Combining the Tactile Resonance Method and Raman Spectroscopy for Tissue Characterization towards Prostate Cancer Detection

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To the memory of my father, Jan-Åke Candejord
Prostate cancer (PCa) is the most common male cancer in Europe and the US, and only lung and colorectal cancer have a higher mortality among European men. In Sweden, PCa is the most common cause of cancer-related death for men.

The overall aim of this thesis was to explore the need for new and complementary methods for PCa detection and to take the first step towards a novel approach: combining the tactile resonance method (TRM) and Raman spectroscopy (RS). First, the main methods for PCa detection were reviewed. Second, to establish a robust protocol for RS experiments in vitro, the effects of snap-freezing and laser illumination on porcine prostate tissue were studied using RS and multivariate statistics. Third, measurements on porcine and human tissue were performed to compare the TRM and RS data via multivariate techniques, and to assess the accuracy of classifying healthy and cancerous tissue using a support vector machine algorithm.

It was concluded through the literature review that the gold standard for PCa detection and diagnosis, the prostate specific antigen test and systematic biopsy, have low sensitivity and specificity. Indolent and aggressive tumors cannot be reliably differentiated, and many men are therefore treated either unnecessarily or too late. Clinical benefits of the state-of-the-art in PCa imaging – advanced ultrasound and MR techniques – have still not been convincingly shown. There is a need for complementary and cost-effective detection methods. TRM and RS are promising techniques, but hitherto their potential for PCa detection have only been investigated in vitro.

In the RS study no evidence of tissue degradation due to 830 nm laser illumination at an irradiance of $\sim 3 \cdot 10^{10}$ W m$^{-2}$ were found. Snap-freezing and subsequent storage at $-80^\circ$C gave rise to subtle but significant changes in Raman spectra, most likely related to alterations in the protein structure. The major changes due to PCa do not seem to be related to the protein structure, hence snap-freezing may be applied in our experiments.

The combined measurements on porcine and human prostate tissue showed that RS provided additional discriminatory power to TRM. The classification accuracy for healthy porcine prostate tissue, and for healthy and cancerous human prostate tissue, was $> 73\%$. This shows the power of the support vector machine applied to the combined data.

In summary, this work indicates that an instrument combining TRM and RS is a promising complementary method for PCa detection. Snap-freezing of samples may be used in future RS studies of PCa. A combined instrument could be used for tumor-border demarcation during surgery, and potentially for guiding prostate biopsies towards lesions suspicious for cancer. All of this should provide a more secure diagnosis and consequently more efficient treatment of the patient.
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Luleå, May 9, 2011
Stefan Candeferd
Part I
In this thesis the following peer-reviewed papers are included and referred to by their Latin letters. My contributions to these papers are shown in Table 1.1.


Table 1.1: The contributions made by Stefan Candefjord to Papers A–D. 1 = main responsibility, 2 = Contributed to high extent, 3 = Contributed.

<table>
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<th>A</th>
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<td>Experimental design</td>
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<td>Performance of experiments</td>
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Other publications of relevance, but not included in this thesis, are listed below.


## Chapter 3

### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
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<tr>
<td>HCA</td>
<td>hierarchical clustering analysis</td>
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<td>MR</td>
<td>magnetic resonance</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MRSI</td>
<td>magnetic resonance spectroscopic imaging</td>
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<td>MTS</td>
<td>micro tactile sensor</td>
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<tr>
<td>NIR</td>
<td>near-infrared</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>principal component</td>
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<td>PCA</td>
<td>principal component analysis</td>
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<td>PCa</td>
<td>prostate cancer</td>
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<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
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<tr>
<td>PZT</td>
<td>lead zirconate titanate</td>
</tr>
<tr>
<td>RS</td>
<td>Raman spectroscopy</td>
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<tr>
<td>SHM</td>
<td>scanning haptic microscopy</td>
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<tr>
<td>SB</td>
<td>systematic biopsy</td>
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<td>SVM</td>
<td>support vector machines</td>
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<td>TRM</td>
<td>tactile resonance method</td>
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This chapter explains the problems prostate cancer poses to public health, describes the theory for the tactile resonance method and Raman spectroscopy, discusses pitfalls for in vitro experiments, and gives an insight into the mathematical tools that were used in this work.

4.1 General background

Prostate cancer (PCa) is the most common form of male cancer in the US and Europe [1, 2]. From the most recent data it is estimated that PCa caused almost 90 000 deaths in Europe in 2008, and only lung and colorectal cancer have a higher mortality among European men [1]. In the US it is the second leading cause of male death due to cancer [2], and the severity of the disease is strongly related to insurance status [3]. The incidence of PCa is expected to increase due to the aging population [4].

PCa is often indolent, more men die with the disease than from it. Considering the large risks for side effects such as impotence and incontinence after radical prostatectomy, i.e. surgical removal of the prostate, active surveillance may be the best option for patients with indolent tumors [5, 6]. On the other hand, to reduce PCa mortality, patients with aggressive tumors likely to metastasize must be treated early on [6]. Current clinical diagnostic tests, the prostate specific antigen (PSA) test, and multiple systematic biopsy (SB), miss many tumors and cannot reliably distinguish between indolent and aggressive PCa [7, 8]. As a consequence, many men are treated either unnecessarily or too late.

The prostate is a deep-sited organ with heterogeneous structure [9, 10]; that makes it difficult to recognize tumors using medical imaging techniques. Advanced techniques for ultrasound and magnetic resonance imaging (MRI) show relatively high sensitivity for PCa detection [11, 12]. However, benign lesions such as prostatitis and benign prostatic hyperplasia (BPH) often give false alarms [11, 12].

The major objective of PCa detection is a more precise disease characterization [13]. Today, there is a lack of information for deciding whether patients that undergo radical prostatectomy would benefit from additional therapy [14]. For high-risk patients further treatment with medical, radiation and/or chemotherapy may be useful [14, 15]. However, selecting appropriate patients upfront is challenging, and delaying adjuvant therapy until
there is evidence of cancer regrowth seems to decrease survival rates [14]. One reason that many patients suffer from cancer recurrence is failure to remove all cancerous tissue at surgery [16]. Positive surgical margins, i.e. cancer present on the surface of the dissected tissue, are found in up to 40% of patients [16,17]. There is currently no accurate technique for analyzing the surgical margins during operation [16,17]. Thus, it becomes challenging for surgeons to remove all cancerous tissue while avoiding damage leading to erectile dysfunction or incontinence [16]. New complementary methods for PCa detection and diagnosis are needed. This thesis takes the first steps towards a novel approach where two experimental techniques are combined, i.e. the tactile resonance method (TRM) and Raman spectroscopy (RS).

TRM was developed to mimic palpation, i.e. to feel the stiffness of a tissue using the fingers, and this is performed by physicians to find tissue abnormalities [18]. The stiffness of many organs are affected by diseases. Tumors are usually stiffer than healthy tissue and can be felt as hard nodules in, e.g. breast and prostate tissue. TRM gives an objective measure of the stiffness through frequency changes of a piezoelectric vibrating element. Several medical applications have been introduced, including measuring the stiffness of single cells to evaluate embryo quality and increase the success-rate of in vitro fertilization [18]. TRM is promising for breast and prostate cancer detection [18]. In vitro studies show that TRM can differentiate soft, healthy prostate tissue from PCa [19–21]. However, the sensitivity is currently insufficient to distinguish between tumors and relatively hard healthy tissue, such as sites with an accumulation of prostate stones.

RS measures the biochemical composition of tissue via laser illumination and analysis of the spectrum of the inelastically scattered light. Disease progression is reflected by changes in the molecular contents of tissue [22]. RS is very promising for a wide range of diagnostic applications [22]. Numerous in vitro studies show that RS can detect many types of cancers, including PCa, with high sensitivity and specificity [22–27]. Despite this high potential, few clinical implementations have emerged. The main reason is a lack of small, flexible and disposable RS fiber optic probes adequate for large clinical trials [28]. RS is very promising for distinguishing indolent and aggressive PCa [24,25,27,29]. The disadvantages of RS are that current fiber optic probes have short penetration depth in tissue (∼0.1 mm [30]), that surrounding light can interfere with the signal of interest, and that intense laser irradiation may damage tissue.

To combine TRM and RS could add up their strengths while minimizing the drawbacks associated with each technique. TRM constitutes a quick, gentle and deep-sensing method that could be used for swift scanning of the tissue. RS could provide complementary information for nodules suspected to be cancerous. In the first place, the combined instrument could be used to probe the surgical margins during radical prostatectomy. In the long term, it could potentially be used for minimally invasive localization and diagnosis of PCa.

In vitro studies are necessary for successful implementation of the combined instrument. To ascertain that the results are transferable to the in vivo situation, it is important that the experimental procedures preserve the native tissue characteristics. Possible degradation of tissue from, e.g. sample preservation and preparation methods, laser
irradiation, and dehydration during measurements, should be investigated.

This thesis gives a background to the difficulties of localizing and diagnosing PCa. It reviews the main methods for PCa detection in clinical use today, and discusses promising novelties that are being developed. The importance of robust in vitro study protocols is discussed, and the effects of snap-freezing and near-infrared laser (NIR) illumination on porcine prostate tissue are investigated using RS. An approach for combining the information from TRM and RS is developed and evaluated on measurements on porcine abdominal tissue. Finally, the accuracy of classification of healthy and cancerous prostate tissue is investigated in experiments on porcine and human samples using a novel experimental setup with a micro tactile sensor (MTS) and an RS fiberoptic probe.

4.2 The prostate

4.2.1 Anatomy and physiology

The prostate is an accessory sex gland whose function is to store and secrete a milky, slightly acidic fluid (pH ~ 6.5), which makes up about 25% of the volume of semen [31]. The gland is about the size of a golf ball and resembles a walnut in shape. It is situated inferior to the bladder, next to the rectal wall that is about 3 mm thick [32], and encircles the prostatic urethra (Figure 4.1). Many prostatic ducts lead the prostatic fluid into the urethra. The prostate is composed of glandular elements that are lined with epithelial cells that secrete prostatic fluid into the glandular lumen (cavity) [10]. The glandular elements are separated by stroma, a supportive framework that consists of smooth muscle tissue and other cellular components embedded in an extracellular matrix rich in collagen [31,33]. There are three anatomical zones in the prostate: the peripheral zone, the transitional zone and the central zone. The composition of the prostate tissue varies between the zones, e.g. the stroma is more or less compact with varying amounts of muscle tissue [10].

The prostate normally increases in volume during specific periods throughout a man’s life. It grows rapidly from puberty until about age 30, remains at a stable size between age 30 and 45, after which it may begin to grow again [31]. The majority of men > 55 years develop BPH, a benign enlargement of the prostate [34]. The formation of prostate stones (corpora amylacea) in the lumen of the glandular elements, due to solidification of glandular secretions, is another common benign occurrence [35,36]. The stones are quite hard and contribute to tissue stiffness, although they make up only a small fraction of the tissue volume [19,37]. They are rarely present in cancerous tissue [36].

The functional role of prostatic fluid is not completely understood, but the following is known [31]:

- It participates in making the semen coagulate after ejaculation, which happens within five minutes (the role of coagulation is unknown).
- It contains protein-digesting enzymes, among them, PSA, which starts to liquefy the semen at 10–20 minutes after ejaculation. This facilitates the movement of sperm through the cervix.
It contains citric acid used by sperm to mobilize energy via ATP (adenosine triphosphate) production.

- It contains an antibiotic, seminalplasmin, which may help to reduce the bacterial content in the semen and in the female reproductive tract.

4.2.2 The porcine prostate

The male reproductive system of the pig is composed of the same structures as in humans [38]. In contrast to the human prostate, the porcine prostate consists of two

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4.2. The prostate

parts, compacta and disseminata [39]. The compact part appears as a number of rounded elevations on the dorsal (towards the back) side of the urethra, whereas the disseminate part surrounds the urethra [39, 40]. The two parts are histologically similar [39]. Prostate stones are present only occasionally in the boar prostate [39]. Nicaise et al. [40] used light and electron microscopy to study the disseminate prostate of 12 boars and 8 barrows, i.e. castrated boars. The prostate of the barrows did not develop normally. The authors concluded that the results permitted the use of the boar prostate as an experimental model for studying the influence of hormones used in human medicine.

4.2.3 Prostate cancer

In Sweden about 10 000 men are diagnosed with PCa each year and more than 2500 die from the disease, making it the most common cause of cancer-related male death [1]. Scandinavia and the Baltic region (Estonia, Latvia and Lithuania) have the highest PCa mortality rates in Europe [41]. PCa is often without symptoms, even in men with aggressive tumors, until severe stages [42]. Estimations based on autopsies show that up to 50% of men harbor PCa by 70 years of age [42, 43]. However, the vast majority of tumors are indolent [8]. In its most aggressive form PCa disperses metastases and is very dangerous; the 5-year survival rate is only 34% [44]. In contrast, survival is 100% if the cancer has not spread beyond the structures adjacent to the prostate or metastasized to distant lymph nodes [44].

Almost all, 95%, of prostate tumors form in the prostatic ducts in the glandular epithelium [44]. The majority develop in the posterior part of the gland (the peripheral zone) [45, 46], which is situated towards the rectum (Figure 4.1). PCa is usually multifocal and provides little contrast to healthy tissue using standard clinical imaging methods, such as ultrasound and MRI. This makes the cancer nodules difficult to detect [47, 48].

The causes of PCa remain largely unexplained [41]. Age, ethnicity and family history have been established as risk factors, and diet and genetic susceptibility may contribute [41].

4.2.4 Detection and diagnosis of prostate cancer

The clinical tests that are used for detection and diagnosis of PCa are the PSA test and SB. Historically, digital rectal examination, i.e. the physician palpates the prostate via the rectum, was the most important test [49, 50]. This method has low accuracy [48] and can usually only detect severe forms of PCa [50]. It is still used as a complement [50]. A high concentration of PSA in the blood indicates cancer [51]. However, the PSA level can be elevated also for men without PCa, often due to BPH [51], and men with normal levels may still have cancer [52]. A multicenter European randomized study including 182 000 men found that PSA-based screening reduced the PCa mortality by 20% [8]. However, it also caused unnecessary treatments and overdiagnosis, i.e. confirming cancer in patients with indolent tumors that would never cause clinical symptoms in their lifetime. To prevent one death 1410 men would have to be screened and 48 additional patients would have to undergo treatment. A similar study in the US, which enrolled almost 77 000 men,
did not find any significant benefits of PSA screening [53]. One possible explanation for the different outcomes is that the European study used a PSA cutoff of 3 ng mL$^{-1}$ in most centers, as compared to 4 ng mL$^{-1}$ in the US study. In addition, about 50% of the patients in the control group in the US were screened as part of usual care [53]. It has been estimated that the rate of overdiagnosis due to PSA screening is 50% [54].

If PCa is suspected from elevated PSA or digital rectal examination SB is performed [50]. An ultrasound probe equipped with a spring-loaded biopsy gun is inserted into the rectum, and the biopsy needle is directed to at least six predetermined sites according to the SB protocol [50]. SB fails to detect 20–30% of present tumors [7]. This can be appreciated since the volume of a biopsy typically is less than one thousandth of the prostate volume [55].

The diagnosis of PCa is determined through histological analysis of tissue sections from the biopsy samples or from the removed prostate when surgery has been performed. Montironi et al. [56] give a detailed description of a recommended procedure for preparation of radical prostatectomy specimens. In brief, first the removed prostate is fixed by injection of formalin at multiple sites using a needle. The surface is inked, and the prostate is immersed into formalin for 24 hours. The prostate is then cut into 4-mm thick slices, which are embedded in paraffin. A microtome is used to cut a 5-µm thick specimen from each of the embedded slices. The specimens are stained with hematoxylin and eosin to induce contrast for histological examination. Under a light microscope a trained observer can recognize different tissue types and distinguish healthy and cancerous tissue (Figure 4.2). The aggressiveness of detected tumors can be assessed from the histological appearance of the prostatic glands following the Gleason grading system [55]. This method is subjective, and the rates of intra- and interobserver disagreement are high [55]. The severity of PCa is clinically rated using a standardized system that defines different stages due to Gleason score and the spread of the primary tumor and metastases [44]. Today the patients diagnosed with PCa have tumors of lower grade and lower stage than 20 years ago, but there is still a wide range of aggressiveness [7]. Most patients have tumors of medium Gleason score, and due to the deficiencies in the practice of the Gleason system predictions of disease progression are often uncertain [55]. The physicians are then faced with a weak foundation for choosing an appropriate treatment.

The main imaging methods for detection of PCa are transrectal ultrasound and MRI. Due to a number of limitations, these techniques are not yet routinely used clinically for direct PCa detection [7,57]. New advances are very promising, but further clinical trials are needed [57,58].

4.2.5 Treatments of prostate cancer

Radical prostatectomy is the recommended treatment for men with aggressive, localized (no metastases present) PCa [15]. It has excellent long-term PCa-specific survival rates [59]. Unfortunately, serious side effects are common. Coelho et al. [60] estimated that > 40% of the patients were impotent and about 20% were incontinent 12 months after surgery. Today a rapidly increasing amount of radical prostatectomy procedures are performed using robotic assistance, which shows promise for improving surgical quality and decreasing
4.3 Tactile resonance method

4.3.1 The piezoelectric effect

The piezoelectric effect was discovered by the brothers Pierre and Jacques Curie in 1880 [65]. They demonstrated that when pressure was applied to a crystal, such as quartz or topaz, an electric potential was generated. The inverse effect also applies, a piezoelectric element changes shape if exposed to an electric field, and will therefore oscillate in response to a sinusoidal voltage variation. A piezoelectric element works as a transducer between electric and kinetic energy. The phenomenon originates from the fact that the unit cells of a piezoelectric material behave like electric dipoles, i.e. a non-uniform

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Figure 4.2: A scan (ScanScope CS, 20× objective, Aperio, Vista, CA, USA) of a histology specimen from the prostate of a 67-year old man who was diagnosed with PCa and underwent radical prostatectomy. The dimensions of the scanned area are 4 × 1.4 mm. The black line indicates the border between healthy and cancerous tissue.

side effects [60,61].

After surgery the PSA level is monitored to assess the effectiveness of the treatment. For approximately 35% of patients, PSA will be detectable within ten years after surgery, which indicates clinically significant cancer recurrence [62,63]. The risk is increased for aggressive cancers and if positive surgical margins are present [16]. Whether patients with aggressive, localized tumors who underwent radical prostatectomy would benefit from additional treatment using radiotherapy, chemotherapy, hormonal therapy or combinations of these is controversial [15, 64]. About 50% of those patients are cured with surgery alone, and they are then spared the side effects and toxicity of additional therapy [15]. Interestingly, van der Kwast et al. [62] found that adjuvant radiotherapy was significantly beneficial only for patients with positive surgical margins. The study included 1005 men. Thus, to reduce the rate of cancer recurrence while minimizing the use of unnecessary adjuvant therapy a method for intraoperative analysis of the surgical margins is greatly needed.

4.3 Tactile resonance method
charge distribution arises because the elementary cells have no center of symmetry. If pressure is exerted on the material the shape of the dipoles is altered, and this will induce a net electric potential in the material. Resonance sensors are typically made from a ceramic piezoelectric material, e.g. lead zirconate titanate (PZT), which can be pictured as a mass of tiny crystals, so-called crystallites, exhibiting dipole characteristics. The unit cells of the crystallites are non-centrosymmetric below the Curie temperature (the critical point below which the material is ferromagnetic), which usually is of the order of 1000 K (∼ 700°C) [66]. A ceramic can be given its piezoelectric properties by heating it to just below the Curie temperature and applying a strong electric field over it. The ceramic is then polarized in the direction of the applied field, and the dipoles are locked when the field is withdrawn.

### 4.3.2 Principle of the tactile resonance method

The principle of the TRM was presented by Omata & Terunuma in 1992 [67]. It is based on a piezoelectric PZT transducer divided into two parts, a driving element that generates vibration, and a pick-up element that detects the frequency of vibration. The transducer is set into oscillation by an electronic feedback circuit consisting of an amplifier, a bandpass filter and a phase-shift circuit, as shown in Figure 4.3 [67]. The signal from the pick-up is fed back to the circuit. The phase-frequency characteristics of the PZT transducer and the electronic circuit determine the oscillation frequency of the whole system. The phase-shift circuit establishes resonance at a user-selected frequency by ensuring that the sum of the phase shifts in the system is zero. To obtain a high sensitivity it is advantageous to choose a frequency close to the inherent resonance frequency of the PZT element. A probe tip is glued to the end of the PZT element (Figure 4.3). It is made in a shape and from a material suitable for the measurement task at hand. As the tip comes into contact with an object the resonance frequency changes, and the shift is related to the stiffness of the material [67]. The absolute frequency shift increases with the stiffness of the probed material. For relatively soft objects, such as silicone gum and the palm of a hand, the shift is negative, whereas it is positive for hard materials such as teeth and glass [67]. Murayama & Omata [68] developed an MTS by using a tip in the form of a 30 mm long, tapered glass needle with a very small spherical tip, from 1 mm down to 0.1 µm in diameter.

### 4.3.3 Theory

Kleesattel & Gladwell introduced a surface hardness tester called the contact-impedance meter in two publications in 1968 [69,70]. Their theoretical explanations could later be applied to describe the characteristics of the TRM [67,71]. A piezoelectric tactile resonance sensor can be modeled as a finite rod vibrating at its resonance frequency in the direction of its length [67,69–71]. The probe tip is assumed to be hemispherical. The frequency change as the sensor comes in contact with an object can be expressed as

\[
\Delta f = -\frac{V_0\beta_x}{2\pi lZ_0}
\]  

(4.1)
4.3. Tactile resonance method

where $V_0$ is the wave velocity in the rod, $l$ is the length of the rod, $Z_0$ is the acoustic impedance of the rod and $\beta_x$ is the reactance part of the acoustic impedance

$$Z_x = \alpha_x + i \beta_x$$  \hspace{1cm} (4.2)

of the probed object, where $\alpha_x$ is the resistance. $\beta_x$ can be written as

$$\beta_x = m_x \omega - \frac{k_x}{\omega}$$  \hspace{1cm} (4.3)

where $\omega$ is the angular frequency, $m_x$ is the contact mass and $k_x$ is the contact stiffness. $m_x$ and $k_x$ depend on the surface contact area $S$ and can be written as

$$m_x = \frac{4a_{11}}{\pi^{3/2}(1 - \nu)} \rho S^{3/2}$$  \hspace{1cm} (4.4)

$$k_x = \frac{2E}{\pi^{1/2}(1 - \nu^2)} S^{1/2}$$  \hspace{1cm} (4.5)

$\nu$ is Poisson’s ratio, $\rho$ is the density, $E$ is the elastic modulus (Young’s modulus), and $a_{11}$ is a coefficient that depends on $\nu$ [70]. $S = \pi r^2$, where $r$ is the radius of the contact area. From (4.3)–(4.5) we see that for large contact areas $m_x$ will dominate, whereas $k_x$ will dominate for small contact areas [67]. Furthermore, at high frequencies the contribution from $m_x \omega$ increases, whereas $k_x/\omega$ becomes more important at low frequencies.

Jalkanen et al. [71] examined the theoretical model of the finite rod for the Venustron® system. The resonance frequency was 58 kHz and the probe had a hemispherically shaped
tip with 5 mm diameter. They showed that, since \( m \omega^2 \gg k_x \omega \) for that system, the surface stiffness term \( k_x \omega \) in (4.3) can be neglected. (4.1)–(4.4) then give

\[
\Delta f \propto \rho S^{3/2}
\]

for a specific rod vibrating at a constant frequency, if Poisson’s ratio is assumed to be constant [71]. The surface contact area \( S \) depends on the contact force between the sensor tip and the measurement object, \( F \), according to \( F \propto ES^{3/2} \) [71]. Substituting this relationship into (4.6) results in

\[
\Delta f \propto \frac{\rho F}{E}
\]

A stiffness sensitive parameter \( \partial F/\partial \Delta f \) can then be derived as

\[
\frac{\partial F}{\partial \Delta f} \propto \frac{E}{\rho}
\]

Jalkanen et al. [71] experimentally verified this theoretical model in measurements on human prostate tissue. Their study showed that density variations were small and mostly non-significant, validating the use of \( \partial F/\partial \Delta f \) as a stiffness sensitive parameter.

For the MTS the contact area is small and the inertia term \( m \omega^2 \) will be smaller than the surface stiffness term \( k_x \omega \) and can usually be neglected [72]. (4.1) can then be written as

\[
\Delta f = \frac{V_0k_x}{2\pi lZ_0\omega}
\]

According to Murayama & Omata [68] Hertz theory can be applied for small indentation depths \( \delta \), and the contact area \( S \) can then be modeled as a function of \( \delta \). They showed that from (4.5) and (4.9) a stiffness sensitive parameter can be obtained as \( \Delta f/\delta \), which is related to Young’s modulus [68]. They calculated Young’s modulus from the slope of the frequency versus indentation curve, and verified that \( \Delta f/\delta \) correlated highly with \( E \) in experiments on silicone samples.

