Towards new sensors for prostate cancer detection - combining Raman spectroscopy and resonance sensor technology

Stefan Candefjord
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To my family
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 licentiate work was to explore the need for new and complementary methods for PCa detection and to take the first step towards a novel approach: combining Raman spectroscopy and resonance sensor technology. Firstly, the main methods for PCa detection were reviewed. Secondly, to establish a robust protocol for Raman experiments in vitro, the effects of snap-freezing and laser illumination on porcine prostate tissue were studied using Raman spectroscopy and multivariate statistics. Thirdly, measurements on pork belly tissue using both a resonance sensor and a Raman fiberoptic probe were evaluated regarding correlation of the data.

It was concluded 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. Raman spectroscopy and resonance sensor technology are promising alternative techniques, but hitherto their potential for PCa detection have only been investigated in vitro.

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

The combined measurements on pork belly tissue showed that Raman spectroscopy provided additional discriminatory power to the resonance sensor. The Raman data explained 67% of the variability of the stiffness parameter. The differentiation of tissue types using the resonance sensor was relatively poor, likely due to its large sample volume compared to the Raman sensor. A smaller resonance sensor tip may improve the results.

In summary, this work indicates that an instrument combining Raman spectroscopy and resonance sensor technology is a promising complementary method for PCa detection. Snap-freezing of samples may be used in future Raman studies of PCa. A combined instrument could potentially be used to guide prostate biopsies towards lesions suspicious for cancer, and for tumor-border demarcation during surgery. All of this should provide a more secure diagnosis and consequently more efficient treatment of the patient.
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The study was performed within the CMTF network.

My warmest thanks to my family and my girlfriend Linda for support and being who you are.
Part I
In this licentiate report 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.

(A) S. Candefjord, K. Ramser and O. A. Lindahl, “Technologies for localization and diagnosis of prostate cancer”, Submitted to Journal of Medical Engineering and Technology.

(B) S. Candefjord, K. Ramser and O. A. Lindahl, “Effects of snap-freezing and near-infrared laser illumination on porcine prostate tissue as measured by Raman spectroscopy”. Submitted to Analyst.

(C) S. Candefjord, M. Nyberg, V. Jalkanen, K. Ramser and O. A. Lindahl, “Evaluating the use of a Raman fiberoptic probe in conjunction with a resonance sensor for measuring porcine tissue in vitro”, Submitted to The World Congress on Medical Physics and Biomedical Engineering.

Table 1.1: The contributions made by Stefan Candefjord to Paper A, B and C. 1 = main responsibility, 2 = Contributed to high extent, 3 = Contributed.

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Other publications of relevance

Other publications of relevance, but not included in this report, are listed below.


Chapter 3

Abbreviations

BPH  benign prostatic hyperplasia
MR   magnetic resonance
MRI  magnetic resonance imaging
MRSI magnetic resonance spectroscopic imaging
NIR  near-infrared
PBS  phosphate buffered saline
PC   principal component
PCA  principal component analysis
PCa  prostate cancer
PSA  prostate-specific antigen
PZT  lead zirconate titanate
SB   systematic biopsy
Chapter 4

Introduction

4.1 General background

Prostate cancer (PCa) is the most common form of male cancer in the US and Europe, and only lung and colorectal cancer have a higher mortality among European men [1, 2]. Due to the aging population the incidence of the disease is expected to increase [3]. The clinical diagnostic tests available today, the prostate specific antigen (PSA) test and multiple systematic biopsy (SB), are unreliable [4, 5]. PCa is often indolent, more men die with the disease than from it. Due to the risks associated with surgically removing the prostate, active surveillance may be the best option for patients with indolent PCa. On the other hand, aggressive tumors with high metastatic potential must be identified and removed at an early stage. Current diagnostic tests cannot reliably distinguish between indolent and aggressive PCa. As a consequence, many men are treated either unnecessarily or too late.

The prostate is a deep-sited organ with heterogenous structure [6], and it is therefore difficult to detect tumors. Advanced techniques for ultrasound and magnetic resonance imaging (MRI) show relatively high sensitivity for PCa detection [7, 8]. However, tumors are often confused with benign lesions, such as prostatitis and benign prostatic hyperplasia (BPH) [7, 8]. Furthermore, a more precise disease characterization is needed, this is the major objective of PCa detection [9]. Today, no clinical method can assess a tumor’s potential to metastasize, and that information would be most useful for deciding whether adjuvant therapy should be implemented [10]. New methods are needed for PCa detection and diagnosis. This work takes the first steps towards a novel approach where two experimental and complementary techniques for PCa detection are combined, i.e. resonance sensor technology and Raman spectroscopy.

The resonance sensor can measure the stiffness of a material through frequency changes of a piezoelectric vibrating element. Resonance sensors have been used in several medical applications [11]. The method has been shown to distinguish between soft, healthy prostate tissue and PCa in vitro [12–14]. It is a promising candidate for PCa detection in vivo. However, the sensitivity is currently insufficient to differentiate tumors
and relatively hard healthy tissue, such as sites with an accumulation of prostate stones.

Raman spectroscopy assesses the biochemical composition of a sample via inelastic scattering of light. A laser illuminates the sample and the spectrum of the backscattered light is analyzed. Numerous in vitro studies show that Raman spectroscopy can detect many types of cancers, including PCa, with high sensitivity and specificity [15–19]. Despite this high potential, few in vivo studies have been reported, the main reason being the difficulties with developing fiberoptic Raman probes for clinical use [20]. Raman spectroscopy is very promising for distinguishing indolent and aggressive PCa [16,17,19].

The disadvantages of Raman spectroscopy are that laser irradiation may damage tissue, that measurements may be sensitive to surrounding light, and that current fiberoptic probes have too short penetration depth to noninvasively detect PCa in vivo.

To combine the two methods could minimize the drawbacks associated with each technique. The resonance sensor constitutes a gentle deep-sensing method that could be used to scan the tissue. The Raman sensor could provide complementary measurements on areas that are likely to be malignant. In the first place, a combined instrument could be used during cancer surgery, to assist surgeons in removing the whole tumor and only a minimum of healthy tissue. It may also be used to guide prostate biopsies. In the long term, it could potentially be used for minimally invasive localization and concurrent automatic diagnosis of PCa in vivo.

In vitro studies are necessary for successful implementation of the combined instrument in vivo. To assure that results of in vitro studies are transferable to the in vivo situation, it is important that preparation protocols and measurement procedures for in vitro studies preserve tissue characteristics close to the native state. It should be established whether the laser irradiation damages the tissue, since this may distort the results. Snap-freezing of tissue is a common preservation method that is considered to affect the tissue minimally. However, only a few Raman spectroscopic studies confirm that, and prostate tissue was not included in those experiments [21–23].

This licentiate report gives a background to the difficulties of localizing and diagnosing PCa. It reviews the main methods for PCa detection in clinical use today, and promising novelities. The importance of robust in vitro study protocols is considered, and the effects of snap-freezing and near-infrared laser illumination on porcine prostate tissue are investigated using Raman spectroscopy. The combination of resonance sensor technology and Raman spectroscopy is discussed and evaluated in preliminary experiments on pork belly tissue.

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 slightly acidic, milky fluid, which makes up about 25% of the volume of semen [24]. The gland is about the size of a golf ball and resembles a walnut in shape [24]. It is inferior to the urinary bladder, and envelops the upper portion of the urethra, as shown in Figure 4.1.
Many prostatic ducts lead the prostatic fluid into the urethra. Fibromuscular stroma, the supporting framework consisting of connective tissue and smooth muscle tissue, encloses the prostate. However, the apex and the base are continuous with adjacent tissue. 70% of the prostate is composed of glandular elements and 30% is fibromuscular stroma. There are three anatomical zones in the prostate: the peripheral zone, the transitional zone and the central zone.

