Advanced Raman Techniques for Real Time Cancer Diagnostics

Submitted by

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Martha Z. Vardaki
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Abstract

Cancer is one of the greatest causes of death in modern societies, affecting over 350,000 new cases every year in the UK. Although there are currently more than 100 different cancer types, breast and prostate cancer remain the most common types for women and men respectively. A number of different cancer types follow, with bladder cancer being the ninth most significant type, accounting for 3% of the total new cases.

The currently employed techniques aim to diagnose the cancer at an early stage, where the symptoms are easier to be treated and the disease more likely to be cured. A further issue is that many cancers diagnosed will not affect a patient in their lifetime. The current gold standard for cancer diagnosis, biopsy followed by histopathology, is an invasive, restrictive technique and the screening tests suffer from low specificity, the need for a novel diagnostic concept is vital. Furthermore, the current clinical approach does not identify those patients most at risk of advancing disease. A promising approach consists of molecular vibrational spectroscopy techniques, which are based on the interactions of light with matter. One of these is Raman spectroscopy, a technique with wide applications in research and industry, which has the advantage of being non-invasive and chemically highly specific.

In this thesis we explore the potential of a group of minimally invasive diagnostic techniques, based on Raman scattering, for prostate, breast and bladder cancer. In the case of the two most prevalent types of cancer, prostate and breast cancer, deep Raman spectroscopy has been employed to study the origin of Raman scattering (Chapters 5 and 6) in animal tissue and tissue phantoms, containing highly scattering materials resembling suspicious features found in tissues (calcifications). The spatial distribution of the Raman signal through the sample volume has been studied in relation to the optical properties and the composition of the sample, showing that a couple of transmission measurements would potentially cover the measuring volume of prostate of typical dimensions. Deep Raman measurements were also extended to animal and human tissue samples, in order to investigate the feasibility of collecting Raman scattering from human prostate tissue and its major tissue components (Chapter 6).
Further improvements on these measurements were attempted by introducing the “photon diode” element (Chapter 7) in order to achieve signal enhancement, which proved to be in the range of ×1-2.4, depending on the optical properties of the tissue and the depth of the probing element. The same “photon diode” concept was utilised to attempt depth prediction of a calcification feature in sample volume (Chapter 8).

Regarding bladder cancer, the minimally invasive approach adopted was Raman spectroscopy on urine samples, rather than deep Raman spectroscopy. Raman microscopy was employed in order to discriminate pathological features of bladder cancer between healthy and malignant urine samples. For that reason, the potential differences in urea’s distribution and interactions in urine from healthy and patients with bladder cancer were studied, resulting in promising diagnostic values (73% sensitivity, 80% specificity).

The results presented in this thesis are expected to lead to a better understanding of the Raman scattering signals collection through biological tissues and help in this way the future design of Raman instruments aiming to target disease specific signals. This study shows promise for future application of Raman spectroscopy and paves the way towards the future integration of Raman spectroscopy in a non-invasive cancer diagnosis.
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Chapter 1: The disease

1.1 Introduction

Cancer is one of the greatest death causes in modern societies, affecting over 14 million people worldwide (statistics 2014). There are currently more than 100 different cancer types, but only four of them (lung, breast, bowel and prostate cancer) account for 4 out of 10 cases diagnosed worldwide\(^1\).

Although mortality for the most common types of cancer has decreased the last few years, the number of cancer cases newly diagnosed continues to rise every year. Only in 2014 in UK, there were 296,863 cancer cases registered, a number which accounts for 813 cases per day\(^2\).

Between the most common risk factors for cancer is smoking, lack of physical activity, alcohol, unhealthy diet, obesity and several infections\(^1\). In most cancer cases, the combination of genetics, age and risk factors result in an uncontrollable cell growth. This is initiated with the mechanism of carcinogenesis which is the process by which normal cells are transformed into cancer cells. This process is characterized by a group of cellular and genetic changes that eventually reprogram a cell to undergo uncontrolled cell division and thus forming a malignant mass. Many theories about cancer development have been described in the latest decades and it applies to all cancer types.

As a general concept through the years, more men are diagnosed with cancer than women. However, the most frequently diagnosed cancer for men remains prostate cancer and for women breast cancer (Fig.1.1). Due to this, prostate and breast cancer are the two main types this study aims at. For the needs of our study, bladder cancer is mentioned less extensively in the last part of this thesis. In this chapter we will provide a theoretical background on the statistics, physiology and cancer pathology of all three types of cancers. This knowledge will support the comprehension of the results presented in the last few chapters of the thesis.
**Figure 1.1:** The number of cancer registrations by the 24 major sites of the body, England, 2014\(^3\).
1.2 Prostate cancer

1.2.1 Statistics (incidence, mortality, survival rates)

Prostate cancer is nowadays the most common male cancer in UK (2010), as a quarter of all new cases of cancer diagnosed in UK men are prostate cancers. There are more than 40,000 new cases every year (47,000 only in 2013) and 54% of men who are diagnosed are over 70 years old\(^4\). Only 1 in 14 men who are diagnosed with prostate cancer die from the disease (10,837 deaths in 2012). Over the last 20 years, the number of men who have been diagnosed with prostate cancer has increased. However, the mortality only slightly decreased, which means that many of those men may not have benefitted from that diagnosis (fig. 1.2).

![Prostate cancer incidence and mortality rates, England, 1971-2010](image)

**Figure 1.2:** Prostate cancer incidence and mortality rates, England, 1971-2010\(^5\).

The prevalence of this disease is so high that it could be considered a normal age related phenomenon\(^6\). On the whole, 70% of men over 80 years have histological evidence of cancer in the prostate\(^7\). Therefore, a very high number of men diagnosed with prostate cancer die with the disease, but not from it.
1.2.2 Anatomy and physiology of prostate gland

Prostate location and function

In a healthy adult man, the prostate gland has the size of a walnut (weighing 20-40g) and the shape of an inverted cone. It is located within the pelvis, with its base proximally at the bladder neck and its apex distally at the urogenital diaphragm. It lies anterior to the rectum, with the urethra running through its centre (Fig. 1.3) and serving as an important reference landmark.8

Figure 1.3: Side view of the prostate9

The prostate gland has multiple functions as it helps to control the flow of urine and in the same time secretes fluid that nourishes and protects sperm. During sexual activity, the seminal vesicles, which are attached to the prostate produce a protein that mixes with prostatic fluid which forms semen. The tubes (vasa deferentia) from the testicles carry sperm up to the prostate where sperm is mixed with the seminal vesicle and prostatic fluids. During ejaculation, the prostate squeezes this fluid into the urethra and therefore it is expelled.
Physiology of the prostate gland

In 1930, Lowsley described human prostate gland as a composition of diverse lobes: 5 main (posterior, middle, anterior and two lateral) and 2 smaller which do not exist in adults\textsuperscript{10}. Although that was a very useful topographic description, the specific separation was not consistent histologically. Fifty years later J. E. McNeal described a different concept which was based on the separation of prostate into zones rather than lobes\textsuperscript{11-13}. This description consists of the most widely accepted concept at the moment (Fig. 1.4).

![Diagram of prostate zones](image)

**Figure 1.4:** Zonal anatomy of the normal prostate as described by McNeal\textsuperscript{14}.

The peripheral zone comprises all the prostatic glandular tissue at the apex as well as all of the tissue located posteriorly near the capsule (Fig. 1.5). The transition zone comprises only 5-10\% of the glandular tissue in the young male and consists of two equal portions of glandular tissue lateral to the urethra in the midgland. The central zone, a cone-shaped area of the adult gland, forms part of the base of the prostate and it is traversed by the ejaculatory ducts. The anterior fibromuscular stroma (AFMS) forms the convexity of the anterior external surface. The apical half of this area is rich in striated muscle, which blends into the gland
and the muscle of the pelvic diaphragm. Toward the base, smooth muscle cells become predominant, blending into the fibers of the bladder neck\(^{15}\).

![Anatomy of the prostate](image)

**Figure 1.5: Anatomy of the prostate\(^{8}\).**

*Cell types in prostatic gland*

At histological level, the two generic categories of prostate cell types are epithelial and stromal cells. The stromal to epithelial ratio tends to be higher in healthy human prostate compared to cancerous\(^{16}\). The epithelial cell layer forms glands which are composed of differentiated basal, secretory luminal, transit-amplifying and small neuroendocrine cells\(^{17}\). The luminal cells are the major cell type in prostate and form a layer over the basal layer (Fig. 1.6). Contrary to basal cells, the luminal ones are androgen dependent. They also produce the prostate specific antigen (PSA) and are terminally differentiated\(^{18}\). The stroma surrounds the epithelial layer forming a boundary on the periphery of the prostate gland. The stromal layer contains muscle cells and fibroblasts\(^{19}\). Additional prostate elements include vascular cells, nerve cells, basement membrane plus tissue matrix.
1.2.3 Cancer pathology and grading

During prostatic carcinogenesis, both prostatic epithelium and smooth muscle dedifferentiation is promoted due to androgen-mediated signal loss between the two different types of cells\textsuperscript{19}.

Most prostate carcinomas are detected in the peripheral (75\%), but some of them (20\%) arise from the transitional zone of prostate gland\textsuperscript{20-23}. Cancer areas in the transition zone tend to be characterized by large irregular glands with tall columnar cells, pale-to-clear cytoplasm and basally located nuclei, whereas prostate cancer in the peripheral zone show small round glands with cuboidal cells, amphophilic cytoplasm and nuclei located to the centre\textsuperscript{24} (Fig. 1.7). On the other hand, anterior fibromuscular is not a usual layer for detection of pathological lesions mainly due to its lack of glands\textsuperscript{25}.

**Figure 1.6:** Prostatic cellular compartments and different prostatic cells\textsuperscript{18}. 

\[\text{Diagram showing prostatic cellular compartments and different prostatic cells.}\]
Figure 1.7: Prostatic adenocarcinoma of different zonal origins. (a) Mid-prostate transverse section with two separate foci of prostatic adenocarcinoma on transition (left) and peripheral (right) zone (b) Prostatic adenocarcinoma in the transition zone (c) Prostatic adenocarcinoma in the peripheral zone.

Prostate calcifications
A common finding during histological assessment of prostate gland is the presence of calcifications, especially in older ages (over 50). Although the mechanism of calcification formation is not fully understood yet, it is believed that they are a result of altered secretions of the luminal cells in the gland, leading to clogged ducts and inflammation. These secretions can be either related to the production of corpora amylacea in benign glands or crystalloids or mucin in cancerous glands. However, some of these processes are pathophysiological phenomena occurring during the aging process.

The pathogenesis of prostatic calcifications still remains unclear. Their size, number or location does not seem to be related to their malignancy. Many
reports support their association with inflammation of the prostate (prostatitis)\textsuperscript{30} or bacterial infection\textsuperscript{31}. Most of the calcifications in prostate are located in the transition zone (77.7\% of the total calcifications detected)\textsuperscript{32}. This supports their relationship to benign prostatic hyperplasia (BPH), since the majority of BPH arise from this zone\textsuperscript{33}. However, there is also evidence that prostate calcifications can occur in direct association with prostatic adenocarcinoma\textsuperscript{32}, although less frequently.

![Figure 1.8: Patterns of calcification associated with benign hyperplasia (A, B and C left) and prostatic adenocarcinoma (A, B, C and D right)\textsuperscript{32}.](image)

\textit{Gleason grading system}

In order to assess the stage and the progression of prostate cancer, Dr Donald F. Gleason developed the Gleason grading system in 1966, based on the study of nearly 3,000 patients’ biopsies. Nowadays, it is the strongest prognostic factor of a patient’s time to progression. The natural history of the disease correlates well with its histological grade, assessed by Gleason score.

Gleason grading system is based entirely on the histological pattern of arrangement of carcinoma prostate cells in H&E-stained sections, observed under low magnification. The Gleason scoring system is defined by a scale of 1
to 10. The five basic grade patterns which are used to generate a histological score are shown in Figure 1.9.

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Small, uniform glands with minimal nuclear changes</td>
</tr>
<tr>
<td>2</td>
<td>Medium-sized acini, still separated by stroma but more closely arranged</td>
</tr>
<tr>
<td>3</td>
<td>The most common finding in prostate cancer biopsies, show marked variation in glandular size and organisation with infiltration of stroma and neighbouring tissues</td>
</tr>
<tr>
<td>4</td>
<td>Markedly atypical cells with extensive infiltration into surrounding tissues</td>
</tr>
<tr>
<td>5</td>
<td>Sheets of undifferentiated cancer cells</td>
</tr>
</tbody>
</table>

**Figure 1.9:** Gleason grades basic patterns\(^{34}\).

The five basic patterns above are used to generate a histological score, which can range from 2 to 10, by adding the primary grade pattern and the secondary grade pattern. The primary pattern is the one that is predominant in area (most common cell pattern), by simple visual inspection. The secondary pattern is the second most common cell pattern. In that way, the primary pattern is added to the secondary grade to arrive at a Gleason score. Consequently, the Gleason score possibilities range from 2 (1 + 1) up to 10 (5 + 5). If only one grade is in the tissue sample, that grade is multiplied by two to give the score\(^{34, 35}\).

In general, **low-grade cancers** (Gleason 6 or less) are typically small and slow-growing, confined to the prostate gland. **High-grade cancers** (Gleason 8 or more) grow faster and frequently invade through the prostate capsule, can directly infiltrate adjacent organs (seminal vesicles, bladder, rectum), and may disseminate to regional lymph nodes and by vascular invasion typically to bone, but also occasionally to lung and liver\(^{36}\).

Although the Gleason system is now widely accepted, there are several issues concerning it as a prognostic factor. Most notably, Gleason grading has inherent sampling errors since it is observer dependent and may vary depending on the level of experience\(^{15}\). Other issues that often arise are specimen being
undergraded\textsuperscript{37} or inability of Gleason score to represent efficiently prostate cancer heterogeneity\textsuperscript{38}.

\section{1.3 Breast cancer}

\subsection{1.3.1 Statistics (incidence, mortality, survival rates)}

Breast cancer is the most frequently diagnosed female cancer and the leading cause for cancer mortality in women worldwide, accounting for 1.38 million of newly diagnosed cancer cases and 14\% of the total cancer deaths in 2008\textsuperscript{39}. In UK, breast cancer is the most common type of cancer, with around 53,400 cases diagnosed only in 2013. Almost half (46\%) of breast cancer cases are diagnosed in females over 65, whereas the disease seems to be less common in the most deprived areas\textsuperscript{40}.

The risk factors related to breast cancer in developed countries are reproductive and hormonal factors, tobacco, alcohol and lack of physical activity\textsuperscript{41}. It has been estimated that exposure to modifiable lifestyle and environmental factors (alcohol, obesity, physical activity) is linked to at least 17\% of female breast cancer cases in UK (2010)\textsuperscript{42}.

Although the last twenty years, the survival rates have risen by 40\% (Fig. 1.10) due to early detection and improved treatment, the mortality rates still remain high. In 2012, around 11,600 women died from breast cancer. This number accounts for 32 women every day\textsuperscript{40}. 

25
Male breast cancer is far less common, with 1 in 870 men to be diagnosed with the disease during their lifetime (the equivalent frequency for women is 1 in 8). This accounts for 0.26% of the overall breast cancer cases.

1.3.2 Anatomy and physiology of breast

Breast location, function and physiology

The breast is a gland existing in both men and women, although more dominant on the latter. The female breast develops at puberty and changes through life in response to hormonal stimulation. Each breast lies over a pectoral muscle and consists of glandular, fatty and connective tissue. The anatomical structure of the breast reflects its function which is the production of milk.

In the adult woman, the mammary gland is composed of approximately 15-20 lobes, supported and held together by a loose mesh of fibers, called Cooper’s ligaments. Each lobe is divided into multiple lobules, which consist of clusters of alveoli containing mammary secretory epithelial cells. These cells, under the influence of hormone prolactin, are producing milk which is transferred to the nipple through small tubes, the lactiferous ducts (Fig.1.11). While the glands are dispersed through the adipose tissue, which consists the main volume of the breast, a thin layer of connective tissue (or fascia) surrounds it on the outside.
Figure 1.11: During lactation, milk moves from the alveoli through the lactiferous ducts to the nipple.

Other structures found in the breast are sensory nerves, blood vessels which are connected to the internal mammary artery and lymph vessels. The lymph vessels are a part of the lymphatic system, a vital part of the immune system useful for fighting infections. The lymph vessels circulate lymph fluid away from the breast and they drain it into small masses of lymphatic tissue in the area around the breast, the lymph nodes.

**Cell types in breast**

Breast tissue is composed of a number of different cell types. The ductal network of the gland is made of epithelial cells, whereas the fat area where the ducts are embedded, consists of adipocytes. In the gland, there is also a number of vascular epithelial cells, making the blood vessels, stromal cells (including fibroblasts) and immune cells. The main lactiferous ducts are lined by stratified squamous epithelium near the nipple and two-layer cuboidal epithelial cells in greater depths. The rest of the duct system consists of two different types of epithelium: one layer of luminal epithelial and one layer of basal/myoepithelial
cells. The luminal epithelium forms the ducts, whereas in the alveoli, the myoepithelial cells surrounds the cuboidal (also luminal epithelial) cells in order to help them secrete milk by contracting. The two different types of epithelium cells form a double layer which is embedded within the fatty stroma.

The mammary stroma consists of a number of components, such as adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells and extracellular matrix (ECM). The ECM contains myoepithelial basement membrane and connective tissue and its role is very important since it allows communication between stromal and epithelial cells. Disruption of this communication can promote breast cancer as we will see in the following section.

1.3.3 Cancer pathology and grading

Breast cancers can be divided into two groups depending on their origin: carcinomas which arise from the epithelial component and the sarcomas that originate from the stromal component of the breast. Carcinomas is the most frequently diagnosed type of breast cancer, with sarcomas to account for less than 1% of the total cases. Carcinoma can be either in situ or invasive, depending on if the abnormal cells have expanded outside of the mammary lobules and ducts. In situ carcinomas, which have not metastasized outside of the milk ducts yet, are believed to be the precursors of the invasive type.

Breast carcinomas can also be classified as ductal and lobular depending on if they have developed in the breast ducts or lobules respectively (Fig.1.12). The most frequently diagnosed invasive breast carcinoma is the invasive (or infiltrating) ductal carcinoma and represents 75-80% of the total invasive breast cancer cases. The second most frequently observed type is the infiltrating lobular carcinoma (10-15%). Although implied by the name, both of the carcinoma types have been suggested to originate in the terminal ductal-lobular unit.
Clinically, human breast cancers are also classified with three markers: estrogen receptor (ER⁺), progesterone receptor (PR⁺) and human epidermal growth factor receptor 2 (HER2⁺). These three markers can classify many breast tumours into one of the three molecular categories based on the presence or absence of the specific receptors on the breast cells: luminal (ER/PR positive), HER2 (HER2 positive), or basal-like (ER/PR negative and HER2 negative). Depending on the different subtype of the cell receptors, the breast tumour can be approached using a more specific treatment.

**Breast calcifications**

A number of primary breast cancer cases (30-50%) give evidence of calcified formations in the mammary gland, which are detectable with mammography and in many cases are the only evidence of malignancy before cancer metastasis. These calcifications are believed to be a product of cell mineralisation mechanism and appear in different forms (clustered, linear or scattered) and shapes. Their chemical composition also varies and can be either calcium oxalate (type I) or calcium phosphate (type II)- mainly hydroxyapatite (HAP). Calcium phosphate crystals are basophilic, whitish, generally ovoid or fusiform, and non-birefringent under polarized light, whereas calcium oxalate crystals are
of amber colour, partially transparent and polyhedral in shape\textsuperscript{59} (Fig. 1.13). The different types of crystals found in the breast appear to be directly related to their pathology. Calcifications with the colourless structure of calcium oxalate are not associated with carcinoma or epithelial hyperplasia but are only found in benign cysts or at most in non-invasive lobular carcinomas \textit{in situ}\textsuperscript{60}, whereas calcifications consisting of calcium phosphates and mainly HAP, can indicate either benign or malignant lesions and are a result of cellular degeneration or necrosis\textsuperscript{61}. Regarding HAP calcifications, it has been also suggested that they contain a higher amount of calcium carbonate when they are formed in malignant ducts rather than in healthy ones\textsuperscript{62, 63}.

![Figure 1.13](image)

\textbf{Figure 1.13:} Type I (A) and II (B) breast micro-calcifications observed in sclerocystic mastopathy and adenocarcinoma respectively\textsuperscript{59}.

\section*{1.4 Bladder cancer}

\subsection*{1.4.1 Statistics (incidence, mortality, survival rates)}

Bladder cancer was the ninth most common diagnosed cancer worldwide in 2012 with 430,000 new cases diagnosed\textsuperscript{64}. In UK, the disease seems to follow the same trend\textsuperscript{65}, rated as the ninth more frequently diagnosed cancer in 2013, with 28 newly diagnosed cases per day in 2013\textsuperscript{66}. Mortality rates have generally decreased the last twenty years in all of the age groups under 85\textsuperscript{66}. This is due to reductions in both smoking prevalence and occupational exposures known to cause bladder cancer\textsuperscript{67}.
Bladder cancer affects men three to four times more frequently compared to females, with a 14-fold variation in incidence internationally\textsuperscript{38}. The disease is both sex and age-related (Fig. 1.14), but it is also directly linked to many avoidable risk factors, such as smoking which is related to 37% of bladder cancer cases in UK\textsuperscript{66}.

\textbf{Figure 1.14:} Average Number of New Cases Per Year and Age-Specific Incidence Rates per 100,000 Population, UK\textsuperscript{68}. The blue and red curve show how the diagnosis rate changes with age for men and women respectively, whereas the bars indicate the number of cases for each age group.

Different types of bladder cancer can be related to different risk factors. Studies have shown that the majority of squamous cell carcinomas (2% of total bladder cancer cases) are associated with bladder infection specifically caused by the parasite Schistosoma\textsuperscript{69}. On the other hand, transitional cell carcinomas (TCC) which represent the majority of bladder cancer cases (93%), are associated with smoking\textsuperscript{39}. Another major risk factor for bladder cancer is occupation and exposure to certain chemicals\textsuperscript{69}.
1.4.2 Anatomy and physiology of bladder

Urinary bladder is a pear shaped organ located in the pelvis, just behind the pelvic bone, which stores all the urine produced by the kidneys and transferred there via two ureters, one for each kidney. Once the urine is stored there, they pass through the urethra, located at the neck of the bladder, and exit the body (Fig. 1.15).

![Bladder location and function](image)

**Figure 1.15**: Bladder location and function\(^{70}\).

Many tiny folds (rugae) line the inner surface of the bladder when it is empty of urine. When the bladder fills up, these folds disappear and the wall expands in such a way that it can store up to 500-600 ml of urine in a healthy person.

A healthy bladder is composed of multiple layers: urothelium, lamina propria, detrusor muscle and perivesical soft tissue (Fig. 1.16). Urothelium is the innermost lining of the bladder and consists of transitional epithelial cells which provide elasticity and protection from extreme pH urine to the bladder. Below the urothelium lies the lamina propria, a layer of connective tissue rich in blood vessels and nerves which supports and controls the bladder wall. The next layer is detrusor muscle which provides the bladder with the ability of contracting during urination in order to expel urine from the body. The detrusor muscle also forms the internal urethral sphincter, responsible for keeping the urine into the bladder.
The outer layer is perivesical soft tissue and consists of fat, fibrous tissue and blood vessels\textsuperscript{70}.

Figure 1.16: Bladder wall structure\textsuperscript{71}.

Histologically, there are three distinct layers identified in the bladder wall: the mucous membrane (mucosa) which consists of the transitional epithelial cells and the submucous coat (lamina propria), the submucosa, the muscular layer (detrusor muscle) and the adventitia, which is the outermost loose connective tissue\textsuperscript{72}.

1.4.3 Cancer pathology and grading

Most bladder cancers originate from the cells on the inner layer of the bladder, the transitional epithelium. Transitional cell carcinoma (TCC) represents the 90\% of the total bladder cancer cases diagnosed. Other bladder cancer types are squamous cell carcinoma and adenocarcinoma, with the latter to originate from cells that make up mucus-secreting glands infections. There is also papilloma, a type of benign urothelial neoplasm of low malignant potential.

Depending on the aggressiveness of the bladder cancer, it can be in situ or invasive. Invasive cancers start by penetrating through the layer of lamina propria and in a second stage they become “muscle-invasive” by travelling through the
detrusor muscle layer. The cancer can also develop outside of the bladder wall, invading into lymph channels or blood vessels. In that case we have metastatic cancer and it can travel to additional organs throughout the body. The invasive nature of the bladder cancer can be assessed more systematically with the TNM (tumor-node-metastasis) classification system. This however is out of the scope of this chapter.
Chapter 2: Diagnostic techniques

2.1 Introduction

A number of techniques are currently employed for cancer diagnosis at an early stage, where the symptoms are easier to be treated and the disease more likely to be cured. Common diagnostic techniques and treatments for prostate, breast and bladder cancer are presented in this chapter. Between several diagnostic methods, biopsy followed by histopathology, the gold standard in cancer diagnosis, and complementary imaging techniques are explored in terms of advantages and limitations. The background information presented in this chapter will help us evaluate the results and conclusions drawn in the following chapters of the thesis.

2.2 Prostate cancer

2.2.1 Current diagnostic techniques

Despite its ambiguous nature, the most commonly used method for prostate cancer screening is currently the PSA test. Once cancer is suspected, clinical examination (DRE) and biopsy, which is currently the gold standard, will be also performed. Depending on the cancer stage, imaging techniques might also be applied in order to check for metastasis throughout the body.

PSA test: The most common test provided for prostate cancer screening. PSA (Prostate Specific Antigen) is a glycoprotein enzyme secreted by both normal and cancerous prostate cells and the test is measuring the PSA levels in patient’s blood. Elevated PSA blood levels are expected to be connected with raised prostate cancer risk. However, although raised PSA serum levels (usually above 4 μg/l) are an indication for prostatic biopsy, this hypothesis may be misleading as they may be also increased in the absence of prostate cancer, due to a non-cancerous enlarged prostate (benign prostatic hyperplasia-BPH) or prostatitis (inflammation of the prostate gland). Statistically, in 85% of the cases an elevated PSA value is due to BPH and only in 12-15% is due to cancer.
Another limitation of PSA test is that it can be age and ethnicity-dependent and also lead to high rate of false positive results, resulting in overdiagnosis and overtreatment. Rates of overdiagnosis vary between different studies and have been shown to range between 29% and 44% for different ethnicities\textsuperscript{74}. In the past, two major landmark trials studying the effect of screening on prostate cancer mortality have taken place. European Randomized Study of Screening for Prostate Cancer (ERSPC) and US-Based Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial have shown that increase of diagnosis with PSA screening did not have any important impact on mortality rates in any age group\textsuperscript{75}. On the contrary, stress placed on the patients through overdiagnosis and overtreatment has increased.

Due to all these limitations, the application of PSA test can be controversial in some cases and its combination with other techniques is essential in order to accurately diagnose the disease.

**Digital rectal exam (DRE) and transrectal ultrasound (TRUS):** The physician uses either a gloved, lubricated finger (Fig. 2.1) or a small probe (Fig. 2.2) respectively to examine the lower rectum and check for abnormalities on the prostate gland. During digital rectal examination the doctor is looking for abnormalities on the prostate through physical examination by assessing the size and the texture of the gland, whereas during transrectal ultrasound the probe releases sound waves which are used to create a video image of the prostate. Because DRE and TRUS are only able to detect gross morphological changes on the prostate gland but cannot determine if the changes are due to prostate cancer or to a non-cancerous condition, these techniques are prone to false positive results (up to 20\%)\textsuperscript{76}. It should be noted here that the ultrasound can additionally detect calcifications found in the prostate gland\textsuperscript{77}, but without being able to confirm their malignancy.
Imaging techniques: Other techniques used in prostate cancer diagnosis are CT scan (Computed Tomography) and MRI (Magnetic Resonance Imaging). Both of them produce very detailed images of lymph nodes or other areas on patient’s body with the help of X-rays and strong magnetic field respectively. The images allow the doctor to check for swollen or enlarged lymph nodes, which might mean that prostate cancer has spread. A most recent advance is multiparametric MRI which seems to be a promising technique for detection, localization and characterization of prostate cancer, using parameters such as T1- and T2 -
weighted images, dynamic contrast, diffusion weighting, and proton spectroscopy\textsuperscript{80}.

**Biopsy followed by histopathology**: The definite diagnosis and classification of prostate cancer is only confirmed after prostate biopsy (Fig. 2.3), which is currently the gold standard for prostate cancer diagnosis. A prostate biopsy may be performed at the same time as the ultrasound and most commonly through the rectum (transrectal method), through the skin between the scrotum and the rectum (perineal method) and through the urethra using a cystoscope (transurethral method). During the procedure, prostate gland tissue samples from different areas of the gland are removed with a special biopsy needle or during surgery to determine if cancer or other abnormal cells are present. The tissue samples are looked at under a microscope in order to detect cell differentiation assumed that cancerous cells are shaped and arranged differently than healthy cells. The samples may also be submitted to staining so that the differences between them are more obvious.

![Transrectal ultrasound guided prostate biopsy](image)

**Figure 2.3**: Transrectal ultrasound guided prostate biopsy\textsuperscript{81}.
However, a negative prostate biopsy does not guarantee the existence of a healthy prostate gland. During biopsy, multiple samples from different areas of the prostate are taken in order to make an accurate diagnosis, but if a tumour is small enough, it can be missed during the procedure. Since only a very small percentage of the entire gland is sampled every time, there are many chances that the biopsy will conclude to a false negative result. This happens for at least 2-3 in 10 men tested (20–30%)\(^{82, 83}\). If a biopsy is negative it may need to be repeated and this may detect a cancer that was missed the first time.

**Immunohistochemistry:** Even though the diagnosis after biopsy can be usually made on cell morphologic features (growth pattern, nuclear atypia, absence of basal cells) it is sometimes difficult to reach a firm diagnosis by routine histological study\(^84\). Therefore, the application of immunohistochemistry to distinguish prostate cancer from benign growths and to confirm the diagnosis becomes helpful and necessary.

The immunohistochemical diagnosis of prostate cancer mainly depends on a group of biomarkers because there is not an individual highly specific and sensitive enough marker discovered yet. Immunohistochemistry for prostate cancer diagnosis usually includes at least one basal cell-specific marker, usually high- molecular-weight cytokeratin (HMWCK) and p63, and the prostate cancer-specific marker, alpha-methyl-CoA-Racemase (AMACR)\(^85\).

**Nomograms:** A predictive approach for prostate cancer diagnosis which takes into consideration results from the tests described above (biopsy, DRE, etc.) and the patient’s record, is nomograms. Nomograms are graphic representations of an algorithm which incorporates several predictors in order to predict a particular endpoint and they consist of sets of axes\(^86\); each variable is represented by a scale, with each value of that variable corresponding to a specific number of points according to its prognostic significance\(^87\). Nomograms help in assessing the nature of the disease and predict the risk and the possible outcomes of the treatment. Prostate cancer nomograms are regularly used by physicians to decide which treatment approaches will result in the greatest benefit for men at different stages of prostate cancer\(^88\).
2.2.2 Treatment options

Treatment options for prostate cancer are suggested by the doctor and depend on the stage of the disease. In early stages, active surveillance might be the best solution for young and old patients and patients with early disease are less likely to receive a radical treatment such as surgery and radiotherapy. Different parameters such as patient’s life expectancy (age), predicted natural history of the prostate cancer and treatment toxicity are usually taken into account before making a decision. The most common treatment approaches are:

**Radiotherapy:** Radiotherapy is a radical approach which uses high-energy rays (x-rays, gamma rays) or particles to destroy the cancer cells while damaging the nearby healthy tissues as little as possible. Radiation can be emitted either externally or internally (brachytherapy) from radioactive pellets inserted inside the body. Radiotherapy may be included in the treatment options combined with surgery in the case of locally advanced prostate cancer. Common toxicities from radiotherapy include urinary problems, tiredness, impotence in 50% of the cases, lymphedema and rectal bleeding.

**Prostatectomy:** Radical prostatectomy is the main type of surgery for prostate cancer, where the surgeon removes the entire prostate gland and surrounding tissue. Similar to radiotherapy, prostatectomy is usually suggested for cases of locally advanced prostate cancer and its potential outcome is directly related to the patient age. According to a 10-year randomized study carried out by The Scandinavian Prostate Cancer Group comparing watchful waiting versus radical prostatectomy, benefits of radical treatment for prostate cancer are only existent for patients younger than 65 years\(^9\).

For prostate cancer metastasis, radiotherapy and prostatectomy are not usually suggested. In this case, different treatment options could be effective. These options might include either hormones or chemotherapy. **Hormonal therapy** is suggested for the majority of patients, whereas **chemotherapy** may be used if hormones fail to confine the cancer. If the prostate cancer has metastasized to bones, bisphosphonate (zoledronic acid) therapy has been shown to delay symptomatic progression of bone metastases\(^9\).
Photodynamic therapy (PDT): Photodynamic therapy is a treatment that uses a drug (photosensitizing agent) which responds to a particular wavelength of light. When photosensitizers are exposed to a specific wavelength of light, they produce a form of oxygen, become cytotoxic and kill nearby cells. The photodynamic approach has been initially applied to superficial cancer, such as skin cancer, where the light is easier to reach. Attempts for application of PDT to interstitial cancers, such as prostate cancer, require the development of transparent fibers, through which the light will be transmitted and delivered within the tumour.

In the first step of PDT for prostate cancer treatment, a photosensitizing agent is administered to the patient either orally or intravenously. The agent is absorbed by cells all over the body but stays in cancer cells longer than it does in normal cells. After a certain period of time (usually 24 to 72 hours after injection), most of the agent has left normal cells but remains in cancer cells and this is when the tumour is exposed to a low power laser light which activates the drug. The light is usually delivered to the prostate through hollow plastic needles which fit into a perineal template (Fig. 2.4) while the patient is under general anaesthesia. When the photosensitizer becomes active due to light, an active cytotoxic form of oxygen is produced. In addition to directly killing cancer cells, PDT can damage blood vessels in the tumour, preventing in this way the cancer cells from receiving necessary nutrients and also activate the immune system to attack the cancer cells. In terms of side effects, PDT can cause burns, swelling, pain, and scarring in nearby healthy tissue. Although these side effects are usually temporary, further research and clinical trials are required in order to evaluate the use of PDT for prostate cancer treatment.
2.3 Breast cancer

2.3.1 Current diagnostic techniques

The most common technique for breast cancer screening and diagnosis is mammography. Currently screening mammography is suggested to every woman over 50 who has no symptoms in UK, as a routine procedure whereas diagnostic mammograms are used after suspicious signs of breast cancer. For younger ages and denser breasts, breast ultrasound scan can also be an option. Other techniques such as physical exam and MRI can be complementary to the mammography. However, the gold standard for breast cancer diagnosis is biopsy followed by histopathological analysis.

**Clinical examination**: A clinical breast examination is performed by a clinician and involves a visual check of any abnormal texture or lumps on the skin and breast tissue. Signs such as palpable lumps, skin thickening and nipple discharge can be quite significant for an early cancer detection. Clinical examination is always combined with imaging diagnostic techniques as it cannot guarantee alone an accurate diagnosis due to its very low sensitivity rates.
Mammography: Mammography is a low energy X-ray test of the breast, using ionizing radiation to create an image where signs of breast cancer such as characteristic masses and micro-calcifications can be early detected. During the procedure, the breast is compressed using a mammography unit (Fig. 2.5) in order to reduce the tissue thickness that x-rays will penetrate and increase the image quality. Mammography can be used for either screening or diagnostic purposes, with the latter to be more detailed and with additional views. Mammograms are less efficient to high density breast tissue.

![Illustration of a mammogram](image)

Figure 2.5: Illustration of a mammogram\textsuperscript{95}.