### 4.3.4 Sensing volume

Jalkanen et al. [21] investigated the sensing volume of the Venustron® TRM system. They concluded that the tip laterally sensed a larger area than the actual contact area and had an estimated penetration depth of 3.5–5.5 mm for an indentation depth \( \delta = 1 \) mm. There was an approximate linear relationship between the indentation depth and the sensing depth; the sensor probed deeper into the tissue at larger indentation depths. For \( \delta = 2 \) mm the penetration depth was estimated to be up to 10 mm. Using an array with 64 TRM sensor elements Murayama et al. [73] demonstrated that tumors in the breast larger than 10 mm could be detected at depths up to 20 mm. TRM has higher potential for noninvasive detection of PCa than RS.
4.3.5 Detection of prostate cancer

Eklund et al. [37] were the first group to measure the stiffness of human prostate tissue using TRM in vitro. A catheter type sensor was used. The catheter was 2 mm in diameter. A hemispherical tip was formed from epoxy and attached to the PZT element, sealing the end of the catheter. They used a proposed model where the tissue stiffness was linearly related to the amounts of glandular tissue and prostate stones. A correlation of $R = -0.96$ between the measured and the expected stiffness was found. The tissue was fixed in formalin, which in general hardens the tissue. The results indicated that stroma and prostate stones were relatively hard tissue components, while glandular tissue was softer. Jalkanen et al. [19,20] examined fresh human prostate tissue with the Venustron® system. A slice of prostate was measured directly after surgical removal. The authors showed that TRM could distinguish glandular tissue from cancerous tissue. In the first study [20] ten samples from ten patients were tested. A $p$-value $< 0.001$ was obtained for a MANOVA test of the difference between cancerous ($n = 13$) and healthy ($n = 98$) tissue. Only measurement sites consisting of 100% cancerous tissue were significantly stiffer than the glandular tissue. Stroma and sites with an accumulation of prostate stones could not be differentiated from cancer in those studies. However, PCa usually develops in the posterior part of the prostate [45,46], where glandular tissue is abundant [19]. Thus, a stiff nodule in this area could indicate cancer [19]. In a recent study [74] Jalkanen demonstrated that hand-held measurements using the Venustron® could accurately determine the stiffness of gelatin ($R^2 = 0.94$). For hand-held measurements the impression speed is unknown, but Jalkanen showed theoretically and experimentally that this factor is not significantly related to the measured stiffness, which is promising for in vivo measurements. Murayama et al. [75] used an elasticity mapping system with an MTS to scan 300 µm-thick prostate sections from two patients. The tip of the MTS was 10 µm in diameter, and the scanning step-size was 5 µm. They found that the proportion of stiff points was larger for cancerous tissue. However, the stiffness distribution of healthy and cancerous tissue overlapped. No statistic evaluation was performed.

4.4 Raman spectroscopy

4.4.1 The Raman effect

When a laser beam illuminates a tissue sample most photons are elastically scattered, i.e. their energy is conserved. A fraction of the photons are inelastically scattered and lose or gain energy as they interact with the biological molecules. There are three main inelastic scattering events: fluorescence, phosphorescence and Raman. Fluorescence and phosphorescence are associated with electronic transitions of the participating molecules. Raman scattering is a relatively weak process (quantum yield $10^{-8}$–$10^{-6}$) in which incident photons set molecular bonds into vibration [76]. The energy of the scattered photons is shifted corresponding to the difference between the initial and final vibrational energy levels. A Raman spectrum is a plot of the scattered intensity versus the energy shifts.
Since every molecule has a unique set of bond vibrations, the spectrum is like a fingerprint of the sample. By convention the energy shift is expressed as a wavenumber shift termed Stokes shift and measured in cm\(^{-1}\) (the number of wavelengths per cm) \([77]\):

\[
\Delta \tilde{\nu} = \frac{1}{\lambda_i} - \frac{1}{\lambda_e} \tag{4.10}
\]

where \(\lambda_i\) and \(\lambda_e\) are the wavelengths of the incident and emitted photons, respectively.

The Raman effect was predicted by quantum mechanics in publications by Smekal in 1923 and Kramers & Heisenberg in 1925 \([78]\). It was experimentally verified in 1928 by the Indian professor Sir C. V. Raman. He observed the phenomenon in a delicate experiment using filtered sunlight as excitation source, a telescope to collect the scattered light and the eye as detector \([79]\). He was awarded the Nobel prize for the discovery already two years later.

Classical physics explains the basic principles of Raman scattering \([76, 80]\). As a molecule is hit by a photon its electron cloud is distorted by the electromagnetic field. The geometry of the cloud is changed and the molecule is excited to a virtual, higher state of energy. This state is unstable; the nuclei of the molecule cannot establish new equilibrium positions in response to the rearrangement of electrons. As the molecule relaxes the nuclei are set into vibration and a photon is emitted. The process is fast (\(10^{-13}–10^{-11}\) s) compared to fluorescence (\(10^{-9}–10^{-7}\) s) \([81]\). Consider a diatomic molecule irradiated by monochromatic light with frequency \(\nu_0\). The electrical field \(E = E_0 \cos(2\pi\nu_0 t)\), where \(t\) denotes time, induces a dipole moment

\[
P = \alpha E = \alpha E_0 \cos(2\pi\nu_0 t) \tag{4.11}
\]

in the molecule. The polarizability \(\alpha\) is a function of the nuclear displacement, because as the molecule changes shape, size or orientation the electron cloud can become easier or more difficult to distort. If the nuclei vibrate with a frequency \(\nu_m\), the nuclear displacement \(q\) can be expressed as \(q = q_0 \cos(2\pi\nu_m t)\), where \(q_0\) is the amplitude of the oscillation. Since \(\alpha(q)\) can be regarded as a linear function of \(q\) for small amplitudes of vibration, it can be expanded as

\[
\alpha = \alpha_0 + \left(\frac{\partial \alpha}{\partial q}\right)_0 q + \ldots
\]

(4.12)

where \(\alpha_0\) is the polarizability at \(q = 0\). Substituting (4.12) into (4.11), and using the formula \(\cos \gamma \cos \beta = \frac{1}{2} \cos (\gamma - \beta) + \frac{1}{2} \cos (\gamma + \beta)\), we obtain

\[
P = \alpha_0 E_0 \cos(2\pi\nu_0 t) + \frac{1}{2} \left(\frac{\partial \alpha}{\partial q}\right)_0 q_0 E_0 \left[\cos(2\pi(\nu_0 - \nu_m) t) + \cos(2\pi(\nu_0 + \nu_m) t)\right] \tag{4.13}
\]

The three terms in (4.13) symbolize dipoles that oscillate with frequencies \(\nu_0\), \(\nu_0 - \nu_m\) and \(\nu_0 + \nu_m\). They describe elastic, Stokes and anti-Stokes scattering, respectively. In anti-Stokes scattering the photons gain energy. This is possible only when the molecule
4.4. **Raman spectroscopy**

![Energy diagram of elastic and Raman scattering. Incident photons are shown as upward arrows and emitted photons as downward arrows.](image)

Initially is at a higher vibrational energy level (Figure 4.4). At room temperature higher levels are sparsely populated and anti-Stokes scattering is weak. A fundamental property of the Raman effect is understood from (4.13): if \( \left( \frac{\partial \alpha}{\partial q} \right)_0 = 0 \), no Raman scattering will occur. This means that a specific molecular vibration is Raman active only if the polarizability is changed during the vibrational cycle. Symmetric vibrations usually cause the largest polarizability changes and generate the strongest scattering [76]. In general the scattering intensity \( I \) depends on the laser power \( l_p \), the frequency of the laser light, \( \nu_0 \), and the polarizability \( \alpha \), according to [76]:

\[
I \propto l_p \alpha^2 \nu_0^4 \tag{4.14}
\]

Hence, the Raman intensity is much stronger if a laser with short wavelength is used.

Quantum physics can be applied to calculate the frequencies of the molecular vibrations [80]. As an example, consider the vibration of a diatomic molecule. It can be modeled as a harmonic oscillator for a single particle. The chemical bonding between the nuclei is pictured as a Hookian spring with a force constant \( k \), and the potential energy \( V(q) = \frac{1}{2}kq^2 \), where \( q \) is the displacement. The Schrödinger equation for this model becomes

\[
-\frac{\hbar^2}{8\pi^2 m} \frac{\partial^2 \Psi}{\partial q^2} + \frac{1}{2}kq^2\Psi = E\Psi \tag{4.15}
\]

\( \hbar \) is Planck’s constant, \( m \) is the mass of the particle, \( \Psi \) is the wave function and \( E \) is the total energy of the particle. The solutions are eigenfunctions with the corresponding
eigenvalues

\[ E_v = \hbar \nu \left( v + \frac{1}{2} \right) \]  (4.16)

c is the speed of light, \( v = 0, 1, 2, \ldots \) is the vibrational quantum number, and

\[ \tilde{\nu} = \frac{1}{2\pi c} \sqrt{\frac{k}{m}} \]  (4.17)

is the wavenumber [cm\(^{-1}\)] of the vibration. Hence, the rule of thumb is that strong bonds and light atoms will give rise to high vibrational frequencies and vice versa [76]. From (4.16) we see that the energy is quantized with a constant separation between energy levels equal to \( \hbar \nu \). This is a good approximation for lower energy levels, but for actual molecules the separation decreases as \( \nu \) increases [80]. The selection rules of quantum mechanics prohibit many vibrational transitions [80]. For the harmonic oscillator, only transitions that fulfill \( \Delta v = \pm 1 \) are allowed. The transition \( v = 0 \leftrightarrow 1 \) normally produces the most intense peak in the Raman spectrum, since most molecules are in their lowest state of energy \( E_0 \) at room temperature. For polyatomic molecules the vibrational patterns can be very complicated. However, in principle the complicated vibrations can be described as a superposition of harmonic oscillations for all nuclei [80].

4.4.2 Instrumentation

A Raman spectrometer basically consists of a laser generating monochromatic light, a sample illumination and collection system, a filter that separates the elastically- and the inelastically-scattered light, a wavelength selector (e.g. a grating) and a detector [80]. Modern systems for tissue measurements typically use NIR diode lasers and CCD detectors [82]. Microscopes or fiber optic probes in the backscattering collection geometry are commonly used to illuminate the sample and collect the Raman light [30]. Figure 4.5 shows a schematic drawing of an RS fiber optic setup.

The development of RS fiber optic probes enables in vivo measurements. Several factors complicate the realization of fiber optic probes. Fused silica fibers generate a strong signal in the Fingerprint spectral interval, which necessitates the use of extra filters at the probe tip to block this radiation [28]. For clinical use the probes need to be flexible and thin, of the order of 1–2 mm to be incorporated into biopsy needles, endoscopes and other devices [83]. They must withstand clinical sterilization routines [83]. Several different probes have been developed, but so far the manufacturing process has been complicated and expensive [28]. However, several technical advancements in the construction of fiber optic probes have been presented recently [28,84,85]. Furthermore, RS measurements in the high wavenumber region, from 2400 to 3800 cm\(^{-1}\), can be performed using simpler probes without filters, since little Raman signal is generated in the probe itself in this region [86–88].

Komachi et al. [89–92] have developed a 0.6 mm thin probe, and demonstrated promising results in measurements of the esophagus and stomach of the living rat [92]. The probe consists of a central delivery fiber surrounded by eight collection fibers. They
claim that it can be commercially manufactured at a low cost [89]. Day et al. [84] describe the development of a miniature, confocal fiber optic probe. Their aim was to construct a probe capable of sampling tissue layers 100–200 µm below the tissue surface, which would be optimal for early detection of esophageal cancer. The depth of field was 147 µm in measurements on polished silicon. In a recent publication the group attained 66–81% sensitivity and 80–98% specificity for discriminating esophageal cancer from healthy tissue using that confocal probe with an integration time of 2 s [85]. They measured 123 biopsy samples from 49 patients. The accuracy was increased for an integration time of 10 s.

The penetration depth in tissue of RS fiber optic probes using the backscattering collection mode is typically only several hundred micrometers [30]. Hence, deep-sited organs, such as the prostate, are inaccessible for noninvasive examinations. Development of RS techniques that can probe deeper into the tissue, such as time-gated RS, transmission RS, and spatially offset probes, is ongoing [30]. Spatially offset probes increase the accessible depth to several mm [30]. However, the spatial separation between excitation fibers and collection fibers makes the probes bulkier than ordinary probes. Using transmission RS identification of calcified materials buried at depths up to 2.7 cm in a breast cancer phantom have been demonstrated [93]. However, in transmission RS the sample is illuminated from one side and the Raman signal is collected from the opposite side; this approach may be difficult to use in vivo.

4.4.3 Raman measurements of tissue

RS is excellent for measuring the biochemical content of tissue for a number of reasons including:

- The majority of biological molecules are Raman active [81].

![Figure 4.5: A typical RS fiber optic setup.](image-url)
Minimal or no sample preparation is required.

Water is a poor Raman scatterer; it interferes little with the spectra of tissue [76].

RS is sensitive to many factors that affect biomolecules, such as pH, degree of hydration, bacterial attack, etc. [94].

The relative abundance of tissue components is proportional to their contributions to the Raman spectrum [83].

In vivo measurements are feasible via fiber optic probes.

Some of the drawbacks with the method are:

- Tissue autofluorescence can distort the Raman signal.
- Acquisition of high quality spectra often requires long integration times. Therefore, in vivo measurements may be affected by motion artifacts.
- The instrumentation is sensitive to surrounding light.
- Current fiber optic probes have a short penetration depth in tissue [30].

RS measurements of tissue were long hampered by the strong, broadband tissue autofluorescence induced by lasers in the visible region [83]. Modern NIR RS systems have largely overcome this problem, since NIR light has too low energy to initiate most fluorescence processes [83]. Autofluorescence of tissue is believed to be generated mainly by a few fluorophores such as flavins, nicotinamide adenine dinucleotide, aromatic acids such as tryptophan, tyrosine and phenylalanine, and porphyrins [93]. Several different approaches for minimizing fluorescence interference have been demonstrated. Time-gating and wavelength shifting can effectively decrease fluorescence, but these require modifications of the Raman instrumentation [96]. An alternative is to use mathematical methods to subtract the fluorescence signal. However, many algorithms cause spectral artifacts [96]. Polynomial fitting does not distort the Raman peaks to a high degree [96]. Lieber et al. [96] presented an algorithm that automatically subtracts the spectral background by fitting a modified polynomial to the spectrum. This method was further developed by Cao et al. [97].

The origin of many observed peaks in tissue spectra can often be interpreted with help of databases and published spectra of biological molecules [81,98]. Tissue generally produces spectra with relatively narrow bands, typically 10–20 cm$^{-1}$ wide [83]. Stokes shifts from 200–3600 cm$^{-1}$ usually cover the information of interest [76]. The characteristic vibrations of the most common chemical groups have been assigned approximate wavenumber ranges that are valid for the groups in most structures [76]. The spectral region 4000–2500 cm$^{-1}$ is where single bonds (X−H) scatter, the interval 2500–2000 cm$^{-1}$ is where multiple bonds (−N=C=O) occur, and the range 2000–1500 cm$^{-1}$ includes double bonds (−C=O, −C=N, −C=C−). The interval 1900–700 cm$^{-1}$ is referred to as the Fingerprint region. Many molecules exhibit complex vibrational patterns that yield unique spectral features in this region, which is densely packed with sharp bands [81]. Raman peaks below
4.4. Raman spectroscopy

RS can explore the primary, secondary, tertiary and quaternary structure of biological molecules [81]. For example protein structure, DNA conformation and cell membrane conformation can be probed. Databases over characteristic peak frequencies of important biological molecules are available, see e.g. Movasaghi et al. [98]. Figure 4.6 shows an example of a porcine prostate spectrum. Tentative assignments of the major peaks identified in the spectrum are given in Table 4.1.

There is an abundance of diagnostic features for cancer detection in the spectra of various tissues [81]. The ratio of intensities of the amide I vibrational mode at 1655 cm$^{-1}$ to the CH$_2$ bending vibrational mode at 1450 cm$^{-1}$ can be used to differentiate healthy and cancerous tissue in brain, breast and gynecological tissues. Cancer induces a significant increase of the DNA content [27,81,99]. The amide III band at 1260 cm$^{-1}$ may contribute towards cancer identification, e.g. the amide III band is broadened in cancerous gynecologic tissue.

Several in vitro studies [24–27, 29, 99, 101] have investigated the potential of RS to detect and grade PCa. Crow et al. [25] attained 98% sensitivity and 99% specificity for differentiating four cell lines with varying degrees of aggressiveness. Cells were placed onto a calcium fluoride slide, and about 50 spectra from each cell line were measured. A total of 200 spectra were input to the diagnostic algorithm, which used principal component analysis (PCA) and linear discriminant analysis. Taleb et al. [99] attained a 100% accurate classification of healthy and cancerous (derived from metastases) prostate cells ($n = 30$). They concluded that the most significant spectral change due to cancer

Figure 4.6: A spectrum of porcine prostate tissue recorded in our laboratory using a Raman micro spectrometer (Renishaw system 2000, Renishaw, Wotton-under-Edge, UK). The integration time was five minutes.

650 cm$^{-1}$ normally belong to inorganic groups, metal-organic groups or lattice vibrations.
Table 4.1: Tentative assignments [98–100] of the major peaks in the porcine prostate spectrum shown in Figure 4.6.

<table>
<thead>
<tr>
<th>Peak position (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1666</td>
<td>Amide I (proteins)/C=C lipid stretch</td>
</tr>
<tr>
<td>1451</td>
<td>CH(_2) bending mode of proteins and lipids</td>
</tr>
<tr>
<td>1245</td>
<td>Amide III (proteins, 1240–1265 cm(^{-1}))</td>
</tr>
<tr>
<td>1208</td>
<td>Tryptophan and phenylalanine (\nu(C\cdots C_6 H_5)) mode</td>
</tr>
<tr>
<td>1032</td>
<td>C–H in-plane bending mode of phenylalanine</td>
</tr>
<tr>
<td>1004</td>
<td>Symmetric ring breathing mode of phenylalanine</td>
</tr>
<tr>
<td>939</td>
<td>C–C stretching of collagen backbone</td>
</tr>
<tr>
<td>877</td>
<td>C–C stretching (collagen)/C–C–N(^+) stretching (lipids)</td>
</tr>
<tr>
<td>856</td>
<td>Ring breathing mode of tyrosine/C–C stretch of proline ring</td>
</tr>
<tr>
<td>816</td>
<td>C–C stretching (collagen)/proline, tyrosine, (\nu_2) PO(_2)– stretch of nucleic acids</td>
</tr>
<tr>
<td>760</td>
<td>Symmetric breathing of tryptophan</td>
</tr>
<tr>
<td>621</td>
<td>C–C twisting mode of phenylalanine</td>
</tr>
<tr>
<td>534</td>
<td>S–S disulfide stretch in proteins</td>
</tr>
</tbody>
</table>

was an increase in the DNA content and a change in DNA conformation from B-DNA to A-DNA. Crow et al. [24] showed that prostate biopsy samples of BPH and cancer with different Gleason scores could be distinguished with an overall accuracy of 89%. They recorded 450 spectra from biopsies of 27 patients, 14 with BPH and 13 with PCa. Devpura et al. [29] identified > 94% of cancerous regions in RS measurements on 10 \(\mu\)m thick prostate specimens. They found that Gleason scores 6, 7 and 8 could be clearly separated. In the only publication using a fiber optic probe [26], PCa was distinguished from BPH and prostatitis with an overall accuracy of 86%. 38 prostate samples from 37 patients were measured. Stone et al. [27] estimated the gross biochemistry of BPH, prostatitis and PCa of different grades of aggressiveness (Gleason score < 7, = 7 and > 7). This was accomplished by comparing the spectra of the prostate samples to the spectra of pure chemical standards assumed to be the main tissue components. It was shown that the DNA content was increased in cancerous tissue. Furthermore, the cholesterol level increased substantially, the choline level was elevated but remained low, triolein was increased, while oleic acid decreased somewhat with progression of disease.

4.5 Tissue preparation and measurement procedures

In vitro experiments should be carefully designed so that the results and conclusions thereof are applicable to in vivo measurements. It is essential to avoid misinterpretation of results due to artifacts originating from tissue preparation and/or inappropriate measurement procedures.

Fresh tissue samples, immersed in physiological buffer to prevent tissue dehydration,
are ideal for in vitro RS studies [102, 103]. However, preservation of the samples is usually necessary since fresh samples are fragile, difficult to acquire and have a very limited shelf life [103]. Clinically, the most common method for archiving tissue samples is formalin-fixation and subsequent paraffin-embedding [104]. Unfortunately, paraffin-embedded samples are not suitable for RS measurements, since the paraffin generates a very strong signal that swamps the Raman signal from the tissue [104]. Deparaffinization of tissue is feasible, but biochemical information may be lost [104]. It is difficult to remove all paraffin, and the residuals cause interfering peaks in the spectra [104, 105]. Formalin fixation may be a suitable alternative preservation method [103, 104]. However, some studies have reported spectral artifacts in formalin-fixed tissue [102, 105].

Freezing of tissue is considered to be the best preservation method for RS studies [105]. Although it is one of the most commonly used methods [105], only a few studies have in fact confirmed its suitability [102, 105, 106]. Shim et al. [102] evaluated snap-freezing of ten different tissue types from hamster with a Fourier-transform Raman spectrometer, equipped with a 1064-nm laser. They compared fresh tissue to snap-frozen tissue stored at −80°C for 1, 9 and 30 days. The frozen tissue was thawed immersed in phosphate buffered saline (PBS) at room temperature for 15 minutes. No spectral artifacts due to freezing or thawing were seen for the different tissue types, except for fat and liver. Their conclusion was that snap-freezing preserved the biochemical composition well. They pointed out that the results should be confirmed for other tissue types and species. To my knowledge no RS study of snap-freezing of prostate tissue has been presented in the literature before.

Raman spectrometers use relatively powerful lasers that may damage tissue, and the measured signal can then be distorted. Therefore, it is necessary to examine the effects of the laser illumination on the tissue. Modern RS systems adapted for tissue measurements use NIR lasers, which are less prone to harm the sample than lasers in the UV or visible region [107–109]. However, studies investigating effects from visible and NIR light irradiation of tissue at high intensity are rare. To my knowledge the effects of NIR laser illumination on prostate tissue have not been investigated using RS before.

The biomechanical properties of tissue may be different in vitro as compared to in vivo. It is well known that skeletal muscles stiffen significantly after death, so-called rigor mortis. However, less is known about how noncontractile soft tissues are affected [110]. Chan & Titze [110] studied the effects of postmortem changes and freezing-thawing of vocal fold tissues excised from dogs. They found no postmortem changes. Slow freezing in −20°C caused a significant increase of stiffness, whereas no changes were observed when snap-freezing in liquid nitrogen was applied. They attributed the changes from slow freezing to the formation of large ice crystals, which disrupted the structure of the extracellular matrix. In another study on freezing of articular cartilage no stiffness changes were seen for either snap-freezing or slow freezing [111]. Freezing seems to be an appropriate preservation method for studies of tissue stiffness [112]. However, this has not been verified for prostate tissue.

For TRM measurements it is very important to prevent dehydration of the tissue, which will increase the stiffness [113]. Jalkanen et al. [19, 20] applied PBS regularly onto
the prostate slices during measurements using a brush. Murayama, Oie et al. [75, 114] used a moisture chamber at 36 °C for elasticity mapping with the MTS. The group later refined the experimental setup to let the tissue sample and the MTS be fully immersed in PBS [113,115], which enabled measurements to be acquired for several hours without artifacts [115].

4.6 Mathematical tools for analysis and classification

Mathematical tools are essential for facilitating interpretation of complex multivariate data sets, such as the combined outputs from TRM and RS. Raman spectra of tissue contains a multitude of peaks (Figure 4.6), and they are difficult to interpret [98]. Multivariate methods such as PCA have become important for identifying the important spectral features for specific biomedical applications. Statistical analyses of data are necessary to support hypotheses about differences between various groups of tissue, e.g. between healthy and diseased tissue. Finally, characterization of tissue during medical examinations or surgery require efficient classification algorithms that provide relevant clinical information from a large set of variables in near real-time.

PCA is a valuable technique for data reduction and interpretation of data sets with a large number of variables [116]. It can often reveal connections not initially suspected. The principal components (PCs) are linear combinations of the original variables that account for a maximum amount of variability in the data. They are uncorrelated and each successive PC explains as much of the remaining variance as possible. Geometrically this represents a projection of the data onto a new coordinate system (Figure 4.7). The values for the observations in the new coordinate system are called PC scores. A few PCs can often describe a large amount of the total variability. Hence, a data set with \( n \) observations on \( p \) variables can be replaced by \( n \) observations on \( k \) PCs, where \( k \ll p \), without much loss of information. The PCs are often used in subsequent analyses in place of the original data [116]. PCA is a valuable tool for spectroscopic applications [22]. For example, Taleb et al. [99] used PCA on RS data to study the differences between healthy and cancerous prostate cells.

Cluster analysis is an unsupervised technique for identifying natural groups containing similar observations [116]. No prior knowledge of the groups is needed; the algorithm defines the user-selected number of groups based on similarity measures, usually some sort of statistical distance between the observations. Hierarchical clustering analysis (HCA) starts out either by looking at all individual observations, or by considering the whole group, and then applies a number of successive merges or divisions. If we consider the former as an example, first all the distances between all observations are determined. Observations that are close together are grouped together. Next, these groups are merged to larger groups that are nearby, and so on and so forth. This continues until the predetermined number of groups have been defined [116]. Cluster analysis is useful for unsupervised differentiation of healthy tissue types, and for identification of diseased tissue, from spectroscopic measurements [22]. It has successfully been used to produce pseudo-color images of histology specimens from spectroscopic data that compare well
with standard histology images [22,117].

The objective of data classification is to define a rule that can decide to which class a new observation belongs [116]. The rule is found by training the classifier to recognize patterns in data from observations of a relatively large number of samples of known classes. Classification techniques are supervised, in contrast to cluster analysis, where no classes are defined prior to analysis. Support vector machines (SVM) is a powerful classification technique that was introduced by Cortes & Vapnik in 1995 [118]. SVM calculates the hyperplane that separates two classes of data with the largest possible margin (Figure 4.8). Cortes & Vapnik introduced the concept of soft margins that enables classification of nonseparable data. The algorithm aims at minimizing the number of classification errors while separating the correctly classified observations with maximal margin [118]. To construct classification rules that generalize well, i.e. classify unseen data with low rate of error [118], cross-validation techniques can be used to find the optimal parameter settings for the SVM algorithm [119]. SVM can be used to separate more than two classes [120]. One strategy is to apply the binary classifier to distinguish two classes at a time, and determine the final class from the outcomes for all those classifications by a voting procedure [120]. SVM has attained very high classification accuracies in spectroscopic studies of cancerous tissues [121–123].
Figure 4.8: SVM classification of data from two groups, shown in red and blue, that are linearly separable. The solid line is the maximum-margin hyperplane, and the dashed lines show the margins. There are other planes that will separate the groups, but they will have smaller margins. The samples on the margins are called support vectors.
Aims

The overall aim of this thesis was to explore the need for new, complementary methods for PCa detection and take the first step towards a novel approach: the combination of TRM and RS. The specific aims were:

- To review the different methods for localization and diagnosis of PCa, in order to explore the demand for new, complementary methods.
  - This objective was assessed in Paper A.

