino acids and carbohydrates), and 1284 cm^{-1} (proteins). In biologically treated patients, Raman intensities at these peaks approached those of HCs (Figure 6). Other spectral features showed no statistically significant treatment-related differences (Figures S3 and S4).

4 | Discussion

This study demonstrates that RS can detect distinct biochemical alterations in both plasma and intestinal tissue of individuals with CD, revealing consistent changes across amino acids, proteins, carbohydrates, carotenoids, and lipids. These findings highlight the multisystem metabolic disturbances characteristic of CD and underscore the potential of RS as a minimally invasive tool for monitoring disease activity and therapeutic response.

Alterations in amino acid-associated Raman features were among the notable findings (Figure 4). Increases in the 761 cm^{-1} band in plasma, with a decreasing trend in tissue, may reflect

disruptions in carnitine handling or inflammation-driven shifts in tryptophan/indole metabolism. Altered tryptophan–indole metabolism is also consistent with prior observations in CD [37]. The reductions observed in valine (949 cm^{-1}) and arginine (981 cm^{-1}) align with reported depletion of branched-chain amino acids and arginine in active CD [44–47]. These changes likely reflect increased metabolic demand, mucosal damage, inflammation-driven catabolism, and impaired absorption. Notably, valine is lower in CD than in ulcerative colitis, highlighting its potential as a disease-specific biomarker [46]. The observed protein structural changes (decreased amide III and increased amide I levels) are consistent with inflammation-driven protein unfolding, proteolysis, collagen remodeling, and elevated matrix metalloproteinase activity in penetrating CD phenotypes [48]. Changes in plasma proteins may further reflect hypoalbuminemia commonly reported in CD [49, 50].

Significant reductions in carbohydrate-related Raman bands (819, 906, and 1124 cm^{-1}) likely reflect degradation of the intestinal mucin layer and impaired glycosylation (Figure 4). Reduced fucose-related signals are consistent with FUT2 polymorphisms that impair intestinal fucosylation and compromise mucosal barrier function [51, 52]. Similarly, reduced xylose-associated signals reflect malabsorption linked to intestinal inflammation and bacterial overgrowth [53, 54]. Together, these findings highlight the critical role of carbohydrate metabolism in gut barrier integrity and immune regulation. Along with carbohydrates, lipids also play key roles in immune regulation and gut health, often through interactions with the gut microbiota. Although lipid-related Raman peaks showed nonsignificant changes in plasma, the consistent reductions observed in intestinal tissue

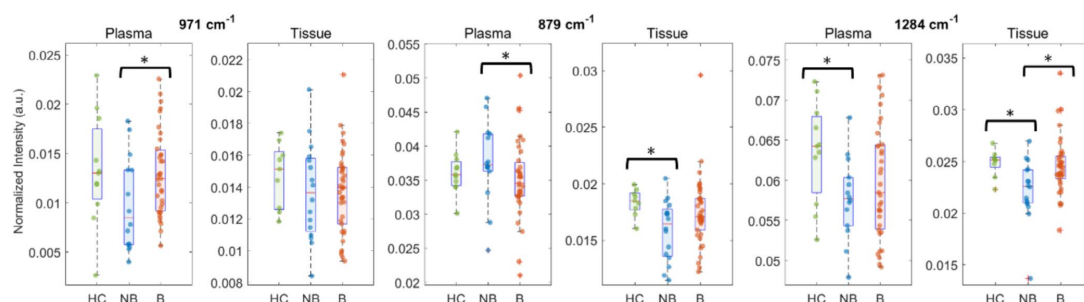


FIGURE 6 | Box plots showing the different Raman intensities of selected peaks with paired samples from both plasma and tissue in HC, non-biological therapy (NB), and advanced therapy (biologics “B”) administered participants. Significant differences ($p < 0.05$) are marked by an asterisk.

suggest altered local lipid metabolism (Figure S1). Dysregulation of microbiota-derived fatty acids, together with inflammation-associated lipid malabsorption, may contribute to these tissue-level changes [55–57].

Carotenoid-associated bands (1154 and 1509 cm⁻¹) were also depleted (Figure 4), consistent with the presence of elevated oxidative stress in CD [37, 58]. Enhanced reactive oxygen species production from activated immune cells, combined with reduced dietary antioxidant availability due to malabsorption, likely contributes to this depletion [59, 60]. These results demonstrate the potential of RS as a tool for noninvasive monitoring of oxidative burden in IBD.