Although mammography is currently considered the standard practice for breast cancer screening in many countries, its application is controversial due its low specificity and high number of false positives obtained\textsuperscript{96}. Mammography is not able to investigate the chemical composition of suspicious elements detected in the breast (eg. calcifications) and therefore discriminate between malignant and benign ones, just by their morphology or location in the gland\textsuperscript{97}. The overdiagnosis of breast cancer results in 21.9% of total women screened for the
disease to be treated unnecessarily\textsuperscript{98, 99}. This constitutes not only a psychological hardship to women but also to a financial burden to the health service. At the same time, mammograms also give a high number of false negative results (around 25\%), especially in younger ages, where breast density is usually higher. As tumours have similar density to fibroglandular rather than fatty tissue, they can be harder to detect in women with denser breasts using mammography\textsuperscript{100}. Another possible risk of mammography is the radiation exposure which seems to be high both in young women, because of radiation’s cumulative effect, and for older women, since their breast epithelial cells are more prone to low dose radiation damage\textsuperscript{101}. Due to the controversy around mammography application, a more individual approach to breast cancer diagnosis has been suggested by the scientific community\textsuperscript{96}.

**Ultrasound:** Ultrasound uses high-frequency sound waves to produce an image of the internal structure of the breast tissue. Contrary to ionizing radiation used by mammography, these waves are not harmful to the cellular DNA. Ultrasound is an inexpensive technique which is more commonly used in combination with other techniques, in women where mammography is not applicable due to their breast tissue density and has a potential to detect small node negative cancers\textsuperscript{102}.

An ultrasound examination is usually able to reveal if an abnormal element in the breast is a solid lump or a cyst but it cannot identify the malignancy of a tumour or detect the presence of calcifications with great accuracy due to lack of contrast between normal parenchyma and the calcifications\textsuperscript{103}. Other disadvantages of ultrasound application for breast cancer diagnosis include the need of highly qualified staff to perform the examination and lack of standardized scanning protocols\textsuperscript{102}.

**MRI (Magnetic Resonance Imaging):** MRI provides an image of the breast tissue using magnetic field and radio waves. MRI has been proven to be significantly more sensitive compared to mammography and ultrasound\textsuperscript{104} as it is not affected by high breast density\textsuperscript{105}. However, due to its low specificity compared to mammography, MRI generates a very high number of false positive results\textsuperscript{106}. Its high cost and rates of overdiagnosis do not allow MRI to be the first
choice for breast cancer diagnosis. It is still very useful though in collecting information about a suspicious area which has already been proved to be malignant using other techniques.

**Biopsy followed by histopathology:** Biopsy is currently the gold standard for breast cancer diagnosis. During the procedure, a small amount of breast tissue is removed from the gland (Fig. 2.6) and examined in terms of morphology under the microscope by a specialized pathologist. There are different types of biopsy: fine needle aspiration biopsy (FNAB), open surgical biopsy and core needle biopsy which can be assisted by x-ray (stereotactic), MRI or a hollow probe communicating with imaging systems (vacuum-assisted).

![Figure 2.6: Fine needle aspiration biopsy for breast cancer diagnosis](image)

In fine needle aspiration biopsy, a very thin hollow needle is inserted into the gland in order to collect a small number of cells or liquid in the case of a cyst. Because the biopsy volume is very small (only a few cells) there is a risk of missing the cancerous area and conclude to false negative results. The chances to detect the malignant area are higher in core needle biopsies, where breast tissue is collected from different locations on the gland, with a bigger needle compared to FNAB. In certain cases, where open surgical biopsy is required, a significant part (incisional) or the entire (excisional) abnormal area may be removed and examined under the microscope. In that case, a complementary amount of tissue (margin) around the abnormal area may also be excised in order to check for cancer cells having metastasized.

Another inherent limitation of breast biopsies is the histopathological interpretation of the samples excised from the gland. This can lead to a high rate
of false negative results especially in the presence of tissue heterogeneity\textsuperscript{108,109}. In order for the biopsy to be as precise as possible, it usually needs to be histopathologically assessed by more than one pathologist and combined with complementary imaging techniques.

2.3.2 Treatment options

Different treatment approaches for breast cancer include surgery, radiotherapy, chemotherapy, hormone therapy and biological (targeted) therapy. These treatments are used individually or in combination, depending on the grade and stage of cancer for each case.

The most common type of treatment for breast cancer is \textbf{surgery}, which involves removing the tumour and nearby margins from the breast tissue. The removal of the margins is important so that tissue further away from the tumour can be checked for cancer cells and therefore for assessing if the cancer is safely removed. The type of surgery for breast cancer can be either lumpectomy or different types of mastectomy, depending on the breast tissue amount required to be removed. Lymph nodes can also be occasionally removed in order to check for possible metastasis.

A second common treatment for breast cancer is \textbf{chemotherapy}. Chemotherapy uses a combination of anti-cancer drugs to attack the tumour and either destroy the cancer cells or slow down their growth rate. Due to the pharmacological targets of the drugs used for breast cancer (DNA, RNA), cells that multiply fast are affected either if they are cancerous or healthy. Because of that, cytotoxic drugs induce a number of side-effects such as hair loss, nausea and vomiting, reduction in blood cells, tiredness and weakness. Chemotherapy is usually combined with other treatments in order to assist, for example, a tumour to shrink just before surgery.

When \textbf{radiotherapy} is used for treatment, high-energy radiation is targeted to a specific part of the body in order to kill cancer cells by permanently damaging their DNA. The main side-effects originate from the damage to adjacent healthy tissue, but are only short-term since the healthy cells are able to repair themselves easier. The radiation therapy can be delivered to the tumour either externally, using a linear accelerator, or internally by inserting a radioactive
material inside the breast tissue and close to the tumour. Radiotherapy is most frequently used in combination with chemotherapy or before and after surgery to minimize the tumour size and destroy any cancer cells left behind, respectively.

A major part of breast cancer diagnosis relies on the identification of hormones receptors on tumour cells and the subsequent treatment with hormone therapy. These hormone receptors can be either estrogen (ER) or progesterone (PR) receptors and their existence on the cell membrane of tumour cells provides potential for the tumour to respond efficiently to hormone therapy. In this case, hormone blockers are used in order to inhibit the hormone supply to the cancer cells. If no hormone receptors are present on the tumour cells, the cancer is “hormone-receptor-negative” and it is more likely that a hormone therapy will be ineffective.

A more recent approach to breast cancer treatment is the targeted therapy. This type of therapy employs targeted drugs, usually monoclonal antibodies, which aim at a specific target on the cancer cells. For example, women who have been identified with HER2 (human epidermal growth factor receptor 2) receptors on their cancer cells can benefit from trastuzumab, a monoclonal antibody specifically targeting this type of receptor. Other targeted therapy drugs include bevacizumab, which reduces cancer cells' blood supply, and lapatinib which interrupts both the HER2 and the EGFR (epidermal growth factor receptor) pathways.

### 2.4 Bladder cancer

#### 2.4.1 Current diagnostic techniques

Similar to breast cancer and prostate, the gold standard for bladder cancer diagnosis is biopsy followed by histopathology. Cystoscopy is the main technique used for both screening and collection of biopsies, whereas a number of imaging techniques are used for assessing the cancer stage.

**Cystoscopy:** Cystoscopy, the current gold standard for bladder diagnosis, is the procedure where the specialist checks for abnormalities on the inner wall lining of the bladder using a cystoscope (a thin flexible tube with a light and camera on the end). Cystoscopy can be accurate in identifying benign over malignant
bladder lesions by their appearance, but if it is not followed up by biopsy, it is less precise in discriminating between different grades of malignancy\textsuperscript{110}. The procedure is performed under local anaesthesia when the patient is checked for the first time and under general anaesthesia when a biopsy collection is performed.

**Imaging techniques:** Techniques such as ultrasound, computerised tomography (CT) and intravenous urogram (IVU) are used to check any abnormalities in the urinary system (bladder, ureters, kidneys). Other imaging techniques such as MRI and bone scans are used to check if the bladder cancer has spread to the bones or rest of the body.

**Photodynamic diagnosis (PDD):** Photodynamic diagnosis is a more advanced approach of standard cystoscopy, where a light-sensitive blue dye is added beforehand and absorbed by the abnormal cells in the bladder. During the procedure, a blue (instead of white) light is shined onto the bladder lining and make the cancer cells fluoresce, helping in this way the specialist to detect the cancerous areas easier. PDD has been mentioned to be superior to white light cystoscopy\textsuperscript{111, 112} in terms of sensitivity, especially for superficial TCC tumours\textsuperscript{113} but it is also characterized by low specificity rates\textsuperscript{113, 114}.

**Biopsy followed by histopathology:** If during cystoscopy any abnormalities are detected in the bladder, a second cystoscopy under general anaesthetic is performed. During this procedure, small samples of tissues are taken from areas that look abnormal or even some healthy areas in order to ensure an accurate diagnosis. Cystoscopy followed by biopsy can usually result in a high rate of false positive, especially when PDD is applied, due to its limited ability of discrimination between cancerous and inflamed areas\textsuperscript{115}.

**Urine tests:** These are non-invasive enzyme immunoassays applied directly to patient’s urine in an attempt to detect urinary tumour markers for bladder cancer diagnosis. Although these markers show high sensitivity, they lack specificity\textsuperscript{116, 117} and a significant positive predictive value\textsuperscript{118}. For example, NMP22 which is a type of nuclear matrix protein released in higher amounts in urine of patients with
TCC, gives a high rate of false positive results because its expression tends to be affected by various pathological conditions\textsuperscript{118,119}, which leads to low specificity rates. Concerning the consistent application of urinary biomarkers, the combination of a number of them seems more promising for an accurate bladder cancer diagnosis, but cannot replace cystoscopy\textsuperscript{120,121}.

2.4.2 Treatment options

Depending on the stage of bladder cancer, different treatment approaches are used. The most common treatment involves Transurethral Resection of Bladder Tumour (TURBT) for non-muscle invasive cancer and cystectomy for muscle invasive bladder cancer.

Non-muscle invasive cancers account for the 70\% of the total bladder cancer cases and is a superficial type of cancer, where the malignancy has not been developed through the deeper muscle layers. These types of cancers are initially treated with Transurethral Resection of Bladder Tumour (TURBT) which is the surgical removal of suspicious or abnormal areas through cystoscopy. TURBT can be followed by complementary treatment in order to minimize the chances of a recurring cancer. One of these treatments include BCG (Bacille Calmette-Guerin), a vaccine for tuberculosis, which can stop the bladder cancer from spreading.

Different approach is adopted for more aggressive types of bladder cancer, which start by penetrating through the bladder wall and eventually reaching and travelling through the muscle layer (muscle invasive cancer) (see chapter 1). For the treatment of this aggressive cancer type, the removal of the bladder (cystectomy) and surrounding organs can be suggested and complementary treatments such as chemotherapy might follow.

2.5 Discussion

The current imaging techniques presented above can significantly assist in prostate, breast and bladder cancer diagnosis, after having their risks and benefits assessed by specialists for each individual case. However, the successful treatment of cancer is directly dependent on the detection of the disease at its earliest stage and one major limitation of the current imaging
methods is the lack of ability to detect an abnormality in an early cancer stage, where all of the early changes during tumorigenesis begin. As a result, cancer is not treated until cancer cells have intruded to surrounding tissues and metastasized\textsuperscript{122}. Additionally, because these techniques have no insight into the molecular tissue level, they cannot discriminate benign from malignant cases.

The biopsy followed by histopathology is the only currently used diagnostic technique which can reveal structural changes on a molecular level during the disease progress. However, even if the biopsy is considered to be the gold standard for cancer diagnosis, it is an invasive technique linked to false negative results\textsuperscript{108} which can render the method partially unreliable. The false negative results derive from the fact that the volume of tissue removed during the biopsy is very small compared to the total tissue volume and not always adequate for an accurate histological evaluation. As a result, if the tumour is small, there is a significant chance of missing it during sampling. Another major limitation of histopathology is relatively low inter-observer reproducibility between different pathologists who are assessing the biopsies\textsuperscript{123, 124}. The evaluation of the cell morphology under the microscope can be quite challenging, especially in intermediate cancer stages, and can therefore be subjectively assessed by different pathologists resulting in a wide variation of results.

Since the current applied techniques do not fully cover the current diagnostic needs, we need to consider different diagnostic approaches which will enable the specialists to detect molecular changes in the potentially malignant tissue more efficiently and in a minimally invasive way. The alternative approach should not only be able to assess the tissue content and provide molecular information of the area, but also to discriminate malignant from benign tissue.

One of the most promising novel diagnostic approaches is comprised of a group of optical techniques which take advantage of light-matter interactions in order to explore the quality and quantity of molecular components in different tissues. One of these techniques is Raman spectroscopy, a promising method which uses Raman scattering in order to analyse vibrational modes of molecules inside tissues. Due to its ability to provide the molecular fingerprint of the area scanned, Raman spectroscopy is able to discriminate between healthy and malignant areas, through their different molecular content. The applications and theoretical
background of Raman spectroscopy and the rest of the optical techniques are described in more detail in the following chapter.
Chapter 3: Novel optical diagnostic techniques

3.1 Introduction

In the last chapter we highlighted the disadvantages of the current diagnostic techniques for cancer. In this chapter we explore the application of optical and more specifically molecular vibrational spectroscopy, and novel approaches for cancer diagnosis.

A number of optical techniques such as elastic scattering spectroscopy (ESS)\textsuperscript{125, 126}, optical coherence tomography (OCT)\textsuperscript{127, 128}, fluorescence spectroscopy\textsuperscript{129, 130}, fluorescence lifetime imaging microscopy (FLIM)\textsuperscript{131} and diffuse reflectance spectroscopy (DRS)\textsuperscript{132, 133} have been studied widely for application in cancer diagnosis. However, these have their limitations, often associated with the application of these techniques. These include low specificity (ESS, fluorescence spectroscopy), low penetration depth and low specificity (OCT), low signal-to-noise ratio (FLIM), low accuracy (DRS) and invasive nature for most of them.

Vibrational spectroscopy, such as Raman and mid-infrared (IR), are strongly sensitive to the chemical composition of the sample and have been tested for application in the cancer diagnosis routine. Between the two techniques, IR is significantly affected by the presence of water in the tissue and therefore cannot be used in non-invasive measurements \textit{in vivo}. In contrast, Raman (near-infrared wavelengths) remains largely unaffected by water content and for that reason it can be used directly in real-time for non-invasive measurements. Due to its high specificity and sensitivity, Raman spectroscopy is currently applied in a number of applications including material characterization, security\textsuperscript{134}, pharmaceutical industry\textsuperscript{135}, art history\textsuperscript{136} and geology\textsuperscript{137}. Between the numerous applications, here we will focus on how this emerging field can contribute in cancer diagnosis.
3.2 Theoretical background

3.2.1 Light propagation in biological samples

Photons propagate through tissues in a number of different ways. They can travel through the sample in a random walk, being scattered from the tissue molecules or being absorbed by them.

In the case of scattering, light photons interact with matter and change their direction, with or without losing energy. The more scattering the medium is, the more the photons will deviate from the original forward direction (Fig. 3.1).

In the case of absorption, the photon will be absorbed only when the energy of the photon matches the energy gap between the initial and the final energy states of the atom or molecule. Energy from the photons which is absorbed, is often dissipated in the tissue as thermal energy. If the energy gap between energy levels in the molecule, is substantially different to the photon energy, then the photon will not be absorbed.

How efficiently the photon will travel through the tissue, depends on the fraction of light which will be absorbed or scattered by it, which is described principally by the scattering and absorption coefficients. The scattering coefficient ($\mu_s$) depends on the probability of the medium per unit length to redirect the incident photons into new directions and therefore prevent the forward on-axis transmission of light. The average distance that a photon travels between scattering events is called mean free path length. Similarly, the absorption
coefficient \((\mu_a)\) describes the probability of the medium per unit length \((\mu_a)\) to absorb the photons\(^{138}\).

Because in biological tissues the light is not scattered in all possible directions but is mainly forward directed (anisotropic scattering)\(^{139, 140}\), it is convenient to define a mean scattering angle which is described by the value of anisotropy \((g)\)\(^{141}\). In order to be more precise in the description of scattering in biological tissues, scattering coefficient and anisotropy are combined into the expression of reduced scattering coefficient which is defined as:

\[
\mu'_s = \mu_s (1 - g) \quad (3.1)
\]

The optical properties described above \((\mu_a, \mu_s, \mu'_s, g)\) are characteristic of tissue in general and range much for different types of human tissue. These can provide useful information on each tissue. For example, absorption coefficient is related to the concentration of various chromophores inside the tissue, whereas scattering coefficient describes the form, size and concentration of the scattering components in the tissue\(^{142}\).

### 3.2.2 The Raman effect

Raman scattering is a specific case of photon scattering, where the photon is scattered in a medium with a change in its energy (inelastic scattering). When incident light falls onto a material surface, it will be typically scattered in random directions. The vast majority of the light will be elastically scattered, maintaining the same energy (i.e. frequency). A very tiny amount of the light (less than 0.001\%)\(^{143}\) will be scattered with slightly different frequency from the incident light (inelastic scattering). The difference in the frequency is characteristic of the bond vibrations of the molecules and provides important chemical and structural information on the material.

The Raman effect was first discovered while C.V. Raman in India was trying to explain the blue colour of the sea in 1921. Since then, the effect was studied and finally established in 1928 in liquids by C.V. Raman and K.S. Krishnan and independently in crystals by G. Landsberg and L. Mandelstam a few years later.
For the discovery of the Raman effect, C.V. Raman was awarded with the first ever Nobel prize to an Asian scientist in 1930.

As light can be both a wave and a particle in nature, the Raman effect can be interpreted in two ways: the wave interpretation and the quantum particle interpretation. In the first case, light is considered to be a wave with an oscillating electric field which interacts with a molecule through its electron cloud. When the light interacts with a molecule, vibrating at a frequency given by its bond strength, it induces a dipole moment ($\mu_{\text{ind}}$) which is equal to the product of the electric field of the incident light ($E$) and the polarizability $\alpha'$ (how easily distorted the molecule’s electron cloud is by an electric field). The polarizability of the molecule depends on the number of the electrons, their distance from the nucleus and how strongly attracted they are to it. The Raman effect depends on polarizability change of molecule during its vibration.

In the quantum mechanics interpretation, light is considered to be a photon which interacts with a molecule and loses or gains energy (inelastic scattering). The possible outcomes from an incident photon hitting a molecule are described in Figure 3.2. Initially the molecule is excited to a virtual state and at a second stage there are three possible outcomes. Most frequently, the molecule will relax back down to the initial ground state, scattering a photon of equal energy to that of the incident photon (Rayleigh scattering) (Fig. 3.3). More rarely, the molecule will either relax to a real vibrational state and emit a photon with less energy than the incident photon (Stokes) or emit back a photon with higher energy after having already been in an excited vibrational state in the first place (anti-Stokes). As most of the molecules are initially found in the ground state at room temperature, it is more likely for a photon to be Stokes rather than anti-Stokes.
Figure 3.2: Three of the ways in which the first electronic excited state can return to the ground state: vibrational relaxation, fluorescence (short-lived photon emission) and phosphorescence (long lived photon emission)\textsuperscript{144}.

Figure 3.3: Jablonski diagram representing quantum energy transitions for Rayleigh, Raman scattering and IR absorption\textsuperscript{145}.
The intensity of the Raman scattered light is proportional to the Raman cross-section of the material \( (\sigma_j) \), which describes the efficiency of the molecule to scatter an incident photon as a Raman-shifted photon with a particular Raman shift\(^{146}\). The Raman scattering intensity is related to the Raman cross-section and laser intensity \( (I_0) \) by the following formula:

\[
I_R = I_0 \sigma_j D dz \quad (3.2)
\]

Where \( D \) is the number density of scatterers and \( dz \) is the path length of the laser in the sample\(^{146}\).

The power of Raman scattering is also linearly related to the intensity of the incident photons and inverse of the photon wavelength to the fourth power:

\[
P_s \propto I_0 / \lambda^4 \quad (3.3)
\]

Unfortunately, as the fluorescence process increases with shorter wavelengths, it is not always possible to achieve the best result as the measurement can be inhibited by fluorescence. The process of fluorescence involves the emission of a photon from an electronically excited state, after absorption of an incident laser photon, after having lost typically some of the (excess) energy through vibrational relaxation\(^{144}\), as shown in Figure 3.2. This process is likely to occur with shorter wavelengths as it necessitates the presence of electronic absorption which are more likely to be present in visible/UV region of the optical spectrum. The emitted fluorescence photons can create excessive background which can swamp weak Raman signals. Another two mechanisms by which an electron in an excited state can return to the ground electronic state are the vibrational relaxation, where the energy of the electron is given away to other vibrational modes as kinetic energy, and the intersystem crossing, where the electron changes spin multiplicity from an excited singlet state to an excited triplet state with the possibility to lead to phosphorescence (Fig. 3.2).

Another type of molecular process, where electrons do not possess enough energy to jump to the first excited state, include Raman scattering, Rayleigh scattering and absorption. Whereas Raman and Rayleigh scattering have been
explained earlier, infrared (IR) absorption process consists of absorption and emission of photons on the vibrational levels of the electronic ground state, (Fig. 3.3). Raman and IR spectra consist of characteristic “fingerprints” for the chemical molecules and can complement one another because different bond vibrations can have different IR and Raman activity. The main difference is that in order for a bond to be IR active, a change in the dipole moment is required. On the other hand, in order for a bond to be Raman active, a change in polarizability is required, as described earlier. Both of the cases usually depend on the elements of symmetry of the molecule. Raman active bonds tend to be symmetric vibrations, whereas IR activity is usually observed with polar bonds and asymmetric vibrations.

The basic bond vibrations that one can find in the molecules can be of two types: stretching vibrations where the bond atoms are successively approaching and moving apart so that the distance between them changes along the axis of the bond and bending vibrations where the angle changes between two bonds. Bending vibrations can be scissoring, rocking, wagging or twisting (Fig. 3.4).
3.3 Instrumentation

After the discovery of Raman scattering, the scientific community took benefit of the new knowledge so fast that Raman spectroscopy was being applied since 1930 and flourished around late 1960s when laser sources became available. Today the principal on which Raman spectrometers operate remains the same (Fig. 3.5) and it is mainly the illumination of the sample and the collection of the
Raman scattering that varies in configuration between the different Raman techniques.

![Simplified diagram of a Raman spectrometer’s operation](image)

**Figure 3.5:** Simplified diagram of a Raman spectrometer’s operation\textsuperscript{147}.

Most of the modern compact Raman spectrometers are either dispersive or non-dispersive depending on if they use a prism/grating or an interferometer (Fourier transform based spectrometers) respectively. Both the types comprise of: a monochromatic light source (laser), a sample area with collection optics, spectral analyser unit and a detector.

**Light sources**

In Raman spectroscopy, any kind of electromagnetic radiation can be used as a light source, including filtered sun light, which was at the core of the discovery of the Raman effect\textsuperscript{148}. Currently, the main light sources used are lasers because they can provide intensity high enough to produce a sufficient amount of Raman scattering. Laser wavelength used in Raman spectroscopy can typically range from UV (<300 nm) to near-infrared (NIR) (1064-1700 nm) (Table I). Lasers of wavelength less than 900 nm are commonly used in Raman dispersive systems, whereas those with 1064 nm and above are usually non-dispersive Fourier transform (FT)-Raman systems\textsuperscript{146}. Shorter wavelengths in near-infrared (NIR) region are generally more efficient since Raman intensity is inversely proportional
to the fourth power of the laser wavelength and also more effective detectors can be used (CCD's). However, as the laser wavelength decreases, the risk of fluorescence increases. This is why for measuring biological samples an intermediate laser wavelength (usually between 532 and 830 nm) is selected as a compromise, depending on the nature of the sample.

<table>
<thead>
<tr>
<th>Laser Type</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubled Ar+</td>
<td>244</td>
</tr>
<tr>
<td>Ar+ (air cooled)</td>
<td>488</td>
</tr>
<tr>
<td>Ar- (water cooled)</td>
<td>351-528</td>
</tr>
<tr>
<td>He-Ne</td>
<td>632</td>
</tr>
<tr>
<td>Kr+</td>
<td>413</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>1064</td>
</tr>
<tr>
<td>Doubled Nd:YAG</td>
<td>532</td>
</tr>
<tr>
<td>Diode</td>
<td>670-865</td>
</tr>
<tr>
<td>Ti:saphire</td>
<td>700-1100</td>
</tr>
</tbody>
</table>

Table I: Commonly available continuous lasers for Raman spectroscopy\(^{146}\).

The most commonly used laser sources for biological samples are the semiconductor diode lasers. These lasers use electric current to excite the active medium and amplify the light. Because diode lasers can be very small in size, power efficient and inexpensive, they are quite attractive for Raman spectroscopy. They also operate in a range of wavelengths in NIR, an area suitable to avoid fluorescence and still achieve high CCD detector performance.

Another type of light source are tunable lasers, which can be, for example, pumped by another laser and their wavelength can be selected by the user (e.g. Ti:sapphire laser). Laser light can be brought to the sample either through a microscope objective (Raman microscopy) or directly, e.g. in a 90\(^{\circ}\) or 180\(^{\circ}\) scattering geometry.

**Sample area with collection optics**

Once the sample is placed on sample stage, the light has to be transferred from the source to the sample. For that reason, optics relaying the laser line onto the
sample and filters rejecting the laser spectral "wings" (amplified spontaneous emission, ASE) are located before the sample holder. In order to achieve this, a bandpass filter transmitting only a narrow range of wavelengths around the laser line, would be typically used.

The light scattered from the sample has to be then collected from the sample. Again depending on the different Raman technique, the Raman scattered light can be collected in different configurations. In any case though, there are filters which are used for rejecting the intense elastically scattered light (Rayleigh) and allowing the relatively weak inelastic Raman scattered light to reach the detector. The above filters used are either notch or edge filters.

An edge filter is a long pass optical filter which rejects wavelengths up to a certain point, and then transmits with high efficiency wavelengths above this point. As a result, an edge filter could block Rayleigh (and anti-Stokes) scattered light and allow Stokes to reach the detector. The notch filters have sharp, discrete absorption which can be chosen to coincide with a specific laser wavelength and is typically a few nanometres wide. As a result, the laser line is reflected but the Raman spectrum (Stokes and anti-Stokes) will be transmitted. The great advantage of notch over edge filter is that it allows both Stokes and anti-Stokes Raman scattered light to pass and reach the detector.

**Light dispersing unit**

In dispersive Raman spectrometers the scattered Raman light is projected and separated into different wavelength components on the detector using a dispersion grating. For example, gratings can be fabricated using densely engraved or imprinted lines or grooves into the surface, which then disperse the incoming light. The higher the number of grating lines per unit length, the higher the dispersion and the higher the spectral resolution achievable.

**Detector**

Dispersive and Fourier transform Raman instruments are using different types of detectors in Raman spectroscopy. Whereas FT spectrometers use liquid nitrogen cooled sensor, the current detectors for dispersive Raman spectrometers typically use Charged-Coupled Devices (CCD). These detectors are often in a form of a two-dimensional arrays of elements (typically < 30μm)
made of semi-conductive material (silicon). When the detector is exposed to light, electron-hole pairs are created in the silicon. The number of photo-electrons (charge) acquired is directly proportional to the number of photons falling on each element. At the end of the measurement, the charge is quantified in an analogue-to-digital convertor. In this way, each pixel acts as an individual detector, so each dispersed wavelength is detected by a different pixel (or closely spaced group of pixels). CCD detectors commonly have a large wavelength response region, routinely extending from 400nm up to approximately 1000nm.

The CCD detector performance can be characterised by generated noise which consists of pixel noise and fixed pattern noise. **Pixel noise** is the variation in the values of a charge read on a pixel and has three main constituents: readout noise, ‘dark’ noise from the dark signal and photon shot noise from the light signal itself. The readout noise is due to the amplifier and electronics and is present on every read out. The ‘dark’ noise depends on the exposure time of the measurement and is due to basic physical laws of charge migration in and out of the measurement region. This noise is dependent on the temperature of the detector which is confronted by cooling of the sensor (e.g. to -75°C). The **fixed pattern noise** is a constant noise which is due to the variation in the dark signal produced by each individual pixel and also small irregularities during the fabrication of CCD. These variations remain stable from measurement to measurement and as such can be subtracted away if accurately measured.

Another important characteristic of CCD detectors is the **quantum efficiency** which is directly related to the spectral response of CCD. The quantum efficiency describes the probability of a photon of a certain frequency to excite a photo-electron in the silicon, i.e. to be detected. The quantum efficiency of a detector depends both on the wavelength of the photons and on the absorption coefficient of the silicon.

Three types of CCD technology are widely used: front-illuminated, back-illuminated and back-illuminated deep depletion. **Deep depletion CCDs**, fabricated from thick silicon substrate of high resistivity, are the ones which offer a maximum efficiency to NIR scattered radiation and are usually an optimum solution for NIR dispersive Raman systems.
3.3.1 Raman microscopy

Raman microscopes are currently the most common configuration of Raman spectroscopy employed in research. A Raman microscope is an optical microscope combined with an excitation laser, laser rejection filters, a spectrometer or monochromator and an optical sensitive detector (usually CCD).

![Schematic drawing of a Raman microscope](image)

**Figure 3.6:** Schematic drawing of a Raman microscope.

In a Raman microscope, a laser beam is focused on the sample through lenses (Fig. 3.6). The scattered light is collected typically in a back-scattering geometry using the same objective that directs the light onto the sample. The scattered light travels through filters in order for the weak Raman light to be isolated from the Rayleigh (elastic) scattering. The collected Raman scattering is then focused on a dispersion grating which separates it into single wavelength components. After the scattering light has been separated into individual spectral components, it is projected onto the detector (usually CCD), which then generates the measured Raman spectrum.
3.3.2 Deep Raman spectroscopy

The conventional Raman microscopy is only able to collect Raman scattering up to a depth of a few hundred micrometres from tissue (depending on the sample). Deep Raman spectroscopy, an emerging set of techniques, is employed for collecting the mean Raman signal from diffusely scattering samples of significant volume and depth (up to several centimetres)\textsuperscript{149}.

Deep Raman spectroscopy can be either of temporal or spatial approach. In the first case, scattered Raman light is being collected from subsurface sample depths (Fig. 3.7) using temporal gating, by taking advantage of a medium which causes light to rotate its polarisation transiently (several picoseconds) by 90\textdegree{} in a nonlinear optical medium (Kerr medium). Kerr gated spectroscopy enables to collect the weak Raman scattering from sublayers, by rejecting the strong surface signal. The Kerr gate setup can be also used to reject sample fluorescence (Fig. 3.8), which usually has a longer lifetime compared to Raman scattering. Because of that, Raman light can change polarization while passing through the active Kerr medium and be detected as soon as the laser pulse strikes the Kerr medium. However, since the Kerr gate anisotropy is only short lived, any fluorescence arriving after the Raman light is not subjected to polarization change and is prevented from going through the second polarizer.

![Figure 3.7](image)

**Figure 3.7:** Schematic diagram of the 4 ps Raman Kerr-gated setup for the discrimination of sub-surface layers\textsuperscript{150}.
Unfortunately, Kerr-gating Raman spectroscopy has limited use in medical applications due to high instrumental complexity, cost and safety limits. For this reason, a range of geometrical optical approaches of deep Raman spectroscopy have been developed\textsuperscript{152, 153}, including: spatially offset Raman spectroscopy (SORS), inverse spatially offset Raman spectroscopy (i-SORS) and transmission Raman spectroscopy (TRS). This group of techniques benefits from both fluorescence suppression (where fluorescence is located near surface) and collecting weak Raman signals from deeper sample layers.

The setup configuration of these techniques is different from Raman microscopy in terms of illumination and collection geometry. In a basic SORS configuration, the laser light is brought onto the sample surface in the centre of a ring and the scattered radiation is collected from a ring or point zone (Fig. 3.9a). In i-SORS case, the sample surface is illuminated in a ring pattern with different diameters and Raman light is collected through a disk within the centre of the ring (Fig. 3.9b). The collection of Raman light can be facilitated using optical fibres or via free-space optical relay system. The ring-shape laser beam can be generated using a conical lens with an adjustable radius of the ring. In the case of transmission Raman spectroscopy, the laser beam illuminates the sample from...
the one side and the mean volume Raman signal is collected on the other side of the sample (Fig. 3.10b).

**Figure 3.9:** Schematic diagram of conventional SORS and inverse SORS (iSORS) showing Raman collection and beam delivery geometries.
Figure 3.10: Basic implementations of the (a) SORS and (b) transmission Raman concepts\textsuperscript{154}.

SORS technique relies on the optical properties of the diffusely scattering material and it is most efficient on samples with low absorption, such as at NIR with the majority of biological tissues. In a turbid medium, as excitation photons reach the sample surface, they are diffusely scattered and travel to deeper sample layers (Fig. 3.11). When the collection area is located in a distance from the excitation one on sample surface, the weak Raman photons from deeper layers can be collected. In SORS, the measurement is performed by collecting the Raman scattering from a spatially offset area to avoid the dominating excitation surface region signals. As the random photon diffusion takes place in the sample, greater depths correspond to a wider spread of photons around the illumination point on the sample surface\textsuperscript{155}. In this way, the greater the ring diameter is, the deeper the Raman photons are collected from.
Lasers commonly used in SORS systems are diode lasers with stabilized wavelength. Typical excitation wavelengths for biological tissues are within NIR region of the spectrum in order to minimize absorption and interfering fluorescence whilst staying within the detection range of CCD.

Conventional SORS technique can be limited in sensitive applications (e.g. \textit{in vivo} measurements) by concentrating all light into one zone. The \textbf{inverse SORS} (iSORS) variant, can comply better with safety limits since it distributes the same light over a wider sample area. For this reason, iSORS is a useful approach that improves certain measurements such as analysis of tissue \textit{in vivo}. In this case, as abovementioned, rather than using a spot collection geometry and a central collection spot for illumination, the collection offset is maintained by illuminating the sample with a ring of light and collecting the Raman signal from a central point.

The ring shaped laser beam can be generated using a conical lens (axicon) where the ring radius can be adjusted by changing the distance between the axicon and the sample\textsuperscript{153}, or by changing the magnification of lenses inserted
between the sample and the axicon\textsuperscript{156}. The radius of the ring defines the spatial offset and can be adjusted to the dimensions and scattering properties of the sample.

SORS techniques can currently achieve depths of many millimetres (~10 mm)\textsuperscript{157}. However, they are generally limited in samples with high absorption coefficients, since this leads to the reduction of photon migration distances and consequently achievable penetration depths. SORS is also limited in the case that the fluorescence from the sample is too high, as the weak Raman scattering cannot stand out and be detected. However, if the fluorescence originates from the sample surface, SORS can effectively avoid measuring it\textsuperscript{155}.

The third variant of deep Raman spectroscopy is \textbf{transmission Raman}. In that case the sample needs to be accessible from both sides, since the laser is shone upon it from one side and the Raman scattering is collected from the opposite side (Fig. 3.10b). During the measurement, the light is diffusely scattered through the sample randomly and as Raman photons can be created at all points that the light passes through, the total Raman signal measured on the opposite side is highly representative of the bulk sample volume. Transmission Raman can be considered as an extreme case of SORS, where the laser beam and the Raman collection zone are completely separated to the extreme (180°) as being on the opposite sides of the sample.

Contrary to SORS techniques, transmission Raman is unable to provide pure signatures of individual layers within a sample, since it mainly offers bulk averaged compositional information for the whole sampling volume within a single spectrum. Because of the bulk information that transmission Raman provides, surface signals can be suppressed\textsuperscript{158}. Up to now, transmission Raman has achieved sampling depths of up to 27 mm\textsuperscript{149}.