- To develop a robust procedure for RS measurements of tissue in vitro, and for mathematical preprocessing and multivariate analysis of RS data. In particular, to evaluate the effects of snap-freezing and NIR laser illumination on porcine prostate tissue using RS.
  - This objective was assessed in Papers B, C and D.

- To develop a multivariate approach for comparing TRM and RS information via measurements on porcine tissue in vitro, in order to investigate the correlation of the data and potential diagnostic power of the combination.
  - This objective was assessed in Paper C.

- To develop a novel setup combining a scanning haptic microscope employing an MTS and an RS fiber optic probe, and adapt the system for measurements of prostate tissue slices.
  - This objective was assessed in Paper D.

- To investigate the accuracy for classification of healthy porcine prostate tissue types, and healthy and cancerous human prostate tissue, using SVM classification of combined TRM and RS measurements with the above-mentioned system.
  - This objective was assessed in Paper D.
Chapter 6

Material and Methods

This chapter summarizes the practical part of the work. It describes the equipment that was used, the measurement procedures and the data analysis. For more detailed information the reader is referred to Papers A–D.

6.1 Literature review

A review of the methods for localization and diagnosis of PCa was performed (A). The review focused on technical methods that can, or have the potential to, directly localize/diagnose PCa in situ via noninvasive or minimally invasive routes. Methods that label the tumor, e.g. with radioactive or fluorescent markers, were excluded. The databases Science Citation Index Expanded® and Social Sciences Citation Index® were searched via Web of Science® for relevant papers using the following combinations of search words:

- prostate AND cancer AND imaging
- ultrasound AND prostate
- prostate AND spectroscopy NOT magnetic
- magnetic AND resonance AND prostate AND cancer
- Raman AND prostate
- resonance AND sensor AND prostate
- infrared AND spectroscopy AND prostate AND cancer
- prostate AND FTIR
- elastography AND prostate
- DWI AND prostate
- DCE MRI AND prostate

http://www.isiwebofknowledge.com
All years of publication, 1975–2009, were included in the searches. Recent publications were favored. The reference lists in the selected articles were scrutinized for additional important papers. 185 publications were eventually selected for the review.

6.2 Experimental setup

Back in 2006 the research subject of Biomedical Engineering had just been established at the university. We did not have our own laboratory at that time. Therefore, a Raman micro spectrometer (Renishaw system 2000, Renishaw, Wotton-under-Edge, UK), which was available at the Dept. of Chemical Engineering and Geosciences, was adapted for measurements of tissue. This was done by installing a NIR laser with 830-nm wavelength (Renishaw HPNIR, 300 mW) and changing the filters of the setup. This system was used to study the effects of snap-freezing and NIR laser illumination on porcine prostate tissue (B). A water-dip objective (NIR Apo 60×/1.0W, Nikon, Tokyo, Japan) was used for spectral acquisition in the Fingerprint region, from 400 to 1800 cm$^{-1}$. The irradiance onto the samples was $\sim 3 \cdot 10^{10}$ W m$^{-2}$. To avoid interference from surrounding light the room was darkened during measurements. The spectrometer was calibrated for wavelength shift daily using the sharp silicon peak at 520 cm$^{-1}$ as reference. The wavelength-dependent sensitivity of the CCD detector was calibrated using a tungsten halogen light source (LS-1-CAL, Ocean Optics, Dunedin, FL, USA).

A sophisticated laboratory for biomedical research was built up during 2006–2009. A state–of–the–art RS fiber optic system was purchased. It was composed of a 0.8 mm thin fiber optic probe (Machida Endoscope Co., Tokyo, Japan), of the same type used in [92], connected to a Raman spectroscope (RXN1, Kaiser Optical Systems, Ann Arbor, MI, USA). The spectroscopy had a holographic grating that enabled simultaneous collection of the complete spectral interval from 100 to 3425 cm$^{-1}$. It incorporated a 400-mW laser at 785 nm (Invictus™, Kaiser Optical Systems). Proprietary software (iC Raman™, Kaiser Optical Systems) controlled the spectrometer. This setup was used for the RS measurements in Papers C and D. All RS measurements were performed in darkness, because spectral distortions from surrounding light were observed. The laser power was limited to avoid damage of the fiber optic probe due to heat building up at the tip. In Paper C it was set to 160 mW corresponding to approximately 80 mW onto the sample. For Paper D the probe was replaced with a more heat-resistant one, allowing use of higher power. The laser power was then set to 270 mW. This corresponded to an output effect at the probe tip of 140–150 mW. The integration time could be lowered from 30 s (C) to 7 s (D) without sacrificing spectral quality. The system was calibrated for wavelength shift and energy sensitivity once a month (HoloLab Calibration Accessory, Kaiser Optical Systems).

For performing the first combined TRM and RS measurements (C) the Venustron® (Axiom Co., Ltd., Koriyama, Fukushima, Japan) TRM system was used in combination with the RS fiber optic setup. A customized setup was constructed (Figure 6.1). The RS fiber optic probe and the Venustron® sensor were mounted next to each other. A three-dimensional translation stage assured that measurements were performed at the
6.2. Experimental setup

same points with both probes. It was composed of three one-dimensional stages (NRT100, Thorlabs, Newton, NJ, USA) with a common control unit (BSC103, Thorlabs). To damp vibrations the whole setup was mounted on an optical table (PBG52513 – Metric UltraLight Series II breadboards, Thorlabs). The translation stage was controlled via a LabVIEW® (version 7.0, National Instruments, Austin, TX, USA) program written in-house. A camera (Powershot S3 IS with close-up lens 500D and LAH-DC20 conversion lens adapter, Canon, Tokyo, Japan) was mounted above the setup. It was used to capture a picture of the tissue sample, which was loaded into the program. The user selected the measurement points from the picture. The coordinate system defined in the program was calibrated to have the origin at the top left corner of the picture. It was important to assure that the sensors measured on the same points of the sample. Their positions were calibrated by visually controlling that each sensor was positioned exactly above a reference mark when the translation stage was moved to the corresponding coordinates.

For Paper D a novel TRM technology was used, the MTS, which was developed by Murayama & Omata [68, 124, 125]. Their research group has developed an elasticity mapping system using the MTS, which has been termed scanning haptic microscopy (SHM) [115]. It has been commercialized, and we purchased such a prototype (P&M Co., Ltd., Aizuwakamatsu, Fukushima, Japan). A unique setup that combined the SHM system with the RS fiber optic setup was developed (Figure 6.2). It was composed of an XYZ-stage with a resolution of 0.01 µm and an actuator mounted on the Z-axis to
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manipulate the MTS. The system incorporated a camera (LifeCam Cinema\textsuperscript{TM}, Microsoft, Redmond, WA, USA) and a USB microscope (Dino-Lite AM413TL, AnMo Electronics Corp., Hsinchu, Taiwan), to capture film and photos of the sample and probes. The temperature was logged by resistance measurement of a platinum resistance element (FK1020 PT1000, Heraeus, Kleinostheim, Germany) with a multimeter (Fluke 45, Fluke Corp., Everett, WA, USA), connected to the computer via GPIB. A labview\textsuperscript{TM} program that controlled the hardware was developed. It automated the measurements as far as possible and collected all data except the RS data, which was recorded by the spectrometer software on a separate computer. The spectral acquisition could not be automatically triggered. Therefore, a photo diode was mounted close to the RS probe (Figure 6.2) to monitor when the laser was illuminating the sample. The shutter to the laser was closed by the spectrometer between the measurements. The spectrometer was set to continuous spectral acquisition, and the translation stage was moved to the next point as the photo diode voltage decreased abruptly. The voltage was measured with a second multimeter (Agilent 34401A, Agilent Technologies, Inc., Santa Clara, CA, USA) via the GPIB interface.

The SHM system was adapted for measurements of prostate tissue (D). The standard settings had to be adjusted and optimizations of the software were done. Since the prostate tissue was rather sticky the MTS was raised relatively far above the tissue after each measurement, approximately 100 µm, so that it detached from the tissue. The impression depth was set to about 40 µm, which made the mappings more stable for samples with uneven surfaces, as compared to the standard setting of ≈ 25 µm (half of the tip diameter). Each MTS had unique characteristics (Figure 6.3). The length and exact shape of the glass needle is very important for obtaining high sensitivity [125]. Some sensors were not suitable for prostate tissue measurements. One reason was that the stiffness range of the prostate tissue was wide, from 2 to 200 kHz, and some sensors did not give a linear response throughout this range. Another reason was that for some sensors the resonance frequency could change abruptly when very stiff points were measured. It was often challenging to identify a frequency where the sensitivity was high, the response linear, and the measurements were stable. The needles were fragile and easily broken due to operator mistakes. Several safety routines were implemented in the labview\textsuperscript{TM} program to avoid this.

The positions of the MTS and the RS probe relative to the stage were determined by using a printed grid with 50 µm line thickness (D). A line intersection was used as reference mark. The MTS was aligned with the reference mark using the USB microscope. Since the RS probe was relatively large it was difficult to calibrate its position visually. Therefore, an RS mapping was performed. A cluster analysis was performed on the data using an HCA algorithm. The measurement points were divided into three groups, and a mapping image was constructed. The line intersection was then clearly visualized (Figure 6.4), and the position of the RS probe was determined with high accuracy.
6.2. Experimental setup

Figure 6.2: The experimental setup for Paper D.
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6.3 Sample preparation

For the study of the effects of laser illumination and snap-freezing (B) two porcine prostates were removed from boars slaughtered at the local abattoir. The prostates were enclosed in a plastic bag and refrigerated. The prostates were not removed from the urethra and the surrounding, protective tissue until cut into smaller samples, which was done within 24 hours after slaughter. Samples were either stored in PBS in the refrigerator (referred to as fresh samples), or snap-frozen in liquid nitrogen and stored at $-80^\circ$C. The frozen samples were allowed to passively thaw immersed in PBS prior to measurement.

In Paper C pork belly tissue was used as a model system. Two pieces were obtained from the local grocery store. They were stored at 6 $^\circ$C. Two samples were cut from each piece, for a total of four samples. The samples were placed on a Styrofoam plate, which was fastened to the translation stage, as shown in Figure 6.1.

In Paper D both porcine and human prostate tissue were investigated. Eight porcine
prostates were collected from slaughtered boars. They were cut into smaller pieces, which were snap-frozen and cut into 500-µm thick slices using a freezing microtome. Adjacent to both sides of each slice 5-µm thin specimens were cut. They were stained for histological analysis. Small samples retrieved from the prostate of a patient undergoing radical prostatectomy were snap-frozen in liquid nitrogen within thirty minutes after surgery. They were prepared in the same way as the porcine samples. Three of the samples contained cancerous tissue and were included in the study. All samples were stored at −80°C until measured.

6.4 Measurement procedure

To study the effects of laser illumination, repeated Raman spectra were acquired from the same point on the fresh tissue samples (B). The integration time was set to 10 s to capture rapidly occurring effects. Spectra were captured subsequently during the first minute, then less frequently during the following four minutes. For the snap-freezing experiments five samples were measured day 1 and used as reference for fresh samples (B). Five snap-frozen samples were measured after 5, 26 and 81 days of storage, to study if prolonged storage at −80°C degraded the tissue. The samples were immersed in PBS during the measurements.

For the combined TRM and RS measurements on pork belly tissue, a grid with 42 measurement points was defined for each sample (C). In total 168 points were measured with each device. The measurement order was randomized with the constriction that
adjacent points were not measured subsequently. This avoided the possibility that viscoelastic effects would influence the TRM measurements. All TRM measurements on a sample were completed before the acquisition of Raman spectra. The tissue was kept moist by brushing it with PBS every fifth minute. The distance from the RS probe to the sample had to be adjusted manually. A magnifying glass was used to facilitate the adjustment.

For the combined measurements on porcine and human prostate tissue (D) dehydration was prevented by putting the samples on a filter paper soaked with PBS, which was put on top of a microscope slide with low spectral background (Calcium Fluoride UV grade, Crystan Ltd., Poole, UK). A shelter made from a rolled-up piece of a Kimwipe paper (Kimtech Science Kimwipes, Kimberly-Clark Inc., Roswell, GA, USA) was put around the sample, and a constant flow of PBS was applied to it (Figure 6.2). The user set the measurement region of interest in the Labview™ program. The MTS was set to the starting position and the surface was detected through the frequency shift. A stiffness map and a topography map were then acquired with the MTS. The step size was 50 µm. Next the RS probe was moved to the starting position and a Raman map was captured using a 300 µm step size. The distance from the RS probe to the sample was adjusted to be constant at 400 µm by using the topography map recorded by SHM.

6.5 Data analysis and statistics

All data analysis was performed using MATLAB® (version R2007b/R2008b/R2010a including Statistics Toolbox version 6.1/7.0/7.3, MathWorks Inc., Natick, MA, USA). The only exception was that MINITAB® (version 15.1.20.0, Minitab Inc., State College, PA, USA) was used to check for autocorrelation in Paper B. The algorithms not included in MATLAB® were written in-house.

The RS data was preprocessed as follows (B–D). The Fingerprint region from 400–1800 cm⁻¹ was selected. Wavelengths < 600 cm⁻¹ were excluded due to interfering Raman scattering generated in the fiber optic probe (C, D) [92]. Spectral spikes due to cosmic rays were removed prior to analysis. Correction for the energy sensitivity of the spectrometers was applied. Each Raman spectrum was lightly filtered using the smoothing algorithm by Eilers [126]. The background was automatically subtracted via the algorithm by Cao et al. [97]. To aid comparison the spectra were vector normalized, so that their integrated areas were equal. In Paper D the variance minimization method [127] was used to remove the Raman signal generated in the fiber optic probe and the interfering signal from the filter paper. The Raman spectra were interpreted by making tentative assignments of the peaks. The database collected by Movasaghi et al. [98] was frequently used. PCA was applied to the preprocessed Raman spectra to reduce the dimensionality of the data (B, C). It was performed on unstandardized data, i.e. the variables were not scaled by dividing them by their standard deviations. The ten first PCs were retained for further evaluation. They explained a large percentage of the total variance.

In Paper B a modified version of Kim’s test [128, 129] was used to determine if the multivariate means of the PC scores of fresh and snap-frozen tissue differed. Three
analogue analyses were conducted, fresh tissue was compared to snap-frozen tissue stored at \(-80^\circ\text{C}\) for 5, 26 and 81 days. If the test showed a significant difference, Yuen’s univariate test [130] was applied to compare the means of the individual PC scores. This was done to investigate which PCs that contributed strongly to the significant multivariate difference. The spectra of these PCs showed the main spectral differences between fresh and snap-frozen tissue.

In Paper C the Raman PC scores were input to an unsupervised HCA algorithm using Ward’s linkage [116, 117]. The data set included all 168 spectra. The cluster analysis divided the spectra into five groups. They were labeled A, B, C, D and E in Paper C, and will here be represented by different colors: black, green, gray, red and blue, respectively. The TRM stiffness-sensitive parameter \(\frac{\partial F}{\partial \Delta f}\) was calculated from \(\Delta f\), \(F\) and \(d\) as [19]

\[
\frac{\partial F}{\partial \Delta f} = \frac{\partial F/\partial d}{\partial \Delta f/\partial d}
\]

and evaluated at \(d = 0.6 \pm 0.1\) mm using linear regression (C). At this depth the sensor measures the tissue near the surface [19]. The nonparametric Kruskal-Wallis test followed by Tukey-Kramer’s multiple comparison test were used to test if the mean stiffness of the groups differed. To evaluate the relationship between the TRM and RS information a linear regression analysis was conducted using the Raman PC scores to explain the stiffness variation. Furthermore, receiver operating characteristic curves were constructed to investigate the ability of TRM to classify each tissue type defined by the cluster analysis of RS data. They showed the sensitivity and specificity for distinguishing the current tissue type from the others for varying stiffness threshold values.

In Paper D three main tissue types were identified in the histologic analysis of the porcine samples: epithelium, lumen and stroma. For the human samples the groups were: epithelium, lumen, stroma, cancerous epithelium, cancerous stroma and prostate stones. The stiffness sensitive parameter for the MTS, \(\Delta f/\delta\), was calculated in MATLAB\textsuperscript{®}. Furthermore, a measure of nonlinear effects was calculated by fitting a second-degree polynomial to the frequency versus indentation curve for each measurement. Differences in the biochemical composition of the tissue types were assessed by analyzing the mean spectra of the tissue types. The Kruskal-Wallis and Tukey-Kramer tests were used to test for significant differences in stiffness. An SVM analysis was performed to find the accuracy of using the TRM alone, and TRM and RS together, to classify the tissue types. Different degrees of tissue homogeneity were analyzed (D).

A \(p\)-value less than 0.05 was considered as statistically significant for all statistical tests. It was assumed that the experimental designs were completely randomized and that the observations were independent.
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Chapter 7

General Results and Discussion

This chapter summarizes and explains the findings in Papers A–D, and discusses their relevance for the development of an instrument combining TRM and RS for PCa detection.

7.1 Literature review

The gold standard for PCa detection and diagnosis, the PSA test and SB, lacks sensitivity and specificity (A). Indolent and aggressive tumors cannot be reliably differentiated, and the rate of overtreatment is high (A).

Grey-scale transrectal ultrasound is used only to guide biopsies to predetermined sites according to SB protocols, since tumors usually cannot be discerned on the ultrasound image. Ultrasound techniques that assess the prostatic blood flow or tissue elasticity are more effective (A). The best results have been obtained with contrast-enhanced ultrasound. Directing biopsies at suspicious lesions detects more clinically significant tumors with fewer biopsy cores, as compared to SB [12,131,132]. However, ultrasound methods still show a low specificity. The subjective interpretation is also a limitation [12].

Advanced MR techniques are very promising (A). The specificity of T2-weighted MRI is merely 50%, but it can be increased significantly by the addition of MRSI [11,133]. A meta-analysis of the literature showed that the sensitivity and specificity of MRSI for PCa detection are 64% and 86%, respectively [134]. MRSI offers objective detection of PCa based on the elevated tumor metabolism. The combination of MRI and MRSI has attained high detection accuracies in many studies. Dynamic contrast-enhanced MRI significantly increases the detection rate of conventional MRI [11]. Diffusion weighted imaging may also improve MRI performance [133]. A combination of several MR techniques has the potential to detect most tumors of clinical significance [133]. The first studies of multiparametric MRI show promising results [135–137]. The main drawbacks of MRI are high costs, limited availability of MR scanners and that MR-guided biopsy is a complex procedure [11,138].

Computer-aided detection and diagnosis is expected to play an important role in the future (A). Intra- and interobserver variability is an issue with both ultrasound and MR techniques. Computerized interpretation have the potential to objectively and efficiently analyze the huge amounts of data generated by new advanced techniques.
General Results and Discussion

Paper A shows that there is a need for new cost-effective and complementary methods for localization and diagnosis of PCa. In particular, it is important to distinguish indolent and aggressive PCa. The proposed instrument combining TRM and RS is interesting as a complementary method for PCa detection, since the individual techniques have demonstrated promising results in vitro (A). However, Paper A concluded that the penetration depth of RS is currently insufficient for noninvasive detection of PCa. Thus, at the present time it is foremost during radical prostatectomy that the combined instrument can make a difference. If the surgical margins can be cleared from cancerous tissue, cancer recurrence could be minimized and adjuvant therapy may be unnecessary, even for men with high-risk tumors. A miniaturized version of the combined instrument could also play a role in biopsy procedures, by analyzing the tissue in proximity to the tip of the needle, and point out regions suspicious for cancer.

7.2 Effects of snap-freezing and laser illumination

7.2.1 Laser illumination

The analysis of difference spectra showed that no changes in the Raman spectra occurred due to the laser illumination \( (n = 5) \) (B). The intensity of the background decreased with the laser illumination time, while the intensity of the Raman signal was unaltered (Figure 7.1). The results were similar for all measurement series. The discussion in Paper B indicates that photobleaching of tissue fluorophores caused the decrease of background intensity. Porphyrins present in myoglobin and hemoglobin were the most likely sources of autofluorescence. If the background is removed during preprocessing of Raman data, this phenomenon should not affect the spectral information significantly.

This study shows that the in vitro measurement protocol was appropriate. NIR illumination of high irradiance did not seem to harm the tissue under the given experimental conditions. For in vivo measurements, the probed tissue will not be immersed in PBS and may not withstand such a high irradiance. However, the irradiance from a fiber optic system will usually be much less than from a microscopic system, due to a larger illumination area, and living tissue may be heat-resistant due to circulating, cooling blood [92]. Hattori et al. [92] used an RS fiber optic probe of the same type used in Paper C to measure the esophagus and stomach of rats in vivo. They found no evidence of degradation of the tissue at an excitation power onto the sample of 80 mW. Assuming that the excitation area equals the area of the delivery fiber \( (\varnothing = 125 \, \mu m) \), this corresponds to an irradiance of about \( \sim 7 \cdot 10^6 \, \text{W m}^{-2} \), which can be compared to the irradiance \( \sim 3 \cdot 10^{10} \, \text{W m}^{-2} \) for the system used in Paper B.

7.2.2 Snap-freezing

Multivariate tests showed that there was a significant difference \( (p < 0.05) \) between the spectra of fresh and snap-frozen tissue (B). The main spectral differences were found through univariate tests of the difference between the PC scores of fresh and snap-frozen
7.2. Effects of snap-freezing and laser illumination

Figure 7.1: The total intensity of the spectral background decreased with the laser illumination time, whereas the intensity of the Raman signal showed small random fluctuations.

Figure 7.3 shows two spectra for day 26 where the differences were relatively large. Significant differences between tissue samples stored at −80°C for different time-spans were found, indicating that the tissue may be affected by the storage. However, the $p$-values were much larger (of the order of $10^2$–$10^6$ times larger) than those found comparing fresh and snap-frozen tissue. Thus, it was most likely the snap-freezing or thawing, and not the storage at −80°C, that was the main cause of the observed changes. As discussed in Paper B, the changes caused by snap-freezing were probably related to alterations of the protein conformation. This may be important to consider for studies that use snap-freezing and find diagnostic information related to protein conformation. Since the changes in cancerous prostate tissue mainly seem to be related to DNA and lipids [24, 27, 99], these findings suggest that snap-freezing may be used to study the difference between healthy and cancerous prostate tissue in vitro.
Figure 7.2: The calculated PCs whose scores were proven to be significantly different for fresh and snap-frozen tissue. Fresh tissue obtained higher scores for these PCs. Peaks characteristic for the differences have been labeled with their wavenumbers.
7.3 Comparing TRM and RS information

Figure 7.3: A spectrum on fresh as compared to snap-frozen prostate tissue for day 26. The difference between the spectra is plotted in black.

7.3 Comparing TRM and RS information

Figure 7.4 shows the result of the cluster analysis of the PC scores of the RS data for one of the samples (C). Visual inspection showed that the spots marked with black were muscle tissue, the red and blue groups fat tissue, whereas the gray and green groups were a mix of muscle and fat tissue. This was confirmed by analysis of the Raman spectral data (C). Figure 7.5 shows the mean spectrum of each group. Analysis of the spectral peaks showed that the blue group contained a higher amount of saturated fatty acids than the red group (C). The spectra were clustered into distinct groups. PC 1, PC 2 and PC 4 contributed strongly to the clustering (Figure 7.6). The interpretations of them were that PC 1 was related to the proportion of fat, PC 2 to the proportion of protein, and PC 4 to the composition of the fat.

The mean stiffness of each tissue group was calculated from the TRM measurements (C), see Figure 7.7. The blue group was significantly stiffer than the other groups ($p < 0.05$). It was explained by the difference in the composition of the fat shown by RS (C). In general, saturated fatty acids have higher melting points than unsaturated fatty acids [139]. Thus, the stiffness is related to the portion of saturated fat.

As shown in Table 1 in Paper C, the RS data explained 67% ($R^2_{adj} = 0.67$) of the total variability of the stiffness parameter. PC 2 and PC 4 were the most important predictor variables, indicating that the proportion of fat and its composition contributed most
General Results and Discussion

Figure 7.4: The outcome of the clustering analysis for one of the pork belly samples. The grid overlay shows the 42 measurements points. The measurement order is shown by the numbers. The group assignment is indicated by the colors. One of the groups (blue) was not represented in this sample. The light area of the sample consisted of fat tissue and the dark area was muscle tissue.

Figure 7.5: The mean spectra of the pork belly tissue groups, which were defined by the cluster analysis. The peaks whose intensity differed substantially between the two fat groups have been labeled with their wavenumbers.
7.3. Comparing TRM and RS information

Figure 7.6: A scatter plot of the scores of PC 1, PC 2 and PC 4 for all RS measurements on the pork belly samples. The colors represent the group assignment from the cluster analysis.

Figure 7.7: The mean stiffness of the five groups defined by the cluster analysis of RS data. A high value indicates a higher stiffness. The blue fat group was significantly stiffer than the other groups ($p < 0.05$).
to tissue stiffness. The analysis of the receiver operating characteristic curves showed that the fat group that was stiffest (blue) was satisfactorily identified using TRM (C). However, the accuracies for identifying the other groups were lower. The Venustron® sensor measured a much larger volume than the RS probe (C). This may partly explain why TRM obtained a relatively poor differentiation of the tissue groups. The TRM sensor probed a mix of several tissue types in many cases, while the RS probe usually measured a more homogeneous area.

In summary, Paper C shows that the TRM and RS information can be compared without prior knowledge of the tissue by a multivariate approach. The method uses PCA and HCA to identify different tissue types at a chosen level of detail. Next, linear regression is used to relate the stiffness to the biochemical composition, and receiver operating characteristic curves assess how the stiffness information can be used to classify the tissue types. As shown in Paper C the method was able to extract a relationship that was not initially suspected, namely that there was a difference in stiffness between two visually similar tissue types, and that it could be explained by the difference in the amount of saturated fat. Furthermore, it showed that the biochemical information obtained from the RS measurements provided additional discriminatory power to the TRM.