A key strength of this study is the paired analysis of plasma and tissue samples. The observed correlations between plasma and tissue Raman markers indicate that several circulating biochemical features reflect local inflammatory alterations within the intestinal mucosa (Figure 5 and Figure S2). This suggests that plasma RS has potential as a minimally invasive proxy for mucosal inflammatory activity. However, the absence of correlations for certain peaks also highlights intrinsic compositional differences between plasma and tissue.

Biologic therapies, including anti-TNF agents such as infliximab and anti-integrins like vedolizumab, target key inflammatory pathways to induce and maintain remission in moderate-to-severe CD [61]. These treatments reduce mucosal injury, prevent complications such as fistulas, and improve quality of life. The recovery of lipid, protein, and amino acid/carbohydrate Raman markers toward healthy profiles in biologic-treated patients supports the potential of RS for monitoring therapeutic response (Figure 6 and Figure S3). These findings are consistent with reports of metabolic normalization accompanying mucosal healing [62]. However, given the limited sample size and mixed biologic regimens in this cohort, these results should be viewed as preliminary. Larger studies are needed to determine the specific effects of individual biologic agents.

4.1 | Limitations

Our results may contribute to the development of minimally invasive approaches for both diagnosis and monitoring of CD. While the findings are encouraging, several limitations must be

acknowledged. Most notably, the relatively small sample size, particularly within the HC group, represents a constraint which may affect the statistical robustness of the findings. On a similar note, systematic evaluation of confounding factors such as diet, medication, physical activity, and lifestyle on blood metabolic profiles will further strengthen the hypothesis of using RS's unique molecular sensitivity for CD.

Patients on biologics in this study were undergoing treatment with various therapies, including adalimumab, vedolizumab, and ustekinumab. However, due to the limited sample size, these patients were analyzed as a single group, precluding a detailed assessment of drug-specific metabolic signature. Future studies with larger longitudinal cohorts will be essential to look into the effects of individual biologics on blood metabolite profiles. Such analyses could contribute to the development of personalized therapeutic strategies by identifying metabolic signatures predictive of response to specific biologic agents.

5 | Conclusion

This study demonstrates the potential of RS to detect plasma spectral markers reflecting molecular changes associated with intestinal inflammation in CD patients. This was further validated through correlation with histological analysis of intestinal tissue biopsies. Raman band assignments and interpretations were made based on measurements of pure biomolecules. The spectral markers identified in this study align with molecular profiles previously reported in multi-omics studies of CD, strengthening the biological relevance of our findings. The rapid molecular-level layer of diagnostic insights offered by RS presents a promising supporting modality to the current gold standard approaches for CD. Its minimally invasive nature is particularly advantageous for patients who require frequent assessments, offering a more comfortable approach for longitudinal follow-up. Future work should aim to expand the patient cohort and validate these findings in a larger cohort.

Acknowledgments

This publication has emanated from research conducted with the financial support of EU Horizon 2020 MSCA-ITN project “Photonics for Healthcare: multiscale cancer diagnosis and therapy” (PHAST), grant

number (860185), and Science Foundation Ireland under grant number (SFI/15/RP/2828 & SFI-12/RC/2276 P2 & SFI-22/RP-2TF/10293). We also acknowledge the funding received from the SEFS Tyndall New Connections Grant. For the purpose of Open Access, the author has applied a CC BY public copyright license to any author-accepted manuscript version arising from this submission.

Funding

This work was supported by H2020 Marie Skłodowska-Curie Actions (860185), SEFS Tyndall New Connections Grant and Science Foundation Ireland (SFI/15/RP/2828, SFI-12/RC/2276 P2, and SFI-22/RP-2TF/10293).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** (A) Box plots showing the different Raman intensity of lipid-specific Raman peaks with paired samples from both plasma and tissue in HC, histologically inflamed (I), and non-inflamed (NI) participants. Significant differences ($p < 0.05$) are marked by an asterisk (B) Correlations between plasma and paired tissue for each participant. The dotted lines indicate the 95% confidence intervals. Correlation coefficients and p values for each intensity ratio are displayed in the individual panels. **Figure S2:** Correlations between plasma and tissue pairs for each participant. Dotted lines indicate the 95% confidence intervals. Correlation coefficients and p values for each intensity ratio are displayed in the individual panels. **Figure S3:** Comparison of averaged Raman spectra of patients on advanced therapy (Biologics 'B'), non-biological therapy (NB), and healthy controls (HC) of (A) plasma and (B) tissue from the same participants. The shaded region represents standard error. (C) and (D) represent the corresponding difference spectra. **Figure S4:** Box plots showing the different Raman intensity of selected peaks with paired samples from both plasma and tissue in HC, non-biological therapy (NB), and advanced therapy (biologics 'B') administered participants. Significant differences ($p < 0.05$) are marked by an asterisk.