A number of different approaches have been applied to transmission Raman setups in order to enhance the signal to noise ratio (SNR) and therefore achieve higher measurement sensitivity and penetration depths. One way of achieving this is to use a “photon diode” which acts as a beam enhancing element. This unidirectional mirror allows laser photons to be transmitted through it on one side whilst laser and Raman photons re-emitted from the diffusely scattering sample to be reflected from it back into the sample due to the angular dependence of its reflectance properties\textsuperscript{159}. In this way, the “photon-diode” prevents the loss of
diffusely scattered photons which re-emerge from the sample at the point where the laser beam enters the sample, and enhances the Raman signal of it\textsuperscript{160}. Additional ways to improve the SNR in deep Raman measurements is the increase of the laser power, the collection time or even the optimization of the collection system efficiency, by using for example wider slit spectrographs in conjunction with high dispersion gratings\textsuperscript{161}.

3.3.3 Fibre optic probes

A number of Raman spectroscopy variations which have also been developed the last few years include: Surface-enhanced Raman spectroscopy (SERS), fibre optic probes, Resonance Raman spectroscopy, Spontaneous Raman spectroscopy (SRS), Optical tweezers Raman spectroscopy (OTRS), Stimulated Raman spectroscopy, Coherent anti-Stokes Raman spectroscopy (CARS), Raman optical activity (ROA) and Tip-enhanced Raman spectroscopy (TERS).

In the context of this thesis, it is worth elaborating on fibre optic probes. Fibre optic Raman probes can be employed to perform measurements in different modalities, as it has been demonstrated through a number of studies. They usually emit a near-infrared laser (750-850 nm) and they contain filters to suppress fluorescence, reflected excitation light and background signal from the fibres. One of the most common Raman probe designs consists of a silicon fibre bundle where the excitation light is emitted to the sample through a central delivery fibre and collected through a number of collection fibres surrounding the central one (Fig. 3.12). The number of collection fibres can vary in a great range\textsuperscript{167-171}, with one to be the minimum \textsuperscript{172,173} and 50 the maximum number demonstrated\textsuperscript{174}. 
Figure 3.12: Schematic of a typical Raman probe tip in a longitudinal view and a transverse cross-section at the fibre–filter interface.\textsuperscript{171}

Fibre optic technology can also be coupled with iSORS\textsuperscript{153} and SORS modality, providing a subsurface signal,\textsuperscript{174} by employing an annular probe where the excitation fibre is spatially separated from the collection ones.\textsuperscript{152, 175, 176} The extreme case of SORS, transmission Raman spectroscopy, can be also performed by employing a collection fibre bundle.\textsuperscript{158, 161}

Figure 3.13: Fibre optic probes employed in a conventional configuration (a), SORS (b) and transmission mode (c).
Raman fibre optic probes have been employed in a number of measurements on skin surface\textsuperscript{166, 177-179}. However, the Raman probe application is more challenging when it comes to accessing and measurement of remote organs, since more flexibility is required. These measurements usually require short acquisition time (less than 1 s), high signal to noise ratio and a limited probe diameter (up to 2 mm)\textsuperscript{171}. Despite their limitations, Raman probes have been employed in a number of studies during the last decade as described in the next section\textsuperscript{157}.

3.4 Novel vibrational spectroscopy in cancer diagnosis

A number of vibrational spectroscopy techniques have shown great potential for cancer diagnosis over the last decade. This is reflected on the literature with studies employing IR\textsuperscript{180, 181} and Raman spectroscopy for the detection of cancer on tissue samples, body fluids and cell cultures\textsuperscript{182-184}. Due to its water content dependence, IR spectroscopy has been mainly used on dried histopathological sections for the detection of several types of cancer such as oesophageal\textsuperscript{185}, gastric\textsuperscript{186}, colorectal\textsuperscript{187} and breast cancer\textsuperscript{188}.

Contrary to IR, Raman spectroscopy is not affected largely by water content since the $–\text{OH}$ vibrational modes of water molecules produce weak Raman signals\textsuperscript{189}. Consequently, Raman spectroscopy can be employed for real-time and non-invasive measurements on fresh samples without the need for fixation. Raman scattering is suitable for both qualitative, since it provides a chemical "fingerprint" of the sample, and quantitative tissue analysis, since the Raman scattering intensity is proportional to the concentration of the sample molecules.

Different modes of Raman spectroscopy have been employed for different types of samples. Conventional Raman spectroscopy, usually coupled with a microscope, is the most common approach to measure tissue sections and biological fluids and has been employed in a number of studies in order to discriminate between pathologies in different cancer types such as oesophagus\textsuperscript{190}, laryngeal\textsuperscript{191}, bladder\textsuperscript{192} and prostate\textsuperscript{193} lymph nodes\textsuperscript{194, 195}, breast\textsuperscript{196, 197}, skin\textsuperscript{198, 199} and liver\textsuperscript{200}. Raman spectroscopy has also shown potential in assessing tumour margins during surgery\textsuperscript{201, 202}, in order to ensure
complete removal of malignant cells and in this way reduce the tumour recurrence risk.

Raman fibre optic probes have been employed for real-time measurements of tissues, inserted through endoscopes or in the form of needle probes. The feasibility of routine clinical endoscopy combined with Raman probe has been demonstrated multiple times for scanning gastrointestinal tract\textsuperscript{170, 203} for Barrett’s oesophagus neoplasia\textsuperscript{204} and colon cancer\textsuperscript{205}. Raman fibre optic probe have also been used for discrimination between benign and malignant samples in prostate\textsuperscript{168} and bladder cancer\textsuperscript{206}, skin cancer\textsuperscript{207}, cervical cancer\textsuperscript{208} and brain cancer\textsuperscript{209, 210}.

However, the need for real time, non-invasive, \textit{in vivo} measurements of significant tissue volumes has led to the application of deep Raman spectroscopy, which includes transmission and spatially offset (SORS) Raman. Up to date, deep Raman includes clinical applications in breast cancer for the detection of calcifications\textsuperscript{157, 161} and assessment of tumour margins\textsuperscript{211, 212} as well as urology\textsuperscript{213}. This promising field of emerging techniques has been employed to conduct the experimental part of this thesis.

### 3.5 Purpose of experiments

As described in the previous three chapters, application of vibrational spectroscopy for cancer diagnosis and disease monitoring exhibit a number of advantages over conventional spectroscopy.

As the thesis mainly focuses on breast, prostate and bladder cancer, a less invasive approach for each one of the cases was explored. In the case of prostate and breast cancer, the transmission Raman mode is most applicable, benefitting from its high penetration depth, as both of the organs can be accessed from opposite sides (Chapter 10- Fig. 10.2). That would include the illumination of the tissue with a laser source on the one side and the collection of Raman scattering from the other side of the tissue. The deployment of transmission Raman geometry might be obvious for the breast cancer screening (Chapter 10- Fig. 10.2a), but less so for the prostate gland analysis, as it is an internal and less
accessible organ. In the latter case, the illumination fibre could be inserted transurethrally and the collection fibre transrectally or the other way round (Chapter 10 - Fig. 10.2b).

To assess the feasibility of such potential measurements, Chapter 5 describes transmission Raman measurements of liquid tissue phantoms similar in size and comparable in optical properties to breast and prostate gland. As calcifications are common features found both in breast and prostate and indicating potential malignancy, the phantoms fabricated contained a calcification-like compound, but of higher Raman cross-section, representing a malignant element in a block of tissue. By studying the magnitude and origin of Raman signal distribution in relation to the calcification position, we explored the possibility of malignancies being detected through the sample volume, in different depths and off the beam axis positions. Fabrication of different phantoms also allowed us to assess the Raman photon migration in relation to the tissue optical properties. This is a necessary step since different optical properties are not only observed between different types of tissues, but also in a single tissue of different malignancy stages. In chapter 6, the measurements are extended to animal samples and their different components as well as human prostate tissue in different modes of deep Raman, in order to assess the feasibility of such a concept.

In an attempt to improve the tissue phantom measurements in transmission Raman geometry, the promising approach of enhancing the Raman signal using the “photon diode” is explored in chapter 7. The signal enhancement is studied in relation to the tissue optical properties and depth of the signal origin in the sample. The same signal enhancement concept is employed in chapter 8 to predict the depth of a potential malignant element in the sample volume.

In the case of bladder cancer (chapter 9), the feasibility of diagnosing the disease in urine samples using Raman microscopy was assessed. In that case, the urine samples were studied in terms of spectral features and their spatial distribution and the results were related to the presence of bladder cancer. Multivariate analysis was also used in order to determine the most significant discriminant features between healthy and malignant group.

The general objective of this thesis is to improve the future design of an in vivo, real-time and non-invasive cancer diagnosis by means of transmission and back-scattering Raman spectroscopy. During our study, we assessed the feasibility of
a non-invasive diagnosis for breast and prostate cancer on tissue samples and phantoms and for bladder cancer on urine samples. In the case of breast and prostate cancer, we assessed the limits of detection of Raman signals from a potential malignancy in simulated \textit{in vivo} measurement conditions. We also explored in which way parameters such as the optical properties and “photon diode” enhancement are contributing to such an application as well as advance the understanding of how the distribution of collected Raman scattering signals change in relation to these parameters.

This study is intended to be a contribution to the future design of Raman instruments targeting disease specific signals within particular regions of the sample, and pave the way towards a real-time, non-invasive cancer diagnosis. The benefits of such an application could involve increased accuracy during cancer diagnosis, improved biopsy targeting and treatment monitoring.
Chapter 4: Materials and methodology

In this chapter the samples employed for measurements with deep Raman setup and Raman microscopy as well as the apparatus characteristics are described. The samples include a number of liquid tissue phantoms, human and animal tissue. The documents submitted for acquiring ethical approval for the human samples can be found attached in the end of the thesis (appendices).

4.1 Sample collection and preparation

4.1.1 Tissue phantoms

Inclusion-containing tissue phantoms with various optical properties are commonly used to simulate tissues in deep Raman spectroscopy. During the experimental part of this thesis, a number of liquid tissue phantoms were fabricated and measured.

4.1.2 Phantom design

The liquid phantoms employed in the study, consisted of a quartz cell (45 mm width × 30 mm length (26 mm internal cell optical path length) × 45 mm height) (ramé-hart instrument co., USA) containing aqueous solutions of Intralipid and Indian ink in various concentrations (Figure 4.1(i)). Intralipid (Fresenius Kabi Ltd, United Kingdom and 20% emulsion, Sigma), a dilute mixture of emulsified fatty acids, served as a scattering medium whereas Indian ink (Histology Stain, American MasterTech), a dispersion of carbon particles, acted as a broad band absorbing agent. The combination of water, Intralipid and Indian ink has been shown not to change the microphysical and optical properties of the constituents when mixed.

The preservation of Intralipid’s quality over time was assured by recording the stability of its UV/Vis absorption at 830 nm before each experiment. The UV measurements were performed using a Thermo Scientific Evolution Array UV-Vis Spectrophotometer with a range of 185-1100 nm, 30 scans and a 1000 ms integration time. A volume of 0.75 ml Intralipid stock solution was diluted down to a 0.15% aqueous solution to avoid saturation in measurements. Since the
Intralipid as a fat emulsion tends to form aggregates which can precipitate over time, recording its absorption frequently reveals any changes in its consistency.

**Figure 4.1:** (i) Liquid tissue phantoms components. (ii) The quartz cell and the vial which were used in our measurements.

Whenever Indian ink was used, it was first subjected to ultrasound in an ultrasonic water bath for 30 minutes. This was necessary in order to prevent suspended carbon particles from forming clusters in the aqueous solution and maintain reproducible ink optical properties\(^{218, 219}\).

In order to simulate a pathobiological formation in the tissue, an inclusion consisting of a thin vial (12 mm width, 4 mm length (2 mm optical path length)) containing hydroxyapatite (HAP) or trans-stilbene, was placed inside the quartz cell (see Fig. 4.1 (ii)). HAP was selected for these experiments in order to represent a calcified formation (calcification) which can usually be found in both breast\(^{220}\) and prostate tissues\(^{63, 221}\). Another important feature of HAP is that it provides a distinct Raman signal from the surrounding phantom (representing the soft tissues here) and therefore the signal distribution in relation to the phantom properties can be better studied. In some cases trans-stilbene was used as an inclusion material instead for its high Raman cross-section properties. By replacing HAP with trans-stilbene it was possible to probe higher optical properties phantoms with the existing Raman apparatus, while having the same expected behaviour as HAP and therefore assess the detection limits of our system.

During the mapping experiments in chapters 5, 6, 7 and 8, the vial was attached to a computer-controlled motorized translation stage (Standa Ltd, Lithuania) in order to move it to different positions inside the liquid phantom.
4.1.2.1 Phantom stability assessment

In order to assess liquid tissue phantom consistency and stability during 2D mapping over time, a series of measurements were recorded for a number of phantoms. Measurements were recorded while the trans-stilbene vial was moved in different positions inside the quartz cell, in a snake-like pattern (Fig. 4.2). After the measurements have been pre-processed as described later in 4.3.1 section, the mean trans-stilbene Raman signal intensity has been calculated on the y axis, in order to plot the intensity as a function of the phantom depth.

Figure 4.2: Recorded measurements while the trans-stilbene vial was moved in a snake-like pattern into the quartz cell.

The movements were realized in a normal (snake-like) pattern and reversed sequence using the motorized x-y translation stage, over different measurement points.
Figure 4.3: Stability measurements in liquid tissue phantom with scattering (1% IL) and no absorption.

Figure 4.4: Stability measurements in liquid tissue phantom with scattering (1% IL) and absorption (0.2μl/ml ink).
Figure 4.5: Stability measurements in liquid tissue phantom with scattering (2% IL) and absorption (0.2μl/ml ink).

The trans-stilbene Raman signal height for 1192 cm\(^{-1}\) band was measured and compared for each phantom in one normal and two reversed sequences with 30 minutes time gap between them. For the only scattering phantom (Fig. 4.3), the trans-stilbene Raman signal between the two time points are identical and the measurements are highly reproducible and accurate. When absorption is introduced in lower (Fig. 4.4) and higher levels (Fig.4.5), the values seem to follow the same trend with an extra variation of less than 10%. This variation is possibly due to the liquid nature of the tissue phantoms, but it is not high enough to affect significantly the experimental results. However, in order to achieve high repeatability between the different measurements, the liquid phantom solution was transferred to a 50 ml falcon tube and mixed by shaking, before the start of every mapping.

4.1.2.2 Correlating phantoms with human tissue
The liquid tissue phantoms employed for this study were fabricated to mimic mainly human breast and prostate tissue both in size and optical properties. In terms of dimensions, the quartz cell was chosen in order to provide an approximation to breast volumes in mammographic screening (1.9–7.2 cm compressed breast thickness)\(^{222}\) and human prostate gland (4–4.5 cm length, 2–3.5 cm width, 1.7–2.5 cm thickness)\(^{223}\).

In terms of optical properties, the Indian ink and Intralipid concentrations in phantoms were determined to match those for human prostate and breast tissue optical properties of absorbance and reduced scattering in 830nm, as found in the literature. The reduced scattering \((\mu'_s)\) and absorption \((\mu_a)\) coefficient values, for normal and cancerous prostate glands, have been calculated through optical extinction and diffusion reflectance measurements. The values reported in the literature for \(\mu'_s\) at 830 nm are 4 cm\(^{-1}\) for healthy and 6 cm\(^{-1}\) for cancerous prostate glands, whereas the values for \(\mu_a\) are 0.027 cm\(^{-1}\) for healthy and 0.002 cm\(^{-1}\) for cancerous prostate respectively\(^{224}\). Svensson et al. used NIR time-resolved spectroscopy to estimate the reduced scattering coefficient between 5.5 and 9.5 cm\(^{-1}\) and absorption coefficient between 0.3 and 0.7 cm\(^{-1}\) for malignant prostate glands with untreated cancer.\(^{225}\). Regarding breast tissue, the reduced scattering coefficient has been calculated for 830 nm wavelength to be \(\mu'_s = 9.84\) cm\(^{-1}\) \(^{226}\). Breast tissue absorption coefficients are not available in the literature for the exact wavelength of 830 nm, but a value of \(\mu_a\) between 0.068 and 0.102 cm\(^{-1}\) in papillary breast cancer is estimated for a very close wavelength of 825 nm\(^{226, 227}\).

Based on the literature values for reduced scattering and absorption coefficients of human prostate and breast tissues, liquid tissue phantoms were built around clinically relevant values of \(\mu'_s\) and \(\mu_a\), using appropriate Intralipid and Indian ink concentrations. In order to calculate the appropriate component concentration required for the phantoms, our stock Intralipid and Indian ink needed to be characterized in terms of reduced scattering coefficient and absorption coefficient respectively. For simplicity reasons we assumed that Intralipid has no absorption and Indian ink no reduced scattering properties.

For the characterization of Intralipid, the fitting parameters for 10%, 20% and 30% Intralipid provided by Michels \(et\ al.\)^\(^{228}\) were used in order to calculate the Intralipid reduced scattering coefficient and converted to our wavelength. From
the fit, we extracted the Intralipid concentrations equivalent to the required reduced scattering coefficient values (Fig. 4.6). Using these values, liquid tissue phantoms with reduced scattering coefficients in a range of 2.3 to 36.9 cm$^{-1}$ were fabricated, using Intralipid concentrations calculated based on the calibration line expression $y = 92.17x$.

![Graph showing Intralipid concentration vs. reduced scattering coefficient](image)

**Figure 4.6:** Percentage Intralipid concentration versus reduced scattering coefficient (cm$^{-1}$).

It should be mentioned here that the Intralipid concentration may not be correlated linearly with the reduced scattering coefficient values in the whole concentration range as assumed from Michels’ paper. However, there is evidence that in low Intralipid concentrations (0.5-1.3%) at 830 nm, there is a fairly linear correlation between the two values$^{228, 230, 231}$.

In terms of absorption properties, different concentrations of Indian ink were used to achieve different absorption coefficients in the phantom. Indian ink from different manufacturers has been widely employed and characterized in various studies$^{232}$. In this study we measured the absorption coefficient of our specific stock volume of Indian ink.
UV/Vis absorption spectra of different ink concentrations were recorded similarly to Intralipid UV measurements earlier.

Data from these measurements were curve fitted for the appropriate wavelength (830 nm) and the ink concentrations required for the human tissue absorption were calculated as below:

If during an absorption measurement we consider an ink aqueous solution with concentration, absorption cross-section $\sigma$ in a cuvette of $x$ thickness, then from Beer-Lambert Law:

$$I = I_0 e^{-\sigma C x} = I_0 e^{-\mu x} \quad (4.1)$$

Where $\mu = \sigma C$ is the linear attenuation coefficient which we can equate with the linear absorption coefficient in the case of ink where there are no scattering

**Figure 4.7**: Absorbance spectra for different concentrations of Indian ink. (laser and Raman photons wavelengths are indicated for citing reasons in Chapter 5).
events present. Because $\mu$ is a function of wavelength, Beer-Lambert Law will also be a function of wavelength:

$$I(\lambda) = I_0(\lambda)e^{-\mu(\lambda)x} \quad (4.2)$$

As absorbance is defined:

$$A = \log\left(\frac{I_0(\lambda)}{I(\lambda)}\right) \quad (4.3)$$

From (1):

$$A = \log\left(\frac{I_0(\lambda)}{I(\lambda)}\right) \Rightarrow \log(I_0(\lambda)/I_0(\lambda)e^{-\mu x}) = \log(e^{\mu x})$$

$$A = \mu x \log(e) = 0.4343 \mu x \quad (4.4)$$

Where: $\mu =$ absorption coefficient depending on the wavelength

$A =$ absorbance value at a specific wavelength

$x =$ cuvette path length (here in cm)

Using the measured absorbance for each ink concentration at 830nm, we plotted the two values against each other in a calibration curve:

**Figure 4.8:** Indian ink calibration curve: ink absorbance versus ink concentrations in aqueous solution ($\mu$l/ml).
Since the cuvette path length was 1 cm in these measurements, we can calculate the respective absorption coefficient for each concentration using (4.3).

Based on the calculations above, a number of liquid phantoms were produced with absorption coefficients in a range from 0 to 2.26 cm⁻¹. Our data were in a very good agreement with the literature in the range of concentrations mentioned²²⁴.

In order to simulate the optical properties of prostate and breast tissues, the scattering and absorption agents were combined in the same sample. The phantoms were made by combining an Intralipid range of 0-4% (corresponding to reduced scattering coefficient: \( \mu_s = 0-36.9\) cm⁻¹) and an Indian ink range of 0-0.5\( \mu_l/ml \) (corresponding to an absorption coefficient: \( \mu_a = 0-2.26\) cm⁻¹).

Finally, the absorbance spectra of water, a main phantom component, has also been recorded (Fig. 4.9).

**Figure 4.9:** Absorbance spectrum of water in the range of 400-1100 nm. Negative absorption values are due to the difference in refractive index between air (used as a blank in the cuvette) and water. The cuvette path length in the measurements is 1 mm.
4.1.3 Tissue samples

4.1.3.1 Animal tissue samples

Animal tissue which has been used in this thesis included chicken breast, beef shin and pork fat, all purchased from a local shop.

4.1.3.2 Human tissue samples

The human samples used in this study were prostate tissue and urine samples and were acquired following fully informed consent of the patients and ethical approval from the Royal Devon & Exeter Tissue Bank. The documents related to this procedure can be found in Appendix 1.

4.1.3.2.1 Prostate tissue

Nineteen tissue sections of prostate biopsies and 9 TURPs have been acquired for the study through biopsy and transurethral resection of prostate, respectively. TURP chips were around 20 mm long and 2.5 mm thick and the biopsies 10 mm long and less than 1 mm thick (Fig. 4.10).

![Figure 4.10: (a) TURP chip and (b), (c) prostate biopsies measured in the deep Raman system.](image)

Once the samples were removed from the patient, they were transferred into cryovials, snapped frozen in liquid nitrogen and stored in an -80°C freezer in Royal Devon and Exeter Hospital. The samples were put into polystyrene boxes filled with dry ice and transferred to Physics School in Exeter University. In the
labs of Exeter University, they were also stored in an -80°C freezer until being measured. The Raman measurements were followed by staining the edge of the sample for orientation reasons. The samples were stained using the following procedure:

a) the end of a dry TURP chip was dipped in ink and let to dry
b) acetic alcohol (100 parts of ethanol mixed with 3 parts of glacial acetic acid) was added on the top of the inked edge
c) the TURP chip was let to dry and then dropped into a formalin pot.

The stained samples were moved into formalin pots and transferred back to the hospital for histopathological analysis. Both the TURP chips and the prostate biopsies were subjected to the same procedure, except that the biopsies were transferred to acetate paper right after the collection, in order to facilitate the sample handling, while their volume remained intact despite their small size.

Figure 4.11: Protocol followed during the collection of prostate samples from the hospital.

For certain measurements described in chapter 6 the TURP chips were cryosectioned while frozen using a freezing- microtome (Fig. 4.11). The sample was kept frozen in ice rather than OCT in order to avoid signal contamination of the tissue. The thickness of the sections was 20 μm.
It should be mentioned that due to the limitations identified while measuring the prostate samples in the deep Raman setup (not sufficient sample size, sample fluorescence induced by the surgical procedure), it was not eventually possible to correlate our results with the histopathological analysis.

### 4.1.3.2.2 Urine samples

Urine samples from 11 healthy and 11 patients with bladder cancer were acquired from the Royal Devon & Exeter Tissue Bank. One of the patients did not have TCC bladder cancer and so the specific urine sample was excluded from the study. All of the samples were provided in 4 ml tubes, flash frozen in liquid nitrogen and stored at -80°C until required for analysis. Part of the samples was initially studied with Mass Spectrometry in Biosciences department of Exeter.
University. As a following step, the remaining urine aliquots were transferred to Biophysics’ lab and studied with Raman spectroscopy. The urine samples used for Raman analysis had not been subjected to any protein removal. During the Raman measurements, the urine samples were measured in drops of 1μl laid on calcium fluoride (CaF₂) substrates (Fig. 4.13).

Figure 4.13: Urine drops measured with Raman microscopy.

4.2 Experimental setups

4.2.1 Deep Raman spectroscopy

The deep Raman setup employed for the measurements at the University of Exeter is a flexible setup which can be easily changed between transmission and iSORS modes. Before the start of the experiments, two different back illuminating CCD cameras were tested and compared in terms of performance, in order to identify the most appropriate one for the deep Raman setup.

4.2.1.1 Camera evaluation

Two CCD detectors with different pixel array configuration, Andor iDus 416 (2000×256) and Andor iDus 420 (1024×255), were compared in terms of fixed
pattern noise. The performance of the two cameras was assessed in terms of stability, oscillations and pattern of dark signal, by acquiring measurements in complete darkness.

Andor iDus 416 model has a greater number of pixels horizontally. In Figure 4.14b the spectral range was adjusted in order to overlap Neon Argon Raman peak positions.

**Figure 4.14:** Neon Argon measured with Andor iDus 420 (green) and 416 (red) detector before (a) and after (b) adjusted spectral range.
The two cameras were also compared in terms of the standard deviation of their dark noise, which was lower for iDus 416 in counts, but higher when calculated in the ultimately relevant photoelectrons (i.e. detected photons) (in iDus 420 the number of photoelectrons per count (2.5) were higher compared with the iDus 416 (0.7)).

Signal to noise ratio was another feature assessed between the two cameras. This is due to photon shot-noise (random and independent of CCD) stemming from the particle-like nature of light and other (fixed) instrumental influences such as etaloning effect (Fabry-Perot) which takes place between the two silicon surfaces of a back illuminating CCD due to the interference of light waves in a constructive or a destructive way (or also present due to laser-blocking filter residual etaloning effect). The etaloning was assessed by calculating the range of regular oscillations divided by the signal, on the white light spectrum measured by each camera (Fig. 4.15). Both cameras exhibit a range of oscillations divided by the signal being around 5%, which is acceptable for our measurements. More specifically, the oscillations for iDus 416 were calculated to be 5.7%, whereas for iDus 420 4.5%.
Figure 4.15: Room light spectrum (acquisition time: 1 s) as measured with Andor iDus 420 (green) and 416 (red).

Another characteristic assessed was the pattern of dark signal which should normally be stable and independent of wavelength. Although, for iDus 416 that was the case, for iDus 420 an uneven elevated background signal was observed when not at fully cooled state (e.g. at ~ -70 °C and higher temperatures) (Fig. 4.16). For that reason, before every measurement it was ensured the CCD camera was cooled fully down to -75 °C in order to minimize the thermal noise.
Figure 4.16: Dark noise pattern as measured in complete darkness (acquisition time: 1s) with Andor iDus 420 (blue and red) and 416 (yellow) at different temperatures (-70 °C and -80 °C) at the lowest charge shift speed (16.25 μs).

Since the two cameras exhibited very similar performance, Andor iDus 420 was eventually chosen for its more suitable pixel configuration for our applications (i.e. it could capture more light in the vertical direction, matching better the fibre bundle slit in our system).

4.2.1.2 Setup description (transmission, iSORS)

The deep Raman system at Exeter University is a flexible setup which can be changed between transmission and iSORS configuration (Fig. 4.17). The setup consisted of a spectrum-stabilized laser (Innovative Photonics Solutions: Innovative Photonic Solutions: I0830MM0350MF-EM) with laser emission at 830 nm and an output power of ~300 mW. The laser was coupled to a Thorlabs 400 μm diameter multimode optical fibre and at the output collimated and filtered by
passing through a pair bandpass laser line filters (FL830-10, Thorlabs) in order to suppress the spectral wings.

**Figure 4.17:** Deep Raman flexible setup for transmission and iSORS measurements in Exeter University.

When the setup was used in a transmission mode, which is the case for most of the measurements described in the experimental section, the laser was directed towards the sample with a mirror and brought onto it with a 25 mm diameter, 70 mm focal length lens (Fig. 4.18a). The sample was illuminated with 280 mW of light in a 3–4 mm diameter spot.
When the setup was used in an iSORS mode, the laser beam was passed through an axicon (conical lens), illuminating the sample in a ring shape, on the same side where the Raman light was being collected (Fig. 4.18b). The size of the ring was adjusted by moving the axicon to different distances from the sample. In this way, different translational positions of the axicon on the rail (0 to 9 cm) correspond to different radii of the illumination ring (1.5 to 7.5 mm), or spatial offsets from the collection centre (Fig. 4.19).
Figure 4.19: Different axicon distances from the sample correspond to different spatial offsets from the collection centre (indicated at the top).

In both transmission and iSORS configuration, the light passing through the sample is either scattered or absorbed by the molecules present. The Raman photons were collected using an AR coated lens (f = 60 mm, dia. = 50 mm, INGCRYS Laser systems). The collimated light was passed through a holographic super notch filter (HSPF-830.0 AR-2.0, Kaiser Optical Systems) to remove the elastically scattered light (laser photons) and imaged onto a fibre probe bundle by a second lens of the identical parameters to the collection lens (i.e. the collection system exhibited no magnification). The fibre bundle (CeramOptec, ‘spot to slit line’ type bundle assembly, active area spot diameter approximately 2.2 mm, slit line 0.2 mm × 14.95 mm) was connected to the entrance port of a Holospec VPH system spectrograph (Kaiser Optical systems Inc, HSG-917.4 custom). Measurements were recorded using a deep depletion CCD camera cooled down to −75 °C (Andor Technology, iDus 420, DU420A-BRD-D, 1024 × 255 pixels). Only a part of the input fibres aligned in the vertical line pattern (around ~6 mm) were imaged onto the CCD. The overall spectral resolution of the detection system was ~8 cm⁻¹.

Through both transmission and iSORS measurements, the shift speed and the readout rate applied on the camera settings were set to the lowest value (16.25 μs and 33 kHz respectively) in order to introduce the least noise possible to the spectra acquired. In the case of accumulated measurements, the cosmic ray removal option in the detector software (Andor Solis) was applied. The system was always calibrated using an aspirin tablet (acetylsalicylic acid) and HAP.
(hydroxyapatite) powder (Fig. 4.20). Their Raman signal was also recorded over a specific acquisition time (3 s for ASA and 1 s for HAP) in order to compare the peak height levels from day to day.

![Figure 4.20: Aspirin (ASA) and hydroxyapatite (HAP) spectra from calibration standards during the deep Raman measurements. Acquisition time: 3 s for ASA and 1 s for HAP. The spectral offset has been adjusted for clarity.](image)

4.2.1.3 Automated stage and mappings

For the recording of the phantom mappings described in the experimental part, a motorized translation stage (8MTF-102LS05, Standa Ltd, Lithuania) was employed. During the mappings, the probing vial was suspended from the motorized stage and moved to different positions. The vial width (12 mm) was always aligned with the y-axis and the 4 mm length was aligned with the optical axis of the system (x-axis). In this way, the vial was moved in a two-dimensional horizontal grid (Fig. 4.21).
During the mappings, the vial was moved in a ‘snake’-like pattern, starting from the laser entrance side and moving towards the exit side of the cell. At each step, a Raman spectrum was recorded and the stage was moved to the next position.

4.2.1.4 “Photon diode” element and reflective filters

The “photon diode” element used in Chapter 7 and 8, is an unmounted 830 nm bandpass filter with 25 mm diameter and 3.5 mm thickness (Semrock, FWHM bandwidth measured as 9.5 nm, BrightLine® singleband bandpass filter) (Fig. 4.22a).
Figure 4.22: (a) Semrock photon diode mounted at the centre of a metal plate wrapped with aluminium for optimum reflectivity and (b) signal enhancement mechanism of photon diode.

The photon diode is a dielectric bandpass filter which increases the collected Raman signal from a highly scattering sample when it is placed in close proximity to the sample surface, at the point where the illumination beam enters the sample. The photon diode is essentially a unidirectional mirror which allows photons of a specific wavelength (in our case: >90% 829-831nm) and angle of incidence (0 ± 5 degrees) to be transmitted. For photons with a different angle of incidence, as the deviation from the right angle increases, the wavelength transmittance distribution is moving to lower wavelengths (Fig. 4.23). Due to this angular dependence, the majority of the photons of a collimated laser beam will enter the sample but will not escape from it backwards once they are scattered or converted to Raman photons, due to the change of angle of incidence and wavelength respectively (Fig. 4.22b). Minimizing the photon loss in the air-sample interface is critical, since more than 90% of the photons can escape typically from the sample by this mechanism. As the laser and Raman photons are forced to remain inside the sample, they have more chances to interact with the Raman
scatterer and travel deeper inside the phantom, towards the collection system. In this way, the sensitivity, penetration depth and overall detectable Raman signal of the experiment are improved.

Figure 4.23: Schematic illustration of the shift of the design wavelength with the angle of incidence for a dielectric bandpass filter used for the enhancement of the coupling of laser radiation into a turbid sample\textsuperscript{235}.

During the measurements, the photon diode was mounted at the centre of a metal plate which was then placed at the front of the quartz cell against its internal wall (Fig. 4.24i). The metal plate outside the active area of the photon diode was covered with an aluminium reflecting foil, in order to achieve the maximum reflectance of re-emerging photons back to the phantom also outside the filter (Fig. 4.24ii).
In order to identify the side of the diode with the effective layer, we recorded a series of measurements from an identical trans-stilbene vial, testing the two different diode sides (A and B) which were facing the laser beam each time. For the test measurements, a combined tissue phantom (1%IL + 0.2 µl/ml ink) was employed and the longitudinal plots of generated Raman signal along the optical axis are presented in Figure 4.25.
From the plot above it is obvious that when side A of the photon diode faces the laser beam, there is a greater transmittance and signal enhancement. This is why in the following experiments the diode was used in this specific orientation.

During the control (no photon diode) measurements in chapter 7 and 8, a quartz window of identical dimensions was placed at the same location in order to make up for the loss of liquid phantom volume and to retain the identical mapping pattern. Additionally, a calcium fluoride disc (20 mm diameter, 1 mm thickness) was placed between the diode/quartz and the cell wall during every measurement, in order to minimize the solution volume trapped in the front interface which would potentially lead to disruption of the incident collimated laser beam.
Both the diode and the quartz layers were cleaned thoroughly before every measurement, using a lens tissue wrapped around a tip and moistened by acetone, as suggested by the manufacturer.

Since a major part of the analysis in Chapters 7 and 8 involves comparison of measurements using different elements (photon diode, quartz layer), it was considered necessary to measure the transmittance of the elements separately on the deep Raman setup using a power meter with no solution and no vial present in the cell (Fig. 4.26).

![Graph](image)

**Figure 4.26:** Transmittance of photon diode and quartz layer used in the experiments, as power meter is set in different distances from them.

The transmitted power though quartz layer and diode individually (without the quartz cell on the sample holder), only shows a significant difference when the power meter is placed closest to the illumination side. However, when the quartz cell was introduced to the measurements (Fig. 4.27), we observed a considerable difference in the power which drops from 275 mW (quartz layer) to 254 mW (photon diode). This reduction of power by ~8% (due to the different transmittance of materials) is affecting signal comparisons between the two of them, as described in chapter 7.
4.2.2 Raman microscopy

4.2.2.1 InVia Raman spectrometer

For the Raman microscopy measurements in chapter 9, we used a Renishaw inVia spectrometer (Renishaw plc, New Mills, Wotton-under-Edge, Gloucestershire, GL12 8JR), with near-infrared diode excitation lasers (785 nm and 830 nm). The system is equipped with reflected light white light imaging and generates 300 mW of power. The laser light is initially focused through the microscope lens (×50 objective) onto the sample on a motorized XYZ stage. Following interaction with the sample, the Raman back-scattered light is collected through the collection optics and focused onto the grating, whereas Rayleigh light is rejected away through filters. The dispersion grating then splits the beam into its constituent wavelengths which are directed onto a CCD detector. The CCD used for converting the photons to electrical signal, is a deep depletion charge-coupled-device detector that comprises an array of 1024 x 256 pixels. Similar schematic overview of a Raman microscope can be found in chapter 3 (Fig. 3.6).