7.4 Classification of prostate tissue

Figure 7.8 shows a typical example of an SHM mapping measurement of porcine prostate tissue, and Figure 7.9 shows one of the mappings on human tissue. For most samples the structures observed in the histology image were clearly reproduced in the stiffness map. The statistical tests showed that the mean stiffness of the porcine tissue types differed ($p < 0.05$). Stroma was stiffest, whereas lumen was softest. Comparison of the mean spectra (Figure 7.10) indicated that the amount of collagen was highest in stroma and lowest in lumen (D). Thus, it is likely that collagen contributes substantially to prostate tissue stiffness. For the human samples the statistical tests showed that lumen was significantly softer than the other tissue types, except for healthy epithelium. This agrees well with the results for porcine tissue. It was also shown that prostate stones were significantly stiffer than cancerous epithelium, healthy stroma, and lumen. That prostate stones are very stiff has been shown by Jalkanen et al. [20] and Eklund et al. [37]. Interestingly, the median stiffness value for cancerous stroma was higher than the healthy tissue types, although it was only significantly stiffer than lumen. This suggests that cancerous stroma may be a cause to the increased stiffness due to PCa.

From the SHM measurements it was observed that there was a predictive value in the shape of the measurement curve, i.e. the degree of nonlinearity (D). The curvature of the measurement curves for the tissue types differed (Figure 7.11). The difference was significant ($p < 0.05$). The classification accuracy was substantially increased when this variable was added to the SHM data. Furthermore, the SHM measurements on human tissue indicated that this parameter added complementary information for tissue discrimination (D). Cancerous stroma showed a significantly higher ($p < 0.05$) deviation from linearity than all healthy tissue types (D). Moreover, cancerous epithelium could be
7.4. Classification of prostate tissue

Figure 7.8: Results for one of the SHM measurements on porcine prostate tissue. In the histologic analysis image epithelium is shown in blue, lumen is white, and stroma is red. The gray points indicate that the tissue type could not be determined. The scale bars show 500 µm.

Figure 7.9: Results for one of the SHM measurements on human prostate tissue. The stiffness map has been noise-reduced using a median filter with a square kernel of 3 × 3 pixels. We see that the cancerous region to the right is associated with increased stiffness. Furthermore, healthy regions rich in stroma are relatively stiff. The area with stones is very stiff. The upper left portion of the sample showed signs of prostatic inflammation at high magnification. This may have increased the stiffness in this region. ca = cancerous.
distinguished from lumen and healthy epithelium ($p < 0.05$). This finding casts new light on the possibilities for tissue characterization using TRM alone. There may be additional diagnostic value, besides the stiffness calculated from the slope of the measurement curve, in the raw data. Further studies are necessary to explain the origin of the nonlinear effects, and assess their possible diagnostic value.

The spectral quality of the RS measurements on human samples was low (D). The reason was that the surgical margin of the removed prostate was covered with blue ink. This is standard clinical procedure; it is useful for determining if all cancerous tissue was removed at radical prostatectomy [17]. If cancerous tissue is found to be in contact with the inked margin, the patient is defined to have a positive surgical margin. The ink gave rise to very strong fluorescence that often saturated the detector in the Fingerprint interval, and swamped the Raman signal from the tissue. Fortunately, it was possible to partly overcome this problem by analyzing the high wavenumber region from 2400–3425 cm$^{-1}$, where the fluorescence was decreased. Although this region does not contain as much biochemical information as the Fingerprint region, the diagnostic value may be similar [86–88]. We found that almost as high classification accuracies were attained for the porcine samples using the high wavenumber region, as compared to the Fingerprint region. However, in comparison with the spectra of porcine tissue the spectral quality was lower for human tissue in the high wavenumber region. Spectra of human tissue were considerably more noisy. Thus, we believe that important diagnostic information may have been lost due to the strong fluorescence.

Figure 7.12 presents the SVM classification accuracies for distinguishing healthy porcine tissue types, and for distinguishing healthy and cancerous prostate tissue, for different
7.4. Classification of prostate tissue

Figure 7.11: The mean SHM measurement curves, for points in homogeneous tissue regions, for the different tissue types for one of the porcine samples. We see that the measurement curves show slight deviations from linearity, and that the curvature for stroma differs from epithelium and lumen.

degrees of tissue homogeneity. TRM alone attained a high accuracy for distinguishing different porcine tissue types, especially for the most homogeneous tissue regions when it was 82%. In combination with RS the accuracy was > 80%, even when the most heterogeneous tissue regions were included in the analysis. For differentiating cancerous tissue the accuracy was 67–70% when using the TRM alone. The analysis of the SHM data indicated that the overlapping stiffness distributions of healthy stroma and cancerous epithelium may have been the main limiting factor (D). The accuracy was increased to 73–77% when RS was added to SHM. The combined accuracy would most likely have increased if the spectral quality for human tissue would have been higher. In future studies alternative experimental approaches should be considered, in order to eliminate or decrease the interference from the application of ink to the surface of the removed prostates.

The results of Papers C and D clearly show that it is feasible and valuable to combine TRM and RS for tissue characterization. Used as a research tool the combination shows promise to extract unknown relationships between stiffness and biochemical composition by the multivariate approach presented in Paper C. This may help to explain the cause for changes in tissue stiffness due to cancer. For the development of a clinical instrument for probing surgical margins Paper D indicates that there is diagnostic information for distinguishing healthy and cancerous tissue using TRM alone. With the addition of RS the diagnostic power is increased. The combination shows high potential for exact tissue characterization, even in complex tissue regions with a mix of different tissue types. This is very promising for the development of a clinical tool to be used during PCa surgery.
Figure 7.12: The five-fold cross-validation accuracies for SVM classification of the porcine prostate tissue types epithelium, lumen and stroma, and healthy and cancerous human prostate tissue.
General Summary and Conclusions

This thesis reviewed the current state-of-the-art in PCa detection. The importance of appropriate in vitro preparation protocols and measurement procedures was discussed and evaluated, and the effects of snap-freezing and NIR laser illumination on porcine prostate tissue were investigated using RS. The combination of TRM and RS was evaluated in experiments on porcine and human tissues using multivariate techniques for explaining the data correlation and assessing the potential for characterization of healthy and diseased tissue.

From the review it was concluded that the specificity and sensitivity of clinical methods currently used for PCa detection and diagnosis, the PSA test and SB, are insufficient. Indolent and aggressive tumors cannot be reliably differentiated, which results in many men being treated either unnecessarily or too late. Clinical benefits of state-of-the-art technology for PCa imaging, advanced ultrasound and MR techniques, have still not been convincingly shown. There is a need for complementary and cost-effective detection methods. TRM and RS are promising complementary techniques.

In the RS study no indications of tissue degradation due to 830 nm laser illumination at an irradiance of $\sim 3 \cdot 10^{10}$ W m$^{-2}$ were observed. Snap-freezing and subsequent storage at $-80^\circ$C gave rise to subtle but significant changes in the Raman spectra, most likely related to alterations in the protein conformations. Since the main changes in cancerous prostate tissue appear to be related to DNA and lipids, these findings indicate that snap-freezing may be used to preserve samples for in vitro studies concerning PCa detection.

The measurements on porcine and human tissue types showed that the combined data can be used to study the causes of differences in tissue stiffness through a multivariate approach. Furthermore, the classification accuracy of healthy porcine tissue types was high using TRM alone, and increased substantially when RS was added. The measurements on human tissue indicate that efficient detection of PCa may be possible.

To summarize, this thesis shows that a combined instrument incorporating both TRM and RS is a promising complementary method for PCa detection. Snap-freezing of samples may be used in future RS evaluations of changes in cancerous prostate tissue. The instrument has potential for assisting surgeons in cancer surgery, and for biopsy guidance. The goal is to provide a more secure diagnosis and consequently more efficient treatment of the patient.
This thesis shows that a future instrument combining TRM and RS may be useful for tumor detection and diagnosis. The TRM sensor would be used to swiftly scan the tissue for stiff lesions. With the additional diagnostic power of RS it may be determined if a lesion is benign or malign, and whether it is indolent or aggressive. This could be accomplished in near real-time through computerized interpretation of measurement data. The combined instrument may be constructed by molding an RS fiber optic probe into a cylindrical resonance sensor element, as depicted in Figure 9.1. This is the thought device for probing surgical margins. Since the RS probe is shielded from surrounding light by the piezoelectric element, measurements may be performed in bright environments. The TRM sensor can be used for tactile feedback to the surgeons and inform them when the instrument touches the tissue. This would facilitate the practical clinical use.

A possible design for a miniaturized version of the combined instrument is shown in Figure 9.2. It uses a single optic fiber inserted into a hollow glass needle, as used for the MTS. This device could be used for possible guidance of prostate biopsies. By using RS measurements in the high wavenumber region it is possible to use a single optic fiber for spectral acquisition [86–88].

Although this thesis focused on PCa, the combined instrument could potentially be used for a variety of biomedical applications, both in vitro and in vivo. Both TRM and RS are versatile methods [18,22], and the combination even more so, since biomechanical and biochemical information are retrieved simultaneously.
Future Outlook

Fiber optic probe Cylindrical PZT element
Hemispherical tip
Quartz window
Rubber mount
Lens
Focal length

Figure 9.1: A possible design of an instrument for scanning of surgical margins.

Glass needle Single optic fiber
Hemispherical tip

Figure 9.2: A possible design for a miniaturized instrument.
References


References


Part II
Technologies for localization and diagnosis of prostate cancer

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The gold standard for detecting prostate cancer (PCa), systematic biopsy, lacks sensitivity as well as grading accuracy. PSA screening leads to overtreatment of many men, and it is unclear whether screening reduces PCa mortality. This review provides an understanding of the difficulties of localizing and diagnosing PCa. It summarizes recent developments of ultrasound (including elastography) and MRI, and discusses some alternative experimental techniques, such as resonance sensor technology and vibrational spectroscopy. A comparison between the different methods is presented. It is concluded that new ultrasound techniques are promising for targeted biopsy procedures, in order to detect more clinically significant cancers while reducing the number of cores. MRI advances are very promising, but MRI remains expensive and MR-guided biopsy is complex. Resonance sensor technology and vibrational spectroscopy have shown promising results in vitro. There is a need for large prospective multicentre trials that unambiguously prove the clinical benefits of these new techniques.

**Keywords**: Prostate cancer; Imaging; Diagnosing; Grading; Targeted biopsy
1. Introduction

Prostate cancer (PCa) has the highest incidence of all cancers, excluding nonmelanoma skin cancers, and is the third leading cause of cancer-related death after lung and colorectal cancer in European men [1]. Due to the ageing population the prevalence of the disease is expected to increase [2], and since 1995 the number of deaths caused by PCa has increased by 16% [1]. Clinically significant PCa is still much more common in Western countries than in Asia [3], although the incidence of PCa observed at autopsy is similar worldwide [4]. However, PCa mortality rates in Asia are rising, which may be explained by gradual Westernization and loss of protective cultural factors such as diet [3].

The gold standard for PCa detection and diagnosis, the prostate-specific antigen (PSA) test and systematic biopsy (SB), has many limitations, and fails to reveal a large percentage of clinically significant tumours [5–7]. To reduce PCa mortality and choose an appropriate therapy, new imaging modalities and diagnostic tools are needed that can accurately localize and diagnose PCa at an early stage [8].

To our knowledge recent reviews in the area have focused on either a specific technology [9–11] or on biomarkers, such as PSA [2, 12, 13]. Furthermore, the area of PCa detection is constantly under debate, and new technologies are emerging and established ones are improved. Therefore, the aim of this review is to give a broad perspective on current state-of-the-art techniques for PCa detection and diagnosis in clinical use today and to mention promising novelties. The review focuses on technical methods that can, or have the potential to, directly localize/diagnose PCa in situ via non-invasive or minimally invasive routes. Methods that label the tumour, e.g. with radioactive or fluorescent markers, are excluded. The included methods are ultrasound and elastography, resonance sensor technology, magnetic resonance imaging (MRI) and vibrational spectroscopy (VS). Each chapter will follow a chronological order that indicates the development of each technique into the current state-of-the-art. Computer-aided detection and diagnosis are discussed in 8. A critical comparison of the techniques is finally given in 9.

2. Anatomy and pathology

The prostate is about the size and shape of a walnut. It surrounds the urethra and the ejaculatory ducts and has a broad base superiorly, situated just below the bladder, and a narrow apex inferiorly. The gland can be divided into three anatomical zones: the peripheral zone (PZ), the transitional zone (TZ) and the central zone (CZ), as illustrated in Figure 1. The PZ, CZ and TZ compose 70%, about 25% and 5–10% of the normal glandular tissue, respectively. Different pathologies occur with different frequencies in the three zones. The majority of tumours, approximately 70%, develop in the PZ [14]. It is generally believed that benign prostatic hyperplasia (BPH), a very common malady in elderly men [15], originates from the TZ. Recent studies indicate that BPH also might develop in the PZ [16, 17].

3. PCa detection and diagnosis

PCa distinguishes itself from many other cancers since it is usually multifocal and not presented as a solitary round mass [18]. Furthermore, the disease progression varies widely [8], and PCa is often asymptomatic until advanced stages have developed [19].

Historically, digital rectal examination (DRE) has been the main method of detection [20], but it is seen today as a rather crude tool [21, 22]. Tumours in the prostate are generally harder than healthy tissue [23–25], owing to the increased cellular density of cancer [18].

The gold standard for detecting and diagnosing PCa is transrectal ultrasound (TRUS)-guided systematic biopsy...
European guidelines of 2007 recommend a minimum of 10 cores [26]. The cores are evaluated using brightfield microscopy, and cancer is detected by its abnormal morphological appearance. It has been estimated that sextant SB, which first was proposed in 1989 [27], fails to find a present cancer in three out of 10 cases [28]. Although the detection rate may be increased by taking additional cores, patients experience increased pain and the costs are elevated [2]. Saturation protocols (≥20 cores) may add little benefit over 10–12 core protocols [29, 30].

Many studies use SB as reference, and due to its shortcomings the true accuracy of the evaluated method cannot be determined. It is preferred to use histological analysis of radical prostatectomy specimens, which is considered superior to SB [31].

The PSA test came into widespread clinical use in the early 1990s [13]. It is the clinical test with the highest positive predictive value for PCa [2]. However, PSA-based screening is controversial [13, 32–34]. The PSA level can be elevated because of benign conditions, and many men with a concentration of PSA < 4 ng ml\(^{-1}\) suffer from clinically significant disease [35, 36]. Furthermore, the use of PSA leads to many men with indolent cancers being unnecessarily treated [13, 34]. Originally 4 ng ml\(^{-1}\) was implemented as a cut-off for recommending biopsy. The latest European guidelines suggest that values around 2.5–3.0 ng ml\(^{-1}\) should be used for younger men [26].

### 3.1. Grading and staging PCa

The biological aggressiveness of PCa is currently histologically evaluated and rated according to the Gleason grading system. The two primary histologic patterns of the cancerous tissue are graded with a score from 1 to 5, as described in detail in [37]. The higher the number the more aggressive is the tumour. The Gleason score (GS) is presented as the sum of these two numbers, resulting in a scale from 2 to 10. The prognostic power of the GS is high. However, the histological examination is subjective, and intra- and inter-observer disagreement is a problem [37, 38]. Moreover, there is a discrepancy between the scoring of biopsy samples and radical prostatectomy specimens [39–42]. That can be expected since most palpable tumours are morphologically heterogeneous and contain multiple grades [43]. Unfortunately, undergrading is the most common problem. This is due both to biopsy sampling error and incorrect histological interpretation, and thus inappropriate treatment strategies can be chosen [37, 44].

There is a standardized system for staging PCa: the tumour-node-metastasis (TNM) system, which rates progression of disease according to the spread of the primary tumour and metastases [6]. Lately, nomograms have increasingly come into clinical practice to predict progression of disease and aid in recommending an appropriate therapy [26]. The nomograms have been developed by statistical treatment of data from large series of patients. Variables such as clinical stage, GS, race, age, tumour size, PSA level and amount of cancer in biopsy, have been incorporated and related to the clinical outcome [20, 37].

Early detection and precise disease characterization are essential for reducing PCa mortality and optimizing patient care. The prognosis of gland-confined cancer of low (<7) GS is good, whereas PCa of high GS (>7) or advanced stage is often incurable. Furthermore, most tumours are clinically insignificant, and no treatment but active surveillance is recommended for these patients [6, 45]. Actually, autopsy reveals that many healthy men harbour PCa [19]. An estimation indicates that almost 50% of men 70–80 years old are affected, and the proportion escalates from the fifth decade of life [46].

### 4. Transrectal ultrasound

Watanabe et al. [47–50] introduced transrectal ultrasound (TRUS) in the middle of the 1970s. The technique has now become the most commonly used modality for imaging the prostate [7]. The main reasons for the widespread use of TRUS is its accurate determination of the volume of the prostate, its safety, ease of use, and above all, its role in guiding prostate biopsies [15].

A TRUS examination of the prostate captures sequences of transverse images from base to apex, and sagittal images from right to left [5]. Figure 2(a) shows a conventional greyscale transverse image through the base of the prostate of a 75-year-old man with confirmed cancer of GS 8 in the left base. Greyscale TRUS can sometimes visualize malignant lesions in the PZ (the TZ is too heterogeneous) since they often appear hypoechoic [5, 51], as exemplified in Figure 2(a). However, many benign occurrences, such as BPH, prostatitis, haemato ma, normal tissue and cysts can also be hypoechoic [52]. Actually, as low as 20% of the hypoechoic lesions turn out to be malignant at biopsy [4, 53]. Moreover, many tumours found at biopsy are isoechoic and cannot be discerned at TRUS [5, 6]. About 50% of non-palpable tumours larger than 1 cm are not ascertained by TRUS [54]. Thus TRUS is insufficient for PCa detection [55]. Another drawback is that TRUS is operator-dependent [56, 57]. Inexperienced readers attain relatively low specificities [7].

### 4.1. Doppler ultrasound

Doppler ultrasound (DU) measures the Doppler shift generated by flowing blood and may disclose PCa due to the neovascularization that often is observed in cancerous areas. Blood vessels with a diameter of about 1 mm can be resolved [58]. There are two different types of DU used for detecting PCa: colour DU (CDU) and power DU (PDU). In CDU a colour map is produced according to the mean Doppler frequency shift, whereas in PDU the colours correspond to the total energy of the Doppler signal [59]. Figure 2(e) shows a CDU image, which can be compared to
the PDU image of Figure 2(f) (these images are contrast enhanced). PDU was developed to address some problems encountered with CDU, such as high noise, angle dependence and aliasing [60].

CDU has demonstrated a high positive predictive value for detecting PCa, but a relatively low sensitivity [61]. According to some studies PDU does not improve the cancer detection rate over sextant SB [62, 63]. Others have shown that if additional tissue areas highlighted by PDU are biopsied, more tumours can be found with an optimized number of biopsy cores [64, 65]. CDU [66] and PDU [67, 68] may be valuable to distinguish BPH from PCa. The resistive index was significantly elevated in patients with BPH as compared to patients who were healthy or diagnosed with PCa [66, 67]. Sauvain et al. [64, 65] showed that PDU can be used to observe if tumours have extended through the capsule. Blood vessels that perforate the capsule are strong indicators of extracapsular spread of cancer.

4.2. Contrast-enhanced ultrasound

The resolution of the prostatic vascular system obtained by DU can be enhanced by injecting a sonographic contrast agent into the system. This makes it possible to resolve blood vessels with diameters down to 50–100 \( \mu m \) [58], and visualize the flow in the microscopic neovessels that multiply in PCa [18]. Both DU and greyscale ultrasound can employ contrast enhancement. Small gas-encapsulated microbubbles with a diameter < 10 \( \mu m \) are commonly used
as contrast agents [2, 58]. The lifetime of the bubbles is \(<\ 5\ \text{min}\ [58].\) How the microbubbles react to the ultrasound depends on its intensity [58]. At relatively low intensities the bubbles are compressed and expanded in a linear manner, and the bubbles then strongly reflect the incoming wave. Nonlinear deformation of the bubbles occurs at higher intensities, giving rise to echoes containing higher harmonics of the incident frequency. If the acoustic power is further increased the bubbles will eventually burst, yielding a strong echo.

High energy ultrasound that makes the microbubbles burst is utilized for the Doppler technique [18]. Figures 2(e) and 2(f) show a contrast-enhanced (CE) CDU and a CE PDU image, respectively. The latter can be compared to the unenhanced PDU image in Figure 2(b), in which the tumour is not visualized.

The use of contrast agents in conjunction with DU augments the PCA detection rate significantly, as compared to SB [18, 61]. Frauscher et al. [69, 70] performed two prospective studies comparing the capability of 10-core SB and CE CDU-targeted biopsies, withdrawing \(<\ 5\ \text{cores},\) to detect PCAs. The targeted approach detected at least as many tumours, and more tumours of high GS, as SB. The patient cohorts were 84 and 230 men, respectively. Mitterberger et al. [71, 72] recently confirmed these findings in two prospective randomized trials examining 100 and 690 men. It was shown that targeted biopsy detected tumours of significantly higher GS as compared to SB [71]. They also found that premedication with dutasteride seemed to reduce blood flow in benign prostatic tissue [73]. This may reduce false positives e.g. from prostatitis [73].

Greyscale ultrasound can efficiently visualize the vascular system using contrast agents and the continuous harmonic imaging (CHI) mode. CHI uses intermediate ultrasound effect, which induces nonlinear microbubble deformation, and reduces microbubble destruction as compared to CE DU. As a result the microbubbles can penetrate further into the microcirculation, and the resolution of CHI is augmented as compared to DU [18]. Higher harmonics of the fundamental frequency, primarily originating from the microbubbles, are filtered out. Modern technology applies broad-bandwidth ultrasound pulses and phase inversion, i.e. two pulses 180° out of phase are emitted, and as a consequence, the fundamental frequency will cancel out [18]. Further prolonged survival of the microbubbles can be achieved by applying intermittent harmonic imaging (IHI), which reduces the frame frequency and thus the ultrasound effect [18]. The efficiency of CHI and IHI is demonstrated in Figures 2(e) and 2(d), respectively.

Evaluations of the CHI and IHI techniques have shown that they perform significantly better than sextant SB per core, but not in total (smaller numbers of targeted biopsies than systematic biopsies have been used) [74, 75]. Halpern et al. [75] examined 301 patients, and cancer was found in 15.5% (175/1133) of the targeted areas versus 10.4% (188/1806) of sextant cores (\(p\ <\ 0.01).\) The directed areas were selected based on images from CE ultrasound using both CHI and IHI, as well as CDU and PDU. The probability that a core was cancerous was doubled for the targeted cores (\(p\ <\ 0.001).\) The area under the receiver operating characteristic curve (AUC) was at best (for IHI) 0.65. False positives, often coupled to BPH, occur frequently when using CE ultrasound [18]. That was also observed in a study including 12 patients where whole-mount prostatectomy was used as reference [76]. Several studies have excluded the TZ to decrease this problem [70, 72].

More sensitive CE ultrasound techniques are being developed, such as the Cadence contrast-pulse sequence technique, which can construct images showing solely microbubble contrast [11, 77]. Detailed information about this technique can be found in [78]. A promising initial study with 20 patients using targeted biopsies found that suspicious lesions were cancerous in 8/11 cases [11]. Yang et al. [79] used the technique to guide additional biopsies to suspicious sites in 115 patients who underwent sextant SB. They concluded that it was more accurate than TRUS and PDU, and could have spared a significant number of patients from unnecessary biopsies.

### 4.3. 3D ultrasound

TRUS can be extended to three dimensions by constructing a 3D image from a series of 2D images [80]. Advanced systems can produce real-time 3D images by means of multiplanar ultrasound probes [7]. 3D images may be easier to interpret than their 2D counterparts [80, 81], and 3D-TRUS can potentially reduce the inter-observer variability experienced for 2D-TRUS [80]. Only a few 3D-TRUS studies on relatively small patient cohorts have been conducted to date. They indicate that 3D-TRUS is not significantly better than 2D-TRUS for detecting PCa [61, 81], but significantly improves the local staging accuracy [80]. The 3D technique can also be applied to DU and CE ultrasound. Hitherto few investigations have explored these possibilities. Examining 282 men with suspected PCa (PSA > 4 ng ml\(^{-1}\)), Sauvain et al. [64] found that 3D PDU achieved 92.4% sensitivity and 72% specificity in detecting PCas as compared to 87.9% and 57.6%, respectively, for standard TRUS. That study showed a remarkably high accuracy, but the authors admit that the accuracy was elevated due to the study design. Bogers et al. [82] correlated 3D CE PDU and biopsy outcome in 18 patients and showed that the sensitivity was markedly higher, 85% as compared to 38%, than for unenhanced 3D PDU images. Using the same technique, Sedelaar et al. [83] detected 86% of the tumours evident at prostatectomy specimens from 70 patients, an improvement over the 61% reached with sextant SB.
4.4. Elastography

Elastography is a technique that applies ultrasound in conjunction with external tissue compression to evaluate the elasticity, or hardness, of tissue [84]. Hard lesions can indicate PCa. A standard TRUS probe can be used for imaging the prostate, and a force that compresses the gland is applied manually by the physician via the probe [85]. The elastogram (strain image) is generated in real time by imaging the prostate, and a force that compresses the gland indicates PCa. A piezoelectric element changes shape if exposed to an electric field, and it will therefore oscillate in response to a sinusoidal voltage variation. The resonance sensor system can be made very small, as demonstrated by Eklund et al. [93], who used a catheter tip sensor to study the stiffness of different prostate tissue types. A miniaturized instrument could potentially be used in conjunction with ultrasound to guide biopsies [94]. Three in vitro studies carried out by Jalkanen et al. [23, 94, 95] have shown that the resonance sensor can distinguish cancerous tissue from healthy glandular epithelium. However, stroma or a large concentration of prostate stones (a benign occurrence) could not be differentiated from cancer [23]. These authors used a system that merges the type of resonance sensor described by Omata and Terunuma with force and position sensors. Lindberg et al. [96] confirmed these results employing another resonance system that incorporates a counter balance arrangement. The sensing volume of the resonance sensor was recently assessed by Jalkanen et al. [95]. It was concluded that the topical system with a hemispherical tip laterally senses a larger area than the actual contact area and has an estimated penetration depth of 3.5-5.5 mm for an impression depth of 1 mm.