The prostate is normally enlarged in periods throughout the life. It grows rapidly during puberty, remains at a stable size between age 30–45, after which it may begin to grow again. The majority of men > 55 years develop BPH, a benign enlargement of the

![Prostate Diagram]

Figure 4.1: The prostate anatomy. Modified from Wikipedia (http://en.wikipedia.org/wiki/Prostate).
prostate [25]. The formation of prostate stones in the lumen of the prostate glands, due to solidification of glandular secretions, is another common benign occurrence. The stones are quite hard and contribute to tissue stiffness, although they only make up a small fraction of the tissue volume [12,26].

The function of the prostatic fluid is not completely understood, but it aids the motility and viability of sperm. It contains citrate, acid phosphatase and several protein-digesting enzymes, such as PSA [24].

4.2.2 The porcine prostate

The male reproductive system of the pig is composed of the same structures as in humans [27]. In contrast to the human prostate, the porcine prostate consists of two lobes and does not surround the urethra [28], and it is relatively small [27].

Nicaise et al. [29] used light and electron microscopy to study the prostate of 12 boars and 8 barrows (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 9,000 men are diagnosed with PCa each year and about 2,300 die from the disease. That makes it the most common cause of cancer-related male death. The disease is often asymptomatic, even in men with aggressive tumors, until the cancer spreads [30]. About 50% of elderly men harbor PCa [31], but the vast majority of cancers are indolent [5]. In its most progressed form PCa disperses metastases and is very dangerous; the 5-year survival rate is only 34%, while it is 100% if the cancer has not spread beyond the structures adjacent to the prostate or metastasized to non-regional lymph nodes [32].

Almost all, 95% of prostate tumors form in the prostatic ducts in the glandular epithelium [32]. The majority develop in the posterior part of the gland (the peripheral zone) [33, 34], which is situated towards the rectum, see Figure 4.1. They are usually multifocal and provide little contrast to healthy tissue using present imaging methods, such as ultrasound and magnetic resonance imaging (MRI), making them difficult to detect.

4.2.4 Localization and diagnosis of prostate cancer

The gold standard for detection and diagnosis of PCa are the PSA test and SB. A high PSA level indicates cancer, but PSA is not a cancer-specific marker. SB fails to detect about 30% of present tumors [35]. Evaluation of the aggressiveness of detected tumors is currently performed following the Gleason grading system, where biopsies are histologically examined. This method is subjective, and the rate of intra- and interobserver disagreement is high [36]. About 70% of men diagnosed with PCa have tumors of a medium Gleason score and a slightly elevated PSA level, and the disease progression is
then unpredictable. Thus, there is currently no accurate method for identifying aggressive tumors at an early stage.

A multicenter European randomized study including 182,000 men recently reported that PSA-based screening for PCa reduced the mortality by 20% [5]. However, it also brought overdagnosis and overtreatment. To prevent one death, 1410 men would have to be screened and 48 additional patients would have to undergo treatment. It was estimated that the rate of overdagnosis, i.e., diagnosing PCa in men with indolent tumors that would not cause clinical symptoms in their lifetime, was 50% [37]. A similar study in the US, which enrolled almost 77,000 men, did not find any significant benefits of PSA screening [38]. Possible explanations for the discrepancy between the two studies include that the European study used a PSA cutoff of 3 ng/mL in most centers, as compared to 4 ng/mL in the US study, and that many patients in the control group in the US were screened as a part of usual care.

The main imaging methods for detection of PCa are ultrasound and MRI. Due to a number of limitations, these techniques are not routinely used clinically for direct PCa detection [4].

### 4.3 Raman spectroscopy

#### 4.3.1 The Raman effect

When a beam of light interacts with a tissue sample, the impinging photons are scattered due to different processes. Most photons are elastically scattered, i.e., the wavelength is not altered by the process. Rayleigh scattering is referred to as the elastic scattering from particles that are small compared to the wavelength of the incident light [39]. A small part of the incoming photons can be inelastically scattered, meaning that the emitted photons have less energy than those absorbed. Raman scattering is an inelastic process in which a tiny fraction, $10^{-8}$–$10^{-6}$, of the incident photons can go into setting molecular bonds into vibration [40]. The emitted photons lose (or gain) energy corresponding to the specific vibrational energy levels of the probed molecules. In a Raman spectrum the wavelength difference between the incident and the scattered photons is plotted as a function of the intensity of the scattered light. Since each molecule has a unique set of bond vibrations, the spectrum is like a fingerprint of the sample.

The Raman effect was discovered in 1928 by the Indian professor Sir C.V. Raman, who observed the phenomenon in a delicate experiment using filtered sunlight as excitation source and the eye as detector [41]. He was awarded the Nobel prize for the discovery already two years later.

Raman scattering can be explained as follows [40]. As a molecule is hit by an incoming photon its electron cloud is distorted by the electromagnetic field. The photon can be treated as an oscillating dipole of the size of the wavelength of the light. This dipole is much larger than the molecule; the wavelength of visible light is 400–700 nm, which can be compared to the size of 0.3–0.4 nm for a small molecule. The electron cloud is polarized by the dipole, its geometry is changed, and a virtual, higher state of energy is reached.
This state is unstable and therefore very short-lived ($\sim 10^{-14}$ s). The nuclei of the molecule cannot respond to the rearrangement of electrons and establish new positions of equilibrium. They are set into vibration during the lifetime of the virtual state. Almost immediately the molecule emits a photon and returns to its ground state. In most cases the emitted light has longer wavelength than the incident light, because energy is required to move the relatively heavy nuclei. The wavelength difference is termed a Stokes shift and corresponds to the frequency of the molecular bond vibration. Scattered photons can sometimes gain energy. This is called anti-Stokes scattering and is possible only when the molecule initially is in an excited energetic state. At room temperature the excited states are much more sparsely populated than the lowest energetic state.

There are certain conditions that have to be fulfilled for a molecule to be Raman active. The selection rules follow from a quantum-mechanical treatment of the molecular vibrations [41]. As an example, consider the vibration of a diatomic molecule. The vibration can be modelled as a harmonic oscillator, i.e. the potential energy of the nuclei as a function of displacement is a parabolic function. In this model the chemical bonding between the nuclei is pictured as a Hookian spring with a force constant $K$. If the Schrödinger equation for this system is solved, eigenvalues $E_\nu$, shown in equation 4.1, and corresponding eigenfunctions are obtained.

$$E_\nu = \hbar c \tilde{\nu} \left( \nu + \frac{1}{2} \right)$$  \hspace{1cm} (4.1)\hspace{1cm}

$h$ is Planck’s constant, $c$ is the speed of light and $\nu \in \mathbb{N}_0$ is the vibrational quantum number. $\tilde{\nu} = \frac{1}{2\pi} \sqrt{\frac{K}{\mu}}$ is the wavenumber [cm$^{-1}$] of the vibration, where $K$ is the force constant and $\mu$ is the mass of each nucleus. Hence, strong bonds and light atoms will give rise to high vibrational frequencies and vice versa [40]. The selection rules of quantum mechanics prohibit many vibrational transitions [41]. For the harmonic oscillator, only transitions that fulfil $\Delta \nu = \pm 1$ are allowed. The transition $\nu = 0 \leftrightarrow 1$ produces the most intense peak in the Raman spectrum, because normally most molecules are in their lowest state of energy $E_0$.