InVia Raman spectrometer can operate in both traditional point Raman mode and StreamLine Raman imaging mode, which is a fast mapping method for data

Figure 4.27: Measuring photon diode and quartz layer transmittance on deep Raman setup.
In Streamline mode, a laser line is employed to scan a sample area and produce a spectrum which is made of an accumulation of 6 point spectra. Whenever Streamline was used, Slalom mode was also activated. Slalom mode ensures that data from the whole of the area defined for analysis is sampled, since it zigzags StreamLine’s laser line. The single point measurements presented in chapter 9 were recorded with traditional Raman mode, whereas the mappings were recorded with the Streamline mode.

Renishaw InVia microscope is equipped with three different gratings (300, 600 and 1200 lines/cm). For the measurements recorded in this thesis, we used the 1200 l/cm grating centred at 1300 cm$^{-1}$. The spectral resolution for 1200 l/cm grating and 785 nm excitation laser was calculated to be 2.5-3 cm$^{-1}$.

Although the system is equipped with internal calibration routines, both internal and external standards (Si, NeAr) were measured prior to each measurement in order to ensure optimum conditions for reproducibility and comparison between the samples.

### 4.2.2.2 Other Raman systems

For the urine maps analysed with multivariate analysis in Chapter 9, measurements were recorded on a Renishaw System 1000 using 830 nm as the excitation wavelength. This is operating in a similar way as InVia$^{237}$. For the polarization measurements described in Chapter 9, polarizers were added in the path between the laser light and the sample on Renishaw System 1000, in a parallel and perpendicular orientation.

For a single measurement of urine samples with 532 nm excitation laser in Chapter 9 (Fig. 9.1), confocal Raman microscope (Witec alpha300) has also been used.

**Raman spectroscopy substrates**

Irrespective of Raman systems employed, urine samples were always measured in drops of 1 μl laid on calcium fluoride (CaF$_2$) substrates (Fig. 4.13). Calcium fluoride has been shown to be a suitable substrate for Raman
measurements, as it only exhibits a peak at 321 cm\(^{-1}\) (Fig. 4.28), lacking significant background in the rest of the fingerprint region.

![Raman Spectrum](image)

**Figure 4.28:** Raman spectrum of CaF\(_2\) discs, used as substrates in Raman microscopy measurements. The peak at 1540 cm\(^{-1}\) corresponds to atmospheric oxygen.

### 4.3 Data processing and analysis

#### 4.3.1 Spectral analysis

Single Raman measurements shown for comparison reasons in this thesis, are plotted using Origin8 (OriginLab, Northampton, MA, USA).

For deep Raman data analysis (Chapter 5, 6, 7, 8), the maps recorded on the deep Raman system, were loaded into Matlab R2013a (The Mathworks Inc., Natick, Massachusetts, USA) for data pre-processing. The spectra were subjected to baseline correction using asymmetric least squares smoothing\(^{238}\), principal component (PC) noise reduction and subsequent reconstruction from the first seven principal components into 1D and 2D images. The noise in the principal components were assessed individually by eye. PC noise reduction is a
useful method for minimizing the noise in a set of spectra by reconstructing it only from the significant noise-free principal components. In this way, all of the important spectral information is retained, whereas the background noise is being removed\textsuperscript{239, 240}. For the 1D plots only, following pre-processing, the mean intensity on the y-axis was calculated in order to directly compare the values between the different maps.

For urine analysis (Chapter 9), the maps data were also processed using Matlab. The set of spectra for each map were subjected to baseline correction with asymmetric least squares smoothing and cosmic ray removal\textsuperscript{241}. The mean spectra for each sample were calculated from the processed individual spectra and scaled to a 0 to 1 scale. The 1542 cm\textsuperscript{-1} band attributed to the atmospheric oxygen has been removed from the spectra which were finally normalized, mean centred and analysed with multivariate techniques.

### 4.3.2 Multivariate analysis

Multivariate analysis is a very useful tool for data analysis in spectroscopy commonly used for data reduction and quality improvement (signal-to-noise ratio, resolution), classification of information of the data and appropriate display of information (visualization of information content and its statistical validity)\textsuperscript{242}. In this thesis, Principal component analysis (PCA) and partial linear discriminant analysis (LDA) were performed over the pre-processed Raman spectra. In the deep Raman data PCA was employed for noise reduction in the spectra, whereas in the urine sample PCA and LDA have been used for revealing the discriminant features between the two pathology groups.

#### 4.3.2.1 Principal Component Analysis (PCA)

PCA is an unsupervised multivariate technique, as it “ignores” the pathology class of each sample. PCA is used to explore the inherent structure of the data and involves a mathematical procedure which uses an orthogonal transformation in order to reduce the dimensionality of a dataset with possibly correlated observations (e.g. Raman shifts) and converts it into linearly uncorrelated (orthogonal) components which are called principal components\textsuperscript{243, 244}. The first principal component (PC1) accounts for the greatest data variance from the mean and each succeeding component accounts for as much of the remaining spectral
variability as possible in a descending order. The original matrix is decomposed by PCA into two smaller matrices, one of scores and another of loadings\(^{245}\).

The loadings describe decreasing amounts of variance present in the dataset\(^{246}\) and when they are plotted as a function of the variables, the plot reveals the most important diagnostic features related to the differences in the dataset. When loadings are multiplied by their corresponding scores and summed, the original spectra can be reconstructed. When scores are plotted against each other, they reveal relationships between the groups of observations. In that way PCA allows the identification of groups of variables that are correlated via underlying phenomena that cannot be observed directly\(^{247}\).

In the deep Raman data, PCA was used for noise reduction in the spectra, by analysing the principal components of the dataset and reconstructing them by only combining the limited noise components.

**4.3.2.2 Linear Discriminant Analysis (LDA)**

Contrary to PCA, LDA is a supervised technique which tries to maximize the separation between multiple classes, by identifying the optimum linear combination of features (vectors) that characterize/discriminate the classes. LDA results in linear discriminant functions that maximize the variance in the data between separate pathological groups and minimize the variance between the members of the same pathological group.

While analysing urine samples in Chapter 9, PCA was initially performed to obtain the first 15 principal components and the PC scores were then used to perform LDA on the dataset. During LDA, each principal component was assessed in terms of ability to provide an optimum separation of the pathology classes. Once the optimum combination is identified, the model can be tested by the "leave-one-out" cross-validation method. This method involves leaving a sample from a group out of the dataset, performing PCA followed by LDA on the dataset and then test the resulting model with the sample which has been left out initially\(^{246}\). In this way a confusion matrix is being created to describe the performance of the classification model, from where the sensitivity and specificity of the diagnostic algorithm can be calculated.
Chapter 5: Deep Raman spectroscopy on tissue phantoms for cancer diagnosis

5.1 Introduction

Deep Raman spectroscopy (Spatially Offset Raman Spectroscopy (SORS) and Transmission Raman Spectroscopy (TRS)) is a group of techniques developed for biomedical, pharmaceutical and security applications\textsuperscript{152, 161} and which in the last decade have found application also in cancer diagnostics\textsuperscript{154}.

In this chapter we are exploring the potential of transmission Raman in the diagnosis of two of the most prevalent types of cancer, prostate and breast cancer, using equivalent tissue phantoms. As previously mentioned in Chapter 3, the potential clinical application of transmission Raman for cancer scanning of breast and prostate glands would include the illumination of the tissue with a laser source and the collection of Raman scattering from the other side of sample. The deployment of transmission Raman geometry might be obvious for the breast cancer screening, but less so for the prostate gland analysis, which is an internal and less accessible organ. In the latter case, the illumination fibre could be inserted transurethraly and the collection fibre transrectally or the other way round as shown in Figure 5.1.

![Figure 5.1: Potential transmission Raman geometry applied to prostate cancer detection (the peripheral zone is marked in blue) (recreated from\textsuperscript{248}).]
A first step in order to assess the potential of this approach is to test its feasibility with transmission Raman measurements on tissue phantoms with similar size and comparable optical properties to breast and prostate. Similar studies on tissue and phantoms for diagnosis\textsuperscript{149, 155, 214, 249, 250} and surgical margins assessment\textsuperscript{212} have been reported in the literature. However, in these studies the Raman signal was only explored in terms of sample thickness, whereas aspects such as its spatial distribution throughout the sample volume and its dependence on the optical properties, still remain unexplored.

In this study, in order to assess these parameters, we are recording the Raman scattering of calcification-like inclusions (often found in cancerous tissues) while its position inside the phantoms is changing and their optical properties vary. In this way we can explore the origin of Raman signal currently in our phantoms and in a later stage in tissue areas of different consistency, understanding the relative signals measured from lesions (calcifications) buried in different depths in real tissues. With these measurements we assess the feasibility and the limits of detection of Raman signals from a calcification in simulated \textit{in vivo} measurement conditions in order to help the future design of optical sampling methods to maximise the excitation and collection of these signals.

### 5.2 Development of liquid tissue phantoms

In order to test the feasibility of measuring liquid tissue phantoms in a transmission mode of the deep Raman setup, their development had to be assessed step by step.

\textit{Proof of principle:} 

First, the capability of the system to collect Raman photons emerging from deeper layers of the sample was tested. For that reason, Raman measurements were recorded from individual and overlaid vials (Fig. 5.2) containing different materials with high Raman cross-section such as calcium hydroxyapatite (HAP), calcium oxalate monohydrate (COM) and polytetrafluoroethylene (PTFE) (Fig. 5.3).
Figure 5.2: Transmission Raman measurement of two overlaid vials containing two different materials.

Figure 5.3: Raman signal from individual and overlaid vials containing different materials, in a transmission setup. The spectral offset has been adjusted for clarity.

The two layer measurements show that even if the sample is opaque, it is still possible for the laser photons to pass through the first layer via diffuse light scattering and interact with the second layer, generating in this way Raman photons which can be detected on the other side of the sample. In all of the overlaid measurements (COM in front of HAP, HAP in front of COM, HAP in front
of PTFE), the Raman peaks of the individual materials can be observed in Figure 5.3. These peaks are more intense or weaker depending on the Raman cross-section of each individual material.

To extend our hypothesis, a plastic container filled with amino acid (L-methionine) was measured on a transmission mode on the deep Raman setup (transmission mode). The plastic container and its content (amino acid in powder) were also measured separately on a conventional reflectance Raman microscope (Renishaw System 1000- Chapter 4).
Figure 5.4: Amino acid powder and plastic container measured on transmission deep Raman instrument (photo) and Raman microscope for an acquisition time of 30 s x 3 accumulations and 10 s x 3 accumulations respectively. The spectral offset has been adjusted for clarity.
We can observe that in both cases, the Raman signal acquired from deep Raman configuration is a composite signal from both the plastic container and the amino acid powder. Contrary to this, the measurements acquired on a conventional Raman microscope are single point spectra of either the plastic container or the amino acid powder.

This proves the ability of deep Raman to acquire signal from deep layers. This is in contrast with the capability of Raman microscopy that only collects effectively Raman photons typically from a few hundred micrometres depth, depending on the sample optical properties and exact optical configuration of the microscope.

**Zero diffuse scattering and zero absorption measurements:**

In order to develop and spectroscopically assess the liquid tissue phantoms (Chapter 4), we started by testing the Raman signal of the HAP (hydroxyapatite) vial in different positions inside the quartz cell filled with water (26 mm optical path) and no diffuse scattering present. The specifications of the vial and cell are described in Chapter 4. No added absorbent was present although water itself induced notable absorption at different laser and Raman detection wavelengths.

The HAP was placed in three different positions (front, middle, back) on the laser beam axis (Fig. 5.5). The Raman signal acquired from these positions is presented in Fig. 5.6.

![Figure 5.5: A schematic diagram of the different positions of the HAP vial inside the quartz cell.](image)
Figure 5.6: Raman signal acquired from HAP vial in three different positions of the beam axis (middle, front, back) in a quartz cell filled with water (transparent sample). The values indicate the heights of the phosphate peak (959 cm\(^{-1}\)). Acquisition time: 1 s x 30 accumulations. The spectral offset has been adjusted for clarity.

We notice that the HAP vial has the maximum signal at the back of the cell, where the focusing point of the laser beam is and the majority of the Raman photons generated are collected by the fibre bundle. The second higher HAP signal is observed when the HAP vial is in the middle of the cell, followed by the position at the front as expected. The change of HAP intensity in different positions in the quartz cell when the medium lacks any scatterer or absorber (plain water), is due to the change of the solid angle of collection. When the HAP vial is closer to the collection side, the collection angle is the largest possible and more of the scattered photons are collected.
Introducing scattering (Intralipid):

To bring the tissue phantoms closer to the real tissue properties, scattering and absorption agents had to be introduced in the model. For that reason, Intralipid, a scattering medium of emulsified fatty acids which is described in Chapter 4, was added into the quartz cell surrounding the HAP vial. In order to explore the effect of scattering on the Raman signal, measurements were recorded with the HAP vial in different positions (front, middle, back) in the same way as in Fig. 5.5 and in a solution of fixed scattering concentration (0.3%).
Figure 5.7: Raman signal before (blue line) and after spectral denoising (red line) acquired from HAP vial (960 cm\(^{-1}\)) in positions front (a), middle (b) and back (c) on the beam axis and while the quartz cell was filled with 0.3% Intralipid solution. Acquisition time: 10 s x 10 accumulations.
We notice that the behaviour of Raman signal changes compared to the water measurements earlier (Fig. 5.6). The maximum signal is still observed when the HAP vial is at the back of the cell, but the second maximum is now at front, and they have both comparable values. The minimum was absent when the vial is in the middle of the quartz cell, where no HAP peak is observed.

In the next step we explored how the concentration of the scatterer affects the Raman signal of HAP. A series of measurements were recorded for different concentrations of Intralipid while the HAP vial was in a fixed position (back). Background measurements were also recorded for the same concentrations of Intralipid in the absence of a HAP vial and were subtracted afterwards from the initial measurements (Figure 5.8). No absorber was added to the solution at this stage.

![Figure 5.8: Raman signal acquired from HAP vial at the back of the cell while the concentration of the Intralipid (IL) in the aqueous solution was increased gradually (from 0.05% to 0.3% IL). Acquisition time: 10 s.](image)

It is important to observe here how the intensity of HAP decreases as the scatterer concentration and therefore the diffuse scattering are increasing. It is
obvious that the more Intralipid molecules there are in the phantom, the more difficult it is for the laser photons to reach the HAP vial and for the generated Raman photons to reach the collection optical fibres. In even higher IL concentrations, the transmittance of the sample would be so low that almost no laser photons would reach the HAP vial to generate Raman signal (which would presumably drop to zero).

**Introducing absorption (Nigrosin, haemoglobin, Indian ink):**

In order to simulate real tissue optical properties, absorption also needed to be introduced in the phantoms. Since many different absorbing agents have been used for phantom fabrication in various studies, it was useful to characterise spectroscopically our potential absorbers first, in order to identify the most suitable one for our purposes. For that reason, Raman spectra were recorded from nigrosin, haemoglobin and Indian ink in the deep Raman setup (Fig. 5.9).

![Raman spectra of HAP vial in an aqueous phantom of (a) Nigrosin (10 s x 10 accumulations), (b) haemoglobin (10 s x 10 accumulations) and (c) Indian ink (6 s x 5 accumulations) on a transmission mode. No scatterer is present. The spectral offset has been adjusted for clarity.](image)

**Figure 5.9:** Raman spectra of HAP vial in an aqueous phantom of (a) Nigrosin (10 s x 10 accumulations), (b) haemoglobin (10 s x 10 accumulations) and (c) Indian ink (6 s x 5 accumulations) on a transmission mode. No scatterer is present. The spectral offset has been adjusted for clarity.
From Figure 5.9 shows that Nigrosin exhibits a high fluorescence background. Since the absorber would be chosen in order to provide broad band absorption without fluorescence contributions, Nigrosin was not suitable as an absorbing agent for measurements in this range of wavelength. The second option was haemoglobin (Hb) which has a suitable Raman spectrum but it is also linked to stability issues when mixed with Intralipid. Indeed, the integrity of haemoglobin in an Intralipid aqueous solution was observed over time, showing a formation of aggregates on the solution surface (Fig. 5.10c) and a subsequent change in clarity of the initial solution (Fig.5.10a-b). For all these reasons Indian ink was chosen as an absorbing agent in the tissue phantoms.

![Image](image.png)

**Figure 5.10:** Observation of haemoglobin stability in an Intralipid aqueous solution over time. The solution before (a) and after (b) a set of measurements that lasted 3 hours. Formation of aggregations can also be observed on the surface of the solution (c) after a long interaction time between Intralipid and haemoglobin.

We can now explore how the concentration of Indian ink affects the Raman signal of HAP when HAP vial is in a fixed position (back). Measurements of phantoms of different absorber concentrations in absence of Intralipid were recorded in the transmission deep Raman setup.
Figure 5.11: Raman signal acquired from HAP vial on the back of the cell while the Indian ink concentration in an aqueous solution was increasing gradually (from 0.1 µl/ml to 0.8 µl/ml). The values indicate the heights of the phosphate peak (959 cm$^{-1}$) in a.u. Acquisition time: 0.25 s x 120 accumulations. The spectral offset has been adjusted for clarity.

The intensity of HAP decreases as the Indian ink concentration increases, because both of the laser and the generated Raman photons are being absorbed and therefore fewer photons reach the collection fibre optics.

**On and off the beam axis measurements:**

The effect of the HAP position on the collected Raman signal has only been explored in terms of the vial position when this is aligned with the beam axis. However, due to the diffuse scattering we expect the Raman signal to be detectable outside the beam limits. In order to explore the spatial detection limits and the Raman scattering distribution more consistently and in greater detail, the
phantom transmission signal was recorded while the HAP vial was at 300 different positions on a grid inside the quartz cell (Fig. 5.12) as described in Chapter 4.

Figure 5.12: The top view of the phantom with the HAP vial inside the quartz cell, in a 300 position grid (less positions are shown here for simplicity purposes).

The measurements took place in a set of phantoms with varying optical properties, in order to simulate the range of clinically relevant optical properties of prostate and breast tissue (Chapter 4 and Chapter 7 - Table II). The results of these measurements are presented below.

5.3 Individual maps of scattering and absorption in transmission mode using HAP

Raman scattering mappings were first recorded in scatterer (Intralipid) only phantoms, in order to assess the effect of scattering alone on the signal distribution of the maps. The amount of scattering was defined by the concentration of the Intralipid added to each phantom, which was in turn related to the scattering coefficient of the phantom (see Chapter 4 for calculations).

Initially, a plain water mapping was recorded for each one of the two sides of HAP vial (Fig. 5.13), in order to check that the orientation of the vial does not have any effect on the signal distribution throughout the phantom.

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Figure 5.13: The two different sides of HAP vial and their corresponding 2D plots in a water solution where laser photons are injected on the left of the image and Raman signal is collected on the right. Every pixel represents a different location of the HAP vial in the cell whereas the colour of the pixel indicates the intensity of ~960 cm\(^{-1}\) phosphate Raman band of HAP.

Between the two different sides of the HAP vial, no significant difference in Raman signal intensity or distribution is observed. This means that any differences on spatial signal distribution between the phantoms, cannot be attributed to the orientation of the vial enhancing in this way the reproducibility of the measurements.
In a next step, Raman scattering maps for tissue phantoms with increasing Intralipid (IL) concentrations and in the absence of absorption were recorded. Both 1D and 2D plots of the mappings were reconstructed (Fig. 5.14). In the 2D plots, every pixel represents a different location of the HAP vial in the cell whereas the colour of the pixel indicates the intensity of ~960 cm\(^{-1}\) phosphate Raman band of HAP for this position. The 1D plots represent the generated Raman signal along the optical axis ('on axis' Raman intensity).
**Figure 5.14:** The origin of Raman signal in different Intralipid concentration tissue phantoms when no absorber is present. The Intralipid concentration and the equivalent scatterer coefficient ($\mu'_s$) of each phantom is indicated on the 2D plots. The intensity scales of the 1D plots have been chosen to highlight the change of HAP intensity across the phantom depth.
In the plain water mapping (Fig. 5.14a) significant Raman signal is only observed on the beam axis positions and not outside of the laser beam boundaries, therefore no diffuse scattering is present as expected. Once the scattering agent of Intralipid is introduced into the system (Fig. 5.14b), laser photons are elastically scattered between its suspended lipid droplets and therefore they are able to reach the HAP vial and interact with it in more distant off axis positions compared to the plain water mapping. This is why Raman photons can be detected outside of the boundaries of the illuminating beam axis, with detection limits that depend on the reduced scattering coefficient (Intralipid concentration) of the tissue phantom.

As the concentration of the scatterer in the phantom increase (moving from Fig. 5.14a–g), the scattering distribution pattern changes. In phantoms with low reduced scattering coefficients (Fig. 5.14b) we observe a form of symmetry in the Raman signal between the illumination (left) and the collection (right) side. It should be considered here that most of the Raman photons in the phantom are expected to be generated on the illumination side because this is where most of the laser photons are entering the cell without having being scattered by the medium yet and as a result they interact with the HAP vial with the greatest probability of inducing Raman events. However, the Raman photons which are generated on the illumination side, need to propagate across the entire width of the cell through the turbid medium to the right side in order to be detected; undergoing loss due to scattering and absorption. On the other hand, when the HAP vial is on the collection side of the phantom, the Raman photons generated there are most efficiently collected, since this is where they have the least distance to travel in order to reach and be detected by the collection optics.

The Raman scattering distribution through the phantom volume will always be a balance between these two phenomena. As Intralipid molecules in the tissue phantom increase in concentration (Fig. 5.14c and d), it is progressively less likely for the Raman photons generated at the illumination side to pass through the phantom volume and be detected on the other side of the cell prior to being absorbed or scattered out of the sample (signal balance moves to the collection
As Intralipid concentration rises further (Fig. 5.14e–g), the diffuse scattering and therefore the path lengths for both laser and Raman photons increase considerably. Because of the water presence in the phantoms, as the photon path length increases, its absorption will have a more significant impact on the Raman signal distribution pattern. As mentioned in the literature and also confirmed with our measurements (Chapter 4- Fig. 4.9), water absorbs the Raman photons generated in our setup (at 902 nm) more than the laser photons (830 nm). This may explain why the signal balance moves to the collection surface (right), as it is easier for laser photons to traverse diffusely the cell than Raman photons, when the scattering increases.

Another important feature evidenced by the maps is that the higher the Intralipid concentration is, the broader the measurable signal region becomes. This means that a lesion located off the illumination–collection axis would be more likely to be detected in tissues with higher turbidity (modelled with higher Intralipid concentrations) than a lower one. This becomes clear if we compare maps with low Intralipid concentrations (Fig. 5.14b and c) with the ones with increased Intralipid (Fig. 5.14d–g).

**Ink maps**

Raman scattering mappings were also recorded in absorber only phantoms in order to observe the individual influence of absorption on the map signal distribution. The absorber of our choice was Indian ink for the reasons described earlier. Three different concentrations of Indian ink, which correspond to different absorption coefficients calculated in Chapter 4, were explored (Fig.5.15).
Figure 5.15: The origin of Raman signal in different Indian ink concentration tissue phantoms when no scatterer is present. The Indian ink concentration and the equivalent absorption coefficient ($\mu_a$) of each phantom is indicated on the 2D plots. The intensity scales of the 1D plots have been chosen to highlight the change of HAP intensity across the phantom depth.

In the ink mappings above, we observe that no Raman signal is recorded outside of the boundaries of the laser beam because of the lack of diffuse
scattering. However, when the ink concentration is doubled, the signal intensity drops by at least half. The ink absorbs both laser and Raman photons and the higher its concentration is, the less signal is recorded. In higher concentrations (Fig. 5.15c), we observe a maximum signal on the collection side which is a result of the laser focus on the system.

The maps above support our understanding of Raman scattering distribution throughout the phantom volume, when absorber and scatterer are added individually. In the next step, we explore how this distribution changes under the influence of both scattering and elevated absorption, which is the case of real tissue.

5.4 Combined scattering and absorption maps in transmission mode using HAP

In this section, both scattering (Intralipid) and absorbing (Indian ink) agents were combined in liquid tissue phantoms in order to simulate the real tissue optical properties of breast and prostate (Chapter 4 and Chapter 7- Table II). Similar to the previous measurements, the recovered Raman signals from various locations of the HAP vial inside the phantom, were explored in terms of its optical properties (Fig. 5.16).
Figure 5.16: Raman scattering 2D(i) and 1D(ii) spatial distribution plots of HAP in phantoms with different Intralipid/ink concentrations. The intensity scales of the 1D plots have been chosen to highlight the change of HAP intensity across the phantom depth.

In the combined scattering and absorption maps, when scattering and absorption agents are both present at low concentrations (Fig. 5.16a), we observe a strong Raman signal from both the illumination and the collection sides.
As explained earlier this is because of the balance between the most efficient Raman photon generation on the illumination side and the most efficient photon detection on the collection side. If we double the density of scatterer but maintain the absorber concentration constant (Fig. 5.16b), the signal on the collection side is being significantly suppressed. This is due to the impact of the ink absorption in the phantom. Indian ink in aqueous solution has been shown through UV/Vis absorbance measurements (Chapter 4 - Fig. 4.7) to absorb the laser photons (830 nm) more than the generated Raman ones (902 nm), i.e. the opposite net effect to that induced by water alone (Chapter 4 - Fig. 4.9). Consequently, the detected signal is much less likely to originate from the collection side of the phantom, as there are relatively less laser photons reaching it.

As a matter of fact, when we increase the scattered concentration, the photon path length is significantly increased and in the presence of ink this directly increases the chances of the laser and Raman photons being absorbed by it. For the same reason, when we increase the ink concentration, we expect a similar effect on the mapping pattern (Fig. 5.16c) where IL concentration was kept constant as in (a) but the concentration of the ink was increased.

The significant impact of absorption on the phantoms is also demonstrated by the fact that in similar experimental\textsuperscript{252} and theoretical measurements\textsuperscript{253, 254} of samples with no or minimal absorption, such as pharmaceutical tablets, the Raman scattering distribution is quite different. Indeed in these experiments the maximum Raman signal originates from objects buried in the middle of the tablets rather than towards its edges. Overall because of the size (extended/infinite lateral dimensions) and optical properties (absence of significant absorption in tablets) differences between maps in biological tissue and pharmaceutical formulations are expected to be significant.

### 5.5 Scattering and absorption maps in transmission mode using trans-stilbene

In order to characterise the system at the maximum range of prostate tissue relevant absorption and scattering conditions, which are higher than those used in the phantoms earlier, we had to resort to a stronger Raman scatterer, trans-stilbene. By replacing HAP with trans-stilbene it was possible to probe higher
IL/ink concentration solutions with the existing Raman apparatus and while recording trans-stilbene’s peak at 1192 cm$^{-1}$ (921 nm).

![2D Raman Intensity of trans-stilbene](image1)

![1D Raman Intensity of trans-stilbene](image2)

(a) Phantom 4: 1% IL and 0.2 μl/ml ink ($\mu'_s=9.2$ cm$^{-1}$, $\mu_a=0.8$ cm$^{-1}$)

(b) Phantom 5: 1% IL and 0.5 μl/ml ink ($\mu'_s=9.2$ cm$^{-1}$, $\mu_a=2.26$ cm$^{-1}$)

**Distance from the illumination side in mm**

**Figure 5.17:** Raman scattering 2D(i) and 1D(ii) spatial distribution plots of trans-stilbene in phantoms with different Intralipid/ink concentrations. The intensity scales of the 1D plots have been chosen to highlight the change of HAP intensity across the phantom depth.

In the figures above, we can still observe the same distribution pattern with the two signal maxima on the illumination and collection side. However, when we increase the absorption by more than twice (Fig. 5.17b), the collection side becomes more dominant. We should mention here that the absorption coefficient in Fig. 5.17b is well above the absorption range of human prostate and breast
tissue (Chapter 4 and Chapter 7 - Table II) and was only tested to assess the
detection limits of our system. Additionally, with this level of absorber present, the
Raman signal measured was of the order of the noise, which makes the data
processing prone to errors.

Another reason why we might observe this artefact is the liquid phase of tissue
phantoms. As the particles in the solution are suspended rather than immobilized
in the phantom phase, their position is changing during the mapping, potentially
resulting in precipitation of ink and Intralipid molecules with time. Given that the
measurements are recorded starting from the illumination (left hand) side and the
solution is not stirred during the mapping, by the time that the measurements on
the collection side have finished, many of the particles in the solution will have
precipitated from the surface to the bottom of the phantom, leaving an optically
clearer solution behind, with less absorption and scattering on its main volume.
However, after the assessment of the phantom stability (Chapter 4 - 4.1.2.1), the
Raman signal variation due to the liquid nature of the phantoms was found to be
present only in combined (scattering + absorption) phantoms and in a range of
less than 10%. This variation is high enough to affect the experimental results
only when the signal to noise ratio is really low and the recorded Raman peak is
close to noise levels, as in Fig. 5.17b. As a conclusion, although this solution
instability exists in all of the phantoms recorded, it only makes a difference here,
where the absorption and scattering is quite high and hence the HAP Raman
signal is very low in intensity and barely visible above the background baseline.

5.6 Scattering and absorption maps in an iSORS
mode using trans- stilbene

Similar mappings were also recorded in an iSORS mode and in different offsets
with approximate radius of 1.5 mm (minimum), 4.5 mm (middle) and 7.5 mm
(maximum). The phantom used had a reduced scattering coefficient of 4.6 cm⁻¹
and absorption coefficient of 0.37 cm⁻¹ (Figure 5.18).
Figure 5.18: Raman scattering 2D(i) and 1D(ii) plots of one trans-stilbene phantom (μ'\(_s\): 4.6 cm\(^{-1}\) and μ\(_a\): 0.37 cm\(^{-1}\)) in three different offsets (minimum, middle and maximum) on an iSORS mode. The intensity scales of the 1D plots have been chosen to highlight the change of HAP intensity across the phantom depth.

From the maps above we observe that the distribution of the Raman signal is higher in the centre of the ring as expected, and lower as the distance from the
centre increases. In minimum offset, where the radius of the collection circle is minimized, the depth of recovered Raman signal seems to be smaller compared to the maximum offset (7.5 mm), where it is possible to recover signal from a bigger area of the phantom. We can also observe that the maximum signal in iSORS is orders of magnitude higher compared to similar phantoms recorded in transmission (Fig. 5.14). This is because the phantom volume through which the photons have to travel in iSORS is much smaller compared to the full phantom length in transmission mode.

5.7 Discussion

In order to assess the potential of measuring the Raman scattering of breast and prostate gland in vivo in a transmission mode, we fabricated and measured the transmitted Raman scattering from liquid tissue phantoms that mimic human prostate and breast tissue. To achieve maximum efficiency of the phantoms and similarity to the human tissue, all of the separate components had to be chosen and assessed spectroscopically, a procedure which is described in the start of the chapter.

As the phantom design was established and assessed with preliminary measurements, detailed Raman scattering maps were recorded for the equivalent in size and optical properties tissue phantoms, when a calcification vial was moved inside them in 300 different positions. In this way we explored the spatial distribution of the Raman signal over the phantom area, not only in a variety of optical properties, but also when the calcification was out of the beam axis and on different distances from it.

In the clinical arena, these measurements can be of importance in assessing the origin and detection limits of Raman scattering of a malignant area when scanning for prostate or breast cancer. The malignant area can be either a group of calcified formations, as it is very often observed in breast cancer, or simply an area with a different Raman signal from the surrounding normal tissue which would in this way stand out from the total volume Raman signal.

In terms of the detection limits, which are different for each system and tissue environment, it was shown that if the measurement parameters (tissue optical properties, Raman profile of the malignant area/lesion, laser spot diameter) are
known, it is possible to assess the minimum distance from the beam axis in which the signal can be detected. In the case of our study the effective measurement area would be around 30 mm x 30 mm for both prostate and breast tissue. Assuming a 45 mm x 30 mm mean dimension human prostate gland, it would be possible to cover its measuring volume using only a couple of transmission measurements in a potential scanning for prostate cancer (Fig. 5.19).

![Figure 5.19: Spatial distribution of Raman scattering intensity of a potential calcification (HAP) throughout the prostate gland.](image)

In terms of the Raman signal origin, it has been clearly shown how the distribution of the collected Raman scattering signals changes under different values of absorption and scattering coefficients of the phantom and hence in different types of tissues. It has been demonstrated that the highest signals are likely to be obtained from nearer surface lesions (either on collection or illumination side) than those at depth using transmission Raman. Even in this case though, relative signals are likely to be as high as 40–60% for ‘lesions’ found in the centre of organs compared to those found towards the edges.

In conclusion, this chapter demonstrates the potential for future applications of transmission Raman, either in the breast or applied through fibre probes, to the prostate. The tissue phantom measurements have expanded our understanding of how the distribution of collected Raman scattering signals change in relation to a range of expected optical properties of tissues. This is expected to support
the future design of instruments which will target the disease specific signals within particular regions of the sample and pave the way towards the ultimate application of non-invasive deep Raman diagnosis of prostate and breast cancer.
Chapter 6: Deep Raman spectroscopy on prostate tissue

6.1 Introduction

A significant amount of research has been conducted on the study of prostate tissue using Raman spectroscopy. Different modalities of Raman spectroscopy have been used in the past to acquire Raman signal from different depths of prostate samples (TURPs)\cite{213} and discriminate between benign and malignant (adenocarcinoma) human prostate tissue\cite{168, 193, 256, 257}. Prostate cell lines of different aggressiveness have also been assessed in terms of aggressiveness by means of Raman spectroscopy\cite{258}.

The molecular differences between different pathologies of tissue show reduced concentration of glycogen and increased concentration of nucleic acids in malignant tissue compared to benign hyperplasia\cite{193}. Similar studies on healthy and different pathologies of prostate tissue have revealed that DNA content increases as the tissue progresses from normal to malignant\cite{259}, as the nuclear to cytoplasm (actin) ratio increases. The collagen amount which is abundant in extracellular matrix can either increase or reduce, depending on the tumour size. On the other hand, choline which has been identified as a malignant disease marker before, and cholesterol are both increasing with disease progression probably as a response to increased cell necrosis and membrane synthesis due to increased proliferation respectively. The same study also provides evidence on more components, such as oleic acid, cholesterol, triolein and actin\cite{192}.

However, transmission and iSORS Raman spectroscopy, which can be used more easily as minimally invasive and real-time tools, have never been used for prostate tissue studies. Having already set the basis for measuring tissue phantoms of similar size and optical properties on the same setup (Chapter 5), in this chapter human prostate tissue is explored.

For this reason, we first assess the feasibility of measuring animal tissue individually and then layered with a stronger Raman scatterer (HAP vial). At a later stage, we approach the study of TURPs and prostate biopsies with both conventional and deep Raman spectroscopy.
6.2 Measuring animal tissue

The Raman signal of three major tissue components (protein, fat and muscle) were assessed on the deep Raman setup by measuring chicken breast, pork fat and beef shin respectively. Animal tissue blocks were cut in thickness of 0.5 and 1 cm and measured in a transmission mode. The results are presented in Fig. 6.2.