6. Magnetic resonance imaging (MRI)

6.1. T1- and T2-weighted MRI

MRI can depict the interior human body by analysing the magnetic resonance (MR) signal that is generated by the hydrogen* nuclei in the examined tissue, when the tissue is being exposed to a strong external magnetic field and is irradiated with a radio wave [97]. There are two main types of signal-generating schemes used in MRI, corresponding to the two MR relaxation times: T1-weighted (T1W) and T2-weighted (T2W) sequences. T1W sequences cannot depict the interior of the prostate but are useful for observing post-biopsy haemorrhage inside the gland, as seen in Figure 3(b) [56]. The anatomy and the internal architecture of the gland can be clearly visualized on T2W images, as demonstrated in Figure 3(a). Most prostate examinations are currently carried out using a pelvic coil in combination with an endorectal coil, at a field strength of 1.5T [98]. In the T2-mode PCa may be seen as areas with low signal strength. However, it is hard to discern tumours in the central gland (the TZ and the CZ are together termed the central gland) due to the overall low intensity in this region [99]. MRI shows a high staging accuracy for PCa [56].

*In human tissue it is predominantly the hydrogen nucleus, abundant in water and lipids, that yields a signal [97].
Tumours are often confused with BPH in the central gland [31, 56]. Furthermore, some benign features, such as prostatitis, haemorrhage and scarring, as well as sequelae of various therapies, can also be characterized by low intensity [2]. Consequently, the specificity of MRI to detect PCa is reduced to about 50% [31]. The estimated sensitivity of MRI to detect PCa varies widely in the literature; figures of 50–96% were demonstrated during the 1990s and 2000s using endorectal coils and whole-mount prostatectomy specimens as reference [31]. The discrepancies probably arise mainly because different criteria for a positive result have been applied, whether or not incidental (many studies exclude tumours < 0.5 ml) and/or TZ tumours are included, and how many segments the prostate is divided into for the analysis [31]. Inter-observer variability is also likely to affect the outcomes [56], and the employment of different techniques [100]. Accordingly, studies that directly compare SB and MRI are particularly valuable [31]. Wefer et al. [101] compared MRI, magnetic resonance spectroscopic imaging (MRSI) and SB for sextant localization of PCa in 47 patients, referring to prostatectomy histology as reference. MRI was more sensitive, 67% (MRSI 76%) versus 50%; but less specific, 69% (MRSI 57%) versus 82%, than SB. Bias may have been introduced since almost half of the patients had their biopsy performed elsewhere. In a retrospective study including 106 men with PCa, Mullerad et al. [102] showed that MRI added significant incremental value to DRE and sextant SB ($p < 0.0001$).

A study published in 2006 [103] sheds new light on the role of MRI in detecting TZ PCa. Two readers achieved sensitivities of 75% and 80% and specificities of 87% and 78%, respectively, for identification of patients with diagnosed TZ PCa by recognizing specific characteristic imaging features of malignant lesions. That study was retrospective and the proposed imaging features have to be further tested [103].

### 6.2. Magnetic resonance spectroscopic imaging

Magnetic resonance spectroscopic imaging (MRSI) extends the possibilities of MRI to detect PCa by allowing assessment of molecular constituents of the tissue. The resonance frequency of the hydrogen nucleus depends on the chemical environment, which determines the characteristics of the local magnetic field. For example, the frequency of hydrogen situated in fat is shifted slightly from the frequency of hydrogen in water. The fact that PCa has a higher metabolism than healthy tissue is manifested in changes in the metabolic spectra. MRSI can quantify the metabolites citrate (Cit), choline (Cho), creatine (Cr) and various polyamines (PA). PCa exhibits an increased concentration of Cho and often a reduction of Cit, whereas the level of Cr does not seem to be affected by the disease [104]. PCa is also distinguished by lower levels of PA [105]. The peak of PA lies just between the Cho and Cr peaks in the MRSI spectrum and therefore usually cannot be clearly

![Figure 3. Gland-confined PCa. (a) Axial MRI of the prostate using an endorectal coil demonstrates a tumour (white arrow) presenting as focal low signal in the PZ on T2W imaging (a) and intermediate signal on the T1W imaging (b). The tumour appears as a more focal lesion than the thin, curvilinear, low signal structures representing collagen strands in the PZ (arrowheads) on the T2W image. The periphery of the gland is well defined and the fat plane (black arrow) between the gland and the neurovascular bundle on the left is retained, implying no capsular penetration including no neurovascular bundle invasion. (Reprinted from Kundra, V., 2006, Prostate cancer imaging. Seminars in Roentgenology, 41, 139–149. Copyright © 2006, with permission from Elsevier).](attachment://image.png)
resolved unless state-of-the-art equipment is used [104]. However, a relatively deep dip between the Cho and Cr peaks is indicative of PCa [105]. Figure 4 shows how MRSI is used in conjunction with MRI to detect PCa.

The most commonly used method to detect PCa due to changes in the metabolic spectrum is to calculate the ratio \[
\frac{\text{Cho} + \text{Cr}}{\text{Cit}}
\]
where the amount of each metabolite is determined from the area of the corresponding peak [104]. The Cr peak is included for ease of quantification because it is adjacent to the Cho peak [106]. Jung et al. [107] suggested a standardized interpretation system for metabolic spectra. Adjustments were made to the initial scoring to account for elevation of the Cho/Cr ratio and for decreased concentrations of PA, and to consider poor S/N. In the study, 37 men scheduled for radical prostatectomy were evaluated with MRI and MRSI in the PZ. An accuracy of 74.2–85.0% with AUC of 0.87–0.89 for the two readers was attained.

However, only clearly benign and malignant voxels were analysed, and 15 out of 37 patients were excluded because the spectral quality was poor. The inter-observer agreement was excellent. Fütterer et al. [108] used the standardized interpretation system presented by Jung et al. [107], in a prospective study using MRI and MRSI to localize PCa in the whole prostate. The patient cohort consisted of 32 men that would undergo radical prostatectomy. The resulting accuracies were 81% and 87%, the AUC 0.85–0.86 and 0.86–0.91 (three readers) for the PZ and the central gland, respectively. However, many voxel regions were assigned unusable. Good inter-observer agreement was observed.

Wang et al. [109] performed a meta-analysis of the literature to assess the accuracy of MRSI for PCa detection, based on the \[
\frac{\text{Cho} + \text{Cr}}{\text{Cit}} > 0
\]
used as a threshold for definitive tumour, the pooled weighted sensitivity, specificity and AUC with corresponding 95% confidence intervals were 64% (55, 72), 86% (79, 91) and 82.7% (68.73, 96.68), respectively.

The specificity of MRI can be increased substantially by the addition of MRSI [10, 110]. Scheidler et al. [111] reported, on the basis of a sextant division of the prostate, a sensitivity of 95% and a specificity of 91% when MRI was used in conjunction with MRSI to retrospectively detect PZ PCa in 53 patients. The MR findings were compared to prostatectomy histology. The MRI detection accuracy of extracapsular extension, as interpreted by less experienced readers, is also significantly increased (AUC = 0.86 vs AUC = 0.75, \( p < 0.05 \)) by inclusion of MRSI [112]. The study population was essentially the same as in Scheidler et al. [111].

MRSI may also be valuable for assessing the aggressiveness of PCa. It has been shown that the \[
\frac{\text{Cho} + \text{Cr}}{\text{Cit}}
\]
correlates with the GS [108, 113, 114]. Zakian et al. [114] examined 123 patients and performed prostatectomy histology.

Two studies recently demonstrated that new clinical models that include MRI and MRSI data can predict the probability of organ-confined [115] and indolent [116] PCa, defined as small organ-confined low-grade tumours, better than the nomograms currently in use. A recent study showed that MRSI may potentially be used to predict the
absence of PCa in men with PSA of 4–10 ng ml\(^{-1}\) [117]. Thus, unnecessary repeated biopsies may be avoided.

### 6.3. Dynamic contrast-enhanced MRI

MRI can take advantage of the increased vascularity of PCa with the help of contrast agents. Dynamic contrast-enhanced MRI (DCE-MRI) captures T1W images of high spatial resolution while a dose of gadolinium chelate is injected [5, 10]. Tumours can be discerned since they usually show early signal enhancement and washout of signal intensity, but not all tumours fit into this pattern [5]. Besides, BPH often exhibits similar enhancement curves [31]. There is no standardized way to analyse the enhancement curves, but many studies indicate that tumours are best depicted 30–90 s after injection [31]. Several prospective studies using DCE-MRI in addition to T2W sequences, correlating their findings with prostatectomy histology, have shown that the technique significantly enhances the PCa detection rate using only T2W MRI [31, 118–121]. Sensitivities up to 90% were reported for detecting PZ PCa. In addition, Hara et al. [118] demonstrated a sensitivity of 93% and a specificity of 96% in a patient cohort of 90 men not previously diagnosed with PCa, but with elevated PSA level (2.5 < PSA < 10 ng ml\(^{-1}\)). A 14-core SB was used as reference. Note that each core was divided into three subcores for a total of 42 subcores, which partly accounts for the excellent specificity [31]. A study by Vos et al. [123] indicates that it is feasible to apply computerized analysis of prostate lesions based on DCE-MRI. Using prostatectomy histology correlation, they reached a PCa detection accuracy of 0.83 (AUC) in a patient group consisting of 34 men with PZ PCa. Among the MR techniques, DCE-MRI is in particular expected to benefit from the use of 3T MR scanners [9, 124].

### 6.4. Diffusion-weighted imaging

An interesting MRI technique is diffusion-weighted imaging (DWI), which can measure the diffusion of water molecules in tissue, and hence provide information on the structural organization of the tissue. Cancer generally shows restricted diffusion, probably due to increased cellular density [125]. Several recent studies, some using 3T MR systems, show that DWI used in conjunction with T2W MRI significantly improves PCa detection as compared to MRI alone [126–132]. Only three of the studies [126, 127, 132] used prostatectomy histology correlation. In the study by Morgan et al. [129], an inexperienced reader did not obtain better results by adding DWI to T2W MRI, raising concerns about inter-observer disagreement. Mazaheri et al. [133] examined 38 men scheduled for prostatectomy, and found that DWI in combination with MRSI performed significantly better than MRSI alone. Recent studies also show that DWI findings may indicate tumour aggressiveness [134, 135]. DWI shows high spatial resolution and high contrast between cancerous and healthy prostate tissue, and enables short acquisition time [10, 136]. However, considerable individual variations of the apparent diffusion coefficient (ADC) have been reported [137, 138]. Another limitation is susceptibility-induced distortions [139].

### 7. Vibrational spectroscopy

Raman spectroscopy (RS) and infrared (IR) spectroscopy, jointly termed vibrational spectroscopy (VS), utilize the fact that a molecule can start vibrating at a specific frequency in response to light irradiation [140]. The vibrational motion requires energy and a scattered photon undergoes a wavelength shift that equals the frequency of the excited vibrational mode of the particular molecule. Hence, different molecular compositions produce unique spectra. The biochemical information extracted by VS can reveal the presence of cancer, which alters the molecular composition and structure of tissue [141].

Many in vitro studies indicate that IR spectroscopy can differentiate PCa and healthy tissue [142–147]. Gazi et al. [148] showed that diagnostic classification (linear discriminant analysis) of Fourier transform IR spectroscopy (FTIR) spectra correlated to the GS (< 7, = 7 and > 7) in paraffin-embedded prostate tissue. A sensitivity and specificity of ≥ 70% and ≥ 81%, respectively, were obtained. Furthermore, the method performed similarly to Gleason grading in predicting metastatic tumours according to the TNM system. This has also been shown by Malins et al. [149]. A study conducted by Bogomolny et al. [150] suggested that FTIR can detect malignancies early on. Cells in culture were infected by a virus that induces malignant transformation, and spectral changes occurred several days earlier than visible morphological alterations. Fernández et al. [143] and Bhagwati et al. [142] developed a set-up capable of high throughput assessment of cells and tissues, reaching extremely efficient classification of different tissue types and pathologies. In vitro applications are impeded by the very short penetration depth of IR fibre optic probes, due to the fact that water very strongly absorbs IR radiation [141].

The group of Crow and Stone [151–155] have evaluated the potential of RS to detect and grade PCa in several recent studies. They have shown that using a Raman micro spectrometer with near-IR (832 nm) laser and linear discriminant analysis, BPH and PCa of different biological aggressiveness (GS < 7, = 7 and > 7) can be identified with an overall accuracy of 89% [152]. The in vitro samples were obtained from biopsies of 27 patients, 14 with BPH and 13 with PCa. Studying cultured PCa cell lines the group also found that the stationary Raman system can almost perfectly, with an overall sensitivity of 98% and a specificity of 99%, differentiate between cell lines of varying
8. Computer-aided detection and diagnosis

Many studies have shown that computerized interpretation of data can improve the accuracy of PCa detection and prediction of the pathologic stage [20]. Artificial neural networks and support vector machines are powerful tools for handling complex and possibly nonlinear relationships between variables, and these are promising for improving the accuracy of nomograms. High accuracies have been demonstrated in some studies, but the full potential has not been elucidated yet [20]. Computerized methods for identifying cancerous tissue in images obtained by ultrasound and MRI are being investigated intensively [11, 123, 157–161]. For example, Mohamed et al. [57] reached an accuracy of 93.75% for automatic detection of PCa in greyscale ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest, ranks ultrasound images. They used an algorithm that segments the ultrasound image into regions of interest. The group has also recently examined the biochemical basis of different pathologies within the prostate with RS [155]. The gross biochemistry of BPH, prostatitis and three grades of PCa (GS < 7, = 7 and > 7) was estimated. It was found that the DNA content increased in cancerous tissue. Moreover, the cholesterol level increased remarkably, choline was elevated but remained low, triolein was increased, whereas oleic acid decreased somewhat with progression of disease.

9. Discussion

Table 1 provides an overview and comparison of the methods included in this review. The advantages and disadvantages of each technique, and concluding remarks, are given. This discussion section is based on Table 1.

CE ultrasound has been shown to outperform today’s gold standard by detecting more clinically significant tumours with fewer cores [11, 162, 163]. However, the image reading is subjective and the inter-observer agreement is not satisfactory [11, 18, 75]. Work is ongoing to develop computer-assisted detection for CE techniques [11, 164]. Contrast agents bring high costs per core, but CE techniques may be cost-effective due to a reduction of the number of cores [69]. Frequent false-positive findings have been reported. Premedication that reduces blood flow in benign lesions can potentially reduce the number of false positives [73]. A report on a multicentre European coordination project [77] provides good evidence of the safety of CE ultrasound. Unfortunately, the project did not achieve its goal of initiating a multicentre study that could prove an additional clinical value of CE ultrasound. However, the authors state that such a study will be initiated.

Elastography is a promising new method that may improve PCa detection. It is not yet feasible, due to technical limitations, to implement an absolute tissue hardness threshold that indicates cancer and enables objective interpretation [88]. No data have been presented that evaluate intra- and inter-observer variability. The main limitation of elastography seems to be that benign lesions, primarily prostatitis and BPH, can be hard to distinguish from tumours [86, 88].

As can be deducted from Table 1 the new MR techniques are very promising. They show good potential for highly accurate detection, and for excluding disease and decreasing the need for repeated biopsy. The performance of T2W MRI is not sufficiently accurate for its use to be advised in screening [2, 31, 61]. MRSI in conjunction with MRI has shown high accuracies for detecting PCa in many studies, and the combination holds promise for non-invasively grading tumours and predicting indolent disease. New metabolic biomarkers may further improve the grading accuracy of MRSI [165, 166]. Furthermore, interpretation of the metabolic profile can be standardized and computerized to eliminate inter-observer disagreement. Drawbacks of MRSI are the long acquisition time, the high frequency of spectral artefacts and low spatial resolution at 1.5T [10, 136, 167]. The addition of DCE-MRI to T2W sequences significantly improves the detection rate [31]. DCE-MRI shows limitations in differentiating PCa from prostatitis and BPH [139]. There are concerns about the safety of gadolinium-based contrast agents [168]. Moreover, DCE-MRI has difficulties discerning TZ tumours [136]. DWI shows good potential for improving MRI detection, but more studies are needed to assess its accuracy [10, 167]. Individual variations of the ADC may impair diagnostic performance [136]. The diagnostic performance of MR techniques may be further improved at 3T [9].

A combination of several MR techniques (MRI, MRSI, DCE-MRI and DWI) is likely to detect PCa most efficiently. According to Kirkham et al. [31] it is reasonable to hope that multiparametric MRI will detect about 90% of
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>CDU</td>
<td>Resolves blood vessels down to 1 mm in a colour map according to mean Doppler frequency shifts due to blood flow.</td>
<td>High positive predictive value.</td>
<td>Low sensitivity. Resolution insufficient to visualize neovascularization. Inter-observer disagreement.</td>
<td>Yes</td>
<td>No improvement over SB.</td>
</tr>
<tr>
<td>PDU</td>
<td>Like CDU, but colour map is produced according to the total energy of the Doppler signal.</td>
<td>Low noise. Some studies show beneficial value of targeted biopsy. Can detect extracapsular spread of cancer.</td>
<td>Resolution insufficient to visualize neovascularization. Inter-observer disagreement.</td>
<td>Yes</td>
<td>No substantial advantage over CDU.</td>
</tr>
<tr>
<td>CE DU</td>
<td>Microbubbles injected into the vascular system. Augments Doppler signal and enables resolution of microscopic vessels.</td>
<td>High resolution visualizes neovascularization. Detects more clinically significant tumours than SB with fewer cores.</td>
<td>False positives frequent. High costs per core. Inter-observer disagreement.</td>
<td>Yes</td>
<td>Targeted biopsy approach outperforms SB.</td>
</tr>
<tr>
<td>CE greyscale ultrasound</td>
<td>Echoes from microbubbles contain higher harmonics and can thus be distinguished from signals from tissue.</td>
<td>Intermediate energy ultrasound ensures prolonged survival of microbubbles, gives higher resolution than CE DU. Outperforms SB.</td>
<td>False positives frequent. Inter-observer disagreement.</td>
<td>Yes</td>
<td>Targeted biopsy approach outperforms SB.</td>
</tr>
<tr>
<td>Elastography</td>
<td>TRUS in conjunction with manual tissue compression evaluates tissue elasticity. Hard lesions indicate PCa.</td>
<td>High sensitivity. Based on standard TRUS. No need for contrast.</td>
<td>False positives frequent. No data on inter-observer variability.</td>
<td>Yes</td>
<td>Promising for targeted biopsy. A high number of false positives seems to be the main limitation.</td>
</tr>
<tr>
<td>Resonance sensor technology</td>
<td>The resonance frequency of a piezoelectric element changes when set in contact with a material. Can assess tissue stiffness.</td>
<td>Tumours developing in soft glandular epithelium, abundant in posterior area, can be detected. Catheter tip sensor can be miniaturized.</td>
<td>Cannot distinguish cancer from hard prostate tissue (stroma, prostate stones). Hard to set the borders of cancerous tissue.</td>
<td>No</td>
<td>Experimental technique with high potential for in vivo PCa detection.</td>
</tr>
<tr>
<td>MRI</td>
<td>Hydrogen nuclei exposed to a strong external magnetic field generate MR signal when irradiated by a radio frequency wave.</td>
<td>High staging accuracy. MR1/MRSI data show potential to predict indolent disease.</td>
<td>Low specificity. High costs. Inter-observer disagreement. Targeted biopsy is time-consuming and costly.</td>
<td>Yes</td>
<td>Low specificity (~50%) means insufficient accuracy for detecting PCa.</td>
</tr>
<tr>
<td>MRSI</td>
<td>MR technique allowing assessment of molecular constituents—metabolites. PCa shows an increased metabolism.</td>
<td>MRSI/MRI shows high accuracy. Potential to non-invasively grade tumours and predict indolent disease. Objective method—good inter-observer agreement.</td>
<td>Long acquisition time. Spectral artefacts common. High costs. Coarse spatial resolution at 1.5 T.</td>
<td>Yes</td>
<td>Due to its high accuracy and potential non-invasive grading capacity, MRSI is a very promising MR technique.</td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>CE MRI enables visualization of prostate vasculatization.</td>
<td>Addition of DCE-MRI to T2W sequences significantly improves BPH and prostatitis cause false positives. Limited visibility of TZ.</td>
<td></td>
<td>Yes</td>
<td>The addition of DCE-MRI to T2W sequences</td>
</tr>
</tbody>
</table>
significant cancers. Early studies show promising results [169–171]. However, it remains to be solved how such large amounts of data should be optimally analysed [10].

Although MRI could be used for guiding biopsies, the procedure is more complex and time-consuming than TRUS, and the clinical practicability is questionable [31, 172, 173]. Presently, there is a lack of accuracy of fusing MRI and TRUS images; better methods are needed to utilize MR data in TRUS-directed biopsy [167, 174]. In the future MR-guided biopsy may be performed via robotic systems, and preliminary studies have shown that accurate needle placement is feasible [173, 175]. High costs and limited availability of MR scanners at present impedes the feasibility of MRI [139].

Experimental techniques, which are summarized in Table 1, such as resonance sensor technology and RS, are also interesting. These methods have so far only been evaluated in vitro. The resonance sensor has potential for non-invasively detecting PCA, but the diagnostic accuracy must be improved. Spectroscopic techniques are promising for objective PCA detection and diagnosis. RS has shown an excellent capability to distinguish between cancerous and healthy tissue as well as grading PCs. However, RS cannot detect PCs non-invasively due to a shallow penetration depth [176]. Development of techniques that can sense deeper into the tissue, such as time-gated RS [176] and spatially offset probes [177], is ongoing. Minimally invasive examinations of the prostate are possible since Raman fibre optic probes thinner than the diameter of common clinical devices such as needles and endoscopes, typically 1–2 mm thick [178], have been demonstrated [179]. Spectroscopic techniques have great potential for aiding histopathologic examinations [180], which are subjective and time-consuming. A questionnaire indicates that the long waiting time for biopsy results causes the most anxiety in patients undergoing screening for PCA [181]. Novel techniques for PCA detection are needed, since both ultrasound and MR techniques show many limitations [182].

Computer-aided detection and diagnosis is likely to play an important role in the future. Intra- and inter-observer variability is a problem with both ultrasound and MR technology. Morphologic and/or functional images are hard to interpret. The high and increasing clinical incidence of PCs presents the urologist with an ever increasing amount of data to analyse. Computer-aided detection and diagnosis have the potential to evaluate complex data objectively, consistently, and in a time- and cost-efficient manner.

To distinguish between indolent and aggressive tumours is of utmost importance for improving patient care. There is a range of treatment options available today, and minimally invasive therapies are gaining interest among patients since radical prostatectomy is associated with potential morbidity [183, 184]. More precise disease

Table 1. (Continued).

<table>
<thead>
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<tr>
<td>DWI</td>
<td>MR technique that can measure the ADC of tissue. PCa shows reduced diffusion.</td>
<td>Initial studies indicate significantly higher detection rate using DWI + T2W MRI. Fast. High contrast between cancerous and healthy tissue. High spatial resolution.</td>
<td>Variation of the ADC between different individuals. Susceptibility-induced distortions. High costs.</td>
<td>Yes</td>
<td>DWIN seems to improve MRI detection accuracy.</td>
</tr>
<tr>
<td>RS</td>
<td>Monochromatic light changes polarizability of molecules, which start to vibrate—inelastic scattering of light induces wavelength shift.</td>
<td>In vitro studies show very high accuracy. Can objectively distinguish different grades of malignancy.</td>
<td>Low penetration depth of current fibre optic probes excludes non-invasive detection. Laser light might affect tissue.</td>
<td>No</td>
<td>Experimental technique with high detection and grading accuracies.</td>
</tr>
<tr>
<td>IR spectroscopy</td>
<td>Measures absorption of polychromatic IR radiation, which changes the dipole moment of the interacting molecules.</td>
<td>In vitro trials demonstrate potential to objectively detect and grade PCa. Some studies indicate similar predictive capacities as the GS.</td>
<td>Difficult to examine tissue with fibre optic probes.</td>
<td>No</td>
<td>Experimental technique with potential to detect and grade PCa. In vivo measurements difficult.</td>
</tr>
</tbody>
</table>
characterization is a major objective of PCa imaging [8]. CE ultrasound and elastography data show correlation with the GS. However, in our opinion MR techniques are more promising for grading cancer, since MRs enables assessment of the chemical contents of the tissue.

A large number of studies on PCa detection and diagnosis are published every year. However, only a few studies follow a rigid study protocol, and the majority of publications contribute little to assessing the clinical value of the methods. This was stressed in the meta-analysis of MRs reports performed by Wang et al. [109], where only seven MRs studies met the inclusion criteria, and merely two studies qualified as grade A according to the criteria of diagnostic research published by the Cochrane centre. Long-term follow-up of the clinical significance of the detected tumours is rarely carried out [18]. The heterogeneity of the design and outcome of different studies blurs the picture of whether new ultrasound and MR methods add significant clinical value. The discrepancy of the reported sensitivity of T2W MRI, reviewed in [6.1], is a clear example. Studies that correlate the imaging findings with SB do not estimate the sensitivity reliably [109]. Comparisons between SB and targeted biopsy are valuable, however, and relatively many studies using ultrasound techniques have been published. The first studies using MR-guided biopsy, which has not been feasible before, are now being published.

To obtain a clear picture of the clinical usefulness of different techniques large-scale multi-centre studies of high quality are necessary [77, 109]. The lack of such publications may be explained by several factors including limitations of funding, intellectual property rights, sharing of data, refinement of the technique during the study period, and parochial interests such as individual career advancement [185]. To gain clinical acceptance of new methods they must be standardized, and clinical benefits and cost-effectiveness must be clearly shown. There are several ultrasound and MR techniques that have the potential to increase the clinical detection rate and diagnostic accuracy for PCa.