Classical theory can be used to further explain some basic features of Raman scattering [41]. Consider a diatomic molecule that is 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 $P = \alpha E = \alpha E_0 \cos (2\pi \nu_0 t)$ 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 harder 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 $\alpha$ for small amplitudes of vibration, it can be expanded as:

$$\alpha = \alpha_0 + \left( \frac{\partial \alpha}{\partial q} \right)_0 q + \ldots$$  \hspace{1cm} (4.2)\hspace{1cm}

where $\alpha_0$ is the polarizability at $q = 0$. Substituting equation 4.2 into the expression for the dipole moment, and using the formula $\cos \gamma \cos \beta = \frac{1}{2} \cos (\gamma - \beta) + \frac{1}{2} \cos (\gamma + \beta)$, we
obtain:

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

(4.3)

The three terms in equation 4.3 symbolize dipoles that oscillate with frequencies \(\nu_0\), \(\nu_0 - \nu_m\) and \(\nu_0 + \nu_m\). They describe Rayleigh, Stokes and anti-Stokes scattering, respectively. Note that the Stokes shift equals the vibrational frequency of the molecule, \(\nu_m\). A fundamental property of Raman scattering is understood from equation 4.3: if \(\left( \frac{\partial \alpha}{\partial q} \right)_0 = 0\), no Raman scattering will occur. This means that a specific vibration of a molecule is Raman active only if the polarizability is changed during the vibrational cycle. The Raman radiation generally becomes more intense as the term \(\left( \frac{\partial \alpha}{\partial q} \right)_0\) increases [41]. Usually, symmetric vibrations cause the largest polarizability changes and generate the strongest scattering [40].

### 4.3.2 Raman instrumentation

A Raman spectrometer basically consists of a laser generating monochromatic light, a sample illumination and collection system, a filter that separates the elastically (Rayleigh) and the inelastically scattered light, a wavelength selector (e.g. a grating) and a detector [41]. Modern systems for tissue measurements typically use near-infrared (NIR) diode lasers and CCD detectors sensitive in the NIR region. Microscopes or fiberoptic probes in the backscattering collection geometry are commonly used to illuminate the sample and collect the Raman light [42]. Figure 4.2 shows a schematic drawing of a Raman fiberoptic setup.

![Figure 4.2: A typical Raman fiberoptic setup.](image)
The development of Raman fiberoptic probes enables in vivo Raman measurements. Several factors complicate the realization of fiberoptic probes. In the Fingerprint region fused silica fibers generate a strong signal, which necessitates the use of extra filters at the probe tip to block this radiation [20]. 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 [43]. They must withstand clinical sterilization routines [43]. Several different probes have been developed, but so far the manufacturing process has been complicated and expensive [20]. However, several technical advancements in the construction of fiberoptic probes have been presented recently [20].

Komachi et al. [44–47] have developed a 0.6 mm thin probe, and demonstrated promising results in measurements of the esophagus and stomach of the living rat [47]. The probe consists of a central delivery fiber surrounded by 8 collection fibers. They claim that it can be commercially manufactured at a low cost [44].

The penetration depth in tissue of Raman systems using the backscattering collection mode is typically only of the order of one hundred micrometers [48]. Hence, deep-sited organs, such as the prostate, are inaccessible for noninvasive examinations. Development of techniques that can probe deeper into the tissue, such as time-gated Raman spectroscopy and spatially offset probes, is ongoing [48]. Minimally invasive examinations via, e.g. endoscopes, are feasible [20].

4.3.3 Raman measurements of tissue

Raman spectroscopy is excellent for measuring the biochemical content of tissue for a number of reasons including: (i) The majority of biological molecules are Raman active [49]. (ii) Minimal or no sample preparation is required. (iii) Water is a poor Raman scatterer, it interferes little with the spectra of tissue components [40]. (iv) Raman spectroscopy is sensitive to many factors that affect biomolecules, such as pH, degree of hydration, bacterial attack, etc. [50]. (v) The relative abundance of tissue components is proportional to their contributions to the Raman spectrum [43]. (vi) In vivo measurements are feasible via fiberoptic probes. Some of the drawbacks with the method are: (i) Tissue autofluorescence can distort the Raman signal, (ii) Acquisition of high quality spectra often requires long integration times, thus in vivo measurements may be affected by motion artifacts, (iii) The instrumentation may be sensitive to surrounding light, and (iv) Current fiberoptic probes have a short penetration depth in tissue [48].

Raman measurements of tissue were long hampered by the strong tissue autofluorescence induced by lasers in the visible region [43]. Modern NIR Raman systems have largely overcome this problem, since NIR light has too low energy to initiate most fluorescence processes [43]. 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 [51]. Several different approaches for minimizing fluorescence interference have been demonstrated. Time-gating and wavelength shifting can effectively decrease fluorescence interference, but require modifications of the Raman instrumentation [52]. Instead, mathematical methods can
be used. However, many algorithms cause spectral artifacts [52]. Polynomial fitting does not distort the Raman bands to a high degree [52]. Lieber et al. [52] 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. [53].

By convention a Raman spectrum is presented with intensity on the vertical axis and the Stokes shifts, measured in cm$^{-1}$, on the horizontal axis. Stokes shifts from 200–3600 cm$^{-1}$ usually cover the information of interest [40]. Biological tissue generally produces spectra with relatively narrow bands, typically 10–20 cm$^{-1}$ wide [43]. The characteristic vibrations of the most common chemical groups have been assigned approximate energy ranges that are valid for the groups in most structures [40]. 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 Finger print region. Many molecules exhibit complex vibrational patterns that yield unique spectral features in this region, which is densely packed with sharp bands [49]. Raman peaks below 650 cm$^{-1}$ normally belong to inorganic groups, metal-organic groups or lattice vibrations. Raman spectroscopy can explore the primary, secondary, tertiary and quaternary structure of biological molecules [49]. 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. [54].

Figure 4.3 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 [49]. 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 normal and cancerous tissues including brain, breast and gynecologic tissues. Cancer induces a significant increase of the nucleic acid content. 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 [16–19, 56, 57] have investigated the potential of Raman spectroscopy to detect and grade PCa. Crow et al. [17] attained 98% sensitivity and 99% specificity for differentiating four cell lines with varying degrees of biological 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. [57] attained a 100% accurate classification of benign and malignant prostate cells ($n = 30$). The results suggested that the most significant spectral change due to cancer was an increase in the DNA content, with a concomitant change in DNA conformation from B-DNA to A-DNA. Crow et al. [16] also showed that prostate biopsy samples of BPH and cancer with different Gleason scores could be distinguished with an overall accuracy of 89%. 450 spectra were recorded from biopsies of 27 patients, 14 with BPH and 13 with PCa. The authors suggested that a Raman needle probe could
Figure 4.3: A spectrum of porcine prostate tissue recorded in our laboratory using a Raman microspectrometer (Renishaw system 2000, Renishaw, UK). The integration time was 5 min.

Table 4.1: Tentative assignments [54, 55] of the major peaks in the porcine prostate spectrum shown in Figure 4.3.

<table>
<thead>
<tr>
<th>Peak position (cm$^{-1}$)</th>
<th>Major assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1666</td>
<td>Amide I / C=C lipid stretch</td>
</tr>
<tr>
<td>1451</td>
<td>CH$_2$ bending mode of proteins and lipids</td>
</tr>
<tr>
<td>1240–1265</td>
<td>Amide III</td>
</tr>
<tr>
<td>1208</td>
<td>Tryptophan and phenylalanine $\nu$(C−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 proline, valine, protein backbone / glycogen</td>
</tr>
<tr>
<td>877</td>
<td>C−C−N$^+$ symmetric stretching (lipids) / C−O−C ring (carbohydrate)</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>
be passed into the prostate in vivo. In another study [18], a fiberoptic probe was used to
distinguish PCa from BPH and prostatitis. 38 prostate samples from 37 patients were
measured, and the overall accuracy was 86%.