Figure 6.1: Three different types of animal tissue: chicken breast (a), pork fat (b) and beef shin (c), on the sample holder of the deep Raman setup.
In the measurements outlined above we can observe characteristic peaks from main components of chicken, pork fat and beef shin. More specifically, peaks observed in the chicken spectrum such as 1657 cm\(^{-1}\), 1450 cm\(^{-1}\), 1003 cm\(^{-1}\) and 875 cm\(^{-1}\) are representative of protein and correspond to \(\nu(C=O)\) of \(\alpha\)-helix conformations, CH\(_2/\text{CH}_3\) bending and amino acids phenylalanine and hydroxylproline respectively\(^{260}\). In beef shin we can notice a peak at 477 cm\(^{-1}\) which corresponds to the muscle glycogen due to the glycogen skeletal deformation vibration\(^{261}\). This peak is more obvious in chicken spectrum which also contains an amount of muscle tissue. In the pork fat Raman spectrum, peaks at 1063 cm\(^{-1}\) (out-of-phase aliphatic C–C stretch), 1124 cm\(^{-1}\) (in-phase aliphatic C–C stretch), 1265 cm\(^{-1}\) (in-plane \(\text{cis}\) olefinic hydrogen bend), 1298 cm\(^{-1}\) (\(\delta(\text{CH}_2)_{\text{hw}}\) methylene twisting deformations), 1448 cm\(^{-1}\) (\(\delta(\text{CH}_2)_{\text{sc}}\) methylene scissor deformations),
1657 cm$^{-1}$ ($\textit{cis}$ $\nu$(C=C) olefinic stretch) and 1745 cm$^{-1}$ ($\nu$(C=O) carbonyl stretch) were observed and assigned to vibrations of adipose tissue$^{262}$.

In a next stage, we assessed the signal from the animal tissue above, layered with hydroxyl-apatite (HAP), a material of high cross-section and biological affinity to the tissues due to the presence of calcifications in some of them. A HAP vial of dimensions described in Chapter 4, was layered with beef shin (Fig. 6.3), chicken breast (Fig. 6.4) and pork fat (Fig. 6.5) of 0.5 and 1 cm.

![Figure 6.3: Transmission Raman spectra of beef shin tissue with and without an extra layer of the HAP behind the tissue section. The spectral offset has been adjusted for clarity.](image-url)
Figure 6.4: Transmission Raman spectra of chicken breast tissue with and without an extra layer of the HAP behind the tissue section. The spectral offset has been adjusted for clarity.
Figure 6.5: Transmission Raman spectra of pork fat tissue with and without an extra layer of the HAP behind the tissue section. Their baselined difference has been also plotted (blue line) in order to confirm the presence of 959 cm\(^{-1}\) peak. The spectral offset has been adjusted for clarity.

The results show that for the same tissue thickness, chicken breast and beef shin allow the HAP signal to be detected more easily. In the case of the pork fat, the Raman signal of the HAP is still barely detectable, only after the subtraction of the two spectra (pork fat with and without HAP). This is because adipose tissue has a larger Raman scattering cross-section relative to protein (chicken)\(^{196}\). Indeed, higher peak intensity is observed in pork fat spectrum, although the measurements were recorded for the same acquisition time and the thickness of the tissue was the same.

Smaller pieces of animal tissue were also measured on both transmission and inverse SORS (iSORS) mode of the deep Raman setup, in order to simulate
dimensions similar to prostate tissue samples (20 mm length x 2.5-3 mm width) and compare the performance of the two modes.

**Figure 6.6:** Measurements from chicken breast and bacon on transmission and iSORS mode of deep Raman setup (The acquisition time for transmission and iSORS mode is 1 s and 60 s respectively, however the Raman intensity of the measurements has been adjusted for comparison reasons). The spectral offset has been adjusted for clarity.

Measurements with both transmission and iSORS show that the Raman signal for the same tissues is reproducible between the two modes. The collected Raman scattering on iSORS tends to be weaker compared to the transmission one. On the other hand, longer acquisition times and hence better signal quality can be applied here due to lack of CCD saturation issues.
6.3 Measuring TURPs

**Transmission Raman:** After the signal from the various biological tissues had been assessed on the deep Raman setup, we recorded measurements of prostate samples collected with the procedure of transurethral resection of the prostate (TURP), a surgical procedure commonly used to treat benign prostate hyperplasia (BPH).

![Image of TURP chips](image)

**Figure 6.7:** One of the TURP chips and its dimensions measured on a transmission deep Raman mode.

For the transmission mode, the laser spot size (3-4 mm diameter) was bigger than most of the TURP chips width (3-5 mm) at many points across their length. Although a considerable amount of light was being transmitted through the tissue sample, no Raman photons were successfully collected, whereas a CCD saturation issue was present in transmission measurements for different acquisition times and powers. That would be attributed either to laser light not interacting (scattered or absorbed) with the sample or high fluorescence levels generated from the laser-sample interaction, or both. In order to explore further, the same TURP chips were also measured in an iSORS mode of the deep Raman setup.
**iSORS mode:** A number of TURP chips which were measured in an iSORS mode, have shown that the Raman signal was dominated by a fluorescence background in these measurements (Fig. 6.8).

![Graph showing Raman shift vs. Raman intensity for different TURP chips from different patients](image)

**Figure 6.8:** Fluorescence background from TURP chips from 4 different patients during 1 s acquisition time on iSORS mode.

The presence of fluorescence while measuring TURP chips has been confirmed by M. Prieto before. Different positions measured on the same TURP chip, using different acquisition times (Fig. 6.9), also did not change the fluorescence background emerging from the sample.
Figure 6.9: Fluorescence background from the same TURP chip during different acquisition times on iSORs mode. Acquisition time: 10 min, 5 min and 2 min for pink, green and red plot respectively. The spectral offset has been adjusted for clarity.

The presence of fluorescence can be attributed to the procedure which is followed for the TURP sample collection. These samples are collected during transurethral resection of the prostate (TURP), a surgical procedure where tissue is being resected with a hot electrical wire loop that cuts through it. Hence we expect the tissue to be burnt and as such exhibiting high absorbance followed by fluorescence/luminescence.

**Raman microscopy:** In order to explore the origin and distribution of fluorescence on the TURP chips, the samples were also measured with conventional Raman microscopy on a Renishaw System 1000 (Chapter 4).

Measurements were first recorded from random points on the surface of a TURP chip (PC006), giving the same fluorescence background as deep Raman (Fig.
In order to check the tissue inside the TURP for fluorescence, a couple of TURP chips (PC005, PC007) were sectioned in the middle and measured on the Raman microscope. Raman spectra from different areas on the tissue of the chips are presented in Fig. 6.10.

Figure 6.10: Raman spectra and characteristic white light images from (i) not burnt and (ii) burnt areas on TURP chips from 3 different patients. Acquisition time: (i) 15 s, 2 s x 5 and 2 s x 5 for PC007, PC005 and PC012 respectively, (ii) 3 s, 3 s and 1 s for PC007, PC005 and PC012 respectively. The spectral offset has been adjusted for clarity.
Two types of areas were observed on the TURP chips: (i) burnt areas exhibiting fluorescence and (ii) non-burnt areas with a clear Raman signal and biologically relevant peak positions. Both of the areas and their Raman spectra are shown in Figure 6.10. Peaks observed in areas free of fluorescence, have been assigned to protein bond vibrations (1251 cm$^{-1}$, 1651 cm$^{-1}$)$^{264}$.

In order to study in greater detail the location of the burnt locations over the sample area, two of the TURPs (PC011, PC014) were cryosectioned, and sections from the middle of the TURP chip volume were laid onto calcium fluoride substrates and mapped using 830 nm wavelength on a Renishaw inVia Raman spectrometer (procedure in Chapter 4).

The measurements from Raman microscopy mapping were preprocessed as described in Chapter 4 and analyzed using Matlab (Fig. 6.11).
Figure 6.11: White light images (A) and Raman microscopy maps (B) of cryosectioned TURP chips (PC011, PC014). The maps (1200 l/mm grating centered at 1300 cm$^{-1}$, 6 s/line acquisition time, 50 μm step, Streamline mode) show the distribution of saturated spectra which correspond to burnt areas over the chip surface. For PC014, a higher resolution smaller map (red line square) was recorded to assess the fluorescence limits in detail.

Results from Raman microscopy measurements locate the saturated spectra, characteristic of the fluorescence background, on the periphery of the sample section. This shows that only the outside surface of the TURP is affected by the
surgical procedure for the TURP collection. We should also observe the lack of fluorescence spots on the top side of PC014 on the Raman map (Fig. 6.11). This is because PC014 was the first chip to be collected from the patient during the TURP procedure and hence only the one side of it was cut with the hot wire, leading to the burnt area.

6.4 Measuring prostate biopsies

Prostate tissue samples collected through Trans-Rectal Ultrasound guided biopsy were measured on deep Raman setup in a transmission and iSORS mode. Once the biopsies were collected, they were laid on acetate paper which, although not an ideal substrate for Raman spectroscopy, it is frequently used in pathology for sample orientation and microtome sectioning.

Initially, measurements were recorded from a biopsy (TB1335 L) on acetate paper (Fig. 6.12-A) as the biopsy was too thin to be removed onto a different substrate. The sample was measured on deep Raman setup and in conventional Raman (Fig. 6.13), on successive points across its length.

Figure 6.12: Prostate biopsies laid on (A) acetate paper and (B) calcium fluoride substrate.
The Raman measurements on the first biopsy show that it is not possible to collect sufficient Raman scattering signal in deep Raman configuration, potentially due to the very small amount of tissue that the biopsy contains and does not allow sufficient photon propagation. Most of the signal in both transmission and iSORS mode seems to derive from the acetate paper. In contrary, Raman microscopy, provides a mixture of Raman signal from both the biopsy tissue and the acetate paper as it is strongly biased towards imaged biopsy layer.

A following attempt has been made to measure a second prostate biopsy on a calcium fluoride (CaF\textsubscript{2}) substrate (Fig. 6.12-B), in order to avoid any additional Raman signal. CaF\textsubscript{2} substrates are commonly used in Raman spectroscopy as they are free of Raman signal in the main fingerprint region\textsuperscript{265}. The biopsy has
been measured both in deep Raman setup and in conventional Raman across its length (Fig. 6.14).

![Raman intensity vs Raman shift graph]

**Figure 6.14:** Raman measurements of the biopsy on calcium fluoride substrate in transmission, iSORS and conventional Raman microscopy. Acquisition time: 1 s, 15 s and 2 s x 4 for transmission, iSORS and inVia modes respectively. The spectral offset has been adjusted for clarity.

The results of Raman measurements from the second biopsy samples are similar to the previous one, as deep Raman configuration again fails to collect any Raman scattering. On the other hand, the confocality of Raman microscopy provides well-shaped Raman spectra lacking any signal from the substrate.

The peaks observed in the Raman microscopy spectra from both of the biopsies correspond to peaks characteristic to prostate tissue as described in literature\textsuperscript{264} with proteins (854 cm\textsuperscript{-1}, 937 cm\textsuperscript{-1}, 1003 cm\textsuperscript{-1}, 1033 cm\textsuperscript{-1}, 1450 cm\textsuperscript{-1}, 1659 cm\textsuperscript{-1}), DNA (1337 cm\textsuperscript{-1}) and lipids (1450 cm\textsuperscript{-1}, 1659 cm\textsuperscript{-1}) to be prevalent.
We should also notice the difference between the Raman microscopy signals from the two biopsies. The biopsy laid on the acetate paper seems to provide a stronger Raman signal compared to the one laid on the calcium fluoride substrate. This is largely because in the case of the transparent and minimally scattering substrate (CaF$_2$), the photons will escape the sample easier by travelling across the other side of the substrate. In the case of the acetate paper, the photons travelling through the biopsy will be scattered partially back by the diffusely scattering acetate paper on the other side of it. This would enforce both laser and Raman photons to change their direction towards the collection side, with the first ones to be given higher chances to interact with the sample and generate Raman scattering – both processes leading to the enhanced detection of Raman signal at the collection biopsy surface.

6.5 Discussion

In this chapter we investigated the feasibility of collecting Raman scattering from different types of prostate tissue. In order to assess the spectral response of major tissue components (protein, fat, muscle), we first started by measuring animal tissue (chicken breast, pork fat and beef shin) in both transmission and iSORS modes, confirming fat yields stronger Raman signal due to its higher Raman cross-section. The different types of animal tissue were also overlaid with a component biologically similar to tissue calcifications (HAP), proving that it is more detectable under muscle and protein compared to fat.

Moving over to prostate samples, the first measurements were attempted on TURP chips which usually consist of benign tissue derived from prostate hyperplasia. Measurements on both modes of deep Raman setup (transmission and iSORS) were dominated by fluorescence. This is a consequence of the transurethral resection of prostate, the surgical procedure where a hot electrical wire is used to collect the samples. In this way, the surface of the chip results in being burnt and exhibiting fluorescence.

This was further confirmed by measuring the same samples with Raman microscopy, where single and map measurements were recorded on the outer and interior tissue of the TURP chips. White light images and single Raman spectra showed a morphological and spectral difference between outer (burnt)
and inner (not burnt) areas of sectioned chips. Mapping measurements on TURP cryosections proved that the saturated spectra which correspond to burnt tissue are distributed on the outline of the TURP section, leaving all of the inner prostate tissue fluorescence free.

Prostate biopsies collected were also measured on deep Raman setup, failing to provide any sufficient Raman signal. This is due to the very low thickness of the specific biopsy. As the prostate biopsies are collected with TRUS biopsy, from 12 different locations of the human prostate, they tend to be very limited in dimensions (around 12 mm long × 1 mm thickness). As a result, the technique used for the measurements needs to be able to focus and collect the Raman scattering from small areas near the surface on the sample (confocality), rather than acquiring a volume signal as in deep Raman mode. This was confirmed by the measurements recorded using Raman microscopy on the same biopsies.

The results of this chapter highlight the limitations of the deep Raman spectroscopy, which include the presence of significant absorption and fluorescence as well as the need for a sufficient sample volume for the laser photons to efficiently propagate through sample and produce Raman scattering which can be effectively collected in deep Raman setups. At the same time, we demonstrate the efficiency of deep Raman on samples which lack the limitations above, as was shown with the initial measurements on animal tissue. The deep Raman study proved the feasibility of collecting Raman signal from the prostate gland which contains similar major tissue components (fat, protein, muscle) found also in the measured animal tissue, whereas Raman microscopy demonstrated that prostate gland is not intrinsically fluorescent and hence deep Raman measurements will not be inhibited by this issue.
Chapter 7: Signal enhancement in deep Raman scattering measurements in tissue phantoms using photon diode

7.1 Introduction

In previous chapters we explored the origin, distribution and quality of Raman signals through liquid tissue phantoms and mammary tissue respectively. In this chapter, we are looking into the enhancement of this signal as a function of both sample depth and optical properties, using a “photon diode” element\textsuperscript{159} (described in chapter 4- section 4.2.1.4).

The photon diode has been applied in deep Raman spectroscopy, enhancing the weak Raman signals present with these techniques. As the photons propagate through a sample of considerable volume, they can be either diffusely scattered or absorbed. Collecting these photons and eventually recovering the Raman signal from large depths can be quite challenging. Different approaches such as the increase of laser power, collection time, improvement of collection efficiency, etc.\textsuperscript{149}, can improve the Raman photon collection and hence the signal to noise ratio. An alternative passive approach is the use of a signal enhancing “photon diode” element, which has already been shown to provide a multifold enhancement of Raman signal.

The photon diode element has been used in a number of deep Raman studies in the past, mainly in order to improve the signal to noise ratio in pharmaceutical tablets. Literature studies involving a signal enhancement with photon diode are presented in Table I below.
<table>
<thead>
<tr>
<th>Signal enhancement</th>
<th>Type of demonstration</th>
<th>Modality</th>
<th>Sample thickness (mm)</th>
<th>Sample</th>
<th>Probing</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>x8.5</td>
<td>experimental</td>
<td>transmission</td>
<td>6-7</td>
<td>Paracetamol powder</td>
<td>bulk</td>
<td>269</td>
</tr>
<tr>
<td>x9.9</td>
<td>experimental</td>
<td>transmission</td>
<td>3.3</td>
<td>paracetamol tablets</td>
<td>bulk</td>
<td>234</td>
</tr>
<tr>
<td>x3.8</td>
<td>experimental</td>
<td>transmission</td>
<td>3.9</td>
<td>paracetamol tablet</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x3-5</td>
<td>numerical simulations</td>
<td>transmission</td>
<td>&lt;20</td>
<td>tissue (no absorption)</td>
<td>bulk</td>
<td>235</td>
</tr>
<tr>
<td>x6</td>
<td>numerical simulations</td>
<td>transmission</td>
<td>&gt;20</td>
<td>tissue (no absorption)</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x3-7</td>
<td>numerical simulations</td>
<td>transmission</td>
<td>1-4</td>
<td>pharmaceutical tablets</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x6.3</td>
<td>experimental</td>
<td>transmission</td>
<td>4</td>
<td>segmented tablet with PET-TiO2 layer in different depths</td>
<td>bulk</td>
<td>270</td>
</tr>
<tr>
<td>x4.3</td>
<td>numerical simulations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x6.5</td>
<td>experimental</td>
<td>transmission</td>
<td>4</td>
<td>paracetamol</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x8.3</td>
<td>experimental</td>
<td>transmission</td>
<td>5.8 (2 trans-stilbene + 3.8 paracetamol)</td>
<td>paracetamol + trans-stilbene (front layer)</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>x9.4</td>
<td>numerical simulations</td>
<td>transmission</td>
<td>4</td>
<td>paracetamol</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x6.7</td>
<td>experimental</td>
<td>SORS</td>
<td>4</td>
<td>paracetamol</td>
<td>bulk</td>
<td></td>
</tr>
<tr>
<td>x4.4 - 4.5</td>
<td>experimental</td>
<td>SORS</td>
<td>4.2</td>
<td>paracetamol overlaid with trans-stilbene</td>
<td>bulk</td>
<td>271</td>
</tr>
<tr>
<td>x1.5</td>
<td>experimental</td>
<td>transmission</td>
<td>27</td>
<td>Breast phantom (porcine tissue)</td>
<td>trans-stilbene</td>
<td>149</td>
</tr>
<tr>
<td>x1.6</td>
<td>experimental</td>
<td>transmission</td>
<td>14</td>
<td>chicken breast</td>
<td>bulk</td>
<td>235</td>
</tr>
</tbody>
</table>

**Table I**: Use of photon diode and enhancement factors achieved through the literature.
As transpires from the table above, the photon diode has been mainly used with pharmaceutical tablets. The concept has also been explored with biological tissue samples\textsuperscript{149, 235} but not as a function of the depth of the signal origin. Because of important differences in the optical properties between tablets and tissues, such as the lack of significant absorption in tablets and considerably longer mean free scattering path lengths in tissues (at least by an order of magnitude) due to the difference in spatial scales, we cannot assume identical behavior and enhancement.

In this chapter, we will explore the photon diode signal enhancement on phantoms and its dependence on both the depth of the signal origin and the sample optical properties. The phantoms which were employed simulate various human tissues in optical properties and particularly breast and prostate in size, due to the prevalence of the corresponding types of cancer. Apart from absorption and scattering agents, the phantoms also consisted of a high Raman cross-section compound representing a malignant element found in many cancerous conditions (e.g. calcifications). Since this element was located at different depths, we were able to explore the enhancement effect of the photon diode as a function of the inclusion depth inside each phantom and also relate it to the range of optical properties (absorption and scattering) of the tissue matrix.

### 7.2 Optical properties of various tissue types and phantoms

The tissue phantoms employed in this chapter were fabricated and measured similarly to the ones described in Chapter 4 and 5 respectively. The optical properties of the phantoms were adjusted to a clinically relevant range of human tissue. For this reason, a number of different types of tissue were studied in terms of optical properties (absorption and reduced scattering coefficient) through literature.

The optical property values that we took into consideration are presented in the table below and their relative distribution and relation to the phantom properties in Fig. 7.1. The reduced scattering coefficients were calculated as a function of the excitation wavelength\textsuperscript{226}, whereas the absorption coefficients are based on
literature values for skin, dermis, brain, white matter, breast, fat, bone, liver, at 850 nm, muscle, small bowel, aorta, stomach wall. For prostate tissue both the reduced scattering and absorption coefficients were extracted from separate studies.

**Figure 7.1:** Reduced scattering and absorption coefficient for different types of mammalian tissues obtained from literature data. Each sphere represents the range of optical properties for each individual type of tissue. The cross (shown with labels in Fig. 7.2) indicates the range of optical properties of the phantoms in the current study.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reduced scattering coefficient (cm(^{-1}))(^{226})</th>
<th>Absorption coefficient (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6.58</td>
<td>0.6- 1(^{278})</td>
</tr>
<tr>
<td>Prostate</td>
<td>4-9.5(^{224, 225})</td>
<td>0.002- 0.7(^{224, 225})</td>
</tr>
<tr>
<td>Aorta</td>
<td>7.07</td>
<td>3.16(^{280})</td>
</tr>
<tr>
<td>Muscle</td>
<td>8.13</td>
<td>0.2- 0.3(^{276})</td>
</tr>
<tr>
<td>Small bowel</td>
<td>8.80</td>
<td>0.05- 0.2(^{279})</td>
</tr>
<tr>
<td>White matter brain</td>
<td>9.41</td>
<td>0.75- 1.15(^{275})</td>
</tr>
<tr>
<td>Breast</td>
<td>9.84</td>
<td>0.068- 0.102(^{227})</td>
</tr>
</tbody>
</table>
### Table II: Optical properties of the different types of mammalian tissue as found in the literature. The reduced scattering coefficient values are presented in ascending order.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean Absorption (μm)</th>
<th>Mean Reduced Scattering Coefficient (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (mean)</td>
<td>10.7</td>
<td>0.4274</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>11.7</td>
<td>2.281</td>
</tr>
<tr>
<td>Fat (mean)</td>
<td>13.1</td>
<td>0.02276</td>
</tr>
<tr>
<td>Bone</td>
<td>15.9</td>
<td>0.2277</td>
</tr>
<tr>
<td>Skin (mean)</td>
<td>22.4</td>
<td>0.25272</td>
</tr>
<tr>
<td>Dermis</td>
<td>23.5</td>
<td>3.273</td>
</tr>
</tbody>
</table>

In order to cover most of the optical properties of different tissues (Fig. 7.1), the phantoms employed for this set of experiments were selected to cover the tissue range in both absorption and scattering. More specifically, the tissue phantoms fabricated ranged from 0- 1.63 cm⁻¹ in absorption and 6.4- 23.04 cm⁻¹ in reduced scattering coefficient (Fig. 7.2). More details on phantom fabrication can be found in Chapter 4.

**Figure 7.2:** Scatterer and absorber concentrations (a) and optical properties (b) of the liquid tissue phantoms prepared and measured with transmission Raman spectroscopy.
7.3 Effect of photon diode on the origin of Raman signal within phantom

In order to evaluate the impact of the photon diode on the signal enhancement and the origin of Raman scattering, the Raman signal was recorded while the high Raman cross-section inclusion (HAP or trans-stilbene vial) was in several different positions inside each phantom. For this reason, a number of representative tissue phantoms with different optical properties were measured and the results are presented below.
Figure 7.3: Transmission Raman scattering 2D plots of Phantom A ($\mu'_s = 4.6$ cm$^{-1}$, $\mu_a = 0.37$ cm$^{-1}$), B ($\mu'_s = 6.9$ cm$^{-1}$, $\mu_a = 0.37$ cm$^{-1}$) and C ($\mu'_s = 13.82$ cm$^{-1}$, $\mu_a = 0.8$ cm$^{-1}$) with (i) and without (ii) the photon diode in a transmission mode. Laser photons are injected on the left of the image and Raman signal is collected on the right. The plots depict the intensity of $\sim 1193$ cm$^{-1}$ Raman band of trans-stilbene for phantom C and 959 cm$^{-1}$ phosphate peak of hydroxyapatite for
phantoms A and B, as the vial moves to different positions in the phantom. The 2D plots without photon diode (ii) have been normalized against the maximum intensity of the diode maps (i).

In the 2D maps above, as the amount of scatterer in the quartz phantoms (ii series) increases from 4.6 cm\(^{-1}\) to 13.82 cm\(^{-1}\), the signal in the middle area becomes stronger and can be detected in more distant positions outside of the illumination beam (Chapter 5). When the “photon diode” element is introduced, all of the phantoms exhibit a signal enhancement throughout their volume. This enhancement is stronger when the vial is at the closest proximity with the diode, as expected, since this is the area benefitting the most due to the great photon loss on the air/ sample interface. The factor of the signal enhancement depends on the optical properties of the phantom and will be explored in the next section. The general distribution pattern does not change after the photon diode application. The front and the back of the phantoms still exhibit the maximum intensity, whereas the middle phantom area is where the minimum Raman scattering is collected.

### 7.4 Diode enhancement in phantoms with varying optical properties

In a second set of experiments, in order to evaluate the diode signal enhancement as a function of the tissue optical properties and phantom depth in a quantitative way, measurements were recorded for a wide range of absorption and scattering, as described in Figure 7.2. In order to make most efficient use of the experimental time, Raman signal was recorded from a \textit{trans}-stilbene vial only on the main beam area of the phantoms. The 1D maps reconstructed from the measurements reveal the exact range of enhancement that a probed object can benefit from, depending on the optical properties of the surrounding medium and its depth in the sample.

First, the diode signal enhancement was studied in phantoms with varying reduced scattering coefficient (Fig. 7.4) and absorption coefficient (Fig. 7.5). For clarity and brevity, the ratio of the \textit{trans}-stilbene signal with the diode over the signal without the diode, was plotted against the phantom depth of the \textit{trans-}
stilbene vial, for phantoms with different optical properties, in the same plot. Additionally, in order to provide confidence in the results, each phantom was mapped three times at the same locations. The mean and the standard deviation were calculated and displayed as error bars in the plots.

**Figure 7.4:** Ratio of *trans*-stilbene Raman signal intensity with diode over signals without the diode versus the *trans*-stilbene vial position along the x-axis in the phantom (mm), for phantoms with the same absorption but different scattering coefficients. The coefficients are presented in cm⁻¹.
Figure 7.5: Ratio of trans-stilbene Raman signal intensity with diode over signals without the diode versus the trans-stilbene vial position along the x-axis in the phantom (mm), for phantoms with the same reduced scattering but different absorption coefficients. The coefficients are presented in cm$^{-1}$.

In the plots above we notice that the ratio of trans-stilbene Raman signal is dropping below one in certain positions (especially at the back) of high optical properties phantoms. That would mean that the signal intensity with the diode is lower than 1 without the diode (quartz), which is not justifiable to such an extent. Further investigation of this issue revealed that a significant volume of solution was trapped at the front of the photon diode, between the diode and the cell wall- (see Chapter 4), resulting in a disruption of the laser beam collimation before it incidents on the photon diode. Because of this, the laser output through the diode was gradually reduced with increasing concentration compared to the quartz layer as only collimated light can pass through. In high scattering and absorption phantoms this issue was more severe, resulting in enhancement ratios considerably below 1.

This issue has been addressed by adding a calcium fluoride disc between the cell wall and the diode, with size big enough (20 mm diameter, 1 mm thickness) to cover the laser illumination area and most of the diode area, in order to
minimize the solution volume trapped in the front of the diode / sample interface. This resulted in improvement of the enhancement ratios in the whole depth of the phantoms apart from the positions on the back (closer to the collection side) (Fig. 7.6, Fig. 7.7) where the Raman signal does not benefit from any diode enhancement (enhancement ratios below 1). The updated results are presented below.

**Diode enhancement in phantoms with varying reduced scattering coefficient**

After the introduction of a calcium fluoride disc, the trans- stilbene generated Raman scattering has been recorded along the optical axis in phantoms with varying reduced scattering and a constant concentration of Indian ink.

![Figure 7.6](image)

**Figure 7.6**: Ratio of trans-stilbene Raman signal intensity with diode over signals without the diode versus the trans-stilbene vial position along the x-axis in the phantom (mm), for phantoms with the same absorption but different scattering coefficients. The coefficients are presented in cm⁻¹.

In the plot above, the reduced scattering coefficient values explored are in the range of 4.6 – 23.04 cm⁻¹. The greatest enhancement factor for all of the
phantoms with varying scattering except for the extreme scattering one ($\mu_s' = 23.04 \text{ cm}^{-1}$), is observed on the measurement points with the closest proximity to the diode (0 mm). At this depth, phantoms with lower absorption or scattering coefficients exhibit higher Raman signal with the diode, as the generated Raman photons cross the phantom bulk on the other side of the vial and are collected from the optics more efficiently in low scattering values. When the vial is positioned in a certain distance from the diode, the phantoms with the higher scattering benefit from the photon diode the most, with enhancement factors ranging between 1.17 and 1.83. The reason for this is that the lower scattering phantoms do not benefit as much from the back-scattering enhancement mechanism of the diode. As a result, in a higher scattering environment, there is greater enhancement from the photon diode as the photons which re-enter the phantom volume reach the vial more easily due to diffuse scattering. In this way, in an extremely high scattering environment, the bulk volume of the phantom is the zone benefiting most from the presence of the photon diode. The signal enhancement decreases as we move away from the photon diode (0-4 mm) and it seems to plateau when the trans-stilbene vial is located around the middle of the phantom (4-16 mm). The enhancement factors in this area appear to fall to a similar range (1.0-1.2) for most of the phantoms. Towards the back of the phantom and closer to the collection side (16-20 mm), the diode enhancement of the signal seems to be decreasing even more. This drop is in some cases so sharp (e.g. Phantom 8: $\mu_s' = 23.04 \text{ cm}^{-1}$ and $\mu_a = 0.8 \text{ cm}^{-1}$) that the diode might not be beneficial for signal enhancement at all. It should be noted that at high scattering coefficient values (18.4 cm$^{-1}$ and 23.04 cm$^{-1}$), the enhancement at the back of the phantom does not follow the same trend as at the front. The drop is most likely due to the overall lower transmittance of the diode compared to quartz layer (by around 10%).

**Diode enhancement in phantoms with varying absorption coefficient**

Similar trends are observed in phantoms with varying absorption coefficients and constant reduced scattering coefficient (concentration of Intralipid) (Fig. 7.7). The signal enhancement from the diode here seems to be almost constant through many vial depths in each of the different phantoms. The absorption
appears to affect the diode enhancement to a greater extent compared to the scattering case in our ranges, since the enhancement ratio drops quicker when we increase the absorption coefficient (ink concentration) rather than the scattering coefficient. Comparing the two plots (Fig. 7.6 and Fig. 7.7), it seems that a middle value of absorption ($\mu_a = 0.8 \text{ cm}^{-1}$) inhibits the signal enhancement in the middle of the phantom more compared to a middle scatterer value ($\mu_s' = 13.82 \text{ cm}^{-1}$). This means that it is less likely for the ‘enhanced’ laser photons to generate a Raman photon which will get detected on the collection side in the presence of ink. On the other hand, when only Intralipid concentration is increased, the absorption increases indirectly due to the path length change too, but not to such an extent.

**Figure 7.7:** Ratio of trans-stilbene Raman signal intensity with diode over signals without the diode versus the trans-stilbene vial position along the x-axis in the phantom (mm), for phantoms with the same reduced scattering but different absorption coefficients.

In terms of the enhancement factor in the varying absorption phantoms, it takes a wide range of values through the entire phantom depth as the vial is moved across towards the collection side. The highest enhancement factor is again observed at the point closest to the diode (0 mm) and it takes different values.
between 0.95 and 2.34, depending on the absorber concentration introduced in each phantom. As expected, the highest signal enhancement is observed in the phantom with no absorption and as soon as 0.1 μl/ml of ink (equivalent to μa of 0.37 cm⁻¹) is present, the signal enhancement drops from 2.34 to 2.21. In the middle area of the phantom (4-16 mm), the signal enhancement has gradually decreased and reached a plateau. Towards the back of the phantom (16-20 mm), the signal enhancement seems to be decreasing even more and for the extreme ink concentration (Phantom 5: μa= 0.8 cm⁻¹ and μs'= 13.82 cm⁻¹), the benefit of using a photon diode is limited, as the signal enhancement ratio constantly remains close or under 1. As mentioned before, the values below 1 can be attributed to the reduced transmittance of photon diode which becomes especially significant in high optical properties phantoms with low signal to noise ratios.

It should be noted that a reduced signal on the edge of the sample is less detrimental to the detection sensitivity than it would be in the centre of the sample, as signals tend to be 10-50% higher at the edges than in the centre for tissue optical properties²⁸².

### 7.5 Discussion

In this chapter we were able to relate the diode signal enhancement with both the tissue optical properties and the probing depth of the sample, for the first time. The enhancement factors observed through the measurements are in the range of 1- 2.4. Studies on samples with similar optical properties (e.g. lack of absorption) and thickness, such as chicken and porcine tissue, simulating breast phantoms, have exhibited a signal enhancement of ×1.6²³⁵ and ×1.5¹⁴⁹ respectively. These observations are in broad agreement with our results but they do not assess the signal enhancement in terms of specific tissue optical properties.

In our study, it was also demonstrated that the gradual introduction of increasing absorber agent affects the signal enhancement more strongly compared to a scatterer increase in our ranges. In terms of enhancement in different sample depths, the overall signal through the sample seems to decrease as the absorption goes up. In contrast, an opposite relation is observed when scattering
is introduced, as the signal enhancement in the bulk sample volume increases with the scattering agent concentration. This is attributed to the fact that the diffuse scattering is reinforcing the backscattering enhancement mechanism of the diode to a greater extent. With this observation it is useful to observe that tissues with higher scattering and less absorption are expected to benefit the most from a photon diode for the signal enhancement in their bulk volume.

Indeed, from figure 7.1 we can conclude that the tissues which benefit the most in terms of signal enhancement are generally the ones with high scattering and low absorption coefficients as fat, bone and whole skin. In these types of tissues, a signal enhancement of at least ×1.4-2.2 at the front and ×1.2-1.7 in the bulk volume can be achieved. Tissues with lower scattering and moderate absorption, such as breast, brain, muscle and small bowel can also benefit from a significant diode signal enhancement of ×1.8-2.2 at the front and ×1-1.5 at the middle of the sample. Tissues with even lower scattering but considerable absorption, such as prostate, white matter brain and liver would still benefit from a photon diode but mainly in regions of interest near the illumination side, with a signal enhancement of around ×1.8. There are finally certain types of tissues such as stomach wall and aorta which would not see any benefit due to their both high optical absorption and scattering properties.

However, even in the case where certain types of tissue do not seem to benefit from a diode signal enhancement, there is still potential for signal to noise ratio improvement, with the use of additional filters/mirrors on the remaining tissue/phantom sides (right, left, top, bottom and the collection sides), as has been proven in the past. In conclusion, when traditional setup parameters (laser power, collection time, collection efficiency) have been maximized, the photon diode approach can contribute with a passive enhancement of a considerable factor, leading to further enhanced detection sensitivity and penetration depth for transmission Raman measurements. This can be proved quite beneficial in the case of an in vivo real time measurement with a Raman probe, where after an assessment of the optical properties of the tissue measured, a simple introduction of a photon diode filter at the front of the Raman probe can enhance the signal by a considerable amount in order to increase the sensitivity in the measurements.
Chapter 8: Depth prediction of an inclusion buried in turbid media using a photon diode

8.1 Introduction

As described previously, deep Raman spectroscopy is capable of providing information on the chemical composition of distinct layers and inclusions at different depths of a sample. However, measuring the depth of the detected inclusion can also be important, for example in the case of breast cancer diagnosis where calcifications are an important diagnostic factor.

Of the deep Raman techniques, only SORS has been studied extensively for depth prediction of layers\textsuperscript{283} or inclusions in animal tissue and tissue phantoms\textsuperscript{216}. Raman tomography with SORS has also been employed in the past in a series of studies in order to assess the location of an inclusion inside tissue phantoms. This concept however involves a large number of collection fibres in different orientations around the sample, and in some modalities additional use of an imaging technique (e.g. MRI, CT) and complex algorithms in order to reconstruct the 3D image\textsuperscript{214, 284, 285}.