10. Conclusion

The current gold standard of PCa detection, SB, has limited sensitivity and difficulties evaluating tumour aggressive- ness. PSA screening leads to over-treatment of many men and its value for reducing PCa mortality is controversial. To detect and grade PCa early on remains challenging. Recent developments of ultrasound techniques indicate that it is possible to disclose more clinically significant cancers with a reduced number of cores by means of targeted biopsy approaches. Advances of MR techniques significantly improve the detection accuracy of MRI, and multiparametric MRI is very promising. Inter-observer disagreement is an issue with both conventional MRI and ultrasound. MRIs is the first method to offer an objective way of assessing PCa, but computer-aided detection based on CE and standard ultrasound and MR techniques is emerging as well. Limited availability of MR scanners, the questionable practicability of MR-directed biopsy, and high costs limit the usefulness of MR methods at present. There are also alternative experimental techniques, resonance sensor technology and VS that have shown promising results in vitro. Appropriate tools for updating the gold standard are available, but large-scale prospective multi-centre studies are needed to determine the clinical benefits of these new methods.

Acknowledgements

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References

Localization and diagnosis of prostate cancer


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Authors:
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Effects of snap-freezing and near-infrared laser illumination on porcine prostate tissue as measured by Raman spectroscopy†

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Most Raman spectroscopic studies on tissue are performed in vitro. To assure that the results are applicable to in vivo investigations, it is of utmost importance that preparation protocols and measurement procedures of tissue for in vitro studies should preserve tissue characteristics close to the native state. This study had two aims. The first was to elucidate if photoinduced effects arise during 5 minutes’ continuous illumination of tissue with an 830 nm laser at an irradiance of ∼3 × 104 W/m2. The second was to investigate the effects of snap-freezing of porcine prostate tissue in liquid nitrogen and subsequent storage at −80 °C, by means of multivariate analysis. 830 nm laser illumination of the specified irradiance did not affect the Raman spectra. A decrease of the spectral background was observed, likely due to photobleaching of tissue fluorophores. Snap-freezing and subsequent storage at −80 °C gave rise to subtle but significant alterations in Raman spectra, most likely related to changes in the protein conformations.

1 Introduction

Raman spectroscopy is a valuable tool for the detection of various forms of cancer, one of them being prostate cancer.1−3 However, many Raman spectroscopic studies on tissue are performed in vitro.4−6 To assure that the results are likely to be applicable to in vivo investigations, it is of utmost importance that preparation protocols and measurement procedures of tissue for in vitro studies preserve tissue characteristics as close to the native state as possible. Changes of the examined tissue induced by inappropriate preparation protocols can obscure differences between malign and healthy tissue.

Correct interpretation of tissue spectra requires good knowledge about how laser light affects the tissue. Otherwise, photoinduced effects may be confused with spectral fingerprints of different pathologic states. For in vivo measurements, it must be ensured that the laser illumination causes no harm to the examined tissue. It is well-known that ultraviolet and near-visible radiation can damage DNA and cells, but there is less literature available for visible and near-infrared (NIR) light of high intensity. Studies on single cells indicate that wavelengths ≤568 nm cause sample degradation, whereas longer wavelengths cause less or no damage.7−8 For example, Puppels et al.7 studied single cells and chromosomes and showed that sample degradation occurred with 514.5 nm but not with 660 nm laser light. There appears to be only a single publication† using NIR light (1064 nm) examining photoinduced effects on excised fresh tissue.

Only a few studies have examined the effects of different tissue preparation protocols such as snap-freezing,4,9,10 which is generally considered to be a good preservation method.10 However, the method should be applied with caution to other types of tissue that have not been previously studied.9

This Raman spectroscopic study aimed to elucidate if any photoinduced effects arise during 5 minutes’ continuous illumination of tissue with an 830 nm laser at an irradiance of ∼3 × 104 W/m2. Furthermore, the aim was to investigate the effects of snap-freezing of porcine prostate tissue in liquid nitrogen and subsequent storage at −80 °C, by means of multivariate analysis of Raman spectra.

2 Materials and methods

2.1 Sample preparation

Porcine prostate tissue, recovered from 2 healthy boars that were slaughtered at the local abattoir, was used for all experiments. Directly after slaughter a veterinarian removed the urethra with the attached prostate gland. It was enclosed in a plastic bag and refrigerated for <24 hours. To minimize sample degradation the prostate tissue was not removed from the urethra, and was protected by the fascia and surrounding tissue, until just before it was cut into smaller samples.

2.1.1 Photoinduced effects. One prostate was cut into tissue slices of approx. 1.5 × 5 × 3 mm, which were stored in phosphate buffered saline (PBS) and refrigerated.

2.1.2 Snap-freezing. One prostate was cut into small slices, approx. 2 × 3 × 2 mm. Five randomly picked samples were stored in PBS and used as reference for fresh samples. The remaining samples were snap-frozen concurrently to guarantee as comparative studies as possible. They were put into 2 mL...
cryovials and lowered into liquid nitrogen. To avoid local heating the cryovials were constantly moved during the freezing. They were then stored at −80 °C. Before measurement the snap-frozen samples were allowed to passively thaw immersed in PBS.

2.2 Raman spectroscopy

A Raman micro spectrometer (Renishaw system 2000), equipped with a 300 mW 830 nm laser and a water dip objective (Nikon NIR, Apo 60 ×1.0 W), was used for all experiments. This spectrometer has two acquisition modes: the extended mode, where a broad spectral window (in this study from 400–1800 cm⁻¹) is acquired by directing the Raman scattered light by a moving prism onto the grating; or the static mode, where fast spectral acquisition is recorded with a static prism. This mode only enables a small spectral window of ~500 cm⁻¹. The irradiance onto the samples was ~3 × 10⁷ W/m². The spectrometer was calibrated for wavelength shift daily by using a single Raman standard, a Raman micro spectrometer (Renishaw system 2000), equipped with a 300 mW 830 nm laser and a water dip objective (Nikon NIR, Apo 60 ×1.0 W), was used for all experiments. This spectrometer has two acquisition modes: the extended mode, where a broad spectral window (in this study from 400–1800 cm⁻¹) is acquired by directing the Raman scattered light by a moving prism onto the grating; or the static mode, where fast spectral acquisition is recorded with a static prism. This mode only enables a small spectral window of ~500 cm⁻¹. The irradiance onto the samples was ~3 × 10⁷ W/m². The spectrometer was calibrated for wavelength shift daily by using a single Raman standard. Principal component analysis (PCA) on unstandardized data was used as a first step to reduce the dimensionality of each data set. The first 10 principal components (PCs) were kept. Next, it was evaluated if there was any significant difference between the multivariate means of the PC scores for fresh and snap-frozen tissues. An adapted version of the original test proposed by Kim, described by Lix et al. was used. In the modified version, trimmed means and Winsorized variance-covariance replace the standard formulas. Trimming between 10 and 20% is recommended, and in this study 15% was chosen.

If a significant difference was found between the multivariate means of the two groups, univariate tests of the equality of means of the individual PC scores were performed. This was done to elucidate which PCs contributed strongly to the significant multivariate difference. Yuen's test was used for this purpose.

Matlab was used for the multivariate analysis, and most of the tools were written in-house. A value of p < 0.05 was regarded as statistically significant. It was assumed that the experimental design was completely randomized and that the observations were independent. Minitab (version 15.1.20.0) was used to check the validity of the assumption of independency by means of testing if significant autocorrelations were present.

3 Results

3.1 Photoinduced effects

No changes in the preprocessed Raman spectra due to the laser illumination during 5 min were observed. This was evaluated across the wavelength interval 400–1800 cm⁻¹, requiring three measurement series using the static mode. Fig. 1 shows the first spectrum of a measurement series from 459 to 961 cm⁻¹. Fig. 2 shows difference spectra for this series. No residual Raman peaks were seen in difference spectra. The total intensity of the background, calculated by integrating the fitted piecewise modified polynomial that was subtracted from all preprocessed spectra, diminished with increased laser illumination time. Fig. 3(a) shows a typical example of the diminishing background. The total intensity of the Raman signal, i.e., the integral of un-normalized background-reduced spectra, was unaltered by the laser illumination. Only random fluctuations were seen, as shown in Fig. 3(b) and confirmed by Fig. 2.

2.3.2 Investigation of snap-freezing effects. The Raman signal was integrated for 30 s and measured over a spectral range of 400–1800 cm⁻¹. The microscope focus was moved approx. 10–20 μm into the sample. Five samples were measured on day 1 and used as a reference of fresh samples. The snap-frozen samples were measured after 5, 26 and 81 days of storage to investigate if subsequent spectra were taken at the same tissue spot. All measurement series were reproduced five times and carried out within 4 days after slaughter.

The samples were immersed in PBS during the measurements to prevent dehydration of the tissue, which could disrupt the protein vibrational modes. To guarantee good signal quality, the microscope focus was moved 20 μm into the sample with a motorized z-stage. The samples were immobilized with needles. A bright-field microscope image was taken before and after each measurement series to ensure that subsequent spectra were taken at the same tissue spot. All measurement series were reproduced five times and carried out within 4 days after slaughter.

2.3 Measurement procedure

2.3.1 Investigation of photoinduced effects. The integration time was set to 10 s in the static mode to enable short measurement times. Spectra were acquired subsequently from the same tissue spot during the first minute, to capture rapidly occurring effects, and thereafter less frequently during an additional 4 min. To prevent dehydration of the tissue, which could disrupt the protein vibrational modes, the microscope focus was moved approx. 10–20 μm into the sample. Five samples were measured on day 1 and used as a reference of fresh samples. The snap-frozen samples were measured after 5, 26 and 81 days of storage to investigate if subsequent spectra were taken at the same tissue spot. All measurement series were reproduced five times and carried out within 4 days after slaughter.

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2.4 Preprocessing

Spectral spikes due to cosmic rays were removed prior to analysis. Correction for the energy sensitivity of the spectrometer was performed by measuring the spectrum of a calibrated light source and calculating the intensity wavenumber response curve. Each Raman spectrum was lightly filtered by the noise-reduction algorithm by Eilers. The background was automatically subtracted using the algorithm by Cao et al., which fits a piecewise modified polynomial to the spectrum. The spectra were vector normalized so that their integrated areas were equal. All preprocessing algorithms, except the one by Eilers, were written in-house and implemented in Matlab (version R2007b including Statistics Toolbox version 6.1).

2.5 Multivariate analysis on snap-freezing data

The preprocessed Raman spectra of fresh and snap-frozen tissues were compared by means of multivariate analysis. Three analogous analyses were carried out, fresh tissue was compared to tissue that had been preserved at −80 °C for 5, 26, and 81 days. Principal component analysis (PCA) on unstandardized data was used as a first step to reduce the dimensionality of each data set. The first 10 principal components (PCs) were kept. Next, it was evaluated if there was any significant difference between the multivariate means of the PC scores for fresh and snap-frozen tissues. An adapted version of the original test proposed by Kim, described by Lix et al. was used. In the modified version, trimmed means and Winsorized variance-covariance replace the standard formulas. Trimming between 10 and 20% is recommended, and in this study 15% was chosen.

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3.2 Snap-freezing

The first 10 PCs, explaining almost 90% of the variance, were selected for further evaluation. A Pareto chart for the PCA of fresh tissue and snap-frozen tissue stored for 5 days is shown in Fig. 4. The corresponding plots for snap-frozen tissue stored 26 and 81 days were similar (data not shown).

Applying the modified Kim’s test on the multivariate means of the PC scores showed a significant difference between the spectra of the fresh and the snap-frozen tissue. The $p$-values obtained were $1 \times 10^{-2}$, $2 \times 10^{-2}$ and $2 \times 10^{-6}$ for snap-frozen tissue stored at $-80 \degree C$ for 5, 26 and 81 days, respectively, as compared to fresh tissue. To elucidate which PCs gave rise to the multivariate difference Yuen’s univariate test was used on the individual PCs. The scores of a few PCs for each measurement series were significantly different. The $p$-values are shown in Table 1. The accompanying PCs to the significant scores are plotted in Fig. 1.

![Fig. 1](image1.png)

**Fig. 1** The first Raman spectrum of one of the measurement series evaluating 459–961 cm$^{-1}$.

![Fig. 2](image2.png)

**Fig. 2** Difference spectra for one measurement series. The spectra are spatially offset for clarity and plotted in order of increasing accumulated laser illumination from bottom to top. All spectra were compared to the spectrum in Fig. 1.

![Fig. 3](image3.png)

**Fig. 3** (a) The total intensity of the spectral background decreased with the laser illumination time. (b) The total intensity of the Raman signal did not change with the laser illumination time, only random fluctuations were seen.

### 3.2 Snap-freezing

The first 10 PCs, explaining almost 90% of the variance, were selected for further evaluation. A Pareto chart for the PCA of fresh tissue and snap-frozen tissue stored for 5 days is shown in Fig. 4. The corresponding plots for snap-frozen tissue stored 26 and 81 days were similar (data not shown).

Applying the modified Kim’s test on the multivariate means of the PC scores showed a significant difference between the spectra of the fresh and the snap-frozen tissue. The $p$-values obtained were $1 \times 10^{-2}$, $2 \times 10^{-2}$ and $2 \times 10^{-6}$ for snap-frozen tissue stored at $-80 \degree C$ for 5, 26 and 81 days, respectively, as compared to fresh tissue. To elucidate which PCs gave rise to the multivariate difference Yuen’s univariate test was used on the individual PCs. The scores of a few PCs for each measurement series were significantly different. The $p$-values are shown in Table 1. The accompanying PCs to the significant scores are plotted in...
Fig. 5. from which some common characteristics due to snap- freezing can be listed (tentative assignments from refs 18 and 19):
- decreased intensity of the phenylalanine (1006 cm⁻¹) and tryptophan (1548 and 760 cm⁻¹) peaks;
- the amide I peak is shifted: fresh tissue yields a more intense signal towards ~1690 cm⁻¹, whereas snap-frozen tissue shows higher intensity towards ~1660 cm⁻¹;
- increased intensity at ~1441 and ~1299 cm⁻¹, which may be assigned to various CH₂/CH vibrations of lipids and/or proteins;
- the amide III peak at ~1244 cm⁻¹ is decreased.

The changes are subtle and could not be seen when comparing mean spectra. Fig. 6 compares two spectra for day 26 where the differences are relatively large. These spectra were selected because their PC5 scores differed markedly.

No signs that the PC scores were time dependent were seen when studying the autocorrelation function in Minitab; the assumption of independent observations was thus valid.

Analyses comparing snap-frozen tissue stored at ~80 °C for different time-spans were also performed. They showed significant differences between these groups. The \( p \)-values obtained applying the modified Kim’s multivariate test were in the order of \( 10^{-9} \) larger than the \( p \)-values obtained comparing fresh and snap-frozen tissues.

**Table 1**

<table>
<thead>
<tr>
<th>Storage time</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
<th>PC6</th>
<th>PC7</th>
<th>PC8</th>
<th>PC9</th>
<th>PC10</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days</td>
<td>0.51</td>
<td>0.19</td>
<td>0.11</td>
<td>0.014</td>
<td>0.63</td>
<td>0.56</td>
<td>0.0074</td>
<td>0.12</td>
<td>0.26</td>
<td>0.34</td>
</tr>
<tr>
<td>26 days</td>
<td>0.56</td>
<td>0.31</td>
<td>0.056</td>
<td>0.47</td>
<td>0.0004</td>
<td>0.17</td>
<td>0.36</td>
<td>0.037</td>
<td>0.93</td>
<td>0.63</td>
</tr>
<tr>
<td>81 days</td>
<td>0.24</td>
<td>0.90</td>
<td>0.12</td>
<td>0.066</td>
<td>0.57</td>
<td>0.82</td>
<td>0.22</td>
<td>0.22</td>
<td>0.033</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5 The calculated PCs whose scores were proven to be significantly different for fresh and snap-frozen tissues. Fresh tissue obtained higher scores of these PCs. Note that PC8 for day 26 has been inverted because snap-frozen tissue obtained higher scores of this PC.

**Table 1** The \( p \)-values obtained when Yuen’s test was applied on the two group means of the individual PC scores, for snap-frozen as compared to fresh tissue. \( p \)-values < 0.05 have been written in bold.
4 Discussion

4.1 Photoinduced effects

No photoinduced effects on the Raman signal were observed at an irradiance of $\sim 3 \times 10^{10}$ W/m² at 830 nm during 5 min. Subsequent spectra were captured at relatively short intervals to study if effects occurred rapidly. To get a reasonable signal quality the integration time could not be less than 10 s. Measurements were acquired temporarily closer than in comparable studies.6-9 The total time was limited to 5 min as relevant integration times usually are <1 min. Our results agree with those of Notingher et al.10 who used 785 nm illumination at 1064 nm at an irradiance of $\sim 2 \times 10^6$ W/m² and found no evidence of heat damage in fat, muscle or liver. In lung tissue they observed changes in the microstructure.

The spectral background diminished with increased illumination time. It has been shown that autofluorescence is generated mainly by a few tissue fluorophores such as flavins, nicotinamide adenine dinucleotide, aromatic acids such as tryptophan, tyrosine and phenylalanine, and porphyrins.48 Which molecular species that are involved depend on the excitation wavelength, as shown in the ultraviolet and the visible wavelength range.22,26 Only a few studies deal with NIR excitation, but it has been suggested that in the NIR region, the intrinsic fluorescence is primarily caused by porphyrins.48 Photobleaching of tissue fluorophores in general and porphyrins in particular is a well-known phenomenon.26,27 Hence, in the present study, it is likely that the registered fluorescence background and its decrease was due to the myoglobin and hemoglobin present in the prostate tissue. However, other fluorophores cannot be excluded.

4.2 Snap-freezing

4.2.1 Sample preparation. To isolate the effects of snap-freezing the frozen and the fresh tissues should be handled as similarly as possible, and tissue degradation should be minimized. Prior to snap-freezing, the prostate was protected by the fascia and adjacent tissue that surround the gland. As the prostate was cut into small samples, focus was on minimizing the time the tissue was exposed to air.

Ideally, to maintain conditions as close to the in vivo state as possible, tissue should be snap-frozen immediately after being removed from the animal body. However, this also requires that the samples used as a reference of fresh samples can be measured immediately. This was not feasible in the present study since the laboratory facility and the abattoir were not at the same location.

4.2.2 Preprocessing. To extract the Raman signal objectively all preprocessing was computerized. A light smoothing was employed that reduced the noise efficiently while minimizing distortion of the Raman peaks. The background-reduction algorithm by Cao et al. was chosen because it selects the polynomial orders of the piecewise polynomial that is fitted to the background based on the fluorescence-to-signal ratio.12

Potentially large differences between the backgrounds of different spectra may affect the results, since no background-reduction algorithm can perfectly differentiate the Raman signal. However, assuming that the fluorescent species are uniformly present throughout the tissue, little variation is expected. The spectral background was in fact consistent. This was checked by plotting and comparing all backgrounds that were subtracted from the spectra in the preprocessing (data not shown). PCA on the backgrounds confirmed this, showing that >98% of the total variance was explained by a single PC.

4.2.3 Multivariate analysis. PCA was performed on unstandardized data since all variables had the same unit and the largest standard deviations in the spectra coincided with the Raman peaks. Only the 10 first PCs were selected for further exploration since the aim was to examine the main differences between fresh and snap-frozen tissues. The variance of all these PCs was much higher than the variance of the original variables, justifying their inclusion. It could be argued that more than 10 PCs should have been included. However, when more than 10 PCs were included, the results showed even stronger significant differences between the multivariate means of fresh and snap-frozen tissues in all comparisons. For example, the $p$-value obtained for comparing snap-frozen tissue stored at $-80\, ^\circ\mathrm{C}$ to fresh tissue was $3 \times 10^{-10}$ when including 20 PCs, explaining about 94% of the total variance, as compared to $1 \times 10^{-7}$ when 10 PCs were included.

This study examined macroscopically thick samples, hence changes due to the freezing process should preferably be investigated using homogeneous tissues. O Faolain et al.4 chose to examine tissue from the placenta due to its homogeneous nature to study the effects of freezing. However, it cannot be excluded that different tissue types react differently from the snap-freezing process. Furthermore, the use of thin-section microscopy would have permitted histopathologic, and probably also spectroscopic, identification of different tissue types. However, that was not an option as preparation of thin sections affects the tissue and hence alters the result. To study thick slices of heterogeneous tissues, such as prostate tissue,49 necessitates the use of statistical methods that can extract the changes due to the freezing process in a heterogenous set of spectra. The

![Fig. 6](image) A spectrum on fresh (blue) as compared to snap-frozen prostate tissue (red) for day 26. Note, for example, that the phenylalanine (1004 cm$^{-1}$) and tryptophan (758 cm$^{-1}$) peaks are increased for fresh tissue.
The amide III peak is located at 1227–1247 cm⁻¹, which could have induced photoinduced effects. Shim and Wilson examined snap-freezing and OCT freezing of several different tissues from hamster using 1064 nm excitation light. They concluded that both freezing methods provided adequate preservation of biochemical composition, although they noted changes of the intensity ratio \( I_{1444}/I_{1637} \) for several tissue types. The differences between studies investigating freezing emphasize that the results for one tissue type may not be fully valid for another.

The analyses comparing snap-frozen tissue stored at −80 °C for different time-spans showed statistically significant differences between these groups. However, the difference between fresh and snap-frozen tissues was much larger than variations between snap-frozen groups. Thus, the main differences probably arise when the samples are snap-frozen or during thawing.

Many in vitro studies investigating, for example, changes in prostate tissue induced by cancer use snap-freezing as a preservation method. More knowledge of the snap-freezing process is needed to elucidate if the results of such studies also apply to in vivo conditions. Although the changes due to snap-freezing found in this study are subtle, a change in protein conformation might potentially have a large influence on the interpretation of findings concerning in vitro studies.

5 Conclusion

The 830 nm laser irradiation of PBS-immersed porcine prostate tissue of high irradiance does not appear to affect Raman spectra. The spectral background decreased with increased laser illumination time, and this was probably caused by photobleaching of tissue fluorophores. For techniques that incorporate the background information into discriminative algorithms it could be worth investigating this in further detail.

Snap-freezing of porcine prostate tissue seems to alter the protein conformation. The changes could not be seen comparing mean spectra, which can be explained by the inhomogeneity of the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. Although the changes in the spectra on the whole were subtle, changes of the protein structure may affect the prostate tissue. 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Combining fibre optic Raman spectroscopy and tactile resonance measurement for tissue characterization

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Combining fibre optic Raman spectroscopy and tactile resonance measurement for tissue characterization

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Abstract

Tissue characterization is fundamental for identification of pathological conditions. Raman spectroscopy (RS) and tactile resonance measurement (TRM) are two promising techniques that measure biochemical content and stiffness, respectively. They have potential to complement the golden standard—histological analysis. By combining RS and TRM, complementary information about tissue content can be obtained and specific drawbacks can be avoided. The aim of this study was to develop a multivariate approach to compare RS and TRM data. The approach was evaluated on measurements at the same points on porcine abdominal tissue. The measurement points were divided into five groups by multivariate analysis of the RS data. A regression analysis was performed and receiver operating characteristic (ROC) curves were used to compare the RS and TRM data. TRM identified one group efficiently (area under ROC curve 0.99). The RS data showed that the proportion of saturated fat was high in this group. The regression analysis showed that stiffness was mainly determined by the amount of fat and its composition. We concluded that RS provided additional, important information for tissue identification that was not provided by TRM alone. The results are promising for development of a method combining RS and TRM for intraoperative tissue characterization.

Keywords: tissue characterization, multivariate analysis, Raman spectroscopy, tactile resonance measurement, biological tissues

1. Introduction

Tissue characterization is very important for the determination of pathological conditions such as cancer. The golden standard is histological analysis using bright field microscopy. The method allows a trained observer to identify diseased tissue through staining of tissue samples. However, the method is subjective and the inter- and intra-observer agreement is
unsatisfactory [1]. Furthermore, it is time-consuming and cannot be used in vivo. Therefore, complementary methods are needed. There are several promising technologies for precise and swift tissue characterization [1–5]. However, to our knowledge, none are ready to be used clinically at present.

Recently, the combination of different techniques has gained increased interest [6]. We have earlier suggested that it may be beneficial to analyze both the biochemical and biomechanical properties of tissue to achieve efficient characterization [1, 7]. Raman spectroscopy (RS) and tactile resonance measurement (TRM) measure the biochemical content and the stiffness of tissue, respectively. These experimental methods have shown promising results in vitro for tissue characterization [2, 4], e.g. prostate cancer detection [1, 4]. Used together, the two methods may compensate for the specific drawbacks of each method alone, e.g. the limited penetration depth of current fibre optic RS probes [8] and TRM’s lack of detailed information about tissue content [9].

RS is an optical method in which a beam of monochromatic light is directed at the examined material. The backscattered light forms a spectrum reflecting the biochemical content of the tissue [10]. By using multivariate analysis of the RS data many pathological conditions can be recognized in an objective manner [11]. For example, several in vitro studies using RS microscope setups show that diagnostic algorithms can differentiate healthy and cancerous prostate tissue with high accuracy [12–16]. The method also holds promise for determining tumour aggressivity [12, 15]. Recently, multivariate analysis of RS and infrared spectroscopic data has been used to characterize healthy tissue as a first step to develop a diagnostic algorithm [17, 18]. Andrade et al [18] used clustering analysis to distinguish different tissue types in normal coloректal samples.

RS can be performed using optical fibres to deliver the exciting laser beam and collect the Raman scattered signal. Crow et al used a fibre optic RS probe and differentiated benign and malignant prostate samples in vitro with an overall accuracy of 86% [13]. RS has not yet become clinically useful due to the lack of suitable fibre optic probes [2], relatively long acquisition times and concerns that the laser illumination may cause photo-induced degeneration [19]. Furthermore, RS usually requires that the measurements should be performed in darkness. It may be possible to overcome these problems by combining RS with TRM.

The TRM mimics palpation since it senses the stiffness of an object. TRM gives an objective reading of the stiffness, as opposed to the subjective manual palpation. The transducer of TRM is a resonance sensor element consisting of a piezoelectric ceramic. The resonance frequency shifts as the probe is pressed against an object, and the shift (Δf) is related to the stiffness of the object [4]. TRM generates an ultrasonic wave in the tissue, and the method is more gentle and deep-sensing [20] than RS. Several medical TRM applications are under development [4], e.g. breast cancer [21] and prostate cancer detection [9, 20, 22, 23]. In vitro studies show that healthy glandular tissue and cancerous prostate tissue can be differentiated using TRM [9, 23]. Stiff prostate tissue indicates the presence of a tumour, although increased stiffness is not necessarily due to cancer. TRM alone cannot yet distinguish between cancerous tissue and stiff healthy prostate tissue such as stroma and prostate stones [9]. With the addition of RS to TRM the diagnostic accuracy may be improved.