Stone et al. [19] estimated the gross biochemistry of BPH, prostatitis and PCa of dif-
ferent 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 stan-
dards assumed to be the main tissue constituents. It was shown that the DNA content
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.

4.4 Resonance sensor technology

4.4.1 The piezoelectric effect

The piezoelectric effect was discovered by the brothers Pierre and Jacques Curie in
1880 [58]. 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 os-
cillate in response to a sinusoidal voltage variation. Hence, a piezoelectric element can
work 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 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 typically
utilize a ceramic piezoelectric material, e.g. lead zirconate titanate (PZT), which can
be pictured as composed of a mass of 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 [59]. 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
will then be polarized in the direction of the applied field, and the dipoles are locked
when the field is withdrawn. The procedure gives rise to a permanent deformation of the
ceramic, as understood from the relation between polarization and mechanical stress.

4.4.2 Piezoelectric resonance sensor principle

The resonance sensor theory used in this work was presented by Omata & Terunuma in
1992 [60]. 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 vibrational fre-
quency. 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 4.4 [60].
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 frequency is set by the phase-shift circuit, which ensures that the sum of the phase shifts in the system is zero. When this condition is fulfilled, resonance is established. It is advantageous to choose an oscillation frequency close to the inherent resonance frequency of the PZT element, since a high sensitivity is then obtained.

![Figure 4.4: The principle of the resonance sensor.](image)

As the tip of the resonance sensor comes into contact with an object the resonance frequency changes, and the shift is related to the stiffness of the material [60]. 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, while it is positive for hard materials such as teeth and glass [60].

### 4.4.3 Explanatory models

The model of vibration modes in a finite rod can be used to approximately describe the characteristics of the resonance sensor [60,61]. The frequency change as the sensor comes in contact with an object can be expressed as

\[
\Delta f = -\frac{V_0 \beta_x}{2\pi l Z_0} \tag{4.4}
\]

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 impedance \(Z_x = \alpha_x + i\beta_x\) of the probed object, where \(\alpha_x\) is the resistance. \(\beta_x\) can be written as

\[
\beta_x = m_x \omega - \frac{k_x}{\omega} \tag{4.5}
\]
where $\omega$ is the angular frequency, $m_x$ is the inertia term and $k_x$ is the surface stiffness term. $m_x$ and $k_x$ can be written as

$$m_x = \frac{4a_{11}}{\pi \beta^2 (1 - \nu)} \rho S^{3/2} \quad (4.6)$$

$$k_x = \frac{2E}{\pi^{1/2} (1 - \nu^2)} S^{1/2} \quad (4.7)$$

$\nu$ is Poisson’s ratio, $\rho$ is the density, $S$ is the surface contact area, $E$ is the elastic modulus (Young’s modulus), and $a_{11}$ is a coefficient that depends on $\nu$ [62]. Jalkanen et al. [61] examined the theoretical model of the finite rod for the Venustron® resonance sensor system. They showed that, since $m_x \omega \gg k_x \omega$ for the system, the surface stiffness term can be neglected. Equations 4.4–4.6 then give

$$\Delta f \propto \rho S^{3/2} \quad (4.8)$$

for a specific rod vibrating at a constant frequency, if Poisson’s ratio is assumed constant [61]. The surface contact area, $S$, depends on the contact force between the sensor tip and the measurement object, $F$, according to $F \propto E S^{3/2}$ [61]. Substituting this relationship into equation 4.8 results in

$$\Delta f \propto \frac{\rho F}{E} \quad (4.9)$$

A stiffness sensitive parameter, $\frac{\partial F}{\partial \Delta f}$, can then be derived as

$$\frac{\partial F}{\partial \Delta f} \propto \frac{E}{\rho} \quad (4.10)$$

Jalkanen et al. [61] 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 $\frac{\partial F}{\partial \Delta f}$ as a stiffness sensitive parameter.

### 4.4.4 Sensing volume

Jalkanen et al. [14] investigated the sensing volume of the Venustron® resonance sensor system. The sensor had a hemispherical tip of 5 mm diameter. 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 impression depth of 1 mm. The impression depth affects the depth-sensing capacity; the deeper impression depth the deeper sections of the tissue that can be measured. The resonance sensor generates vibrations in the ultrasound range. As ultrasound propagates through tissue some of the sound energy is absorbed. There is an almost linear relationship between the absorption coefficient of tissue and the sound frequency [63]. Thus, a lower resonance frequency increases the penetration depth.
4.4.5 Detection of prostate cancer

Eklund et al. [26] was the first group to measure the stiffness of human prostate tissue with a resonance sensor in vitro. A correlation of $R = -0.96$ with the proposed stiffness based on the histological composition of the tissue was obtained. They used a catheter type resonance sensor with a cylindrical PZT element having a diameter of 1.2 mm. 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. [12,13] examined fresh human prostate tissue with the Venustron® resonance sensor system. Directly after surgical removal the prostates were cooled with ice and transported from the surgical unit to the laboratory facility. The authors showed that the resonance sensor could distinguish relatively soft glandular prostate tissue from cancerous tissue. In the first study ten prostate samples from ten patients were tested and a $p$-value $< 0.001$ was obtained for a MANOVA test of the difference between cancerous ($n = 13$) and healthy ($n = 98$) tissue [13]. Only measurement sites consisting of 100% cancerous tissue were significantly stiffer than the glandular tissue, indicating that the resonance technique has difficulties in demarcating the borders of tumors. 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 [33,34], where soft glandular tissue is abundant [12]. Thus, a stiff lesion in this area could indicate cancer [12].

4.5 Preparation protocols and measurement procedures for in vitro studies in general

It is essential to avoid misinterpretation of experimental findings in vitro due to artifacts originating from tissue preparation and/or inappropriate measurement procedures. A group in Umeå has performed experiments on human prostate tissue using different resonance sensor systems [12, 13, 26, 64]. Appropriate study protocols are described in those publications. This report therefore mostly focuses on investigating measurement protocols and preparation procedures for Raman measurements.

Fresh tissue samples, immersed in physiological buffer preventing tissue dehydration, are ideal for in vitro Raman spectroscopic studies [21,65]. However, preservation of the samples is usually necessary since fresh samples are fragile, hard to procure and have a very limited shelf life [65]. Clinically, the most common method for archiving tissue specimens is formalin-fixation and subsequent paraffin-embedding [66]. Unfortunately, paraffin-embedded samples are not suitable for Raman measurements, since the paraffin generates a very strong signal that swamps spectral information from the tissue [66]. Deparaffinization of tissue is feasible, but biochemical information may be lost [66]. It is difficult to remove all paraffin, and the residuals cause interfering spectral peaks [22,66]. Formalin fixation may be a suitable alternative preservation method [65,66]. However, some studies have observed spectral artifacts in formalin-fixed tissue [21,22].

Freezing of tissue is considered the gold standard for preservation methods [22]. Only
4.5. Preparation protocols and measurement procedures for in vitro studies in general

A few studies have evaluated different protocols for freezing and subsequent thawing using Raman spectroscopy [21–23]. Shim et al. [21] studied snap-freezing of 10 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 $-80^\circ\text{C}$ for 1, 9 and 30 days. The frozen tissue was thawed immersed in phosphate buffered saline (PBS) at room temperature for 15 min. No spectral artifacts due to freezing/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 Raman spectroscopic study of snap-freezing of prostate tissue has been presented in the literature.