In this study, we are using a simple concept of determining the depth of a single inclusion or “calcification” in transmission Raman geometry which in itself does not render depth specific information in its basic form. To provide depth sensing capability we take advantage of photon diode signal enhancement. The photon diode has been predominantly used to achieve signal enhancement in pharmaceutical tablets\textsuperscript{235} and tissue samples\textsuperscript{149} as described in Chapter 7. In this chapter, the photon diode is employed to assess if the depth of signal origin within the sample can be ascertained, since it has been shown that the signal enhancement it provides is a function of depth\textsuperscript{160}. In this way, we explore the potential of predicting the depth of a chemically distinct object (inclusion) buried within a turbid matrix by performing only two measurements in an identical sample orientation, with and without a beam enhancing element, ‘photon diode’. The measurements were carried out in animal tissue samples and liquid tissue phantoms with and without absorption. Wherever both absorption and scattering
were introduced, the optical properties were in the range of those in human breast tissue.

### 8.2 Depth prediction in liquid tissue phantoms with no absorption

In order to assess the depth of the signal origin inside the phantom, measurements were carried out using the deep Raman system in a transmission mode, with and without the photon diode, configured as described in Chapter 7. The tissue phantoms employed were fabricated as described in Chapter 4, in order to achieve desirable values of optical properties.

The phantoms studied initially were free of absorption but were characterized by a reduced scattering coefficient of $\mu'_s = 13.82 \text{ cm}^{-1}$, as this is a middle range value of the reduced scattering coefficient in biological tissues. Sets of repeated runs were performed in three different phantoms with the same optical properties, while the depth was varied with and without the photon diode. In the latter case, a quartz window replaced the photon diode in order to make up for the volume loss in the phantom. The intensity of the trans-stilbene Raman band at $1193 \text{ cm}^{-1}$ was derived from each measurement and used in analysis below.

Three independent measurement replicates with the photon diode element have been plotted versus phantom depth (Fig. 8.1). Each replicate involved the full replacement of the liquid phantom with a freshly made one and re-setting up the experiment.
Figure 8.1: Raman signal intensity of trans-stilbene vial in photon diode measurements, as a function of its depth inside the liquid phantom.

In order to make up for the errors acquired from resetting the experiment in a repeat and for the power differences in the setup, the intensity values from the photon diode measurements in each data set have been normalized to the intensity recorded from trans-stilbene when it was located at the back of the cell and while using the quartz window (Fig.8.2).
Figure 8.2: Normalized Raman signal intensity of trans-stilbene vial in photon diode measurements as a function of the phantom depth.

A calibration curve has been created using the mean values from the first two data sets (Phantom 1 and Phantom 2) and an exponential line was fitted to the data (Figure 8.3).

Figure 8.3: Mean Raman signal intensity of trans-stilbene in the photon diode data sets of the first two phantoms with exponential fitting ($y = 0.2418e^{0.1037x}$)
Using the equation of the calibration curve \( f(x) = 0.2418e^{0.1037x} \), the depth values of the third set were predicted by using the correspondent normalized photon diode intensity. The predicted versus the actual depth values were plotted against each other, showing a very high correlation and a great potential for depth prediction (Fig. 8.4), with a root mean square error of prediction (RMSEP) of 0.7 mm. The value of the RMSEP (3.5%) is justified by the error margin of the experimental procedure, such as the preparation of the solutions (±6.25%). The fact that the predicted depth values are so close to the actual depth values across the whole phantom depth is very promising for the application of the depth prediction concept in phantoms/samples exhibiting mainly scattering.

**Figure 8.4:** Prediction of depth derived from the normalized Raman intensity values with photon diode. The dotted line represents a 100% accurate depth prediction.

### 8.3 Depth prediction in combined liquid tissue phantoms

In a next stage, depth prediction was attempted in combined phantoms with both absorption and reduced scattering agents added. Four different liquid tissue
phantoms of identical optical properties ($\mu'_s = 9.2 \text{ cm}^{-1}$ and $\mu_a = 0.8 \text{ cm}^{-1}$) were measured with and without the photon diode in transmission mode, while the *trans*-stilbene vial was in different positions inside the phantom volume. The intensity of the *trans*-stilbene Raman has been determined for each measurement and the ratio of signal intensity with photon diode over the one without the photon diode was calculated for each depth (Fig. 8.5). The values were then normalized to the signal derived from the quartz set when the vial was at the back of the phantom (Fig. 8.6).

**Figure 8.5:** Ratio of *trans*-stilbene Raman signal intensity with diode over signal without the diode versus the phantom depth of the vial.
Figure 8.6: Normalized ratio of *trans*-stilbene Raman signal intensity with diode over signal without the diode, as a function of the phantom depth.

From the first three independent data sets, a calibration curve has been drawn using the mean values of Raman intensity. The data points were then fitted with an exponential line $f(x) = 0.73e^{-0.69x} + 1.065$ (Fig. 8.7), which was calculated by running a least square regression analysis on Excel.
Figure 8.7: Mean value of the normalized ratios of trans-stilbene Raman signal intensity with diode over signal without the diode as a function of the phantom depth, in the data sets of the first three phantoms with exponential fitting: \( f(x) = 0.73e^{-0.69x} + 1.065 \).

The depth values for the fourth data set have been predicted using the equation of the calibration curve: \( f(x) = 0.73e^{-0.69x} + 1.065 \). The predicted depth values versus the actual ones were plotted against each other, exhibiting a low prediction potential (Fig. 8.8), with RMSEP to be 4.9 mm. For predicted depths where values could not be defined from the equation, we assumed the maximum depth value (20 mm).
Figure 8.8: Prediction of depth derived from the normalized ratios of trans-stilbene Raman intensity with beam enhancer over intensity without beam enhancer.

From the prediction curve above, we can observe that although the depth values can accurately be predicted when the inclusion is located at the front of the phantom (0-4 mm), the prediction capability is lost when we are moving towards the middle and back of the sample. This is also suggested by Figure 8.7, where the intensity values of the exponential fitting drop very slowly in relation to the depth, as we move towards the main phantom volume. Therefore, a very high precision and reproducibility between the measurements and the different phantoms would be required in order to successfully predict the depth values in this area. Due to the liquid nature of phantoms and the instability in optical properties of scattering agent (Intralipid), in both stock solution and phantoms, such a high reproducibility could not be achieved and consequently our model fails to accurately predict the middle and back areas.
8.4 Depth prediction in tissue sections

Similar measurements were performed on chicken tissue sections. As tissue has both absorption and scattering properties, it was expected to provide similar results to those of combined tissue phantoms.

In order to test the feasibility of depth prediction through tissue, transmission measurements were recorded while a calcification standard (hydroxyapatite-HAP) vial was placed between four different layers of chicken tissue (10 mm total thickness), in positions aligned with the laser beam axis (Fig. 8.9).

![Figure 8.9: Transmission Raman measurements for depth prediction in chicken tissue.](image)

A series of five measurements were recorded both with and without photon diode on five different blocks of chicken tissue sections. The intensity of the HAP Raman band at 960 cm⁻¹ has been derived from each single measurement and the ratios of the intensity with photon diode at the same positions over the intensity without photon diode have been calculated for each single depth and group of tissue sections. The values were then normalized to the signal derived from the quartz set when the vial was at the back of the phantom ((Fig. 8.10).
Figure 8.10: Normalized ratios of HAP Raman signal intensity with diode over signal without the diode, as a function of the phantom depth for each individual set of measurements.

The mean values from four of the independent data sets were then used to create a calibration curve and fit an exponential line to the data which was calculated by running a least squares regression analysis (Figure 8.11).
Figure 8.11: Mean value of the normalized ratios of HAP Raman signal intensity with diode over signal without the diode as a function of the phantom depth, in the data sets of the first four sets with exponential fitting ($y = 8.06e^{-4.59x} + 1.74$).

Using the equation of the calibration curve above ($f(x) = 8.06e^{-4.59x} + 1.74$), we attempted to predict the depth values of the fifth data set. For predicted depths whose values could not be defined from the equation, we assumed the maximum depth value (10 mm). The predicted versus the actual depth values were plotted against each other (Fig. 8.12), confirming the very low correlation of collected Raman signal and tissue depth and highlighting the limitations of this attempt in tissue samples (RMSEP = 4.2 mm). Although the RMSEP is not higher compared to the one in combined tissue phantoms, the prediction model fails because most of the depth values have to be assumed, since they cannot be defined in an exponential scale and hence are not in the prediction range of the calibration curve. Although a number of different fittings have also been tested for calibration in this dataset, they resulted in worse prediction values.
Figure 8.12: Prediction of depth derived from the normalized ratios of HAP Raman intensity with beam enhancer over intensity without beam enhancer.

Similarly to the combined tissue phantoms, although the depth is predicted reasonably well when the calcification is at the front of the tissue block (0 mm), it fails in the middle and back. This can be attributed to a deviation between the independent sets of measurements (Fig. 8.10), which relates to the different optical properties (within a range) and slightly varying thicknesses of different tissue chicken sections and therefore lower reproducibility between the data sets.

8.5 Discussion

In this experimental section, we attempted to predict the depth of a high Raman cross-section scatterer (inclusion) buried in different positions in a turbid matrix by using enhancement ratios achieved with photon diode in a transmission Raman geometry. This prediction has shown to be successful across the whole sample depth when no absorber agent was present, but not when absorption is either physically introduced (liquid tissue phantoms) or endogenous (chicken tissue). Similar studies on pharmaceutical tablets which also lack any distinct
absorption at visible-NIR wavelengths, confirm the strong dependence of signal as a function of sample depth by exhibiting a maximum enhancement when the inclusion is closest to the illumination side\textsuperscript{270}. In the same study, numerical simulations also came to the conclusion that the introduction of absorption, which is equal for laser and Raman photons, reduces the enhancement levels through the sample depth, leading to a more symmetrical depth profile which does not favor the depth prediction.

This study demonstrates the limitations of the depth prediction on samples with absorption (tissue phantoms and tissue sections). In this case we are only able to accurately predict the depth when the inclusion is close to the front (illumination side), whereas the depth prediction is affected by a number of factors. The first one is the shape of the curve when absorption is introduced which exhibits an almost flat area in the middle volume and towards the back (Fig. 8.7). In order for the depth to be predicted through a calibration curve with such a shallow slope, a very high reproducibility between the independent samples and data sets is required. This is because depths which correspond to intensities very close to each other need higher precision in order to be predicted.

However, because of the liquid nature of the tissue phantoms and the reduced stability of higher concentration scattering agent over time especially in the presence of ink, it was not possible to achieve such a high reproducibility. Phantom stability has been already assessed in Chapter 4 (Fig. 4.3, 4.4, 4.5), showing that the 10\% deviation observed in signal levels does not affect our results in the previous chapters. However, in this chapter, due to the very high precision required for the depth prediction in combined tissue phantoms, this deviation has a much more significant effect on our results. The tissue samples (chicken) also failed to provide high precision in measurements, since it is not possible to control precisely the optical properties or thicknesses of each different section of fresh tissue and therefore achieve a highly reproducible set of measurements.

In conclusion, this study exhibits the potential of predicting the depth of an inclusion in samples of significant thickness (10-30 mm) and zero or very low absorption. The limitations on the optical properties demonstrate that tissue is not the ideal medium for this depth prediction concept. In contrary, pharmaceutical tablets which bear typically no absorption, match the criteria for a promising
prediction model. The photon diode element has only been used on pharmaceutical tablets before in order to achieve a more efficient quantitative analysis of active pharmaceutical ingredients (API)\textsuperscript{234}. The employment of the photon diode for predicting the depth of an API layer or even impurities in the tablet volume could be a new promising approach and would open the way to a number of different applications in the industrial pharmaceutical analysis.
Chapter 9: Bladder cancer and Raman spectroscopy

Much research has been done towards the improvement of the gold standard for bladder cancer diagnosis, which comprises cystoscopy and biopsy followed by histopathology. A number of optical techniques developed, such as fluorescence cystoscopy, electrical impedance spectroscopy\(^{286}\) and virtual spectroscopy with MRI and CT\(^{287}\), enhance the sensitivity of white light cystoscopy by improving the localization of the malignant areas\(^{288,289}\). However, these techniques suffer from a low specificity\(^{290-292}\), are not able to accurately discriminate malignant from benign areas without these being assessed histochemically and come with the invasive nature of cystoscopy.

Attempts for a non-invasive approach rely on urine analysis rather than the study and sampling of the bladder wall. Non-invasive techniques which have been suggested include the detection of biomarkers, such as DNA and proteins\(^{293,294}\). However, as mentioned more extensively in Chapter 2, despite their high sensitivity, these markers usually lack specificity\(^{116-118}\).

Raman spectroscopy has also been used for bladder cancer detection in different studies measuring either the bladder wall with a Raman probe \textit{in vivo}\(^{206}\) or excised tissue samples with Raman microscopy\(^{295,296}\) or a confocal Raman probe\(^{297}\) \textit{ex vivo}. The differences between benign and different malignant conditions are attributed to proteins, lipids and nucleic acids. More specifically, proteins, collagen and certain lipids are more abundant in healthy tissues whereas DNA, glycogen expression and certain amino acids are found to be increased in malignant tissues\(^{206,295}\).

An alternative non-invasive approach for bladder cancer diagnosis described in this chapter, is the direct measurement of patients’ urine using Raman spectroscopy. Artificial urine samples have been generally measured with Raman spectroscopy in the past\(^{298}\). However due to the naturally low concentration of all urine components (uric acid, creatinine, etc.) except for urea, it has been shown that spontaneous Raman spectroscopy’s detection capability can be limited\(^{299}\). In one study conventional Raman spectroscopy, has been employed for measuring bladder tissue epithelial cells found in urine from patients with bladder cancer\(^{300}\),
but not in plain urine. In this chapter, we present the results of our study of the potential differences in urea’s distribution and interactions in urine from healthy individuals and patients with bladder cancer, using Raman microscopy.

9.1 Raman signals of urine samples

Raman spectra of urine samples were initially measured with three different excitation wavelengths in order to assess the most suitable one for subsequent measurements (Fig. 9.1). All of the Raman systems have been calibrated as described in section 4.2.2, except for the Witec system which contains a systematic error. Since the purpose of these measurements was the assessment of the quality of the Raman spectra rather than the exact position of the Raman bands, our results should not be affected.

![Raman spectra](image.png)

**Figure 9.1:** Urine Raman spectra recorded with different laser excitation wavelengths (532, 785 and 830 nm) on different Raman spectrometers. The spatial offset between the individual spectra has been adjusted for clarity.
As shown in the spectra above, urine samples exhibit fluorescence when measured with lower wavelengths (532 nm)\(^3\) but cells inside the urine are clearly detected\(^3\). At higher wavelengths (785 and 830 nm), plain urine does not exhibit any fluorescence and this is why these wavelengths were chosen for the urine analysis.

Spontaneous Raman microscopy has been shown not to be able to detect efficiently most of the urine components at their physiological values in human urine\(^2\) due to their low concentrations. Indeed, as shown in Figure 9.1, the only dominant peak in plain urine is attributed to the symmetric C–N stretching band of urea at 1003 cm\(^{-1}\). For this reason, the study was focused on the analysis of the urea peak position and its spatial distribution over the urea drop area in order to discriminate healthy from bladder cancer urine.

### 9.2 Urea peak position analysis

During measurements in different urine samples, we noticed that the main peak of urea is adopting different positions between 995 and 1011 cm\(^{-1}\) (Fig. 9.2). A number of factors have been considered in order to find the reason behind this shift.
Figure 9.2: Peak shifts of the C-N stretching urea band in urine samples.

**Polarization**

Initially, the urea peak shift in urine samples was studied under different types of polarization. The effect was tested on three different crystals of one urine sample by changing polarization of the collected light relative to the polarization of an 830 nm incident laser beam (parallel, perpendicular and no change of polarization) (Fig. 9.3).
Figure 9.3: Raman spectra under different types of polarization for the same urea crystal. Note that the peak at 325 cm\(^{-1}\) is attributed to the calcium fluoride substrate.

Different types of polarization (perpendicular and parallel to the laser) were found to have no effect on the C-N stretching urea band position in urine samples.

**Drying time**

The effect of drying time on urine samples was also explored on fresh urine (one healthy and one malignant), measured as soon as they were collected and after a period of ten days. The measurements were recorded with a 785 nm laser and analysed with Principal Component Analysis (PCA) in order to assess the stability of the urine components over the drop area. The loading plots and score images are shown in the figures below.
Figure 9.4: Loadings comparison in a healthy urine sample which has been measured as soon as collected (time: 0 days — left) and ten days later (time: 10 days -right). Loading plots are only shown for the first three principal components and the urea peak position is marked in each one of them.
Figure 9.5: Score images on PC1 (a), PC2 (b) and PC3 (c) of a healthy urine sample measured as soon as collected and 10 days later.

In Figure 9.4 we can see that the spectral variation is explained by the same loading plots before and after the drying period. Spectral features of urea in the loading plots, such as the peak position, change between different loading plots for a specific time point (demonstrating the peak shift observed earlier), but is highly reproducible between the two different time points, proving that the urea peak shift does not depend on the drying time of the sample.

In Figure 9.5, where the distribution of the principal components over the drop area is shown, we observe a difference in the intensity of each component before and after the drying period. While the components at 0 time are of higher intensity compared to 10 days later (also reflected on the variance percentages of
loadings), the distribution between the two time points remains similar. More specifically, the crystal shaped formations which are attributed to the higher urea shift (1002 cm\(^{-1}\) for time 0 and 1007 cm\(^{-1}\) for 10 days later), are concentrated in the middle of the drop and represent the greatest variance percentage over the drop area. A component-free ring which surrounds the urine drop also seems to retain its stability over time (Fig. 9.5b). These results demonstrate that the duration of Raman measurements should not impact on the components distribution in the droplet area or the PCA analysis conducted afterwards. Additionally, urine drops measured and left to dry over different periods of time should be safe to be compared with each other.

Similar trends are observed for the malignant urine drop below, where the high wavenumber urea shift is observed in crystal clusters concentrated in the middle of the drop (Fig. 9.7a, 9.7b). This spectral feature is constant both in terms of distribution and position of the urea band. The component-free ring is also present in the malignant urine (Fig, 9.7a).

**Figure 9.6**: Loadings comparison in a malignant urine sample which has been measured as soon as collected (time: 0 days – left) and ten days later (time: 10
days – right). Loading plots are only shown for the first three components and the urea peak position is marked in each one of them.

**Figure 9.7:** Score images on PC1 (a), PC2 (b) and PC3 (c) of a malignant urine sample measured as soon as collected and 10 days later.

### 9.3 Urea vibrations in different environments

As stated in the literature before\textsuperscript{302}, the urea peak shift can be attributed to urea molecule interactions in different environments. Frequency shifts of urea’s
vibrational modes have also been recorded when urea state changes from liquid to solid (with IR) and from solid to gas. It has also been shown that the position of the urea peak is shifting linearly with its concentration in an aqueous solution.

In order to confirm that the position shift of urea band observed in the urine samples is connected to the change of environment and presence of different molecules, urea solid samples and aqueous solutions at physiological concentration were prepared, measured and analysed (Fig. 9.8).

Figure 9.8: Raman spectra of solid urea crystals and aqueous urea solutions (9.3 g/L) measured with 830 nm laser for an acquisition time of 10 s. The Raman intensity of the spectra has been adjusted appropriately for comparison reasons.

Raman spectra recorded from 9.3 g/L urea aqueous solutions showed a urea band with a maximum at 1006 cm$^{-1}$, whereas for the same band in solid urea spectra the maximum was located at 1013 cm$^{-1}$, in agreement with earlier studies. Comparison of the two urea bands demonstrates a peak shift similar...
to the one observed in the urine samples earlier. However, it should be noted that the left shifted urea band in artificial urea solutions (1006 cm\(^{-1}\)) did not match accurately the one in the urine samples (995 cm\(^{-1}\)) and for this reason further investigation was needed.

The urea molecule is able to only bind strongly to one water molecule using two molecular bonds, by donating an electron from a nitrogen atom and accepting one onto the urea oxygen atom\(^{307}\). However, each urea molecule can also link up to five different water molecules more weakly through hydrogen bonding, or to cations through electrostatic forces via the electron-rich oxygen and nitrogen atoms (Fig. 9.9). In absence of sufficient water molecules, urea binds to itself through hydrogen bonds (a urea dimer is a particularly stable species) (Fig. 9.9c).

![Figure 9.9: Urea molecule in different environments can interact with water molecules (a), cations (b) or neighbouring urea molecules (c).](image_url)

When urea molecules are connected to each other rather than to water molecules, the CN bond becomes shorter in the urea resonance forms\(^{304}\) and therefore its vibrational frequency increases. This is why the urea band position in Raman spectra is observed at higher wavenumbers (1011 cm\(^{-1}\)). Conversely, when urea molecules are connected to the water, the CN bond remains single rather than double which is characterized by lower energy and lower frequency (1006 cm\(^{-1}\)). When a urea molecule is connected to a salt cation, a stronger electrostatic bond is formed. This interaction makes the CN bond in urea molecule even weaker, causing a lower wavenumber shift to the Raman band (995 cm\(^{-1}\)).
Validating our hypothesis, recorded Raman spectra from aqueous urea solutions containing NaCl (0.18M), showed a urea band at 1005 cm\(^{-1}\). However, when a curve-fit analysis was applied to this band using OriginLab software, it revealed two different sub-bands: a \(\nu(\text{CN})\)\(_s\) band component with a maximum at 1008 cm\(^{-1}\) due to urea H-bonded to water and/or other urea molecules and a component at 995 cm\(^{-1}\) which can be assigned to urea interacting with Na\(^+\) cations through the carbonyl oxygen and the nitrogen atom (Fig. 9.10).

**Figure 9.10:** Results of curve-fit analysis applied to the urea band of an aqueous urea solution containing NaCl (0.18M) recorded with 830 nm laser excitation wavelength (acquisition time: 10 s).

Based on this hypothesis, different urea peak positions in urine samples are a result of urea interactions with different molecules. Peak positions lower than 1005 cm\(^{-1}\), such as 995 cm\(^{-1}\) and 998 cm\(^{-1}\), can be assigned to urea interactions with cations, whereas high wavenumber positions (1011 cm\(^{-1}\)) are assigned to solid urea in which molecules are bound with each other (crystalline urea).
between, the band with positions 1002 cm\(^{-1}\) and 1005 cm\(^{-1}\) can be assigned to partially hydrated states of urea.

After establishing the reasons behind the urea band shift, urine samples from 21 healthy and patients with bladder cancer were studied using Raman microscopy. Initially, mean Raman spectra were recorded from each drop and analysed with Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) using leave-one-out cross-validation, in order to explore the hypothesis that the urea peak shift – which depends on molecular interactions as described above – is related to the malignancy status. In a second round of measurements, a number of healthy and malignant urine samples were studied more extensively by recording Raman maps, in order to reveal any differences in the distribution of the different urea crystals over the droplet area.

### 9.4 Analysis of urine samples using multivariate analysis

Raman spectra were recorded from 30 different positions on dried drops from 21 urine samples (10 with bladder cancer and 11 healthy) by focusing a near infrared laser line (830nm) through a 50x objective lens of an optical microscope onto the surface of the drop (Fig. 9.11).
Figure 9.11: Urine drop under the white light of Raman microscope. The Raman spectra were recorded in the highlighted mapping area on a grid of 30 points (scale in μm).

Single Raman spectra were pre-processed and mean Raman spectra were calculated for each sample (Fig. 9.12- A).
Figure 9.12: Mean Raman spectra of 21 urine samples over the whole spectral range (A) and in the urea peak region (B), as they were measured with 830 nm laser on a Raman microscope.
As expected, the dominant peak in the spectra is the urea peak (around 1003 cm\(^{-1}\)) which shifts to different positions between different measurement points in different samples (Fig. 9.12-B).

![Graph showing urea peak on mean Raman spectra of healthy and malignant urine samples.](image)

**Figure 9.13:** Urea peak on mean Raman spectra of healthy (green plot at 1000 cm\(^{-1}\)) and malignant (red plot at 1003 cm\(^{-1}\)) urine samples.

This urea position change could be potentially related to the sample malignancy as indicated by the mean healthy and malignant spectra (Fig. 9.13). In order to explore the differences between the two groups further, multivariate analysis (PCA, LDA) was carried out on the dataset. PCA, which is further described in Chapter 4, is an unsupervised multivariate technique used to analyse the inherent structure of the data. In our case, PCA transforms a set of Raman spectra into a number of uncorrelated (orthogonal) variables (principal components) which describe the spectral variance of the dataset.

Initially, spectral features which are not related to the samples, such as the Raman peak attributed to the atmospheric oxygen at 1542 cm\(^{-1}\) \(^{308}\), were eliminated (Fig. 9.14). After the Raman spectra were processed (scaled 0-1, normalized, mean centred), they were analysed with PCA in order to reveal the
most significant diagnostic variables (PC-loadings) related to the spectral differences found in the dataset (Fig. 9.14).

**Figure 9.14:** The first six most significant principal components as they resulted from PCA analysis.

The six first loadings resulting from PCA describe ~83% of the total spectral variance in our samples. The most prominent feature (loading 1- 69.63%) describes the intensity variation of urea peak, whereas the different position of the band through the different loadings shows the variation of the peak position. The first two loadings, which correspond to PC1 and PC2, clearly show the main feature of urea band located at 1000 cm\(^{-1}\), a hydrated urea state. PC4, PC5 and
PC6 loadings describe a shift of the urea peak towards lower (991 and 994 cm\(^{-1}\)) and higher (1005 cm\(^{-1}\)) wavenumbers.

The first three principal components (PC1, PC2 and PC3), which represent the 80.48% of the total spectral variance across the urine samples, were plotted against each other in different combinations, in order to reveal discriminative relationships between healthy and malignant urine samples (Fig. 9.15).
Figure 9.15: Two dimensional PCA scores plots of the urine Raman spectra, with PC1, PC2 and PC3 plotted against each other, showing healthy (green) and malignant (pink) urine samples.
In order to explore further the possible discrimination between the two groups (healthy and malignant), LDA was also performed, based on the first 15 PCs resulted from the PCA, and followed by a leave-one-out cross-validation. The number of principal components used in LDA was decided based on the presence of spectral features rather than plain noise background, in the corresponding loadings. LDA is a supervised model which optimally classifies the data into two classes and during the cross-validation. All mean spectra except one were used to build an LDA model and then to classify the left out spectrum. This process was repeated so that each spectrum is predicted once. The training model demonstrated a 73% sensitivity and 80% specificity, and a clear discrimination was shown between the two distributions (Fig. 9.16).

**Figure 9.16:** Histogram of healthy group (green) against the malignant (red) showing the frequency of values with particular linear discrimination function scores.

It should be mentioned that the discrimination was based on the LD function (shown in Fig. 9.17) which, despite the noisy background, clearly demonstrates that the urea shift is the dominant discriminant factor between the two groups.
After it was established that the main discriminant factor between the two groups is the urea peak shift, Raman maps were recorded in greater detail for two healthy and three malignant urine samples. The purpose was not only to detect where the urea shifts are localized but also to explore the distribution of the main spectral features over the drop area. The detailed Raman maps were run both with 785 nm and 830 nm laser excitation wavelengths and processed using PCA.

**9.5 Mapping of healthy and bladder cancer urine drops using Raman microscopy**

![Graph showing Raman intensity vs. Raman shift (cm⁻¹)](image)

*Figure 9.17:* The LDA discriminant function used from the LDA model for the optimum discrimination between the healthy and the bladder cancer urine group.
analysis. As expected, most of the principal components revealed during the PCA analysis, are related to urea peak shifts in the samples. High density Raman maps reveal that healthy and malignant drops exhibit certain distinct characteristics in the distribution of the two main types of crystals: urea interacting with cations at 992 cm\(^{-1}\) and hydrated urea at 1005 cm\(^{-1}\).

Looking at the principal components in the Raman maps measured, it appears that healthy urine drops generally show the same distribution of hydrated urea molecules in the centre (1005 cm\(^{-1}\)) and mostly the absence of urea···cations clusters over the whole drop area (992 cm\(^{-1}\)) (Fig. 9.18). In comparison with the malignant urine samples, it is also obvious that the urea–cation band at 995 cm\(^{-1}\) is not as abundant in the healthy as in the malignant samples (Fig. 9.19).
Figure 9.18: Distribution of 1005 cm$^{-1}$ (a) and 992 cm$^{-1}$ (b) component eigenvalues in healthy urine samples, as revealed from PCA analysis (loadings and score images on the correspondent Principal Components).

In malignant samples (Fig. 9.19), the distribution based on the band at 995 cm$^{-1}$ which indicates the presence of urea···cation clusters is more dominant and tends to be located all over the droplet area. This is obvious in the score images which correspond to loadings with a dominant spectral feature around 995 cm$^{-1}$ (990.4 cm$^{-1}$, 993.8 cm$^{-1}$, 993.8 cm$^{-1}$) (Fig. 9.18b). It should be also noted that the urea···cation clusters in malignant samples are smaller and scattered in a larger drop area compared to the ones in healthy samples. The centre of the droplet in malignant samples is characterised by large clusters of hydrated urea, showing according to the loadings, a band around 1005 cm$^{-1}$ (1010 cm$^{-1}$, 1005 cm$^{-1}$, 1007 cm$^{-1}$), leaving the periphery of the drop component-free (Fig. 9.19a).
Figure 9.19: Distribution of 1005 cm\(^{-1}\) (a) and 992 cm\(^{-1}\) (b) band in malignant urine samples, as revealed from PCA analysis (loadings and score images on the correspondent Principal Components).

Another general difference observed between the two types of samples is that the dried urine drops from the healthy group are generally more transparent, and hence of lower concentration in urea, than the drops from the malignant group. On the other hand, malignant samples are generally richer in urea and more
abundant in the different clusters that it might form. The observed distributions of urine components sometimes called ‘coffee ring’ effect\(^{309}\).

It has been mentioned before that when urea is interacting with water, a small fraction of the water molecules is strongly immobilized by urea\(^{307}\). Hence, when the urine samples are drying, they trap predominantly the intermediate hydrated states of urea rather than the fully dried form\(^{303}\). In this way, the urea···urea interaction which produces the dry state phase is prevented and this is why shifts corresponding to the dry phase (1013 cm\(^{-1}\)) are not observed in the urine samples, even after they have dried out.

### 9.6 Discussion

In this chapter, healthy and bladder cancer urine samples were studied by the means of Raman microscopy. As demonstrated by previous studies\(^{299}\), the majority of the components in urine cannot be detected using spontaneous Raman, due to their low concentration. Cells found in urine have been used in the past to detect bladder cancer. However, since cells are something uncommon to be found in urine samples, especially with the second urine of the day as used here, our analysis could not be based on them. This is why our study focused on the spectral features and spatial distribution of urea molecules in the urine droplets.

The main distinguishing spectral feature explored was the peak shift of the urea symmetric C–N stretching band. After eliminating a number of possible factors and demonstrating that the stability of urine drops over time only slightly affects urea distribution, it was shown that the main contribution to this shift derives from the different interactions between urea and its surrounding molecules. These interactions are promoted to different extent not only between different urine samples but also between different areas of the same sample depending on how the drop dries.

Detailed Raman maps on a number of healthy and malignant urine samples confirmed the different urea species distribution on different types of drops. The urea···cation was found to be more abundant in malignant urine, with the hydrated urea to be concentrated mainly in the centre of the drops.
Linear discriminant analysis including all of the urine samples, made use of the 15 first principal components as they resulted from previous PCA, in order to establish a linear discriminant function. This function revealed the urea peak shift as the main discriminant feature that leads to the optimum discrimination between the two groups. Employing this function to discriminate between the two groups of our dataset, a sensitivity of 73% and a specificity of 80% was achieved, with a clear separation of the two groups shown in the histogram. Although our observations cannot be conclusive due to the small number of urine samples available, this feasibility study demonstrates significant potential in bladder cancer non-invasive diagnosis. Based on the potential discrimination indicated through this study, further measurements and a bigger number of samples are required in order to establish higher statistical values for the performance of this promising non-invasive approach.
Chapter 10: Discussions

10.1 Assessment of deep Raman application in breast and prostate cancer diagnosis

In the main part of this thesis we explored the feasibility of applying deep Raman spectroscopy for cancer, and specifically prostate and breast cancer diagnosis. As Raman spectroscopy is a chemically specific, non-invasive technique which can scan a significant volume of tissue, it could provide a promising alternative to the current diagnosis.

In the first stage, liquid tissue phantoms were employed in order to assess the feasibility of measurements on samples with optical properties and size comparable to those of breast and prostate. Similar studies on tissue and phantoms for diagnosis\textsuperscript{149,155,214,249,250} and surgical margins assessment\textsuperscript{212} have been reported in the literature. However, in these studies the Raman signal was only explored in terms of sample thickness, whereas aspects such as its spatial distribution throughout the sample volume and its dependence on the optical properties were unexplored. In addition, the spatial distribution of the Raman signal of potentially malignant features (calcifications) has been studied in different phantoms, assessing the signal origin and detection limits of Raman scattering in prostate and breast tissue of specific optical properties and dimensions.

In terms of the detection limits, which are different for each system and tissue environment, it was shown that if the measurement parameters (tissue optical properties, Raman profile of the malignant area/lesion, laser spot diameter) are known, it is possible to assess the characteristic distance from the beam axis from which the signal can be detected. In the case of our study the effective measurement area would be an area of 30 mm x 30 mm from the illumination point for both prostate and breast tissue. Assuming a 45 mm x 30 mm mean dimension human prostate gland, it would be possible to cover its entire volume using only a couple of transmission measurements in a potential scanning for prostate cancer (Chapter 5- Fig. 5.19).

In terms of the Raman signal origin, it has been clearly shown how the distribution of the collected Raman scattering signals changes under different
values of absorption and scattering coefficients of the phantom and hence in different types of tissues. It has been demonstrated that the highest signals are likely to be obtained from nearer surface lesions (either on collection or illumination side) than those at depth using transmission Raman spectroscopy. Even in this case though, relative signals are likely to be as high as 40–60% for ‘lesions’ found in the centre of organs compared to those found towards the edges. This is in contrast to previous experimental and theoretical studies in powders and pharmaceutical tablets with minimal or no absorption on layers of infinite lateral dimensions, which indicate that the maximum Raman signal originates from objects buried in the centre of the tablets rather than towards its edges.

Another aspect of the study was to assess the feasibility of the measurements in terms of tissue content. Numerous studies using conventional Raman spectroscopy have proven not only the potential of acquiring Raman signal from human prostate and breast tissue, but also of discriminating between different pathologies of prostate tissue and breast cancer, respectively. As a part of this thesis, different types of human prostate tissue acquired from the local tissue bank have been assessed in terms of Raman signal collection, using deep Raman spectroscopy. In order to explore the potential of the deep Raman technique as an approach to the clinical practice, the samples have been measured across the whole volume in a transmission mode and not sectioned as in earlier studies. This attempt revealed the limitations of the deep Raman application on small samples of prostate tissue, which includes the presence of fluorescence (TURP samples) and the need for a sufficient sample volume (prostate biopsies). Measurements of prostate samples’ individual components (fat, muscle tissue) and sections of TURPs, confirmed the possibility of collecting Raman photons from samples which lack the limitations above, by proving that the prostate gland is not intrinsically fluorescent and hence a sufficient volume of it can be efficiently studied by means of deep Raman spectroscopy.

Our results demonstrate that failure of acquiring deep Raman signals from prostate tissue was due to the properties of the samples used rather than the actual technique. For that reason, we suggest that measurements of whole prostate glands (potentially removed after prostatectomy) would prove feasibility of the concept. If this is the case, we can set the basis for a non-invasive, real
time prostate cancer diagnosis, where the illumination fibre could be inserted transurethrally and the collection fibre transrectally or the other way round (Fig. 10.1b). The deployment of transmission Raman geometry for this clinical application would be even more obvious in the case of breast, as it is an external and more accessible organ (Fig. 10.1a). In both of the cases, the transmission Raman mode is applicable and provide the advantage of high penetration depth, since both of the glands are accessible from both sides.