Our long-term goal is to integrate RS and TRM into one single probe to be used clinically. For instance, the instrument could be a portable hand-held device used during radical prostatectomy surgery to locate cancerous tissue in the surgical margins. The hand-held resonance sensor concept has recently been demonstrated on tissue phantoms [22]. TRM would be used to scan the prostate tissue and localize stiff areas, which may indicate tumors. The suspicious areas would be analyzed by RS to be confirmed as healthy or diseased tissue. Diagnostic algorithms will be developed that objectively can classify the tissue from the combined RS and TRM information.

A first step toward combining RS and TRM for tissue characterization is to compare measurements from the same tissue. The aim of this study was to develop a multivariate approach to compare the RS information of biochemical content to the TRM information of tissue stiffness.

2. Material and methods

2.1. Sample preparation

We chose to use porcine abdominal tissue for this study. Two packages were purchased from the local grocery store and kept at 6 °C until sample preparation. Rectangular tissue samples were cut 9 mm thick with approximately 40 and 50 mm sides. The tissue samples were fixed to a 19 mm thick Styrofoam plate with needles. A total of four samples, two from each package, were prepared. The samples were taken out of the refrigerator 30 min before each measurement series to suppress temperature drift during the measurement run. All measurements were performed at room temperature (25 °C ± 0.8 °C). Phosphate buffered saline was applied with a soft brush to the freshly cut surface every fifth minute.

2.2. Measurements

2.2.1. Raman spectroscopy. A fibre optic RS probe [24] (Machida Endoscope Co., Tokyo, Japan) was used. It had an outer diameter of 0.8 mm and incorporated a central delivery fibre and eight surrounding collecting fibres. Optical filters at the probe tip rejected Raman scattered light generated in the delivery fibre. The fibre optic probe was connected to an RXN1 spectroscope with a continuous-wave Invictus™ diode laser at 785 nm (both from Kaiser Optical Systems (KOSI), Ann Arbor, MI USA). The system was calibrated for wavelength shift and energy sensitivity using a calibration system (Hololab Calibration Accessory, KOSI). The output effect to the samples was adjusted to approximately 80 mW and the signal was integrated for 30 s. Prior to each measurement, the probe tip was positioned as close to the sample as possible without touching it. The ideal measurement distance for this type of probe is 0.4 mm [24]. In this study, the approximate distance was set by visual inspection using a magnifying glass. This was sufficient since good spectral quality was acquired in a range of several tenths of mm around the optimal distance.
2.2.2. Tactile resonance measurement. A tactile resonance sensor system consisting of a stepper motor, a position meter and a probe tip with force sensor and a piezoelectric element (Venustron®Axiom Co. Ltd, Koriyama Fukushima, Japan) was used [9, 20, 22, 23, 26]. The piezoelectric element was divided into a driving part and a pick-up, which recorded the resonance frequency. The element was set into vibration by an electronic feedback circuit that retained the phase shift at zero. The shift in resonance frequency occurring as the sensor is pressed against a measurement object is related to the stiffness of the object [27].

The resonance frequency of the piezoelectric element was 59 kHz before contact. The resonance frequency shift ($\Delta f$), impression force ($F$) and impression depth ($d$) were sampled at 200 Hz during measurements. The sensor tip was pressed maximally 1.0 mm into the tissue at a speed of 1 mm s$^{-1}$. The tissue surface was detected through the large frequency shift that occurred upon contact. The radius of the hemispherical tip was 2.5 mm.

2.2.3. Combined RS and TRM measurement setup. The RS and TRM probes were mounted above a computer-controlled translation stage (NRT150P1, three NRT100, BSC103, Thorlabs, Newton, NJ, USA), together with a digital camera (Canon Powershot S3 IS with close-up lens 500D and LAH-DC20 conversion lens adapter, Canon, Tokyo, Japan), figure 1. The Styrofoam plate with the tissue sample was fixed to the translation stage. A software program, developed in-house, was implemented in LabVIEW™ (National Instruments, Austin, TX, USA) and ensured that measurements with both probes were performed at the same locations on the tissue sample. The positions of the probes were calibrated before the measurements started, by aligning the probe tips with a reference mark on the translation stage. Each tissue sample was photographed and the position of the sample relative to the reference mark on the translation stage. A grid of 42 measurement points arranged in a 6 × 7 matrix was overlaid on the image of each sample. The total number of measurement points on the four samples was 168. The points were set 5.0 mm apart since this approximately equaled the diameter measured by the TRM probe at an impression depth of 0.6 mm [20]. The measurement run time order was randomized with the constraint that spatially adjacent points were separated in time by at least one intervening measurement. The total measurement time was around two hours, whereof the TRM measurements took approximately 30 min and the RS measurements 90 min.

2.3. Data preprocessing and analysis

Matlab® (version R2009b including Statistics Toolbox version 7.0, MathWorks Inc., Natick, MA, USA) was used for all preprocessing and analysis. Algorithms included in Matlab® were used if possible. Additional algorithms were written and implemented in Matlab®-in-house. Eilers’ algorithm [28] has been made available for Matlab® via the Internet.

All 168 spectra measured on the four samples were collected in one data set. Principal component analysis (PCA) [30] was used to reduce the dimensionality of the data. PCA projects the data onto a new coordinate system so that the first coordinate describes as much of the variance as possible, the second coordinate explains as much of the remaining variance as possible, and so on. The coordinates are called principal components (PCs), and they are orthogonal to each other. The values for the observations expressed in the new coordinate system are called PC scores. The preprocessed spectra were analyzed using PCA, and a selected number of PCs were retained. The reduced data set was then input to a hierarchical cluster analysis (HCA) algorithm. The HCA automatically divided the spectra into groups, where spectra within the same group were similar to each other. Ward’s linkage [30] was used to merge observations (spectra) close to each other, based on the Euclidean distances between the observations.

The spectra were interpreted by tentatively assigning the spectral features to specific molecular vibrations using a database [31].

2.3.1. Preprocessing and analysis of the RS data. The spectral interval 600–1800 cm$^{-1}$, referred to as the fingerprint region [10], was selected for further analysis. Each spectrum was lightly filtered by a noise-reduction algorithm, setting the smoothing parameters $d = 2$ and $\lambda = 10$ [28]. The fluorescence background was automatically subtracted by fitting a piecewise modified polynomial to each spectrum [29]. The spectra were vector normalized so that the integrated area equaled the diameter measured by the TRM probe at an impression depth of 0.6 mm [20]. The measurement run time order was randomized with the constraint that spatially adjacent points were separated in time by at least one intervening measurement. The total measurement time was around two hours, whereof the TRM measurements took approximately 30 min and the RS measurements 90 min.

2.3.2. Analysis of the TRM data. The stiffness parameter $S$ [N Hz$^{-1}$] was calculated from $\Delta f$, $F$ and $d$.

$$ S = \frac{\Delta F / \Delta d}{\Delta f / \Delta d} $$

(1)
where $\partial F/\partial d$ and $\partial \Delta f/\partial d$ were estimated numerically by linear regression in an interval of $d \pm 0.1 \text{ mm}$. $S$ was calculated at $d = 0.6 \text{ mm}$, representing the tissue stiffness near the surface [20]. $S$ has previously been shown to be proportional to Young’s modulus [26].

### 2.3.3. Combined measurements

A linear regression analysis [32] was performed to relate $S$ to the PCs. $S$ was taken as the model response variable, whereas the PCs were the model predictor variables. A residual analysis of zero mean, constant variance, time independence and normality was done for model validation. The adjusted coefficient of multiple determination $R^2_{\text{adj}}$ was used for model control and comparison [32].

The accuracy of TRM to classify different tissue types according to their stiffness was evaluated by the area under the receiver operating characteristic (ROC) curve. The sensitivity and specificity is the proportion of correct negative classification. An area under the curve close to 1.0 indicates an excellent method, whereas an area of 0.5 indicates a noninformative method [33].

### 2.3.4. Statistics

The mean stiffness was calculated for each group as defined by the cluster analysis of the RS data. The nonparametric Kruskal–Wallis test, followed by Tukey–Kramer’s multiple comparison test, were performed to test for differences between groups of stiffness measurements. The Anderson–Darling test was used to test for normality. Statistical test results with $p < 0.05$ were considered statistically significant.

### 3. Results

#### 3.1. RS measurements

The RS data were first analyzed using PCA. The first ten PCs were retained. These explained >99% of the total variance of the RS data, whereof PC 1 alone explained 94%. The spectra were divided into five groups as defined by the HCA, and they were labeled A, B, C, D and E. The mean spectra (figure 2) agreed well with published results on porcine tissue [34]. Analysis of the spectral peaks showed that group A was muscle tissue, D and E fat tissue, while B and C consisted of both muscle and fat tissue (figure 2). Group C contained a larger proportion of fat than group B. Spectra D and E were similar but the intensity of several peaks differed (figure 2 inset). The major differences were assigned to different vibrations in lipids [31]:

- Spectrum D had higher intensity than spectrum E at the peaks at 1659 and 1265 cm$^{-1}$. These peaks were assigned to C=C stretching and vibrations of the C=C and C=H bonds, respectively.
- Spectrum E had higher intensity than spectrum D at the peaks at 1440, 1296, 1128 and 1062 cm$^{-1}$. The first two peaks were assigned to CH$_2$/CH$_3$ and CH$_2$ vibrations, respectively. The peaks at 1128 and 1062 cm$^{-1}$ were both due to C=C stretching.

These differences indicated that group E contained a higher proportion of saturated fatty acids than group D [10]. The composition of the fat differed between the two batches of tissue. One batch consisted only of fat from group D, whereas the other batch consisted mainly of fat from group E (42 out of 44 measurement points). PCs 1, 2 and 4 discriminated the different groups most efficiently (figure 3). PC 1 was interpreted as being related to the proportion of fat tissue, PC 2 to the proportion of muscle tissue, whereas PC 4 described the amount of saturated/unsaturated fatty acids.

#### 3.2. TRM measurements

The stiffness parameter $S$ was calculated from the two derivatives $\partial F/\partial d$ and $\partial \Delta f/\partial d$ (illustrated in figure 4), according to equation (1). $S$ was positively skewed (figure 5) with a mean of 0.40 mN Hz$^{-1}$ and a standard deviation of 0.24 mN Hz$^{-1}$. For the majority of the measurement points $S$ was <0.40 mN Hz$^{-1}$, but some stiffer points on the tissue were observed (figure 5).
Figure 3. A scatter plot of the scores of PC 1, 2 and 4 for all RS measurements. The group division (A–E) from the cluster analysis is shown by the different markers.

Figure 4. The impression force (F) and the frequency shift (Δf) plotted against the impression depth (d) for two representative measurement points. The values of the derivatives ∂F/∂d and ∂Δf/∂d at d = 0.6 were estimated by calculating the line slopes in an impression depth interval of 0.5 to 0.7 mm, marked by crosses (+).

Figure 5. A histogram of the stiffness parameter S distribution of all measurement points (n = 168). A higher value of S indicates stiffer tissue.

Figure 6. The mean stiffness of groups A–E calculated from the TRM measurements. The standard deviations are shown by the bars. The asterisk (*) denotes significantly separated groups (p < 0.05).

Table 1. Model coefficients (βi) from the regression models between the stiffness parameter S and the Raman PCs. The full model included all ten PCs. R^2_adj is the adjusted coefficient of multiple determination. The asterisks (*) mark the coefficients that were significant (p < 0.05). The significant model included the six significant PCs. All model coefficient values are the standardized model coefficients divided by their sum and given as a percentage.

<table>
<thead>
<tr>
<th>Model coefficient, βi (%)</th>
<th>Full model, R^2_adj = 0.673</th>
<th>Significant model, R^2_adj = 0.669</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1</td>
<td>30*</td>
<td>33</td>
</tr>
<tr>
<td>β2</td>
<td>−12*</td>
<td>−14</td>
</tr>
<tr>
<td>β3</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>β4</td>
<td>28*</td>
<td>32</td>
</tr>
<tr>
<td>β5</td>
<td>−7*</td>
<td>−7</td>
</tr>
<tr>
<td>β6</td>
<td>6*</td>
<td>7</td>
</tr>
<tr>
<td>β7</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>β8</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>β9</td>
<td>−5</td>
<td>−</td>
</tr>
<tr>
<td>β10</td>
<td>6*</td>
<td>7</td>
</tr>
</tbody>
</table>

3.3. Comparing RS with TRM measurements

Group E was significantly stiffer than the other groups (p < 0.05), whereas no significant difference was found among groups A–D (figure 6).

The estimated coefficients (βi) from the regression analyses gave the relative impact of the ith PC score on the stiffness parameter S (table 1). A negative coefficient indicated that the specific PC decreased S. The residual
The RS data explained about 67% of the variability in the dataset. Analysis showed that the requirements of zero mean, normality, and independence were fulfilled for both models. The RS data explained about 67% of the variability in the dataset. The threshold value was selected as the average of all measurements compared to the average of each group. The accuracy of TRM to discriminate group E was excellent since the area under the ROC was close to 1.0 (figure 7). The accuracies for detecting the other tissue groups were lower.

Figure 8 shows the result of the cluster analysis on the RS data, and measurement points with increased stiffness, for one of the four samples. This sample was from the batch of tissue that contained fat with a higher amount of saturated fatty acids (group E). The result for the other sample from the same batch was similar. The fat in the two samples from the other batch was less stiff (figure 6, group D), and could not be efficiently discriminated by TRM (figure 7, group D).

4. Discussion

Earlier studies have shown that RS and TRM are individually promising techniques for characterizing tissue content. In this study we have taken an important step toward combining RS and TRM for more exact tissue characterization. We have developed a new multivariate approach to compare the biochemical content and stiffness of tissue by using PCA, HCA, linear regression and ROC curves. The approach was evaluated by measurements on porcine abdominal tissue. The results showed that the approach can provide valuable information about how the RS and TRM data are related. This information will be needed to efficiently combine the methods and to construct a diagnostic algorithm in future studies comparing healthy and diseased human tissue.

Swine share anatomic and physiological properties with humans that make them suitable for use in biomedical research. Porcine abdominal tissue was chosen because it is composed of two well-investigated tissue types, fat and muscle tissue, that could be visually identified. Furthermore, abdominal tissue contains relatively large homogenous areas of the same tissue type, but also heterogeneous areas. This allowed measurements to be performed both on single tissue types and on mixes of different types, which constituted a good basis for comparing the RS and TRM data without performing a histological analysis of the tissue samples. It was expected that the stiffness of the fat and the muscle tissue would differ. The samples were carefully cut to achieve a flat surface of sufficient size. This facilitated the manual adjustment of the distance between the RS probe and the surface. Erroneous TRM results due to surface roughness were also avoided. The needles, which were used to fasten the tissue, may have affected the stiffness measurements. However, no measurement points were positioned close to the needles, i.e. within the spatial measurement range of the TRM. In this way possible artifacts from the needles were minimized. Four tissue samples from two different batches were considered sufficient to develop an approach to analyze the data since statistically significant results were obtained.

### Figure 7.
ROC curves showing the ability of TRM to classify groups A–E defined by the cluster analysis on the RS data. Circled points mark the stiffness threshold value calculated as the average between the mean of the current group and the mean of all measurements in the other groups.

### Figure 8.
A digital photograph of one of the porcine abdominal samples with an overlay showing the measurement points and the group assignment from the cluster analysis on the RS data. The dark area is mainly muscle tissue and the light area is fat. Group A is represented by white squares, B by black upward-pointing triangles, C by black circles, D by white diamonds and E by white downward-pointing triangles. The large circles denote points with increased stiffness. The threshold value was selected as the average between the mean of group E and the mean of all measurements in groups A–D (the circled point in figure 7, group E).

analysis showed that the requirements of zero mean, normality, constant variance and time independence of the residuals were fulfilled for both models. The RS data explained about 67% of the variability in the dataset. The threshold value was selected as the average of all measurements compared to the average of each group. The accuracy of TRM to discriminate group E was excellent since the area under the ROC was close to 1.0 (figure 7). The accuracies for detecting the other tissue groups were lower.

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The TRM measurements were performed before the RS measurements (figure 1). The reason was that the laser irradiation may affect the stiffness of the tissue, e.g. via dehydration, whereas the TRM was unlikely to induce any changes of the biochemical content.

The tissue characteristics were first described by using the RS data to divide the measurement points into groups with similar tissue. The tissue description given by TRM was less detailed and allowed less tissue discrimination than that given by the RS. Cluster analysis of the RS data is an efficient tool to distinguish different tissue types [18]. HCA enables an unsupervised automatic division of the measurement points into a selected number of groups. The number of groups that is appropriate cannot be determined in advance [36]. In general, more details are revealed when the number of groups is increased, but larger tissue structures may then be disguised. After comparing cluster analyses using 2–9 groups, five groups were chosen in this study. The Raman spectral data showed that the measurement points were divided into five distinct groups (figures 2 and 3). In particular, D and E were clearly separated in the PC scores plot (figure 3). These groups were merged using fewer than five clusters in the HCA. Choosing more than five clusters did not aid the comparison between TRM and RS since the mean stiffness of the new subgroups did not differ significantly.

Visual inspection of the samples (figure 8) agreed with interpretations of the mean spectra (figure 2). TRM was expected to discriminate fat from muscle tissue due to differences in stiffness. However, it only separated one type of fat (group E) from the muscle tissue. The spectral data showed that group E contained a larger proportion of unsaturated fat than group D. This agreed with the stiffness measurements showing that group E was stiffer than group D since saturated fat is harder than unsaturated fat at room temperature [37]. The regression analysis (table 1) showed that the biochemical content explained a large proportion (67%) of the tissue stiffness variation that could not be explained by the RS, and that TRM and RS give complementary information for tissue characterization.

The diagnostic accuracy for identifying group E was satisfactory using TRM. However, the other groups could not be distinguished efficiently by TRM (figure 7). The Raman spectral data showed marked differences between groups A–D (figures 2 and 3). This suggests that RS added valuable information to TRM to aid in the differentiation into tissue groups A–D.

If the measurements volumes of the two sensors had been more equal, a better model agreement (table 1) may have been obtained. RS fibre optic probes measuring the backscattered light have penetration depths of the order of 100 μm [8], whereas the sensing depth of the TRM probe has been estimated to be about 2.5–4.5 mm at an impression depth of 0.6 mm [20]. The lateral measurement areas also differ; they are estimated to be ∼0.2 mm² for the RS probe [25] and ∼20 mm² for the TRM probe [20]. The areas with mainly muscle tissue visually appeared more heterogeneous than the areas with fat (figure 8). In heterogeneous areas TRM measured on several different tissue types at once (upper part of figure 8), due to its relatively large sensing volume. This probably impaired TRM’s ability to distinguish between them (figure 6). Measurement points with increased stiffness that did not belong to group E seemed to be positioned close to areas with high fat content (figure 8). The increased stiffness may have been due to the adjacent fat, which was not sensed by the RS probe. Four of these points were assigned to group A, which was interpreted as pure muscle tissue. For the future development of a combined probe, the sensing volume of the two methods could be equalized by using an RS probe with larger penetration depth and a smaller TRM probe tip. TRM can use probe tips in a variety of sizes. Systems using tips with diameters from 10 μm to 5 mm have been presented [4, 38], although few instruments are commercially available. RS spatially offset probes can sense deeper into the tissue. Matousek et al. have demonstrated transcutaneous measurements of human bone in vivo [39].

We have shown that the approach presented in this study could extract which biochemical components affected the stiffness of porcine abdominal tissue. This is promising for future studies on human tissues. By gaining insight into the correlation between stiffness and biochemical composition a deeper understanding of the nature of pathological tissues may be attained.

5. Conclusion

This study shows that RS measurements of the biochemical content provide additional information to TRM stiffness measurements on porcine abdominal tissue for discriminating different tissue types. The RS and TRM data can be successfully compared through multivariate analysis of the RS data, linear regression analysis and the use of ROC curves. Detailed information about biochemical components that affect the stiffness can be extracted. This information could be used to efficiently combine the RS and the TRM methods, and to gain a better understanding of diseases that affect tissue stiffness. The results are promising for further development of an instrument combining RS and TRM to be used for intraoperative diagnosis of, e.g., prostate cancer.

Acknowledgments

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Combining scanning haptic microscopy and fiber optic Raman spectroscopy for tissue characterization

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Combining scanning haptic microscopy and fiber optic Raman spectroscopy for tissue characterization

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Abstract

The tactile resonance method (TRM) and Raman spectroscopy (RS) are promising for tissue characterization in vivo. Our goal is to combine these techniques into one instrument, to use TRM for swift scanning, and RS for increasing the diagnostic power. The aim of this study was to determine the classification accuracy, using support vector machines, for measurements on porcine and human prostate tissue. This was done with the TRM using scanning haptic microscopy (SHM) for assessing stiffness on a micro-scale, and with fiber optic RS measurements for assessing biochemical content. We compared the accuracy for using SHM alone versus SHM combined with RS, for different degrees of tissue homogeneity. The cross-validation classification accuracy for healthy porcine tissue types using SHM alone was 65–82%, and when RS was added it was increased to 80–87%. The accuracy for healthy and cancerous human tissue was 67–70% when only SHM was used, and increased to 73–77% for the combined measurements. This shows that the potential for swift and accurate classification of healthy and cancerous prostate tissue is high. This is promising for developing a tool for probing the surgical margins during prostate cancer surgery.

Keywords: tactile resonance method, Raman spectroscopy, prostate cancer, support vector machines, tissue characterization

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Abbreviations

$\kappa$ curvature parameter

$E$ Young’s modulus

MTS micro tactile sensor

PZT lead zirconate titanate

PBS phosphate buffered saline

RBF radial basis function

ROI region of interest

RS Raman spectroscopy

SHM scanning haptic microscopy

SVM support vector machines

TRM tactile resonance method

1 Introduction

Surgical removal of the prostate is the recommended treatment for patients suffering from high-risk localized prostate cancer [1]. To minimize the risk for cancer recurrence it is important that all cancerous tissue is removed [2]. This is a challenge for the surgeons, because minimal nerve damage is imperative for recovery of erectile function, and they have no instrument for probing the surgical margins [3]. Cancer can generally not be detected by the eye. Therefore, there is a great need for a tool that could detect remaining cancer [3]. The tactile resonance method (TRM) and Raman spectroscopy (RS) are two techniques that are promising for in vivo characterization of prostate tissue [4,5]. Each has certain disadvantages that limit its usefulness. Our research focuses on combining these two methods to benefit from their strengths while minimizing their disadvantages.

TRM mimics the perception of palpation and gives an objective measure of stiffness. It is based on the principle that the contact impedance of a vibrating rod changes when it is applied against an additional load. A change of the vibrational frequency is required to keep the phase shift constant during an application of the sensor to a sample. The frequency shift is proportional to the density and Young’s modulus ($E$) of the sample [6].

The TRM can be used with a variety of sensor sizes. For medical applications, TRM sensors have been developed with diameters ranging from 1.0 to 5.0 mm [5,6]. A recent development has been the invention of scanning haptic microscopy (SHM), which uses a micro tactile sensor (MTS) [7]. The MTS can be made with sensor tips ranging from 0.1 $\mu$m to 1.0 mm. This has made it possible to visualize the elasticity distribution over the surface of sliced tissues of porcine heart muscle [8], porcine vascular tissues [9,10] and human prostate [11], on a micro scale.

RS is a powerful technique for probing the biochemical contents of native tissue [12]. Its principle is to expose the tissue to strong monochromatic light from a laser to make molecular bonds vibrate. The Raman scattered photons obtain a wavelength shift corresponding to the difference between the initial and final vibrational energy levels.
Every molecule has a unique set of vibrational frequencies, hence every tissue type gives rise to a unique Raman spectrum. Numerous in vitro studies have attained high accuracies for identifying cancerous tissue using RS [12]. However, development of clinical applications has been delayed due to a lack of adequate fiber optic probes [13]. The main disadvantages of RS are the shallow penetration depth of current fiber optic probes [14], that surrounding light interferes with the signal, and that strong laser illumination may harm the tissue [15].

By combining TRM and RS we can make use of the fast and gentle scanning capability of TRM to localize lesions suspected to be cancerous. These can then be probed by RS, and a diagnostic algorithm can be used to determine the pathology from the combined data.

Support vector machines (SVM) can be used for reliable tissue classification. They are collections of algorithms used for data analysis and pattern recognition [16]. The method has been used on RS data to classify different types of tissues and to detect cancer [17]. The SVM algorithm calculates the hyperplane that maximizes the separation between groups. It maps the data set into a higher-dimensional space to achieve nonlinear separation, and it works well on data sets with many variables. Therefore, we here apply SVM to the multitude of variables generated from the combination of TRM and RS.

An important step towards an in vivo application is to develop an efficient tissue classification procedure. In a previous study [5], we combined TRM and RS measurements using a TRM probe with $\sim 20 \text{ mm}^2$ sensing area. Further investigations on a smaller scale were suggested. The aim of this study was to investigate the accuracy of characterization of porcine and human prostate tissue using SVM on micro-scale SHM measurements and fiber optic RS measurements for the first time.