Raman spectrometers utilize relatively powerful lasers that may damage the sample. The measurements may then no longer reflect the biochemical composition of the unharmed sample. Therefore, it is necessary to examine the effects of the laser illumination on the sample. Modern Raman systems adapted for tissue measurements use NIR excitation light, which is less prone to harm the sample than light in the UV or visible region [67–69]. However, studies investigating photoinduced effects of visible and NIR light of high intensity on tissue are scarce. To my knowledge the effects of NIR laser illumination on prostate tissue have hitherto not been investigated using Raman spectroscopy.
The overall aim of this licentiate work was to explore the need for new, complementary methods for PCa detection and take the first step towards a novel approach: the combination of Raman spectroscopy and resonance sensor technology. The specific aims were:

(i) 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.

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

(iii) To evaluate the combined information from measurements on porcine tissue in vitro with a Raman fiberoptic probe and a resonance sensor, in order to investigate the correlation of the data and potential diagnostic power of the combination.
   - This objective was assessed in Paper C.
6.1 Literature review of technologies for localization and diagnosis of prostate cancer

A review of the different 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 non-invasive 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®[^1] 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

A manual research of the reference lists cited in the selected articles was also done.

[^1]: http://www.isiwebofknowledge.com
6.2 Experimental setup

To study the effects of snap-freezing and NIR laser illumination on porcine prostate tissue (B) a Raman microspectrometer (Renishaw system 2000, Renishaw, UK) was used. The system incorporated a 300 mW laser (Renishaw HPNIR, Renishaw, UK) with 830 nm wavelength. A water-dip objective (NIR Apo 60×/1.0W, Nikon, Japan) was used for spectral acquisition from 400 to 1800 cm⁻¹. The irradiance onto the samples was \( \sim 3 \cdot 10^{10} \) W/m². To avoid interference from surrounding light the room was darkened during measurements. The wavelength shift of the spectrometer was calibrated daily using the sharp silicon peak at 520 cm⁻¹ as reference. The sensitivity of the detector at different wavelengths was measured using a calibrated light source, and corrected for in the preprocessing of spectra.

To evaluate the combined information from both measurement modalities (C) a Raman fiberoptic system and the Venustron® resonance sensor system were used. The Raman system consisted of a 0.8 mm thin fiberoptic probe (Machida Endoscope Co, Japan), of the same type used in [47], coupled to a spectroscope with a holographic grating (RXN1, Kaiser Optical Systems, USA). The spectroscope analyzed all wavelengths from 100–3425 cm⁻¹ simultaneously. Since the fiberoptic probe produced interfering Raman scattering up to \( \sim 600 \) cm⁻¹ [47], the interval 100–600 cm⁻¹ was excluded. In Paper C, the wavelength region 600–1800 cm⁻¹ was studied. The excitation light at 785 nm was generated by a 400 mW laser (Invictus™, Kaiser Optical Systems, USA). Because spectral distortions from surrounding light were observed, the laboratory was darkened during measurements. The distance from the Raman probe to the sample had to be adjusted manually. A calibration system (HoloLab Calibration Accessory, Kaiser Optical Systems, USA) was used to calibrate the wavelength shift and energy sensitivity of the spectrometer.

The Venustron® (Axiom Co., Ltd., Kōriyama Fukushima, Japan) resonance sensor system (C) consisted of a motorized piezoelectric resonance sensor element, a force sensor and a position sensor. They were incorporated into a common housing, which was mounted to a table stand, as seen in Figure 6.1 (part I). The sensor tip was hemispherical with a diameter of 5 mm. The resonance frequency of the system was 59 kHz. A supplied hardware unit with driving electronics and a central processing unit communicated with the sensors. It was connected to a computer running the Venustron® software (version 2.31a). During a measurement the tip was lowered towards the sample with the motor. The surface of the sample was detected using the frequency change option in the software. The tip was pressed 1 mm into the tissue at a speed of 1 mm s⁻¹. The resonance frequency change \( \Delta f \), the force \( F \) and the impression depth \( d \) were sampled at 200 Hz during the impression of the sensor tip into the sample.

The experimental setup for the combined measurements (C) is shown in Figure 6.1. The Raman fiberoptic probe and the resonance sensor were mounted next to each other. A three-dimensional translation stage assured that measurements were performed at the same points using the two separate instruments. It was composed of three one-dimensional stages (Thorlabs, NRT100) with a common control unit (Thorlabs, BSC103).
6.3 Sample preparation

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® program that was developed in-house. The coordinate system defined in the program was calibrated to have the origin at the top left corner of the picture of the sample. It was important to assure that the sensors measured on the same points of the sample. Their reference coordinates were calibrated by visually controlling that each sensor was positioned exactly above a reference mark when the translation stage was moved to the corresponding position.

Figure 6.1: The experimental setup. I) Venustron® resonance sensor II) Temperature probe III) Raman fiberoptic probe IV) Tissue sample V) Styrofoam plate VI) Translation stage.

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. They 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°C. The frozen
samples were allowed to passively thaw immersed in PBS prior to measurement.

For the combined Raman and resonance measurements (C) pork belly tissue was used as a model system. Two pieces were obtained from the local grocery store. They were stored at 6°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.

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 4 min. The samples were immersed in PBS and immobilized with needles.

For the snap-freezing experiments the Raman signal was integrated for 30 s and measured from 400 to 1800 cm\(^{-1}\) (B). 5 samples were measured day 1 and used as reference for fresh samples. 5 snap-frozen samples were measured after 5, 26 and 81 days of storage, respectively, to study if prolonged storage at \(-80°C\) degraded the tissue. The samples were immersed in PBS during the measurements.

For the combined Raman and resonance measurements a grid with 42 measurement points was defined for each sample (C). In total 168 measurement points were measured with each device. The measurement order was randomized with the constriction that adjacent points were not measured subsequently, to avoid the possibility that viscoelastic effects could influence the resonance sensor measurements. All resonance sensor measurements on a sample were completed before the acquisition of Raman spectra. The tissue was kept moist by brushing it with PBS every fifth min.

6.5 Data analysis & Statistics

All data analysis was performed using MATLAB® (version R2007b/R2008b including Statistics Toolbox version 6.1/7.0, MathWorks, USA). The only exception was that MINITAB® (version 15.1.20.0, Minitab Inc., USA) was used to check for autocorrelation in Paper B. Many of the algorithms were not included in MATLAB®, and were then written in-house.

The stiffness sensitive parameter (C) \( \frac{\partial F}{\partial \Delta f} \) was calculated from \( \Delta f \), \( F \) and \( d \) as [12]

\[
\frac{\partial F}{\partial \Delta f} = \frac{\partial F/\partial d}{\partial \Delta f/\partial d} \tag{6.1}
\]

\( \frac{\partial F/\partial d}{\partial \Delta f/\partial d} \) was evaluated at \( d = 0.6 \text{ mm} \). At this depth the sensor measures the tissue near the surface [12]. \( \frac{\partial F}{\partial d} |_{d=0.6} \) and \( \frac{\partial \Delta f}{\partial d} |_{d=0.6} \) were estimated numerically by linear regression in the interval 0.5–0.7 mm.
6.5. Data analysis & Statistics

The Raman data was preprocessed as follows (B,C). 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 filtered by the smoothing algorithm by Eilers [70]. The background was automatically subtracted via the algorithm by Cao et al. [53]. To aid comparison, the spectra were vector normalized so that their integrated areas were equal.

Principal component analysis [71] (PCA) was applied to the preprocessed Raman spectra to reduce the dimensionality of the data (B,C). The PCA was performed on unstandardized data, i.e. the variables were not scaled by dividing them by their standard deviations. An appropriate number of principal components (PCs), explaining a large percentage of the total variance, was retained. In Papers B and C the 10 first PCs were kept for further evaluation.