**Figure 10.1:** Potential transmission Raman geometry applied to (a) breast and (b) prostate cancer (recreated from).

We should point out that diagnosing cancer with Raman fibre optic probes, mainly in the form of endoscopes, has been studied and experimentally applied for pathology discrimination throughout various studies. In the case of the deployment of fibre optic probes in a transmission mode, our study would contribute to increasing the efficiency of this approach by identifying areas with a maximum distribution of Raman signal. Although the tissue phantoms we employed might be different from tissues in terms of heterogeneity of the content, our results would still be applicable since we took into consideration the relative signals of the samples. However, being aware of the Raman signal distribution, would also apply to a more significant parameter: the detection limits. Being able to evaluate the effective volume of where Raman signal can be collected from, would increase the efficiency and sensitivity of the measurements.
10.2 Application of photon diode for signal enhancement and depth prediction

In order to improve the Raman signal and achieve the maximum possible efficiency in signal collection, a “photon diode” element was introduced in our measurements and its effect has been studied in terms of “calcification” depth in the sample and optical properties. This concept has been applied mainly in previous studies to pharmaceutical tablets\textsuperscript{159,269} and to limited extent to biological tissue\textsuperscript{149,235} but without exploring the parameters mentioned above. Because of important differences in the optical properties between tablets and tissues, such as the lack of significant absorption in tablets and considerably longer mean free scattering path lengths in tissues (at least by an order of magnitude) due to the difference in spatial scales, one cannot assume identical behavior and enhancement. The signal enhancement exhibited by the employment of photon diode in a wide range of phantom optical properties was between ×1 and ×2.4. Our results are in broad agreement with observations from similar studies which demonstrate a signal enhancement of ×1.6 and ×1.5 in chicken\textsuperscript{235} and porcine tissue\textsuperscript{149} respectively, which were performed without assessing the signal enhancement in terms of specific tissue optical properties.

Our measurements would contribute in a potential application of the photon diode element in transmission probe measurements as they indicate the types of tissues which are more likely to benefit from such a concept. From our results, tissues with high scattering and low absorption coefficients, such as fat, bone and skin (all layers) would benefit the most in terms of signal enhancement (Chapter 7). In these types of tissues, a signal enhancement of at least ×1.4-2.2 at the front and ×1.2-1.7 in the bulk volume can be achieved. Tissues with lower scattering and moderate absorption, such as breast, brain, muscle and small bowel can also benefit from a significant diode signal enhancement of ×1.8-2.2 at the front and ×1.5 at the middle of the sample. Tissues with even lower scattering coefficient but considerable absorption, such as prostate, white matter brain and liver would still benefit from a photon diode but mainly in regions near the illumination side, with a signal enhancement of around ×1.8. Finally, there are certain types of tissues such as dermis, stomach wall and aorta which would not
see any benefit due to their high optical absorption and high scattering properties. However, even in the case where certain types of tissue do not seem to benefit from a diode signal enhancement, there is still potential for signal to noise ratio improvement, with the use of additional enhancers/mirrors on the remaining tissue/phantom sides (right, left, top, bottom and the collection sides), as has been proven in the past\textsuperscript{159}.

The photon diode approach can be beneficial when traditional setup parameters (laser power, collection time, collection efficiency) have been maximized. This can prove beneficial in the case of an \textit{in vivo} real time measurement with a Raman probe, where after an assessment of the optical properties of the tissue measured, a simple introduction of a photon diode filter on the front of the Raman probe (Fig. 10.2) can enhance the signal by a considerable amount, increasing in this way the detection sensitivity and penetration depth of the measurements.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure10.2.png}
\caption{Schematic diagram of photon diode element implemented in clinical practice.}
\end{figure}
Apart from significantly enhancing the Raman signals on the phantoms, the photon diode has also been employed to predict the depth of a chemically distinct object buried within a turbid matrix. This novel approach for depth prediction could address the localization of certain suspicious lesions in tissues, a significant clinical feature in breast cancer diagnosis which is currently only available through mammography. The advantage of this potential approach lies in its simplicity, since it only requires two measurements in an identical sample orientation, with and without the photon diode. Earlier studies have attempted depth prediction of layers or inclusions in animal tissue and tissue phantoms. Raman tomography with SORS has also been employed in order to assess the location of an inclusion inside tissue phantoms. This concept however involves a large number of collection fibres in different orientations around the sample, additional use of an imaging technique (e.g. MRI, CT) and complex algorithms in order to reconstruct the 3D image. That makes the technique less flexible and not easy to implement in the clinical practice.

The depth prediction has shown to be successful across the whole sample depth when no absorber agent was present, but not in samples where absorption is either endogenous or physically introduced (exogenous). Similar studies on pharmaceutical tablets which lack any distinct absorption at visible-NIR wavelengths, confirm the strong dependence of signal as a function of sample depth by exhibiting a maximum enhancement when the inclusion is closest to the illumination side. In the same study, numerical simulations also evidence that the introduction of absorption reduces the enhancement levels through the sample depth, leading to a symmetrical depth profile which is not amenable to depth prediction. That demonstrates the limitations of the concept in media with inherent absorption such as tissue phantoms and tissue samples. In this case we were only able to accurately predict the depth when the inclusion was close to the front of the sample (illumination side).

However, the prediction depth concept was very promising for samples with little absorption and a thickness of up to 10-30 mm. That would render this application ideal for pharmaceutical tablets where minimum absorption is present. To date, the photon diode concept has only been used on pharmaceutical tablets in order to achieve a more efficient quantitative analysis of active pharmaceutical ingredients (API). The employment of this concept for predicting the depth of
an API layer or even impurities in the tablet volume could be a promising approach and open the way to a number of different applications in the industrial pharmaceutical analysis.

10.3 Assessment of Raman microscopy in bladder cancer diagnosis

In the final experimental chapter of the thesis, we explored the potential of diagnosing bladder cancer by means of conventional Raman microscopy on urine samples. Raman spectroscopy has been employed for bladder cancer diagnosis in earlier studies by either measuring the bladder wall with a Raman probe \textit{in vivo} \cite{206} or excised tissue samples with Raman microscopy \cite{295, 296} or a confocal Raman probe \cite{297} \textit{ex vivo}. In contrary, our study involved direct and completely non-invasive measurements on patients’ urine samples. From our results, the main discriminant feature was indicated to be the \textbf{urea Raman peak shift} which depends on the interactions between urea and the surrounding molecules. These interactions are promoted to a different extent not only between different urine samples but also between different areas of the same sample. Based on this spectral feature, multivariate analysis on the urine measurements has indicated a \textbf{sensitivity of 73\%} and a \textbf{specificity of 80\%} in the discrimination between the two pathology groups. In similar studies of bladder epithelial cells in urine samples with Raman spectroscopy, sensitivity and specificity was 92\% and 91\% respectively, involving however a higher number of samples.\cite{300} Further \textit{in vivo} and \textit{ex vivo} measurements of the bladder wall of patients with bladder cancer have demonstrated a sensitivity and specificity range of 85-96\% and 79-100\% respectively.\cite{206, 295-297} A number of commercially available urine-based markers for bladder cancer tests have also exhibited a maximum sensitivity and specificity value of 83\% and 86\% respectively.\cite{310} Although our observations in this study cannot be conclusive due to the small number of the urine samples available, these feasibility measurements demonstrate significant potential in non-invasive bladder cancer diagnosis. Further measurements on a bigger number of samples would potentially establish higher statistical values for the performance of this promising Raman approach.
Future work

There are a number of interesting research directions raised from this study which would be worth following up with future work.

In terms of prostate and breast cancer diagnostics, the efficiency of deep Raman has been shown for the respective optical properties. Specifically for prostate, it has also been shown that fluorescence would not inhibit measurements in tissues (unless they had been cut with a hot loop), and consequently in vivo analysis of whole prostate glands may be viable. The latter case would be quite interesting to explore in the operating theatre, since we have additionally shown that the whole prostate volume can be potentially scanned by a couple of transmission Raman measurements.

For this purpose, prostate glands removed through prostatectomies and just before being examined with histopathology for assessing cancer metastasis, could be potentially scanned in a transmission Raman setup on site. A number of measurements mainly around the peripheral zone where most of the carcinomas are diagnosed, would possibly detect calcifications or even suspicious areas with different Raman signal from the rest of the surrounding tissue in the gland. In a next step, the spectroscopical results can be correlated to the histopathological analysis on the same gland and identify signals corresponding to healthy or malignant prostate areas.

If this approach is successful, the next stage would be the real-time, non-invasive scanning of prostate gland using fibre optics for illumination and collection through urethra and rectum, as described in Chapter 3. This concept would have the advantage of being minimally invasive compared to the current standard of biopsy, as no tissue would need to be removed from the gland. Additionally, the tissue volume scanned would exceed those in core biopsy samples, which are usually only a few mm in thickness and length. The small prostate volume tested for cancer with biopsy frequently leads to low sensitivity of the technique as the malignant area might be missed. With a deep Raman approach, the whole gland and mainly the high risk areas (peripheral and transitional zone) which are located in proximity to the rectum and centrally to the gland respectively (Fig. 11.1), would be potentially assessed for malignancies in their whole volume, increasing in this way the sensitivity of diagnosis. The
application of deep Raman on prostate cancer is a part of this study which could not be completed since the ethical and trust approval for this work took longer than anticipated. However, this is likely to be achieved in the months following the end of this doctorate project.

**Figure 11.1**: Potential transmission Raman geometry for prostate cancer detection in relation to the prostate zones (recreated from\textsuperscript{248}) (A) and the side view diagram of the prostate showing the anterior fibromuscular stroma (FM), central zone (CZ), posterior zone (PZ), transition zone (TZ), prostatic urethra (PU), and a seminal vesicle (SV) (B)\textsuperscript{311}.

A similar approach would also be viable in the case of the breast cancer where the relationship between calcifications and disease is more well-established. In that case, transmission Raman which is particularly sensitive to calcifications due to their high Raman cross-section, would detect the nature of the calcified formations in the breast glad in transmission mode, without the need of tissue removal for a biopsy. As there are types of calcifications (type I, calcium oxalate) which are benign and do not provide any risk for the patient, Raman spectroscopy which is sensitive to the molecule vibrations of calcifications, would easily rule these cases out of the need for biopsies. That would reduce the number of women (around 22%) who are unnecessarily going through the stress and the clinical procedure of cancer diagnosis. The research group in Exeter University takes significant steps towards this direction by optimizing the transmission deep
Raman system (for wavelength, etc) which will be used on scanning human breast samples in collaboration with Royal Devon & Exeter Hospital.

The application of transmission Raman spectroscopy on both breast and prostate cancer, can benefit from the work presented in this thesis on photon diode signal enhancement. With the application of the “photon diode” element at the front of the illumination fibre optic probe, we can double the signal enhancement of tissue in close proximity with the excitation side and the rest of the area up to ×1.5. This application can potentially show benefit with similar enhancement for fibre optic probes used alone or inserted though endoscopes on a range of tissues such as small bowel, skin and bone.

Another interesting work to follow up would be the inclusion depth prediction in diffusely scattering samples, using the “photon diode” element. Although the results were not encouraging for tissue media which include absorbing agents, this concept would ideally work for pharmaceutical tablets, where much work has be done with the “photon diode” effect on signal enhancement, but no depth prediction of any inclusion in their volume has been attempted yet. Potential applications in this field can include the depth prediction of one or multiple API layers in a pharmaceutical tablet, detection of impurities or contamination and their depth in the tablet volume.

Finally, the study on bladder cancer urine samples using Raman microscopy, demonstrated significant potential in non-invasive diagnosis. However, that was a feasibility study and our observations cannot be conclusive due to the small number of the urine samples studied. Based on the promising specificity (80%) and sensitivity (73%) achieved through our study, further measurements and a bigger number of samples would be required in order to establish higher statistical values for the application of this non-invasive approach. In a potential continuation of the study, a suggestion would be to collect the first urine of the day instead of the second one from the patients. In this way, more proteins would be detected in the urine volume, providing a greater potential for spectral discrimination between the two pathology groups. If this is the case, spectral features which are different between healthy and malignant samples could be attributed to molecular vibrations deriving from specific biomarkers in bladder cancer. That would provide an entirely non-invasive approach which would be
also possible to use as a screening test on patients’ urine samples for bladder cancer diagnosis.
Conclusions

The aim of this thesis was to explore minimally invasive approaches to cancer diagnosis, using conventional and deep Raman spectroscopy. The application of spectroscopic techniques and the advancing of individual parameters of their development have been assessed for three different cancer types, prostate, breast and bladder cancer.

In the case of prostate and breast cancer, we studied:

- the Raman signal spatial distribution of potentially malignant features (calcifications) in different phantoms
- the origin and detection limits of Raman scattering of the calcifications in prostate and breast phantoms

In terms of detection limits, our results confirmed the feasibility of acquiring sufficient signal to detect malignancy over the volume of an average prostate gland, using only a couple of transmission measurements. In terms of Raman signal origin, it has been demonstrated that the highest signals are likely to be obtained from nearer surface lesions (either on collection or illumination side) than those at middle depth. However, relative signals can be still as high as 40-60% for “lesions” found in the centre of organs, compared to those found towards the edges.

During the measurements of human tissue (TURPs, biopsies) acquired from the local tissue bank, the limitations of deep Raman spectroscopy on prostate tissue were highlighted. These included:

- the presence of fluorescence (TURP samples)
- the need for a sufficient sample volume (prostate biopsies).

At the same time, we demonstrated the feasibility of collecting Raman photons from samples which lack the limitations above, by proving that prostate gland is not intrinsically fluorescent at 830 nm and hence a sufficient volume of it can be efficiently studied by means of deep Raman spectroscopy.

Further improvement of the deep Raman approach was explored in terms of signal enhancement, by introducing the “photon diode” element. The effect of the photon diode has been studied in a wide range of optical properties, showing:
- a signal enhancement between ×1 and ×2.4
- considerable signal enhancement for high scattering and low absorption levels
- certain types of tissue (fat, bone) benefit more compared to other types (stomach, aorta)
- the signal enhancement levels attenuate as the location (depth) of artificial “malignant lesions” in the phantoms increases.

The same “photon diode” concept has also been employed to study another important feature in clinical diagnosis, the inclusion (or calcification) depth. A novel approach for depth prediction was explored while the “malignant feature” was buried in different depths of tissue phantoms and the signal enhancement ratios achieved with the photon diode were used to predict the depth of the ‘calcification’. The depth prediction has specifically shown to be successful in samples where no absorption is present, rendering this application suitable for pharmaceutical tablets were minimum absorption is present.

In the case of bladder cancer, potential diagnosis was also assessed by means of Raman microscopy in healthy and malignant urine samples. The main discriminant feature was found to be the urea Raman peak shift which depends on the interactions between urea and the surrounding molecules. Based on this spectral feature, multivariate analysis on the urine measurements have indicated a sensitivity of 73% and a specificity of 80% in the discrimination between the two pathology groups.
Appendices

Appendix 1- Local ethical approval

Local ethical approval for TURP chips

Exeter Clinical Research Facility

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Telephone: 01392 403202

Ref: CTB21 STB16/SW
10 July 2014

Dear Professor Stone

The Royal Devon and Exeter Tissue Bank Steering Committee has approved your request to collect and use TURP chips taken during routine transurethral resection of the prostate, for prostate cancer diagnosis (approval numbers CTB21 and STB16).

In order to obtain the Tissue Bank paperwork (patient information sheet and consent form) and coded labels required for the samples, please contact Sophie Warren, Research Officer.

Yours sincerely,

Malcolm Crundwell
Tissue Bank Director

cc Lynda Garcia, R&D Facilitator, Noy Scott House, RD&EFT
✓ Martha Vardaki, Research Fellow, School of Physics, Uni of Exeter

The NIHR Exeter Clinical Research Facility is a partnership between
The University of Exeter Medical School and the Royal Devon and Exeter NHS Foundation Trust
Local ethical approval for prostate biopsies

Exeter Clinical Research Facility

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Telephone: 01392 408202
Ref. CTB28 STB27
10th September 2015

Dear Professor Stone

The Royal Devon and Exeter Tissue Bank Steering Committee have approved your request to collect and use prostate biopsies taken during routine prostate care (approval numbers CTB27 and STB27).

In order to obtain the Tissue Bank paperwork (patient information sheet and consent form) and coded labels required for the samples, please contact Chloe Slate, Research Officer.

Yours sincerely,

[Signature]

Malcolm Crundwell
Tissue Bank Director

cc Jo Lowe, R&D Facilitator, Noy Scott House, RD&FET
Martha Vardaki, Research Fellow, School of Physics, University of Exeter

The NIHR Exeter Clinical Research Facility is a partnership between
The University of Exeter Medical School and the Royal Devon and Exeter NHS Foundation Trust
Appendix 2- Publications derived from this thesis

Studying the distribution of deep Raman spectroscopy signals using liquid tissue phantoms with varying optical properties

Martha Z. Vardaki,* Benjamin Gardner,* Nicholas Stone* and Pavel Matousek*

In this study, we employed large volume liquid tissue phantoms, consisting of a scattering agent (lipid), an absorption agent (India ink) and a synthesized calcium phosphate powder (hydroxyapatite, HAP) similar to that found in connective tissues (e.g. breast and prostate), to simulate human tissues. We studied experimentally the magnitude and origin of Raman signals in transmission Raman geometry as a function of optical properties of the medium and the location of calculations within the phantom. The goal was to inform the development of future noninvasive cancer screening applications. In vivo, the results provide insight into light propagation and Raman scattering distribution in deep Raman measurements, exploring also the effect of the variation of relative absorbance of laser and Raman photons within the phantoms. Most notably when modeling breast and prostate tissue, it follows that maximum signal is obtained from the front and back faces of the tissue with the central region contributing less to the measured spectrum.

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Introduction

Deep Raman spectroscopy consists of a group of emerging spectroscopic techniques being developed for biomedical, pharmaceutical and security applications based around Spatially Offset Raman Spectroscopy (SORS) and Transmission Raman Spectroscopy (TRS). In contrast to conventional Raman spectroscopy, deep Raman has been shown to be able to acquire specific molecular signals from calculations from depths up to 2.7 cm, or 5 cm with the use of labelled nanoparticles (SERSORS). The feasibility of non-labelled deep Raman approaches has been already demonstrated for potential application to breast cancer and bone disease diagnostics. A further demonstration of the potential for clinical SORS has been shown in a feasibility study to assess the surgical margins in excised breast tumour samples. In the transmission mode, it has been used for signal recovery in a variety of studies, with examples of Raman and diffuse optical tomography on bone and breast tissue phantoms.

However, in the studies above, the measured Raman scattering has only been explored in terms of signal intensity versus tissue thickness. A major unexplored aspect of deep Raman is the spatial distribution of the Raman scattering throughout a sample volume and its dependence on optical properties of the sample.

Related research has been conducted on pharmaceutical tablets, studying the spatial origin of measured Raman signals changes when both in spatial and time-resolved transmission Raman measurements, and in respective Monte Carlo simulations. However, because of the great difference between biological tissue and pharmaceutical formulations (e.g. presence of absorption in tissue, different spatial scales), the medical applications warrant a specific and tissue-oriented study.

In the field of Raman fiberoptic probes, a needle fibre optic probe has been developed and tested for subcutaneous Raman measurements by being inserted beneath the surface of various ex vivo biological tissues to acquire a Raman signal. Even though Raman needle probes can enable significant depth probing, they are invasive and generally only sample a volume restricted to a zone local to the probe tip.

For the first time, our measurements explore experimentally the signal distributions in tissue phantoms representing prostate and breast tissue. To ensure that our measurements are comparable to real conditions, we produced liquid tissue phantoms with optical properties comparable to these types of tissue.

Prostate cancer is nowadays the third most common male cancer in UK with more than 30000 new cases every year. Some of the most common tests taken to detect prostate cancer (PSA, digital rectal exam, ultrasound) are not always reliable since they can only detect the gross morphological
changes on the gland and often fail to determine if these changes are due to a cancerous condition. As a result, more invasive methods such as prostate biopsy and histopathology are being applied for the definitive diagnosis and classification of the disease. However, if the tumor is small, it can be missed by biopsy since only a very small percentage of the entire gland is sampled.17

Prostate cancer is also one of the commonest cancers in men. Over 100,000 men are diagnosed each year in the UK. While mammography is vastly affected by breast tissue density18,19 and ultrasound exhibits a low sensitivity,20 current diagnostic means seem to be insufficient for an adequate early diagnosis. The gold standard for breast cancer is currently biopsy followed by histopathology. However, even in that case not all of the tissue can be sampled in order to be assessed as cancerous or benign.21

Conventional Raman spectroscopy has already established the feasibility of detecting prostate and breast cancer ex vivo. Studies have shown the possibility of distinguishing between normal prostate, benign prostate hyperplasia and different grades of prostate carcinoma.22,23 In the case of breast cancer, a number of studies have also managed to discriminate between healthy, benign and malignant breast tissue using Raman spectroscopy under the microscope.24,25

Deep Raman techniques have also shown potential in distinguishing between benign and malignant breast tissue, on the grounds of the detection of different types of calcifications which appear in different pathological types of breast tissue.26-28 Therefore, it is clear that methods able to probe Raman signals from the tissue volume of interest such as spatially offset Raman spectroscopy (SORS) or transmission Raman spectroscopy, have the potential to provide a minimally invasive solution for in vivo diagnosis of the disease.

This study has explored the feasibility of acquiring Raman signals from each point of a two-dimensional grid in the tissue phantom. The objectives have been to enable both an understanding of the relative signals measured from lesions buried at different depths in tissues and to help the future design of optical sampling methods to maximise the extraction and collection of these signals. In order to explore how the photon migration of the Raman signals change when the optical properties of the surrounding tissue change, we produced a selection of liquid tissue phantoms within a 30 mm × 45 mm × 45 mm quartz cell. In this way we simulated the potential in vivo measurement conditions for human prostate (transurethrally and transrectally) and breast samples and assessed the feasibility of such measurements.

Results and discussion

Intralipid maps

Raman scattering maps were recorded for tissue phantoms with different Intralipid (IL) concentrations and therefore different scattering coefficients (Fig. 1). Parameters such as
power, laser wavelength, beam size and acquisition time were kept constant during the mapping.

In the plain water mapping (Fig. 1a) no diffuse scattering is present and the measured signal is limited to the boundaries of the laser beam. Once we introduce Intralipid into the system (Fig. 1b), laser photons are able to reach and interact with HAP in more distant off axis positions as they elastically scatter between suspended lipid droplets. Therefore, Raman signal can be detected outside of the boundaries of the illuminating beam with limits that depend on the scatterer (Intralipid) concentration in the tissue phantom.

As the numbers of Intralipid molecules in the phantom increase (moving from Fig. 1c-g), the scattering distribution pattern changes. In phantoms with low scattering coefficients (Fig. 1b) we observe a form of symmetry in the Raman signal between the illumination (left) and the collection (right) side. It should be considered here that the highest number of Raman photons are expected to be generated on the illumination side because this is where most of the laser photons are present in the cell, with the greater probability of inducing Raman events. However, those photons need to propagate across the entire width of the cell through the turbid medium to the right side in order to be detected, undergoing loss due to scattering and absorption. On the other hand, when the sample cuvette is on the collection side of the phantom, the Raman photons generated there are more efficiently collected, since this is where they have the least distance to travel in order to reach and be detected by the collection optics.

The distribution of Raman scattering signals across the maps will always be a balance between these two phenomena. As Intralipid molecules in the tissue phantom increase in concentration (Fig. 1c and d), it is progressively less likely for the Raman photons generated at the illumination spot to pass through the phantom volume and be detected on the other side of the cell prior to being absorbed or scattered out of the sample (signal balance moves to the collection (right) side).

As Intralipid concentration rises further (Fig. 1e-g), the path length laser and Raman photons increase considerably and therefore the water absorption engenders more significant impact on the signal distribution pattern. As mentioned in the literature and also confirmed with our measurements (Fig. 2), water absorbs the Raman photons generated in our setup (at 902 nm) more than the laser photons (830 nm). This explains why the signal balance moves to the collection surface (right), as it is easier for laser photons to traverse diffusely the cell than Raman photons, when the scattering increases.

Another important feature evidenced by the maps is that the higher the Intralipid concentration is, the broader the measurable signal region becomes. This means that a lesion located off the illumination-collection axis would be more likely to be detected in tissues with higher turbidity (modelled with higher Intralipid concentrations) than a lower one. This becomes clear if we compare maps with low Intralipid concentrations (Fig. 1b and c) with the ones with increased Intralipid (Fig. 1d-g).

![Absorbance spectra of water in the range of 400–1100 nm.](image)

The maps above support our understanding of Raman scattering distribution throughout the phantom volume, in the absence of any additional absorber apart from water itself. In the next step, we explored how this distribution changes under the influence of both scattering and elevated absorption, which is the case of real tissue.

Intralipid and Indian ink maps

Intralipid and Indian ink were combined in liquid tissue phantoms to best simulate real human breast and prostate tissue optical properties. In the first set of experiments a cuvette of HAP (calcium hydroxyapatite, a type of calcification found in tissues) was used to explore the effect of varying the optical properties on the recovery of signals from various locations within the phantom volume (Fig. 3).

In these maps, scattering and absorption agents are both present at low concentrations (Fig. 3a), we observe a strong Raman signal from both the illumination and the collection sides. If we double the density of scatterer but maintain the absorber concentration constant (Fig. 3b), the signal on the collection side is being significantly suppressed. This is due to the significant increase in the photon path length which directly increases the chance of the laser and Raman photons being absorbed by the Indian ink. For the same reason, when we increase the ink concentration, we expect a similar effect on the mapping pattern (Fig. 3c) where II concentration was kept constant as in (a) but the concentration of the ink was increased.

Indian ink aqueous solution has been shown through UV/Vis absorbance measurements (Fig. 4) to absorb the laser photons (830 nm) more than the generated Raman ones (902 nm), i.e. the opposite net effect to that induced by water alone. Consequently, the detected signal is much less likely to originate from the collection side of the phantom, as there are relatively less laser photons reaching it.
To characterise the system at the maximum range of prostate tissue relevant absorption and scattering conditions, which are higher than those used earlier in the paper, we had to resort to a stronger Raman scatterer, trans-stilbene. By replacing HAP with trans-stilbene it was possible to probe higher II/ink concentration solutions with the existing Raman apparatus and while recording trans-stilbene’s peak at 1192 cm⁻¹ (921 nm), with this sample, we could still observe the same distribution pattern with the two maxima on illumination and collection side.

However, when we increase the absorption further (Fig. 5b), the collection side becomes more dominant. We should mention here that the absorption coefficient in Fig. 5b is well above the absorption range of human prostate and breast tissues and was only tested to assess the detection limits of our system. Additionally, with this level of absorption present, the Raman signal measured was of the order of the noise, which makes the data processing prone to errors.

The findings from this study enable us to design optimised instrumentation for use with tissue samples. We expect that appropriate modifications in our system (increased laser power, minimised loss of laser photons from the sample using a photon diode, improved collection efficiency and opening slits and using high dispersion gratings) will increase signals by around two orders of magnitude, enabling the probing of samples with weaker signals at these depths.

**Experimental**

**Tissue phantom optical properties**

Inclusion-containing tissue phantoms with specific optical properties are a common approach to simulate tissues in deep Raman techniques.¹⁴,¹⁷ Intralipid (Fresenius Kabi Ltd,
United Kingdom) and Indian ink (Histology Stain, American MasterTech) were used to induce diffuse scattering and absorbance in tissue phantoms respectively. Intralipid, a dilute mixture of emulsified long chain triglycerides, serves as a scattering medium whereas Indian ink, a dispersion of carbon particles, acts as a broad band absorbing agent.

The Indian ink and Intralipid concentrations in phantoms were determined to match those for human prostate and breast tissue optical properties of absorbance and scattering, as found in the literature. However, there is only limited data in the literature on tissue optical properties for the laser wavelength of 830 nm.

The reduced scattering (\(\mu_s'\)) and absorption (\(\mu_a\)) coefficient values for normal and cancerous prostate glands, have been calculated through optical extinction and diffusion reflectance measurements. The values calculated for \(\mu_s'\) at 830 nm were 4 cm\(^{-1}\) for healthy and 6 cm\(^{-1}\) for cancerous prostate glands, whereas the values for \(\mu_a\) were estimated to be 0.027 cm\(^{-1}\) for healthy and 0.002 cm\(^{-1}\) for cancerous prostate respectively.\(^{39}\)

Svensson et al. used NIR time-resolved spectroscopy to estimate the reduced scattering coefficient between 5.5 and 9.5 cm\(^{-1}\) and absorption coefficient between 0.3 and 0.7 cm\(^{-1}\) for malignant prostate glands with untreated cancer.\(^{29}\)

Concerning breast tissue, the reduced scattering coefficient has been calculated for 830 nm wavelength to be \(\mu_s' = 9.84\) cm\(^{-1}\).\(^{30}\) Breast tissue absorption coefficients are not available in literature for the exact wavelength of 830 nm, but a value of \(\mu_a\) between 0.068 and 0.102 cm\(^{-1}\) in papillary breast cancer is estimated for the very close wavelength of 825 nm.\(^{31,32}\)

Based on the literature values for reduced scattering and absorption coefficients of human prostate and breast tissues, we built liquid tissue phantoms using Intralipid and Indian ink concentrations which would provide equivalent values of optical properties.

Intralipid has been well characterized in terms of optical properties in literature. Using the fitting parameters for 10%, 20%, and 30% Intralipid provided by Michals et al.,\(^{28}\) for calculating the Intralipid optical properties and converting for our wavelength, we plotted a fit line through which we extracted the Intralipid concentrations equivalent to the required reduced scattering coefficient values (Fig. 6).

Using these values, liquid tissue phantoms with reduced scattering coefficients of 2.3, 4.6, 9.2, 18.4, 27.7 and 36.9 cm\(^{-1}\) were produced, using Intralipid concentrations calculated based on the calibration line expression (\(y = 52.179x\)).\(^{33}\)

In terms of absorption properties, different concentrations of Indian ink were used to achieve different absorption coefficients in the phantom. Indian ink from different manufacturers has been widely employed and characterized in various studies.\(^{34}\) In this study we measured the absorption coefficient of our specific sample of Indian ink.

After having recorded UV/Vis absorption spectra of different ink concentrations, our data were curve fitted for the appropriate wavelength (830 nm) and the ink concentrations required for the human tissue absorption change were calculated.\(^{35}\) Our data were in very good agreement with the literature, at least in the concentrations mentioned.\(^{36}\) Liquid phantoms were produced with absorption coefficients of 0.37, 0.63 and 2.26 cm\(^{-1}\).

To simulate the optical properties of prostate and breast tissues, the scattering and absorption agents were combined in the same sample. The liquid tissue phantoms which were made to cover the range of optical properties in human prostate and breast tissue, are summarised in the Table 1.

**Table 1** Optical properties of the simple Sony Intralipid and combined Intralipid and Indian ink liquid tissue phantoms constructed in relation to the human prostate tissue range. The right hand column shows the material used to provide the human signal in each of the phantoms.

<table>
<thead>
<tr>
<th>Phantom</th>
<th>(\mu_s') (cm(^{-1}))</th>
<th>(\mu_a) (cm(^{-1}))</th>
<th>Human signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phantom a</td>
<td>0.0</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom b</td>
<td>2.3</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom c</td>
<td>4.6</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom d</td>
<td>9.2</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom e</td>
<td>18.4</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom f</td>
<td>27.7</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom g</td>
<td>36.9</td>
<td>0.0</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom 1</td>
<td>4.6</td>
<td>0.37</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom 2</td>
<td>9.2</td>
<td>0.37</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom 3</td>
<td>4.6</td>
<td>0.08</td>
<td>HAP</td>
</tr>
<tr>
<td>Phantom 4</td>
<td>9.2</td>
<td>0.80</td>
<td>trans-stilbene</td>
</tr>
<tr>
<td>Phantom 5</td>
<td>9.2</td>
<td>3.26</td>
<td>trans-stilbene</td>
</tr>
<tr>
<td>Prostate tissue</td>
<td>4.95</td>
<td>0.002-0.7</td>
<td>--</td>
</tr>
<tr>
<td>Breast tissue</td>
<td>9.84</td>
<td>0.068-0.102</td>
<td>--</td>
</tr>
</tbody>
</table>

**Fig. 6** Percentage Intralipid concentration versus reduced scattering coefficient (cm\(^{-1}\)).

Tissue phantom preparation

The liquid phantoms consisted of a quartz cell (45 mm width \(\times\) 30 mm length (26 mm internal cell optical path length) \(\times\) 45 mm height) (mm-hert instrument co., USA) containing aqueous solutions of Intralipid and Indian ink in various concentrations.

A thin cuvette (12 mm width, 4 mm length (2 mm optical path length)) containing hydroxyapatite (HAP) or trans-stilbene
The quartz cell and the HAP cuvette were used in our measurements.

Deep Raman setup

The deep Raman system at Exeter University used a transmission Raman configuration (Fig. 8). It consisted of a spectrally stabilized laser (Innovative Photonics Solutions) with a laser emission at 830 nm and an output power of ~300 mW. The laser was coupled to a Thorlabs 460 µm multimode patch cord, collimated and filtered by passing through a pair of laser line filters (FL830-10, Thorlabs) in order to suppress the off-centre spectral emission from the laser line. The laser was directed towards the sample with a mirror and brought onto the sample with a 25 mm diameter, 70 mm focal length lens. The sample was illuminated with 280 mW of light in a 3-4 mm diameter spot. The collimated light passing through the tissue phantom is either scattered or absorbed by the molecules present. The raman scattered photons were collected using an AR coated lens (f = 60 mm, dia. 50 mm, INOCHRYS Laser systems). The collimated scattered light was passed through a holographic super notch filter (HSFP-830.0 AR-2.0, Kaiser Optical Systems) to remove the elastically scattered light (laser photons) and imaged onto a fibre probe bundle by a second lens of the identical parameters to the collection lens (i.e. with no magnification). The fibre bundle (CeramOptec, ‘spot to slit line’ type bundle assembly, active area spot diameter approximately 2.21 mm, slit line approximately 0.25 mm × 14.95 mm) was connected to the entrance port of a HoloSpec VPH system spectrophotograph (Kaiser Optical systems Inc, HS19013.4 custom). Maps were recorded using a deep depletion CCD camera cooled down to −75 °C (Andor Technology, DU420A-BR-DD, 1024 × 255 pixels). The overall spectral resolution of the detection system was ~8 cm⁻¹.

The signal was collected using 6 accumulations of 5 seconds (apart from the water mapping where we recorded the signal for 120 × 0.35 seconds to stay below the CCD saturation levels) and the cosmic ray removal option of the detector software (Andor Solis) was applied. The system was calibrated using Raman bands of an aspirin tablet (acetyl salicylic acid).

Raman mapping

In order to explore the effect of Raman scattering and light distribution in different tissue phantoms, the Raman spectra of the tissue phantom when the cuvette was in 300 different positions (in the x-y plane) were recorded. The movements were realised in ‘snake’ mode with the support of a motorised x-y translation stage. To make most efficient use of experimental time, steps were 1 mm in the main beam area (between 10-20 mm on x axis and 8-14 mm on y axis) and
2 mm around the edges of the large cell (the rest of the mapping area). At each step, a Raman spectrum was recorded and the stage was moved to the next position.

Data analysis

All data were loaded into Matlab R2013a (MathWorks), where they were subjected to cosmic sky removal, baseline correction, principal component (PC) noise reduction and subsequent reconstruction from the first seven principal components into 1D and 2D images for better signal distribution visualization.

In these presented maps, the colour of the pixel indicates the intensity above the fit baseline of the characteristic HAP peak (959 cm$$^{-1}$$) or in the case of still bone the peak at 1192 cm$$^{-1}$$.