## 2 Material and Methods

### 2.1 Sample preparation

The prostate gland was removed from eight, healthy, approx. six-month-old boars that were slaughtered at the local abattoir. A veterinarian removed the prostates with attached urethras directly after slaughter. They were enclosed in plastic bags and refrigerated until transportation. Five tissue samples were cut from each prostate. They were covered with tissue freezing medium (TFM-5 (red), Triangle Biomedical Sciences Inc., Durham, USA) and immersed in liquid nitrogen for at least 30 s. After the samples had been frozen three holes ($\varphi = 1 \text{ mm}$) were drilled as position landmarks. Each sample was cut into two $500 \mu m$ thick sections using a microtome (Cryo-Star HM 560 M, Microm, Walldorf, Germany). Adjacent to both sides of each section a specimen of $5 \mu m$ thickness was cut, which was stained for histologic analysis with hematoxylin and eosin according to standard procedure. The $500-\mu m$ sections were stored in $-80^\circ C < 6$ months, until measurements were performed. Prior to measurements they were short-term stored in the freezer at the biomedical laboratory ($-20^\circ C$). All the histology specimens were scanned using a scanning microscope (ScanScope CS, 20× objective, Aperio, Vista, CA, USA).
Samples from four of the prostates were used for tests to optimize the measurement procedure. Ten arbitrary samples were taken from each of the four remaining prostates. Half were used for further optimization tests, leaving 21 samples for the final measurements. To minimize tissue degradation the prostates were kept in sealed containers and refrigerated until measurements. Furthermore, the protective fascia surrounding the prostates was removed just before the snap-freezing. All samples were snap-frozen within two days after slaughter.

A 67-year old man with prostate cancer of Gleason score seven and stage pT3 had his prostate surgically removed. One 5-mm and one 8-mm punch were used to take four samples from the prostate in regions suspected to contain both healthy and cancerous tissue. The samples were snap-frozen in liquid nitrogen within 30 minutes after surgery. The preparation followed the same procedure as for the porcine samples. Only three of the 500-µm sections contained cancerous tissue and were included in the study.

Permission to use animal samples was obtained from the Swedish Board of Agriculture. The study was approved by the ethics committee of Umeå University. The participating patient gave informed written consent.

2.2 Scanning haptic microscopy

Topography and elasticity mapping of the 500-µm tissue sections were measured with SHM, which uses an MTS to scan the surface and measure the elasticity distribution. The basic system configuration and its operation are well described in previous studies [9,10]. The system was originally developed by Murayama et al. [8] and is currently available as a customizable product named SHM (P&M Co., Ltd., Aizuwakamatsu, Fukushima, Japan).

The main part of SHM was composed of a precise XYZ stage and a Z-axis actuator, which was attached on the Z-axis stage to manipulate the MTS. Two cameras, one for monitoring the sensor tip with high magnification, and the other for observing the sample and setting the measurement region of interest (ROI) with low magnification, were mounted on the Z-axis stage. The samples were mounted on the XY-axis stage for horizontal movement. Each axis was controlled using a very fine stepping motor with a resolution of 0.01 µm.

The MTS is a highly sensitive resonator-based contact impedance meter capable of estimating the elastic modulus of soft tissues in micrometer scale. It consists of a cylindrical piezoelectric transducer made of lead zirconate titanate (PZT). The PZT transmits a very weak longitudinal ultrasonic wave (frequency 100–200 kHz) into a glass needle, which has a spherically shaped tip that makes contact with the measurement object. A phase shift circuit is used to drive the MTS to obtain high signal-to-noise ratio. Details of the composition, the electronics, and the detection principle of the MTS are published in [7,8,18]. The articles show that the change in the oscillation frequency Δf (Hz) for a certain indentation or Δf/δ (Hz/µm) is highly correlated to E. Four different sensors were used in this study. They consisted of PZT elements that were 15 mm long and 2 mm wide, onto which a glass needle was attached (φ = 1 mm, length 21–27 mm). The needles had tapered ends with tip diameters between 53 and 65 µm.
2. Material and Methods

The best performance was obtained for frequencies around 200 kHz (188.2–200.8 kHz). Gelatin samples were used to calibrate $\Delta f/\delta$ to $E$ at the end of each day of measurement. Six samples (G9382 Gelatin from bovine skin Type B, Sigma-Aldrich, St. Lois, MO, USA) with gelatin weight percentage ranging from 3 to 25% were prepared once a week. The gelatin was solidified in 53-mm Petri dishes and stored at 5°C for at least 90 minutes before measurement. The samples were probed by SHM within three minutes in room temperature and then replaced in the refrigerator for at least 30 minutes. Next, $E$ was determined by depressing a flat-ended steel rod ($\varphi = 2 \text{ mm}$) perpendicular to the gelatin surface and recording the reactive force. A computer-controlled translation stage (NRT150P1, three NRT100, BSC103, Thorlabs, Newton, NJ, USA) controlled the motion of the rod and recorded the vertical impression depth $W$, which was maximally 0.14 mm, with a step size of 0.01 mm. The reactive force was read by a 0.01-g resolution electronic balance (HT-600H, Elastocon, Borås, Sweden), and a stable reading was acquired after 2 s. $E$ was calculated according to the theory by Sneddon [19] at $W = 0.09 \pm 0.05 \text{ mm}$ to ensure full contact. A linear model of $E$ for all samples was fitted to the mean $\Delta f/\delta$ ($n = 100$) value using a least-squares fit, and the equation for the resulting line was used to calculate $E$ for the corresponding tissue measurements.

2.3 Raman spectroscopy

A fiber optic RS probe [20] (Machida Endoscope Co., Tokyo, Japan) with an outer diameter of 0.8 mm was connected to an RXN1 spectroscope with a continuous-wave Invictus® diode laser at 785 nm (both from Kaiser Optical Systems (KOSI), Ann Arbor, MI, USA). The iC Raman® software (version 2.0, KOSI) was used for spectral acquisition from 100 to 3425 cm$^{-1}$. The output effect to the samples was adjusted to approximately 150 mW, corresponding to a laser input effect of 270 mW. The integration time was set to 7 s. The diameter of the laser spot on the sample was estimated to be 0.3 mm [21]. The system was calibrated for wavelength shift and energy sensitivity using neon emission lines and a NIST traceable white-light source (HoloLab Calibration Accessory, KOSI).

2.4 Combination of RS and SHM

The experimental setup and principle of measurement is shown in Figure 1. The RS probe was mounted on the Z axis of the SHM setup. An in-house developed program written in LabVIEW® (version 8.6, National Instruments, Austin, TX, USA) was used to control the system.

A spatial calibration of the setup was performed using a printed grid with a line thickness of 50 $\mu$m. First, the axes of the camera (LifeCam Cinema®, Microsoft, Redmond, WA, USA) for setting the ROI were carefully aligned with the axes of the stage. Second, the image distortion was established and removed by recording the image and corresponding stage coordinates of nine grid line intersections. Third, the positions of the MTS and RS probes relative to the camera were determined by using a line intersection as reference mark. The high magnification camera (Dino-Lite AM413TL, AnMo Electronics Corp., Hsinchu, Taiwan) of the SHM setup was used to position the probes precisely above
this mark. Because the RS probe was large compared to the mark an RS mapping was conducted to improve the accuracy. A cluster analysis of the spectra clearly showed the exact location of the mark. The probe position accuracy using the camera to set the ROI was tested after calibration and was of the order of 100 \( \mu m \). The center-to-center distance between the MTS and RS measurement areas was estimated to be within 20 \( \mu m \).

After the samples were removed from the freezer they were put on top of a filter paper soaked with PBS, which in turn was mounted on a microscope slide with low Raman background (Calcium Fluoride UV grade, Crystran Ltd., Poole, UK). A simplified humid chamber was made by putting a rolled-up piece of a Kimwipe paper (Kimtech Science Kimwipes, Kimberly-Clark Inc., Roswell, GA, USA) around the sample. A pump system (Nemesys, cetoni GmbH, Korbussen, Germany) was used to supply a constant flow of PBS to the Kimwipe during the measurements. A flow of 0.23 \( \mu L s^{-1} \) was used. The distance from the RS probe to the sample was adjusted to be constant at 400 \( \mu m \), which provides high spectral quality [20], by using the topographic information acquired with SHM.

The ROI was chosen to be 3000 \( \times \) 3000 \( \mu m \) for all measurements on porcine prostate. 60 \( \times \) 60 = 3600 measurement points were acquired with SHM using a 50-\( \mu m \) step size, and subsequently 10 \( \times \) 10 = 100 measurement points were acquired with RS using a 300-\( \mu m \) step size. Measurements were started 15 minutes after the samples were removed from the freezer. The measurement time was less than 45 minutes, of which the SHM took approx. 30 minutes. The same measurement procedure was used for the human samples. However, the ROI was adjusted to the sample size and positioned to cover the regions with cancerous tissue. A total of 275 spectra and 9900 SHM measurement points were acquired from the human samples.
2.5 Data preprocessing and analysis

MATLAB® (version R2010a including Statistics Toolbox version 7.3, MathWorks Inc., Natick, MA, USA) was used for all preprocessing and analysis. The LIBSVM [22] library interface to MATLAB® (version 3.0) was used for the SVM analysis. Algorithms not available in MATLAB® were written in-house. Eilers’ algorithm [23] was available for MATLAB® via the Internet. Two separate analyses were conducted, one for the porcine samples and one for the human samples.

2.5.1 Histologic analysis

The scans of the tissue specimens were randomly assigned and histologically analyzed by two operators (authors S.C. and M.N.). The area of measurement was localized by comparing the histology image to the picture of the measured sample and to the stiffness map acquired by the SHM. Using Photoshop® (version CS5, Adobe®, San Jose, CA, USA) the histology image was rotated, scaled and sometimes skewed so the drill holes and the morphological features overlapped. A grid that defined the measurement points was then superimposed over the histology image. The tissue type of each square of the grid was manually determined. For porcine prostate there were three main tissue types: epithelium, lumen and stroma. For human prostate there were six main tissue types: normal epithelium, cancerous epithelium, lumen, prostate stones, normal stroma and cancerous stroma. Points that could not be determined were excluded from the analysis. The quality of the histologic analysis was ascertained by a board certified pathologist (author A.B.), with over 25 years of experience of prostate histopathology. There was a relatively large uncertainty in the localization of the measurement area. Therefore, we performed the analysis of SHM data on more homogeneous tissue regions by extracting the subset of measurement points whose neighbors were of the same tissue type.

2.5.2 SHM

The stiffness indicator \( \Delta f/\delta \), and an additional curvature parameter \( \kappa \) (Hz \( \mu \text{m}^{-2} \)), was calculated from the frequency shift data. The threshold for surface detection was set to 10 Hz, and only a shift in the direction expected for the current sensor triggered detection. A linear least-squares fit was performed to the five consecutive data points starting from the point of surface detection. A second-degree polynomial was fitted to the same data points. The coefficient for the quadratic term, \( \kappa \), was a measure of the curvature of the frequency-indentation curve, and was related to nonlinear effects. Measurements for which \(< 3\) data points were available, or for which the coefficient of determination \( R^2 < 0.9 \) for the linear fit, were discarded.

2.5.3 RS

For the porcine samples the raw Raman spectra were mainly composed of signals from the tissue, the optic fibers in the RS probe [20], and the filter paper on which the sample
lay. To extract the useful Raman signal a number of consecutive preprocessing steps were applied to the raw spectra:

- The Fingerprint spectral interval $600–1800 \text{ cm}^{-1}$ was selected [12].
- The background generated by the RS probe itself was estimated by measuring on a microscope slide with low inherent background. It was then removed from the raw spectra by applying the variance minimization method [24].
- Light smoothing was employed using Eilers’ algorithm with $d = 2$ and $\lambda = 10$ [23].
- The filter paper generated a number of sharp Raman peaks, which were subtracted using the second-derivative variance minimization method [24].
- The fluorescence background was subtracted by fitting a piecewise polynomial to each spectrum [25].
- The spectra were vector normalized so that their integrated intensities were equalized.

Spectral peaks were tentatively assigned to specific molecular vibrations by using the database [26]. For the human samples the Fingerprint interval could not be used due to strong fluorescence caused by the ink applied to the surgical resection margin. Therefore, the interval $2400–3425 \text{ cm}^{-1}$ was selected, which has been shown to be rich in diagnostic information [27]. Spectra saturated within this interval were removed ($n = 30$) from the analysis. The raw spectra were smoothed ($d = 2$ and $\lambda = 100$), background-reduced, and normalized using the algorithms mentioned above.

2.5.4 Classification

We determined the tissue type of each RS measurement area, which was assumed to be $300 \mu\text{m}$ in diameter, by assigning it to the type most abundant within the area. The tissue types for the human samples were grouped into either healthy or cancerous tissue. Areas that did not contain more than a threshold percentage of the same tissue type were excluded. Analyses were performed for three thresholds: 50%, 67% and 83%. The preprocessed RS data, the mean stiffness of each area, and the mean value of $\kappa$ for each area, were input to the SVM. Each input variable was scaled to the interval $[0 1]$ to give all attributes equal importance regardless of numeric range. To manage the unbalanced data sets, weights were assigned to each tissue type in proportion to their total abundance.

The approach for SVM classification suggested by Chen et al. [28] with the radial basis function (RBF) kernel was adopted. This approach suggests that the kernel parameter $\gamma$ and an additional penalty parameter $C$, which controls the trade-off between complexity of the decision rule and frequency of error [16], can be decided through a grid search in which the highest five-fold cross-validation accuracy is found. Intervals from $2^{-15}$ to $2^1$ and from $2^{-5}$ to $2^{15}$ were searched to determine the RBF width $\gamma$ and the penalty factor $C$, respectively. A too high value of $C$ would overfit the support vectors, and a too low value would underfit them.
2.5.5 Statistics

The Lillie test was used to evaluate data normality. The Kruskal-Wallis nonparametric test followed by the Tukey-Kramer multiple comparison test were used to assess significant differences in stiffness and $\kappa$ values between the tissue types. A $p$-value less than 0.05 was considered statistically significant.

3 Results

The data analysis was conducted on all three human samples and 14 of the 21 porcine samples. Seven had to be excluded for the following reasons: to avoid damage to the MTS, due to tissue adsorption on the MTS tip, or due to an inappropriate MTS driving frequency. For three samples the measurement area could not be identified on the histologic image.

3.1 SHM

The calibration measurements on gelatin showed that the stiffness indicator was linear to $E$ ($R^2 \geq 0.99$). Figure 2 shows a typical example of an elasticity mapping. Tissue structures were clearly observed as differences in their elasticity (Figure 2(c)), where stroma corresponded to high elasticity regions and lumen mostly to low elasticity regions.

For the porcine samples the median values of $E$ and $\kappa$ for epithelium, lumen and stroma were significantly different ($p < 0.05$). There were relatively large overlaps of the ranges of $E$ and $\kappa$ for the tissue types (Figures 3 and 4). The stiffness data was not normally distributed ($p < 0.05$), a positive skewness was observed. $\kappa$ did not follow a normal distribution.

For the human samples there were significant differences between the median $E$ and $\kappa$ values ($p < 0.05$). Lumen was significantly softer than all other groups, except for healthy epithelium ($p < 0.05$). Prostate stones were significantly stiffer than cancerous epithelium, healthy stroma, and lumen ($p < 0.05$). The median $\kappa$ value for cancerous stroma was significantly higher than all other groups, except for prostate stones. Furthermore, $\kappa$ was higher for cancerous epithelium than lumen and healthy epithelium ($p < 0.05$). The median values of $E$ and $\kappa$ are shown in Figure 5. There were large overlaps between the groups. $E$ and $\kappa$ were generally not normally distributed within the groups.

3.2 RS

The mean spectra of the porcine tissue types showed the following differences (Figure 6):

- Changes in the collagen content seen in peaks at 1654 cm$^{-1}$ (Amide I), 1448 cm$^{-1}$ (Amide III), 935 cm$^{-1}$ and 813 cm$^{-1}$. Stroma showed the highest collagen content and lumen the lowest.

- Changes in the DNA content seen in the region 640–850 cm$^{-1}$. Stroma showed the highest DNA content and lumen the lowest.
Figure 2: In the histologic analysis lumen is black, epithelium gray and stroma white. The scale bars show 500 µm.

Figure 3: A box plot of the $E$ values for the different porcine tissue types. The line in the middle of the box shows the median, and the bottom and the top of the box show the 25th and 75th percentile, respectively. The whiskers extend to 1.5 times the interquartile range away from the top or bottom of the box, or to the farthest observations from the box. Data points outside the whiskers are plotted individually.
3. Results

$\kappa$ (Hz $\mu$m$^{-2}$) epithelium ($n = 7964$) lumen ($n = 351$) stroma ($n = 4577$)

-0.4
-0.2
0
0.2
0.4
0.6

Figure 4: A box plot of the $\kappa$ values for the different porcine tissue types. The line in the middle of the box shows the median, and the bottom and the top of the box show the 25th and 75th percentile, respectively. The whiskers extend to 1.5 times the interquartile range away from the top or bottom of the box, or to the farthest observations from the box. Data points outside the whiskers are plotted individually.

Changes in the lipid and fat content seen in the peaks between 1720 to 1790 cm$^{-1}$. Lumen showed the highest content.

For the human samples it was found that the blue ink applied to the surgical resection margin, which is standard clinical procedure, caused a very strong fluorescent signal that often saturated the CCD detector and swamped the signal from the tissue in the Fingerprint interval (Figure 7). However, the fluorescence decreased towards the high wavenumber region.

3.3 Classification

Table 1 shows the classification results for porcine tissue, and Table 2 shows the results for human tissue. The cross-validation accuracy was generally increased for the more homogeneous tissue regions. Furthermore, by adding RS to SHM the accuracy was substantially increased. The values of $C$ and $\gamma$ ranged from 1.1 to 19000 and 0.023 to 4.8, respectively, for porcine samples. For human tissue $C$ was between 50 and 33000, and $\gamma$ was between 0.0013 and 8.0.
Figure 5: The medians of the $E$ and $\kappa$ values for the human tissue types. $e =$ epithelium, $l =$ lumen, $s =$ stroma, $ce =$ cancerous epithelium, $cs =$ cancerous stroma, and $st =$ prostate stones.

Table 1: SVM classification accuracies for determining porcine tissue types from SHM and RS data. CVA = cross-validation accuracy, PA = prediction accuracy.

<table>
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<th>Homogeneity threshold</th>
<th>&gt; 50% $(n=1221)$</th>
<th>&gt; 67% $(n=926)$</th>
<th>&gt; 83% $(n=519)$</th>
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</thead>
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<td></td>
<td>CVA</td>
<td>PA</td>
<td>CVA</td>
</tr>
<tr>
<td>SHM</td>
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<td>65.2</td>
<td>69.5</td>
</tr>
<tr>
<td>SHM and RS</td>
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<td>100</td>
<td>83.5</td>
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</table>

Table 2: SVM classification accuracies for distinguishing healthy and cancerous human prostate tissue. CVA = cross-validation accuracy, PA = prediction accuracy.

<table>
<thead>
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<th>Homogeneity threshold</th>
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<th>&gt; 67% $(n=190)$</th>
<th>&gt; 83% $(n=178)$</th>
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<td></td>
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<td>PA</td>
<td>CVA</td>
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<tr>
<td>SHM</td>
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<td>SHM and RS</td>
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<td>100</td>
<td>73.7</td>
</tr>
</tbody>
</table>
3. Results

Figure 6: The mean Raman spectra of the porcine tissue types. A difference spectrum between stroma and lumen, which differed the most, is shown at the top. Its intensity has been multiplied by four to enhance the differences. arb = arbitrary units.
4 Discussion

In this study SVM was applied to classify porcine and human prostate tissue from SHM and RS measurements. The combined classification accuracy was 80–87% for healthy porcine tissue, and 73–77% for healthy and cancerous human tissue, depending on the homogeneity of the tissue. We have shown that the classification accuracy for using SHM alone is good, and by adding RS to SHM the classification accuracy is substantially increased.

This study confirms the results of Jalkanen et al. [29, 30] and Eklund et al. [31]. They concluded that healthy stroma was stiffer than glandular tissue, i.e. lumen and epithelium, and that prostate stones increased tissue stiffness. Furthermore, Jalkanen et al. [29] showed that cancerous prostate tissue was significantly stiffer than glandular tissue ($p < 0.05$), whereas stroma and prostate stones could not be distinguished from cancer. This agrees well with our findings, which in addition indicate that cancerous epithelium and healthy stroma are the most difficult tissue types to distinguish, whereas cancerous stroma shows increased stiffness (Figure 5). Tuxhorn et al. [32] observed an elevated amount of collagen and loss of smooth muscle in cancerous stroma, which is a possible explanation for the increased stiffness observed in this study. Using SHM, Murayama et al. [11] mapped the elasticity of human prostate and observed that the cancerous node (about 100 µm in diameter) was stiffer than surrounding normal tissues. However, it was technically difficult to compare the elasticity map to the histology image. Thus, the elasticity distribution could not be further analyzed [11].
4. Discussion

In this study, the localization of the measurement area on the histology images was done manually. The histology specimens were often deformed, which made it more difficult to overlap the morphological features of the histologic images and the stiffness maps. Since the step size of the SHM mappings was merely 50 \( \mu \text{m} \), a slight localization error would have caused many measurement points to be misclassified. To minimize the impact of this, we conducted the analysis of SHM data on homogeneous tissue regions. We observed that the separation of \( E \) for the tissue types then increased, and the deviations from normality decreased. This confirmed that many points had been misclassified. For the SVM analysis the measurement areas were 300 \( \mu \text{m} \) in diameter, so the localization errors did not affect the outcome as much. Still, higher classification accuracy may have been attained with smaller localization errors. In future studies an objective and more accurate localization may be provided by developing an algorithm that can assist in identifying the measurement area.

Histology specimens from both above and below the measurement sample were acquired to give a rough estimate of the tissue distribution across the sample. The specimens that were cut below the measurement samples were of low quality. We were only able to conclude that the cancerous tissue to some extent continued across the human 500-\( \mu \text{m} \) sections. Thus, the histologic analysis conveyed information only about the tissue type at the topmost layer of the tissue. The probes picked up signal from deep-laying layers of tissue. The Raman spectra even contained peaks from the filter paper under the tissue. Jalkanen et al. [29] showed that the TRM was sensitive to layers of tissue lying deeper than the impression depth in a weighted tissue proportion model. In this study, it can therefore be suspected that since the MTS was impressed about 40 \( \mu \text{m} \), it also sensed even deeper layers.

Maintaining tissue moistness is fundamental for SHM measurements [10, 33]. In this study we used a simple but effective method in which the sample was placed on a filter paper and surrounded by a shelter to which a constant flow of PBS was supplied. Extensive tests were carried out to find an optimal amount of flow to prevent tissue dehydration during the total SHM measurement time of approx. 30 minutes. Six repeated SHM mappings were acquired on the same area of 10 \( \times \) 10 points on porcine prostate tissue during 30 minutes. The optimal flow was determined by minimizing the change in mean stiffness while maximizing the correlation coefficients between the maps. In the final SHM mappings no general trend of sample dehydration was observed in plots of \( E \) versus acquisition time. However, dehydration/flooding may have contributed to the large spread of \( E \). In future studies the stability of the mappings and the quality of the data may be enhanced by using a moisture chamber, or immersing the sample fully in PBS [10].

Gelatin was chosen as material for stiffness calibration because it is a biological material easily prepared in a stiffness range comparable to that of prostate tissue. A potential disadvantage of gelatin is that its stiffness is temperature dependent. This effect was suppressed by storing the samples in a refrigerator and minimizing the time in room temperature to less than three minutes. No temperature effect was observed in the SHM data. For two of the porcine prostate samples the gelatin calibration from the
previous day was used to calculate $E$ because the MTS tip was broken after the tissue measurement due to operator mistake.

The choice of using SVM for classification was made based on the amount of variables and data, as well as the likelihood of non-separable classifications. SVM is well suited to handle this type of data by using soft margins to deal with non-separable classifications [16]. Furthermore, by using kernel functions the SVM is able to handle a large amount of variables and large quantities of data. The classification accuracy of SVM depends on the kernel function $k(x_i, x_j)$. Several of the non-linear kernels were tested, including the inhomogeneous polynomial kernel of the form $k(x_i, x_j) = (x_i \cdot x_j)^d$, the RBF of the form $k(x_i, x_j) = e^{-\gamma \|x_i - x_j\|^2}$, $\gamma > 0$, and the sigmoid kernel of the form $k(x_i, x_j) = \tanh(\gamma x_i \cdot x_j + r)$, $\gamma > 0, r < 0$ where $x_i, x_j$ are data vectors and $\gamma, d$ and $r$ are kernel parameters. Tests showed that the best results were given by the RBF kernel. This agrees with Sattlecker et al. [17], who compared different SVM kernels for classifying RS measurements on lymph node samples from breast cancer patients.

From Tables 1 and 2 we see that by using SHM alone the cross-validation classification accuracy was 65–82%. Thus, stiffness measurements are valuable for tissue characterization and for localizing stiff nodules. The nonlinear parameter $\kappa$, which was a measure of the curvature of the measurement curve, had a large positive impact on the accuracy achieved by SHM. There was a relatively large separation of the $\kappa$ values for the human tissue types, and we see from Figure 5 that $\kappa$ seems to add complementary information to $E$, especially for differentiating healthy epithelium. This suggests that $\kappa$ has a diagnostic value. Figure 5 also indicates that the similarities of the $E$ and $\kappa$ values for healthy stroma and cancerous epithelium may have been the main factor limiting the accuracy for distinguishing healthy and cancerous tissue. The patient in this study suffered from prostatitis, i.e. inflammation of the prostate gland, which is a common cause of false positives in elastographic examinations of the prostate [15,34]. The inflammation may have increased the stiffness of the healthy tissue in this patient.

The classification accuracy increased substantially when RS was added to SHM (Tables 1 and 2). For the porcine samples it was over 80%, even when the most heterogeneous tissue regions were included. For the human samples the accuracy was lower. The main reason was most likely the low quality of the Raman signal, which was quenched by the fluorescence from the ink applied to the surgical margin. Even though the fluorescence was decreased in the high wavenumber region (Figure 7), the spectral quality was lower and considerably more noisy than the spectra of porcine tissue.

The high classification accuracies for combined SHM and RS measurements on heterogeneous tissue regions indicate that the proposed combined instrument will be strong for investigating tissue boundaries. Furthermore, the results indicate that the rate of misclassification for homogeneous regions will be low. This is very promising for an application where surgical margins are probed for the presence of cancer.
5 Conclusion

The combination of SHM and RS can successfully be used to identify different prostate tissue types by using SVM. The results indicate accurate detection of cancerous prostate tissue. This is promising for the development of an instrument that combines these two methods for tissue characterization during prostate cancer surgery.

Acknowledgments

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References


References