In Paper B, a modified version of Kim’s test [72], described in [73], was used to determine if the multivariate means of the PC scores of fresh and snap-frozen tissue differed. Three analogous analyses were conducted, fresh tissue was compared to snap-frozen tissue stored at $-80^\circ$C for 5, 26 and 81 days. If the test showed a significant difference, Yuen’s univariate test 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 hierarchical clustering analysis [74] algorithm using Ward’s linkage [75]. The data set included all 168 spectra. The spectra were divided into 5 clusters. Different colors were used to label the five groups: black, green, yellow, red and blue. The non-parametric Kruskal-Wallis test followed by Tukey-Kramer’s multiple comparison test were used to test if the mean stiffness of the groups defined from the cluster analysis of Raman data differed.

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.
7.1 Technologies for localization and diagnosis of prostate cancer

The accuracy of the gold standard for PCa detection and diagnosis, the PSA test and SB, is insufficient (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. Ultrasonic methods assessing 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 cores, as compared to SB [8,76,77]. However, ultrasound methods still show a low specificity. The subjective interpretation is also a limitation [8].

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 [7,78]. A meta-analysis of the literature showed that the sensitivity and specificity of MRSI for PCa detection are 64% and 86%, respectively [79]. MRSI offers objective detection of PCa based on elevated tumor metabolism. The combination of MRI and MRSI has attained high detection accuracies in many studies. Dynamic contrast-enhanced MRI significantly augments the detection rate of conventional MRI [7]. Diffusion weighted imaging may also improve MRI performance [78]. A combination of several MR techniques has the potential to detect most tumors of clinical significance [78]. The first studies of multiparametric MRI show promising results [80–82]. The main drawbacks of MRI are high costs, limited availability of MR scanners and that MR-guided biopsy is a complex procedure [7,83].

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.

Paper A shows that there is a need for new cost-effective and complementary methods
General results and discussion

for localization and diagnosis of PCa. In particular, it is important to distinguish indolent and aggressive PCa. The proposed combination of resonance sensor technology and Raman spectroscopy is an interesting alternative technique. As shown in Paper A, both techniques have demonstrated promising results \textit{in vitro}. However, hitherto only a small number of studies have been conducted. Raman spectroscopy holds promise for grading tumors. Furthermore, the objective information obtained from the techniques is suitable for computerized interpretation.

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 due to the laser illumination occurred \((n = 5)\) (B). The intensity of the background decreased with the laser illumination time, while the intensity of the Raman signal was unaltered. Figure 7.1 shows how the intensity of the background drops, whereas the intensity of the Raman signal exhibits small random fluctuations. The results were similar for all measurement series.

![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.](image-url)

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 that was used 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 fiberoptic 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 [47]. Hattori et al. [47] used a Raman fiberoptic probe of the same type used in Paper C, but with 0.6 mm diameter, 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 (125 μm), this corresponds to an irradiance of about $7 \cdot 10^6$ W/m$^2$, which can be compared to the irradiance $3 \cdot 10^{10}$ W/m$^2$ for the system used in Paper B.

### 7.2.2 Snap-freezing

Multivariate tests showed that there was a strong significant difference between the spectra of fresh and snap-frozen tissue ($p < 10^{-5}$) (B). The main spectral differences were found through univariate tests of the difference between the PC scores of fresh and snap-frozen tissue, see Table 1 in Paper B. Figure 7.2 shows the calculated PCs whose scores differed significantly between fresh and snap-frozen tissue. Peaks that were characteristic for the differences have been labeled with their wavenumbers. Some common effects due to the snap-freezing can be listed (tentative assignments from [49,54]):

- Decreased intensity of the phenylalanine (1006 cm$^{-1}$) and tryptophan (1548 cm$^{-1}$ and 760 cm$^{-1}$) peaks.
- The amide I peak (1654–1685 cm$^{-1}$) is shifted towards lower wavenumbers.
- Increased intensity at $\sim 1441$ cm$^{-1}$ and $\sim 1299$ cm$^{-1}$, which was assigned to CH$_2$/CH vibrations of lipids and/or proteins.
- The amide III peak at $\sim 1244$ cm$^{-1}$ is decreased.

The changes due to snap-freezing were subtle and could not be revealed by comparing mean spectra. Figure 7.3 shows two spectra for day 26 where the differences were relatively large.

Significant differences between tissue 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 $10^5$–$10^6$ times larger) than those found comparing fresh and snap-frozen tissue. Thus, the main differences between fresh and snap-frozen tissue most likely arose during snap-freezing or thawing, and not during storage at −80°C.
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 of these PCs. Note that PC8 for day 26 has been inverted because snap-frozen tissue obtained higher scores of this PC.
7.3 Combined Raman and resonance measurements

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 in general that find diagnostic information related to protein conformation. Since the changes in cancerous prostate tissue mainly seem to be related to DNA and lipids [16, 19, 57], these findings suggest that snap-freezing may be used to study the difference between cancerous and healthy prostate tissue \textit{in vitro}.

7.3 Combined Raman and resonance measurements

Figure 7.4 shows the result of the cluster analysis of the PC scores of the Raman 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 yellow and green groups were a mix of muscle and fat tissue. This was confirmed by analysis of the Raman spectral data. Figure 7.5 shows one representative spectrum from each group. The spectra differed substantially and were clustered into distinct groups. PC1, PC2 and PC4 contributed strongly to the clustering, as seen in Figure 7.6. The interpretations of the PCs were that PC1 was related to the proportion of fat, PC2 to the proportion of protein, and PC4 to the composition of the fat.

The mean stiffness of each tissue group was calculated from the resonance sensor.
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. Each cluster was assigned a specific color. 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: One representative Raman spectrum from each of the pork belly tissue groups, which were defined by the cluster analysis. The peaks whose intensity differed substantially between the red and the blue spectra have been labeled with their wavenumbers.
7.3. Combined Raman and resonance measurements

measurements (C), see Figure 7.7. The blue group was significantly stiffer than the other groups ($p < 0.05$). That the stiffness of the blue and the red groups differed was not expected, since the measurement sites generally had the same visual appearance. A possible explanation was found by analyzing the spectra. As seen in Figure 7.5, the intensity of several peaks of the blue and the red spectrum differed markedly. These peaks were prominent in PC4, and as shown in Figure 7.6, the scores of PC4 constituted the main difference between the blue and the red groups. Tentative assignments of the peaks indicated that the content of saturated fatty acids was higher in the blue group. For example, the peak at 1656 cm$^{-1}$ was assigned to the vibration of the carbon–carbon double bond (C=C), and the peaks at 1127 cm$^{-1}$ and 1062 cm$^{-1}$ to the single bond (C–C) [54]. In general, saturated fatty acids have higher melting points than unsaturated fatty acids [84]. Thus, the stiffness is related to the portion of saturated fat.

As shown in Table 1 in Paper C, the Raman data explained 67\% ($R^2_{\text{adj}} = 0.67$) of the total variability of the stiffness parameter $\frac{\partial F}{\partial \Delta f}$. PC2 and PC4 were the most important predictor variables, indicating that the proportion of fat and its composition contributed most to the measured stiffness.

A higher degree of correlation between Raman and resonance data may be obtained if a resonance sensor with a smaller tip is used. The Venustron® resonance sensor measured a much larger tissue volume than the Raman probe (C). This may also partly explain why the resonance sensor obtained a relatively poor differentiation of the tissue groups defined by the cluster analysis of Raman data. The resonance sensor probably sensed a mix of tissue components in many cases, while the Raman probe usually measured a
Figure 7.7: Mean ± standard deviation of the measured stiffness for the five groups defined by the cluster analysis of Raman data. A high value indicates a higher stiffness. The number of measurements (n) is given in the parentheses. The blue group was significantly stiffer than the other groups (p < 0.05).