Conclusions

In this study we explored the Raman scattering distribution in the liquid tissue phantom that mimics prostate and breast tissues in both approximate size and optical properties of the phantom. It has been clearly shown how the distribution of the collected Raman scattering signals changes under different values of absorption and scattering coefficients of the phantom. The results elucidate the distribution of Raman signals obtained from such extended tissue phantoms and demonstrate that the highest signals are likely to be obtained from deeper surface lesions than those at depth using transmission Raman. Even in this case though, we demonstrated relative signals of around 40-60% for lesions found in the centre of organs compared to those found towards the edges. This is contrary to observations reported earlier with pharmacological tablets where layered samples of (extended/ infinite lateral dimensions) were investigated with minimal contributions from absorptions. These studies demonstrated a maximum signal originating from the centre of pharmacological tablets. It shows promise for future applications of transmission Raman, either in the breast or applied through fibre probes, to the prostate. It has advanced the understanding of how the distribution of collected Raman scattering signals change in relation to a range of expected optical properties of tissues. This helps the future test of instruments that we may wish to use to target disease specific signals within particular regions of the sample and pave the way towards the ultimate application of non-invasive deep Raman diagnosis of prostate and breast cancer.

Acknowledgements

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Notes and references

Characterisation of signal enhancements achieved when utilizing a photon diode in deep Raman spectroscopy of tissue

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Abstract: We characterise the performance of a beam enhancing element (‘photon diode’) for use in deep Raman spectroscopy (DRS) of biological tissues. The optical component enhances the number of laser photons coupled into a tissue sample by returning escaping photons back into it at the illumination zone. The method is compatible with transmission Raman spectroscopy, a deep Raman spectroscopy concept, and its implementation leads to considerable enhancement of detected Raman photon rates. In the past, the enhancement concept was demonstrated with a variety of samples (pharmaceutical tablets, tissue, etc.) but it was not systematically characterized with biological tissues. In this study, we investigate the enhancing properties of the photon diode in the transmission Raman geometry as a function of i) the depth and b) the optical properties of tissue samples. Liquid tissue phantoms were employed to facilitate systematic variation of optical properties. These were chosen to mimic optical properties of human tissues, including breast and prostate. The obtained results evidence that a photon diode can enhance Raman signals of tissues by a maximum of $\times$ 2.4, although it can also decrease the signals created towards the back of samples that exhibit high scattering or absorption properties.

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OCIS codes: (300.0500) Spectroscopy; (300.0450) Spectroscopy, Raman

References and links


1. Introduction

Deep Raman spectroscopy (DRS), comprising principally of Spatially Offset Raman Spectroscopy (SORS) [1] and Transmission Raman Spectroscopy (TRS) [2], has been established in recent years as a promising tool for non-invasive disease diagnostics. The encompassing techniques can achieve a depth range of up to two orders of magnitude higher than conventional Raman spectroscopy [3], paving the way for novel medical diagnosis and other medical analytical applications in vivo.

Deep Raman techniques in turbid media are characterized by weak Raman signals due to diffuse scattering (and potentially absorption) of photons propagating through these samples. This poses a particular challenge when it comes to recovering Raman signals from deeper layers in biological tissue. To improve Raman signal to noise ratios, traditional methods, such as the increase of laser power, collection time, improvement of collection efficiency etc., can be used [4]. Further improvements can be made by employing a signal enhancing element, a “photon diode”, which was shown to provide a multifold enhancement of Raman signal in powder samples. This can be beneficial as an additional route to boosting accessible depths and sensitivity of deep Raman spectroscopy.

A photon diode comprises a dielectric bandpass filter which increases the collected Raman signal from a highly scattering sample when placed in close proximity to the sample surface centred on the laser illuminating zone. It acts, in essence, as a unidirectional mirror allowing photons of a given wavelength and angle of incidence (e.g. at normal incidence) to be transmitted through it on one side whilst the photons re-emitted from the diffusely scattering sample (a dominant loss mechanism in TRS of tissue and other diffusely scattering samples) to be reflected from it back into the sample due to the angular dependence of its reflectance properties [5]. In this way, the element prevents the loss of diffusely scattered photons which re-emerge from the sample at the point where the laser beam enters the sample, while it allows the collimated laser beam impacting on it at normal incidence to be transmitted through the filter from the other side.

The photon diode has been used in various deep Raman studies in the past, predominantly to achieve signal enhancement, mainly with pharmaceutical tablets. The photon diode enhancement of pharmaceutical tablets in a transmission mode is typically in the range of \( \times 4-\times 10 \) of the mean volume signal when the tablet thickness varies between 3 and 7 mm [5–8]. The mechanism of enhancement has also been studied using Monte Carlo simulations yielding corresponding enhancement factors [5, 8]. A modified version of the photon diode element is also applicable in the SORS modality where it has been demonstrated for a 4.2 mm two-layer pharmaceutical tablet, exhibiting enhancement by a factor of \( \times 4.5 \) for the front layer and \( \times 2.2 \) for the back layer [9]. Further investigations of the enhanced Raman signal as a function of layer depth has been conducted while probing a thin film of polymer into different depths of a 4 mm tablet, showing an overall enhancement of \( \times 6 \) experimentally and \( \times 4 \) using numerical models [10]. Alternative enhancement methods include hemispherical mirrors also returning emerging photons back into the sample at the illumination zone with reported enhancement factors of up to \( \times 40 \) in 6.35 mm thick pharmaceutical tablets [11].

Studies exploring the dependence of Raman signal on the depth of its origin in biological samples have also been performed [12], but without the use of photon diode. Separate Raman measurements on biological tissue using a photon diode, reported an enhancement factor of \( \times 1.6 \) for the mean volume signal of a 14 mm thick chicken breast [8] and \( \times 1.5 \) for a high-
Raman scatterer buried in a 2.7 mm depth of 27 mm porcine tissue [4], while both of the tissue samples were used to simulate a human breast.

The photon diode enhancement of Raman signals as a function of the depth of their origin is an aspect that has not been explored yet in biological tissue samples. Large differences between the optical properties of biological tissues and pharmaceutical tablets, such as the lack of absorption in tablets and the mean free scattering path length in tissues due to the difference in spatial scales does not allow us to assume identical behavior. In this paper, we explore the dependence of photon diode signal enhancement not only on the signal source depth but also on tissue optical properties using liquid tissue phantoms. The 30 mm thickness phantoms employed were of similar optical properties to various human tissues, and of similar dimensions to particularly breast and prostate. The tissue phantoms consisted of a scattering agent, an absorption agent and a high Raman cross-section compound located at a given depth representing, for example, a malignant element found in many cancerous conditions (e.g. calcifications). The purpose of this study was to characterise the enhancement effect of the photon diode as a function of the inclusion depth inside the phantom in a transmission mode and the optical properties of the tissue matrix (scattering and absorption coefficients).

2. Materials and methods

Tissue phantoms with inclusions and different optical properties are widely used to simulate tissues in optical spectroscopy and deep Raman techniques [13, 14]. The liquid phase of the phantoms allows high flexibility in the control of its optical properties.

In this study, Intralipid and Indian ink were used to induce scattering and absorbance, respectively, in the liquid tissue phantoms. Intralipid, a dilute mixture of emulsified fatty acids serves as a scattering medium whereas Indian ink, a dispersion of carbon particles, acts as a wide spectrum absorbing agent.

2.1 Tissue phantom optical properties

The phantoms were prepared to include most of the range of optical properties found in tissues. Because these values vary considerably and can overlap between different types of tissues, it was important to explore which of the different tissue types would benefit the most from the diode signal enhancement.

The optical properties of various tissues which we took into consideration are presented in Table 1 and their relative distribution and relation to the phantom properties in Fig. 1. The reduced scattering coefficients were calculated as a function of the excitation wavelength [15], whereas the absorption coefficients are based on literature values for skin [16], dermis [17], brain [18], white matter [19], breast [15, 20], fat [21], bone [22], liver [23] at 850 nm, muscle [21], small bowel [24], aorta [25], stomach wall [26]. For prostate tissue both the reduced scattering and absorption coefficients were extracted from separate studies [27, 28].

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reduced scattering coefficient (cm⁻¹) [15]</th>
<th>Absorption coefficient (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6.58</td>
<td>0.6-1 [23]</td>
</tr>
<tr>
<td>Prostate</td>
<td>4.95 [27, 28]</td>
<td>0.002-0.7 [27, 28]</td>
</tr>
<tr>
<td>Aorta</td>
<td>7.07</td>
<td>3.18 [25]</td>
</tr>
<tr>
<td>Muscle</td>
<td>8.15</td>
<td>0.2-0.3 [21]</td>
</tr>
<tr>
<td>Small bowel</td>
<td>8.80</td>
<td>0.05-0.2 [24]</td>
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<tr>
<td>White matter brain</td>
<td>9.41</td>
<td>0.75-1.15 [29]</td>
</tr>
<tr>
<td>Breast</td>
<td>9.84</td>
<td>0.068-0.102 [20]</td>
</tr>
<tr>
<td>Brain (mean)</td>
<td>10.70</td>
<td>0.4 [18]</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>11.68</td>
<td>2 [26]</td>
</tr>
<tr>
<td>Fat (mean)</td>
<td>13.09</td>
<td>0.02 [21]</td>
</tr>
<tr>
<td>Bone</td>
<td>15.93</td>
<td>0.2 [22]</td>
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<tr>
<td>Skin (mean)</td>
<td>22.39</td>
<td>0.25 [16]</td>
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<tr>
<td>Dermal</td>
<td>23.53</td>
<td>3 [17]</td>
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2.2 Tissue phantom preparation

The liquid tissue phantoms consisted of an optically clear quartz cell (45 mm width × 30 mm length (26 mm internal cell optical path length) × 45 mm height) (ramé-hart instrument co., USA) and contained aqueous solutions of Intralipid (20% emulsion, Sigma) and Indian ink (Histology Stain, American MasterTech), in various concentrations.

A thin vial (outer size: 12 mm width, 4 mm length; inner size: 10 mm width, 2 mm length in optical axis) filled with trans-stilbene and suspended from a motorized translation stage (8MTF-102LS05, Standa Ltd, Lithuania), was placed inside the quartz cell (Fig. 2). The vial width (12 mm) was aligned with the y-axis and the 4 mm length was aligned with the optical axis of the system (x-axis). Using the motorized stage, the trans-stilbene vial was moved to a number of different positions (depths) in a two-dimensional horizontal grid, inside the quartz cell during each mapping.

As stated above the liquid tissue phantoms were designed to simulate human breast and prostate tissue (amongst others) both in size [29, 30] and optical properties. Trans-stilbene was selected for its high Raman cross-section and represents a localized region of abnormal tissue, e.g. a zone with micro-calcifications, which are often found in different types of cancerous tissues and have a distinctly different Raman signal from surrounding soft tissues (Intralipid and ink solution here). The purpose of the experiment was to study the effect of the
photon diode on the Raman signal detected from the trans-stilbene vial at each location within the cell. The differences in Raman cross section between micro-calcifications and trans-stilbene are largely irrelevant in this study as the effect of the photon diode is linear in its nature and therefore the same enhancement factors would be achieved irrespective of the magnitude of Raman scattering cross section of the material within the cell (other properties such as scattering and zero-absorption of trans-stilbene powder at the excitation and detection spectral ranges, were assumed to be approximately the same as for micro-calcifications).

In order to elucidate the role of both the absorption and scattering properties on the diode enhancement effect, the liquid tissue phantom studies were divided into two groups (Fig. 3):

a) Liquid tissue phantom measurements with a constant absorption coefficient ($\mu_a = 0.8\ \text{cm}^{-1}$) and a varying reduced scattering coefficient ($\mu_s' = 4.6-23.0\ \text{cm}^{-1}$)

b) Liquid tissue phantom measurements with a constant reduced scattering coefficient ($\mu_s' = 13.8\ \text{cm}^{-1}$) and a varying absorption coefficient ($\mu_a = 0.1-6.3\ \text{cm}^{-1}$)

Fig. 3. Scattering and absorber concentrations (a) and optical properties (b) of the liquid tissue phantoms prepared and measured with transmission Raman spectroscopy.

The liquid tissue phantoms were prepared and characterized in optical properties ranges similar to our previous study [12] in order to match a clinically relevant range. During the phantom fabrication, Intralipid was used as a scatterer and Indian ink as an absorber agent. Using literature values of reduced scattering coefficient for 10%, 20% and 30% of Intralipid [31] and extrapolating to values of lower concentration which agree with other studies [32-34] we calculated the Intralipid concentrations equivalent to the required reduced scattering coefficient values for our phantoms. For the Indian ink characterization, UV/Vis absorption spectra have been recorded for different ink concentrations and the values extracted for the appropriate wavelength (830 nm) to calculate the appropriate ink concentrations required for our phantoms [35]. It should be mentioned here that during the phantom characterization, Intralipid is assumed to have no absorption and Indian ink no scattering.

The optical properties of liquid tissue phantoms used are summarized in Table 2.

Table 2. Optical properties of the liquid tissue phantoms used in the study. The material used to provide the distinct Raman signal at given locations was trans-stilbene.

<table>
<thead>
<tr>
<th>Phantom</th>
<th>$\mu_s' \ (\text{cm}^{-1})$</th>
<th>$\mu_a \ (\text{cm}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phantom 1</td>
<td>13.82</td>
<td>0</td>
</tr>
<tr>
<td>Phantom 2</td>
<td>13.82</td>
<td>0.37</td>
</tr>
<tr>
<td>Phantom 3</td>
<td>13.82</td>
<td>0.8</td>
</tr>
<tr>
<td>Phantom 4</td>
<td>13.82</td>
<td>1.22</td>
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<tr>
<td>Phantom 5</td>
<td>13.82</td>
<td>1.63</td>
</tr>
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<td>Phantom 6</td>
<td>4.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Phantom 7</td>
<td>9.2</td>
<td>0.8</td>
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<tr>
<td>Phantom 8</td>
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</tr>
<tr>
<td>Phantom 9</td>
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<td>0.8</td>
</tr>
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</table>
It should be noted that during the study, the Intralipid absorption at 830 nm was checked before each experiment by recording UV/Vis measurements, for precipitation of lipid aggregates in order to ensure the preservation of its stability and consistency. Additionally, prior to sample preparation, the Indian ink was always subjected to ultrasound agitation in an ultrasonic bath for 30 minutes in order to prevent solution instability and ensure reproducibility of optical properties [36, 37].

2.3 Deep Raman setup

We have used a deep Raman system at the University of Exeter arranged in a transmission Raman configuration. The setup is shown in Fig. 4. It consisted of a spectrum stabilized laser (Innovative Photonics Solutions) with laser emission at 830 nm and an output power of ~300 mW. The laser light was coupled to a Thorlabs 400 µm multimode patch cord, collimated at exit and filtered by passing through a pair of laser line filters (FL830-10, Thorlabs) in order to suppress the amplified spontaneous emission (ASE) from the laser and other unwanted emission born in the optical fibre. The laser beam was directed towards the sample with a mirror and brought onto the sample with a 25 mm diameter, 70 mm focal length lens. The sample was illuminated with 280 mW of light in a 3–4 mm diameter spot. The collimated light passing through the tissue phantom is either scattered or absorbed by the sample components present. The Raman scattered photons were collected on the other side of the phantom using a lens (f = 60 mm, dia. = 50 mm, AR coated, INGCRYS Laser systems). The collimated scattered light was passed through a holographic super notch filter (HSPF-830.0 AR-2.0, Kaiser Optical Systems) to remove the elastically scattered light (laser photons) and imaged onto a fibre probe bundle by a second lens of the identical parameters to the collection lens (i.e. no magnification was present). The fibre bundle (CeramOptec, ‘spot to slit line’ type bundle assembly, active area spot diameter approximately 2.2 mm, slit line of approximately 0.22 × 15.0 mm dimensions) was connected to the entrance port of a Holospec VPH system spectrograph (Kaiser Optical systems Inc, HSG-917.4 custom). The Raman spectra were recorded using a deep depletion CCD camera cooled down to −75 °C (Andor Technology, DU420A-BR-DD, 1024 × 255 pixels). The overall spectral resolution of the detection system was ~8 cm⁻¹.

Fig. 4. Schematic diagram of the deep Raman setup in the transmission mode used.

In order to explore the properties of diode enhancement on Raman signal, a photon diode element (Semrock, 830 nm, with FWHM bandwidth measured as 9.5 nm, BrightLine® single-band bandpass filter – 25 mm diameter with overall 3.5 mm thickness) was mounted at the center of a metal plate which was then placed at the front of the quartz cell against its internal wall (see Fig. 2). The metal plate outside the active area of the photon diode was covered with an aluminum reflecting foil, in order to achieve the maximum reflectance of re-emerging photons back to the phantom also outside the laser illumination zone. During the control (without a photon diode) measurements, a quartz window of identical dimensions was placed at the same location in order to make up for the loss of liquid phantom volume and to retain the identical mapping pattern. Additionally, a calcium fluoride disc (20 mm diameter, 1 mm thickness) was placed between the diode/quartz and the cell wall during every measurement.
in order to minimize the solution volume trapped in the interface which would potentially lead to disruption of the collimated laser beam.

2.4 Raman mapping

In order to explore the diode enhancement effect on Raman signal through the different liquid tissue phantoms, we recorded the Raman spectra of the tissue phantom when the trans-stilbene vial was at 42 different positions on the optical axis of the setup. At each location the signal was collected using 6 accumulations of 5 seconds (30 s overall) and the cosmic ray removal option of the detector software (Andor Solis) was applied. The system was calibrated using Raman bands of an aspirin tablet (acetylsalicylic acid).

A motorized x-y translation stage was used to move the vial between different positions, in a 'snake'-like pattern (starting from the laser entrance side and moving towards the exit side of the cell). While recording the 1D maps, the step size on the x axis varied between 1 mm around the center of the phantom (between 8 and 14 mm from the illumination side) and 2 mm on the edges of the phantom (the rest of the mapping area). On the y axis only the main beam area (between 21 and 23 mm) was scanned, using 1 mm step size. At each step, a Raman spectrum was recorded and the stage was moved to the next position. In order to provide confidence in the results, each phantom was mapped three times at the same locations. The mean and the standard deviation were calculated and displayed as error bars in the plots.

For the 2D plot generated from the Phantom 3 map (Fig. 5), the vial was positioned at 176 different locations inside the quartz cell within the x-y (horizontal) plane. To make most efficient use of the experimental time, the area of the y axis covered was between 12 and 32 mm distance from the top edge of the cell. On the y axis, the step size was 1 mm in the main beam area (between 18 and 28 mm) and 2 mm on the rest of the mapping area. On the x-axis the mapping was performed with a 2 mm step size for the full accessible length of the phantom (0-20 mm).

2.5 Data analysis

All data were loaded into Matlab R2013a (MathWorks), where they were subjected to baseline correction using asymmetric least squares smoothing [38] and principal component (PC) noise reduction. Principal component analysis, a statistical method which is more commonly used to reveal the spectral variance throughout a data set, is also useful for minimizing the noise in a set of spectra by reconstructing it only from the significant principal components (in our case the first seven PCs). In this way, all of the important spectral information is retained, whereas the background noise is being removed [39, 40]. For the 1D plots, the mean intensity on the y axis was calculated in order to directly compare the values between the different maps.

Both one and two dimensional plots of the signal distributions are given. These plots display the trans-stilbene peak intensity, at each spatial location in the phantoms, at 1193 cm⁻¹ for all the phantoms except for Phantom 1 (phantom with zero absorption), where 1594 cm⁻¹ peak has been used instead, in order to avoid saturation issues.

3. Results

3.1 Effect of photon diode on the origin of Raman signal within the liquid phantoms

To evaluate the impact of the photon diode on the signal enhancement and the origin of Raman scattering, we first recorded a full map of Phantom 3 (μs = 13.82 cm⁻¹, μα = 0.8 cm⁻¹) with and without the photon diode (Fig. 5). The color of the pixels indicates the peak intensity of 1193 cm⁻¹ from trans-stilbene at each location.
Fig. 5. Raman scattering 2D plots of Phantom 3 ($\mu_s' = 13.82 \text{ cm}^{-1}$, $\mu_a = 0.8 \text{ cm}^{-1}$) with (a) and without (b) the photon diode in a transmission mode. Laser photons are injected on the left of the image and Raman signal is collected on the right. The plots depict the intensity of ~1195 cm$^{-1}$ Raman band of trans-stilbene as the vial moves to different positions in the phantom. The 2D plot (b) has been normalized against the maximum intensity of the diode map (a).

The photon diode provided an enhancement factor of $\sim \times 1.7$ on the front of the phantom (closer to the illumination side) which decreased and remained stable in the middle of the phantom. This 2D plot is consistent with the results presented in the next part, where a variety of optical properties were explored.

3.2 Diode enhancement in phantoms with varying reduced scattering coefficient

The trans-stilbene generated Raman scattering has been recorded along the optical axis in phantoms with varying reduced scattering coefficient and a constant concentration of Indian ink.

Fig. 6. Ratio of trans-stilbene Raman signal intensity with diode over signal without the diode versus the trans-stilbene vial position along the x-axis in the phantom (mm), for phantoms with the same absorption but different scattering coefficients. The coefficients are presented in cm$^{-1}$. 

#258011  Received 1 Feb 2016; revised 5 Apr 2016; accepted 5 Apr 2016; published 6 May 2016
(C) 2016 OSA 1 June 2016 | Vol. 7, No. 6 | DOI:10.1364/BOE.7.002130 | BIOMEDICAL OPTICS EXPRESS 2138
The reduced scattering coefficient values explored were in the range of 4.6 – 23.04 cm\(^{-1}\). For clarity and brevity, the ratio of the trans-stilbene signal with the diode over the signal without the diode, was plotted against the phantom depth of the trans-stilbene vial, for phantoms with different optical properties, on the same plot (Fig. 6). The greatest enhancement factor for the phantoms with varying scattering, is observed on the measurement points with the closest proximity to the diode (0 mm). At this depth, phantoms with lower optical properties exhibit higher Raman signal with the diode, as the generated Raman photons cross the phantom bulk on the other side of the vial and are collected from the optics more efficiently in low scattering values. When the vial is positioned in a distance from the diode, the phantoms with the higher scattering benefit from the photon diode the most, with enhancement factors ranging between 1.17 and 1.83. The reason for that is due to lower diffuse scattering, the low scattering phantoms do not benefit as much from the backscattering enhancement mechanism of the diode. As a result, in a higher scattering environment, there is greater enhancement from the photon diode as the photons which re-enter the phantom volume reach the vial more easily due to diffuse scattering. In this way, the bulk volume of the phantom is the zone benefiting most from the presence of the photon diode. The signal enhancement decreases as we move towards (0-4 mm) and it seems to plateau when the trans-stilbene vial is located around the middle of the phantom (4-16 mm). The enhancement factors in this area appear to fall to a similar range (1.0-1.2) for most of the phantoms. Towards the back of the phantom and closer to the collection side (16-20 mm), the diode enhancement of the signal seems to be decreasing even more. This drop is in some cases so sharp (e.g. Phantom 8: \(\mu'_s = 23.04\) cm\(^{-1}\) and \(\mu_s = 0.8\) cm\(^{-1}\)), that the diode might not be beneficial for signal enhancement at all. It should be noted that at high scattering coefficient values (18.4 cm\(^{-1}\) and 23.04 cm\(^{-1}\)), the enhancement at the back of the phantom does not follow the same trend as at the front. The drop is most likely due to the overall lower transmittance of the diode compared to quartz layer (by around 10%).

3.3 Diode enhancement in phantoms with varying absorption coefficient

Similar trends are observed in phantoms with varying absorption coefficients and constant reduced scattering coefficient (concentration of Intralipid) (Fig. 7). The signal enhancement from the diode here seems to be almost constant through many vial depths in each of the different phantoms. The absorption appears to affect the diode enhancement to a greater extent compared to the scattering case, since the enhancement ratio drops quicker when we increase the absorption coefficient (ink concentration) rather than the scattering coefficient. Comparing the two plots (Fig. 6 and Fig. 7), it seems that a middle value of absorption (\(\mu_s = 0.8\) cm\(^{-1}\)) inhibits the signal enhancement in the middle of the phantom more compared to a middle scatterer value (\(\mu'_s = 13.82\) cm\(^{-1}\)). This means that it is less likely for the ‘enhanced’ laser photons to generate a Raman photon which will get detected on the collection side in the presence of ink. On the other hand, when only Intralipid concentration is increased, the absorption increases indirectly due to the path length change too, but not to such an extent.
In terms of the enhancement factor in the varying absorption phantoms, it takes a wide range of values through the entire phantom depth as the vial is moved across towards the collection side. The highest enhancement factor is again observed at the points closest to the diode (0 mm) and it takes different values between 0.95 and 2.34, depending on the absorber agent introduced in each phantom. As expected, the highest signal enhancement is observed in the phantom with no absorption and as soon as 0.1 μL/ml of ink is present, equivalent to μₐ of 0.37 cm⁻¹, the signal enhancement drops from 2.34 to 2.21. In the middle area of the phantom (4-16 mm), the signal enhancement has gradually decreased and reached a plateau. Towards the back of the phantom (16-20 mm), the signal enhancement seems to be decreasing even more and for the extreme ink concentration (Phantom 5: μₐ = 0.8 cm⁻¹ and μₐ′ = 13.82 cm⁻¹), the benefit of using a photon diode is limited, as the signal enhancement ratio constantly remains close or under 1. As mentioned before, the values below 1 can be attributed to the reduced transmittance of photon diode which becomes especially significant in high optical properties phantoms with low signal to noise ratios.

It should be noted that a reduced signal on the edge of the sample is less detrimental to the detection sensitivity than it would be in the centre of the sample, as signals tend to be 10-50% higher at the edges than in the centre for tissue optical properties [12].

4. Conclusion

In this series of experiments, we have demonstrated the signal enhancement potential of photon diode in tissue phantoms with varying optical properties. It was shown that the gradual introduction of absorber agent affects the signal enhancement more strongly compared to a scatterer increase.

The enhancement factors observed through the measurements are in the range of 1-2.4. Similar studies on pharmaceutical tablets have exhibited an enhancement as high as × 10 [7]. However, the optical properties of these samples (e.g. lack of absorption) are quite different and their physical dimensions and those of illumination and collection zones are also different to those of biological tissues. To date, biological samples of similar thickness reported in the literature, show an enhancement of ~1.5 times for the entire sample matrix when measured in
a transmission setup using a photon diode [4, 8]. These observations are in broad agreement with our results although these reports do not include any enhancement studies involving inclusions inside the phantoms. Our study also demonstrates how the diode enhancement changes with varying scattering and absorption properties in samples with Raman scattering.

Through our study, it is obvious that different tissue types will benefit to different extent from the use of photon diode. For example, based on the optical properties shown in Fig. 1, tissues such as liver, prostate, breast, muscle, fat, brain, bone and small bowel would benefit with at least \( \times 1.2 \) signal enhancement in the bulk volume, whereas other tissues such as dermis, white matter, skin and stomach wall would not see any benefit due to their higher optical absorption and scattering properties. In those tissues with regions of interest near the illumination side the photon diode may achieve signal enhancements of \( \times 1.5 \) to \( \times 2.3 \).

Where a signal enhancement is achieved it leads to enhanced detection sensitivity and penetration depth for transmission Raman measurements. Furthermore, the use of additional filters/mirrors on the remaining tissue/phantom sides (right, left, top, bottom and the collection sides) as indicated earlier [5] would provide the prospect for further improvements to Raman signal to noise ratios.

Acknowledgments

We thank the STFC BioMedical Network (STFC, STMA00012) and the University of Exeter for their financial support. An EPSRC grant [EP/K020374/1] partly funded the work presented here. We also thank Dr. Ben Gardner (University of Exeter) for his support with data analysis.
# Appendix 3- CV

## CURRICULUM VITAE

### Personal Information

<table>
<thead>
<tr>
<th>Last Name</th>
<th>Vardaki</th>
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<tbody>
<tr>
<td>First Name</td>
<td>Martha</td>
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<td>Date of birth</td>
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<td>Nationality</td>
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<td>Marital status</td>
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<td>E-mail</td>
<td><a href="mailto:mv254@exeter.ac.uk">mv254@exeter.ac.uk</a></td>
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</table>

### Work Address

Biomedical Physics
Physics department
Streatham Campus
University of Exeter,
EX4 4QL, Exeter, UK

### Education

**2013-Present**
PhD Candidate, Department of Physics, University of Exeter, UK
Project title: “**Development of deep Raman techniques as a novel prospect for prostate cancer detection**”
- **Collaboration with STFC-RAL and Royal Devon & Exeter NHS Foundation Trust**

**2011-2013**
MSc in Industrial Pharmacy and Drug Analysis
Instrumental Pharmaceutical Analysis lab, Department of pharmacy, University of Patras, Greece
Thesis title: “**Development of a Novel Diagnostic Method for Bone and Cartilage Diseases**”

**2010**
Pharmacist license
<table>
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<th>Year</th>
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<tr>
<td>2009-2010</td>
<td>Research experience in Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Greece</td>
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<td>Research project: “Effects of bisphosphonates on cancer induced angiogenesis”</td>
</tr>
<tr>
<td>2004-2009</td>
<td>BSc in Pharmacy (5-year curriculum), Department of Pharmacy, University of Patras, Greece</td>
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<td>Diploma work title: “Development of analytical methodology using HPLC for the isolation of the main ingredients of Crocus endemic species.”</td>
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<td>2001-2004</td>
<td>High School of Myrina, Lemnos</td>
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**Training**

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<tr>
<td>2013-Present</td>
<td>Laboratory of deep Raman and conventional Raman spectroscopy, Physics School, University of Exeter, UK, in study of breast and prostate phantoms and tissue samples (prostate TURPs and biopsies, urine samples, spine sections)</td>
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<tr>
<td>2010-2013</td>
<td>Laboratory of Instrumental Pharmaceutical Analysis, University of Patras, Greece, in study of bone and cartilage with spectroscopic techniques</td>
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<tr>
<td>2009-2010</td>
<td>Laboratory of Molecular Pharmacology, University of Patras, Greece, in bisphosphonates’ impact on eukaryotic cell culture and angiogenesis of chicken embryo</td>
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<tr>
<td>1/2010-3/2010</td>
<td>Practical training in Regional General State Hospital of Patras Agios Andreas, Patras, Greece</td>
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<tr>
<td>7/2009-1/2010</td>
<td>Practical training in drug store</td>
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<td>3/2010-7/2010</td>
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</table>
Laboratory instructor in BSc practical courses, Exeter University.

i. Organic Chemistry (Biosciences degree)

ii. Inorganic Chemistry (Biosciences degree)

iii. Physics (Natural Sciences degree)

Laboratory instructor in BSc practical courses on Instrumental Analysis, Pharmacy department, University of Patras.

i. Quantification of NaCl in physiological saline using ion-exchange chromatography.

ii. Determination of the quantity of active pharmaceutical ingredients in commercial tablets (Panadol Extra®, Aspirin® and Depon®) using UV-Vis spectrometry.

iii. Quantification of Cl\(^-\) as an impurity in chlorothiazide using Argentometric titration.

**Foreign languages**

**English**

Certificate of Proficiency in English, University of Cambridge

Certificate of Proficiency in English, University of Michigan

**French**

Diplôme Approfondi de Langue Française, Dalf C2

**Special knowledge**

**Computers**

Microsoft Office Suite (Word, Excel, Powerpoint, Outlook), MicroCal Origin, Peakfit, Adobe Photoshop, SIMCA (Umetrics), Eigenvector SOLO, LaTeX, Matlab
### Specialization in Techniques

<table>
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<th>Analytical techniques</th>
<th>Raman Spectroscopy (FT-Raman and micro-Raman), High Performance Liquid Chromatography (HPLC), FT-IR Spectroscopy, Attenuated Total Reflectance IR, UV-Vis spectrometry, deep Raman spectroscopy (transmission, SORS)</th>
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<td>Bioanalytical and Biochemical techniques</td>
<td>Eukaryotic cell cultures, Isolation of Human umbilical vein endothelial cells (HUVEC), in vivo chicken embryo CAM angiogenesis assay, MTT cell viability assay, Western Blot Analysis</td>
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<td>Other techniques</td>
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### Certifications for laboratory work

1. Lasers and ILS Safety Course, May 2013, Redwood Education Centre, Gloucestershire Royal Hospital
2. Laser Safety Training conducted by Public Health England (PHE)

### Publications in international journals


2. Martha Z. Vardaki, Nicholas Stone, Pavel Matousek, “Characterisation of signal enhancements achieved when..."
utilizing a photon diode in deep Raman spectroscopy of tissue”, Biomedical Optics Express, 7 (2016), 2130-2141

**Participation in Research Programmes**

2009-2011

Greek national research program of Karatheodoris: Chemical analysis of bones using spectroscopic technology. (M. Orkoula)

2013-2016

EPSRC Healthcare technology: A novel Deep Raman spectroscopy platform for non-invasive in situ molecular analysis of disease specific tissue compositional changes. (N. Stone, P. Matousek)

**Scientific Community Involvement**

- organizer of Biophysics weekly group meeting (2013- 2015, Exeter university)

- representative of Physics PGR Community in College Health and Safety committee (2013- 2016, Exeter university)

- Coblentz society member (2013- present)

- Society for Applied Spectroscopy member (2013- present)

- SPIE (International Society for Optics and Photonics) member (2013- present)

**Grants/ Awards**

- August 2015: Royal Society of Chemistry travel grant for attending and presenting research work in SciX, Providence, RI, USA
• April 2015: “Most Innovative Poster” award, Postgraduate Research Showcase, Exeter University

• July 2014: ABS (Association of British Spectroscopists) Trust Bursary for attending SPEC, Poland


10. Sofia Kouvaritaki, **Martha Z. Vardaki**, Sofia Panteliu, Christos G. Kontoyannis and Malvina G. Orkoula. Study of the quality of human bone jaw by Raman spectroscopy. 21st Panhellenic Congress of
Participation in Conferences

Chemistry, Thessaloniki, December 2011. (Oral Presentation)


1. SciX, Rhode Island, Providence, 27 September- 4 October 2015
2. Meeting of the IRDG, Exeter, 26-28 August 2015
4. SPEC, “Shedding new light on disease”, August 2014, Krakow, Poland
5. IRDG 203rd Christmas meeting and Poster Session, December 2013, UCL, London
6. 202nd Meeting of the IRDG, September 2013, Glasgow
7. European Conference on Non-linear Optical Spectroscopy (ECONOS) and CARS workshop 2013, Exeter, April 2013 (organizing assistance)
9. 21st Panhellenic Congress of Chemistry, Thessaloniki, December 2011
10. 15th Panhellenic Pharmaceutical Congress, Athens, May 2011
11. 12th Congress of Medicinal Chemistry, Patras, April 2011
12. 2nd Conference Meeting on “Interaction of cells to extracellular network and biomaterials: Applications on anagenetic medicine and tissue engineering”, Patras, December 2010
13. Course in the fundamentals, applications and instrumentation of capillary ion chromatography and rapid separation LC, Patras, October 2010
15. 36th ESCP European Symposium on Clinical Pharmacy, Istanbul, October 2007
16. 5th Hellenic Forum on Bioactive Peptides, Patras, May 2006
17. 34th European Symposium on Clinical Pharmacy, Amsterdam, October 2005
18. EPSA 2nd Autumn Assembly, Amsterdam, October 2005

- Public talk: “Raman: shining light on disease”, Sidmouth Science Festival, Norman Lockyer Observatory, October 2015

Prof. Nicholas Stone (first PhD supervisor):
Professor of Biomedical Imaging and Biosensing,
Biomedical physics, School of Physics, University of Exeter
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Prof. Pavel Matousek (second PhD supervisor):
STFC Senior Fellow, Central Laser Facility, Rutherford Appleton Laboratory
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Asst Prof. Malvina Orkoula (MSc supervisor):
Assistant Professor of Instrumental Pharmaceutical Analysis, Department of Pharmacy, University of Patras
E-mail: malbie@upatras.gr
References

9. WebMD, Picture of the Prostate).


143. P. Matousek and N. Stone, J Biophotonics, 2013, 6, 7-19.