In summary, Paper C shows that the biochemical information obtained from the Raman measurements provided additional discriminatory power to the resonance sensor. This is promising for the development of an instrument combining the two techniques for cancer detection and diagnosis in vivo.
General summary and conclusions

This licentiate report 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 Raman spectroscopy. The novel idea of combining Raman spectroscopy and resonance sensor technology was presented, and preliminary experiments were performed on pork belly tissue using a resonance sensor, a Raman fiberoptic probe and a translation stage that assured that the sensors measured on the same points on the sample. Multivariate algorithms were implemented and used to analyze the experimental data.

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. Raman spectroscopy and resonance sensor technology are promising alternative techniques. However, these methods have only been evaluated in vitro in a small number of studies.

No evidence of tissue degradation due to 830 nm laser illumination at an irradiance of $\sim 3 \cdot 10^{10}$ W/m² were found. The spectral background decreased with the laser illumination time, and this was probably caused by photobleaching of tissue fluorophores, such as porphyrins. 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 combined measurements on pork belly tissue showed that Raman spectroscopy provided additional discriminatory power to the resonance sensor. The Raman data explained 67% of the total variability of the stiffness parameter. The differentiation of
tissue types using the resonance sensor was relatively poor. The results may be improved by using a smaller resonance sensor tip sensing a smaller volume of the sample.

To summarize, this report indicates that a combined instrument incorporating both Raman spectroscopy and resonance sensor technology is a promising complementary method for PCa detection. Snap-freezing of samples may be used in future Raman evaluations of changes in cancerous prostate tissue. The instrument has potential for \textit{in vivo} tumor-border demarcation and biopsy guidance. The goal is to provide a more secure diagnosis and consequently more efficient treatment of the patient.
The combined instrument may be constructed by molding a Raman fiberoptic probe into a cylindrical resonance sensor element, as depicted in Figure 9.1. Since the Raman probe is shielded from surrounding light by the piezoelectric element, it may be possible to perform measurements in bright environments.

Future work will consist of investigating how the combined information from Raman and resonance measurements should be optimally analyzed. I think that multivariate algorithms will play a central role in these analyses. Combined measurements on human prostate tissue \textit{in vitro} will be performed, preferably using a resonance sensor with a smaller probe tip. The size and shape of the tip will probably affect the measurements to a large degree, and more research is needed to determine what characterizes a good tip.
design. Further evaluations of a robust study protocol for the combined measurements should be performed. If feasible, a system that automatically adjusts the distance from the Raman fiberoptic probe to the sample will be developed.

The development of a combined instrument incorporating a Raman fiberoptic probe and a resonance sensor is ongoing. After a prototype has been produced, it will be investigated whether the two sensors interfere with each other. Experiments in bright conditions will be conducted to find out if the piezoelectric element shields the Raman probe sufficiently from stray light to allow measurements to be conducted without the need to darken the room.

*In vitro*, combined measurements will be performed on the microscopic level by using a miniaturized resonance sensor system [85] and a Raman microscope. Linking information of stiffness and biochemistry on the cellular level could provide new knowledge about the development of cancer. The results may aid the understanding of combined measurements on the macroscopic level.

I believe that potential evaluations *in vivo* will be a major challenge. *In vivo* a number of parameters that may affect the measurements will be difficult to control, including: (i) The angle between the probe and the tissue surface. (ii) The pressure applied to the tissue during measurements. (iii) Motion artifacts. (iv) Contaminations of the probe tip. Moreover, it may be difficult to diagnose patients from Raman spectroscopic measurements, because the Raman method may pick up differences due to the spectral variation between different individuals. This is actually one reason for why Raman spectroscopy has not been implemented clinically for oncological applications yet. Hopefully the combined information can help decrease the variation between patients.

Possible clinical applications for the combined instrument include tumor-border demarcation during cancer surgery, and guidance of prostate biopsies. A miniaturized version may be incorporated into an endoscope, which would make it possible to examine many parts of the human interior body. As a biopsy-guidance tool the instrument may permit that fewer biopsies are taken without compromising PCa detection rate. This would reduce the discomfort for patients, since many consider biopsies very painful and the pain increases with the number of cores taken [3, 86]. It would also significantly reduce the costs [3, 87]. Furthermore, the instrument may shorten the time needed to surgically remove tumors by quickly scanning adjacent tissue for cancer. A very effective surgical procedure for removing tumors is Mohs’ surgery, where the cancer is resected in successive steps [20]. The removed tissue is histologically examined and more tissue is excised if the margins are cancerous. However, the procedure is very time-consuming. If the histological analysis could be significantly shortened or eliminated, the surgery could be performed much faster [20].

The instrument has potential for making accurate diagnoses of PCa, which could help decrease mortality and optimize patient care. Since computerized interpretation of measurement data could be accomplished in near real-time, patients could be given a preliminary diagnose concurrently with a clinical examination. The long waiting time for biopsy results seems to cause the most anxiety in men being screened for PCa [88].


References


Part II
Technologies for localization and diagnosis of prostate cancer

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Technologies for localization and diagnosis of prostate cancer

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Abstract

The gold standard for detecting prostate cancer (PCa), systematic biopsy, lacks sensitivity as well as grading accuracy. PSA screening leads to over treatment 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

List of abbreviations

ADC apparent diffusion coefficient
AUC area under receiver operating characteristic curve
BPH benign prostatic hyperplasia
CDU colour Doppler ultrasound
CE contrast-enhanced
CHI continuous harmonic imaging
CZ central zone
DCE-MRI dynamic contrast-enhanced MRI
DRE digital rectal examination
DU Doppler ultrasound
DWI diffusion-weighted imaging
FTIR Fourier transform infrared spectroscopy
GS Gleason score
IHI intermittent harmonic imaging
IR infrared
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 aging 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 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].

The aims of this review are to present and compare 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 section 8. A critical comparison of the techniques is finally given in section 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, approx. 70%, develop in the PZ [9]. It is generally believed that benign prostatic hyperplasia (BPH), a very common malady in elderly men [10], originates from the TZ. Recent studies indicate that BPH also might develop in the PZ [11,12].

Figure 1: Zonal anatomy of the prostate: (a) The urethra is represented in yellow and the seminal vesicles and ejaculatory ducts in blue. The TZ (dark green) consists of two independent lobes. (b) The CZ (orange) bounds on the posterior surface of the TZ and encloses the ejaculatory ducts. (c) The PZ (red) is the most posterolateral glandular component of the prostate. The ratio of PZ to central gland (CZ + TZ) tissue gradually decreases upward from apex to base. (d) Anteriorly, the prostate is covered by a thick nonglandular fibromuscular stroma (brown).

(Reprinted from Radiotherapy and Oncology, 76, Geert M. Villeirs, Koenraad L. Verstraete, Wilfried J. De Neve and Gert O. De Meerleer, Magnetic resonance imaging anatomy of the prostate and periprostatic area: a guide for radiotherapists, 99–106, Copyright © (2005), with permission from Elsevier.)
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 [13]. Furthermore, the disease progression varies widely [8], and PCa is often asymptomatic until advanced stages have developed [14].

Historically, digital rectal examination (DRE) has been the main method of detection [15], but it is seen today as a rather crude tool [16, 17]. Tumours in the prostate are generally harder than healthy tissue [18–20], owing to the increased cellular density of cancer [13].

The gold standard for detecting and diagnosing PCa is transrectal ultrasound (TRUS)-guided systematic biopsy (SB). European guidelines 2007 recommend a minimum of 10 cores [21]. The cores are evaluated using brightfield microscopy, and cancer is detected due to its abnormal morphological appearance. It has been estimated that sextant SB, which first was proposed in 1989 [22], fails to find a present cancer in 3 out of 10 cases [23]. 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 [24,25].

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 [26].

The PSA test came into widespread clinical use in the early 1990s [27]. It is the clinical test with the highest positive predictive value for PCa [2]. However, PSA-based screening is controversial [27–30]. The PSA-level can be elevated because of benign conditions, and many men with a concentration of PSA < 4 ng/ml suffer from clinically significant disease [31, 32]. Furthermore, the use of PSA leads to many men with indolent cancers being unnecessarily treated [27, 30]. Originally 4 ng/ml was implemented as a cutoff for recommending biopsy. The latest European guidelines suggest that values around 2.5–3.0 ng/ml should be used for younger men [21].

Grading and staging PCa  The biological aggressiveness of PCa is currently histologically evaluated and rated according to the Gleason grading system. The most and second most prevalent pattern of the tumour are graded with a score from 1 to 5, 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 interobserver disagreement is a problem [33,34]. Moreover, there is a discrepancy between the scoring of biopsy samples and radical prostatectomy specimens [35–38]. That can be expected since most palpable tumours are morphologically heterogenous and contain multiple grades [39]. Unfortunately, undergrading is the most common problem. That is due both to biopsy sampling error and incorrect histological interpretation, and thus inappropriate treatment strategies can be chosen [34,40].

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 [21]. 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 [15,34].

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 PCas 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 [6,41]. Actually, autopsy reveals that many healthy men harbour PCas [14]. An estimation indicates that almost 50% of men 70–80 years old are affected, and the proportion escalates from the 5th decade of life [42].

4 Transrectal ultrasound (TRUS)

Watanabe et al. [43–46] introduced 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 [10].

A TRUS examination of the prostate captures sequences of transverse images from base to apex, and sagittal images from right to left [5]. Figure 1.2(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, 47], as exemplified in figure 1.2(a). However, many benign occurrences, such as BPH, prostatitis, haematoma, normal tissue and cysts can also be hypoechoic [48]. Actually, as low as 20% of the hypoechoic lesions turn out to be malignant at biopsy [4, 49]. 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 [50]. Thus TRUS is insufficient for PCa detection [51]. Another drawback is that TRUS is operator-dependent [52, 53]. 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 [54]. There are two different types of DU used for detecting PCas: 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 [55]. Figure 1.2(e) shows a CDU image, which can be compared to the PDU image of figure 1.2(f) (these images...
Figure 2: A 75-year-old man with GS 8 cancer in the left base: transverse images through the base of the prostate.

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are contrast enhanced). PDU was developed to address some problems encountered with CDU, such as high noise, angle dependence and aliasing [56].

CDU has demonstrated a high positive predictive value for detecting PCa, but a relatively low sensitivity [57]. According to some studies PDU does not improve the cancer detection rate over sextant SB [58,59]. 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 [60,61]. CDU [62] and PDU [63,64] 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 [62,63]. Sauvain et al. [60,61] have shown the feasibility of PDU to observe if tumours have extended through the capsule. Blood vessels that perforate the capsule are namely 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. That makes it possible to resolve blood vessels with diameters down to 50–100 μm [54], and visualize the flow in the microscopic neovessels that multiply in PCa [13]. Both DU and greyscale ultrasound can employ contrast enhancement. Small gas-encapsulated microbubbles with a diameter < 10 μm are commonly used as contrast agents [2,54]. The lifetime of the bubbles is < 5 min [54]. How the microbubbles react to the ultrasound depends on its intensity [54]. 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, which gives 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 [13]. Figures 1.2(e) and 1.2(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 1.2(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 [13, 57]. Frauscher et al. [65, 66] performed two prospective studies comparing the capability of 10-core SB and CE CDU-targeted biopsies, withdrawing ≤ 5 cores, to detect PCa. 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. [67, 68] 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 [67]. They also found that premedication with dutasteride seemed to reduce blood flow in benign prostatic tissue [69]. This may reduce false positives from, e.g. prostatitis [69].

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 [13]. 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 [13]. 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 [13]. The efficiency of CHI and IHI is demonstrated in figures 1.2(c) and 1.2(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) [70, 71]. Halpern et al. [71] examined 301 patients, and cancer was found in 15.5% (175/1133) of the targeted areas versus 10.4%
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 [13]. That was also observed in a study including 12 patients where whole-mount prostatectomy was used as reference [72]. Several studies have excluded the TZ to decrease this problem [66, 68].

More sensitive CE ultrasound techniques are upcoming, such as the Cadence contrast-pulse sequence technique, which can construct images showing solely microbubble contrast [73, 74]. Detailed information about this technique can be found in Phillips & Gardner [75]. A promising initial study with 20 patients using targeted biopsies found that suspicious lesions were cancerous in 8/11 cases [73]. Yang et al. [76] 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 [77]. 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 [77, 78], and 3D-TRUS can potentially reduce the interobserver variability experienced for 2D-TRUS [77]. 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 [57, 78], but significantly improves the local staging accuracy [77]. 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), Sauvain et al. [60] found that 3D PDU achieved 92.4% sensitivity and 72% specificity in detecting PCa 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. [79] 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. [80] 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 [81]. 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 [82],
The elastogram (strain image) is generated in real-time by differentiating the spatial displacement distribution between consecutive ultrasound images [19]. König et al. [82] performed a study (the first real-time elastography study to assess PCa) in which 404 men with abnormal DRE and/or increased PSA were examined with elastography. 151 of the men had confirmed PCa, and elastography was able to pinpoint 127 (84.1%) of these. Three criteria for a positive elastography finding were applied: the lesion was marked as being hard, the elastogram was reproducible (e.g. after tilting of the probe), and the lesion was not suspicious for being benign in the conventional TRUS image.

Lately, 2006-2008, a number of studies have been published that all conclude that elastography may be a valuable tool [19, 83–87]. The performance of elastography was rated in 15 patients using prostatectomy specimens as reference [83]. The estimated sensitivity and specificity were 87% and 92%, respectively. A similar study succeeded to disclose 20 out of 27 cancers [87]. Tumours of high grade and large volume were easier to detect. Tsutsumi et al. [86] examined 51 men and used prostatectomy histology correlation, reaching an overall sensitivity of 77%. Pallwein et al. [84] conducted a prospective study including 230 men that compared 10-core SB with targeted biopsy in the PZ, withdrawing ≤ 5 cores per patient. The sensitivities for detecting PCa per patient were 72% (58/81) and 84% (68/81) for the systematic and the elastography-directed procedures, respectively, but the difference was not statistically significant. However, per core the targeted approach was significantly better (p < 0.001), and it was almost 3 times more likely to prove cancer from the lesions pointed out by elastography. All the missed tumours, except one (1/13), were of GS ≤ 6. Using a similar approach the group reported 86% sensitivity and 72% specificity for the entire prostate in a study which involved 492 men [85]. There was only middle correlation between the GS and the elastographic findings. A high rate of false positives, 51.6%, mainly due to prostatitis, was observed. Standard TRUS was not used to identify lesions that might be benign.

5 Resonance sensor technology

The principle of a resonance sensor is based on the fact that the resonance frequency of a vibrating element changes when it is in contact with a material. The observed frequency shift is related to the physical hardness of the object, and the sensor can be used to measure stiffness variations in tissue [88]. In 1992 a resonance sensor based on a piezoelectric element driven at its resonance frequency by a feedback/amplifier circuit was presented by Omata & Terunuma [89]. 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. [90], 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 [91]. Three in vitro studies carried out by Jalkanen et al. [18, 91, 92] 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 [18]. They used
a system that merges the type of resonance sensor described by Omata and Terunuma with force and position sensors. Lindberg et al. [93] 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. [92]. 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 hydrogen¹ nuclei in the examined tissue, when the tissue is being exposed to a strong external magnetic field and is irradiated with a radio wave [94]. 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 postbiopsy haemorrhage inside the gland, as seen in figure 3b [52]. The anatomy and the internal architecture of the gland can be clearly visualized on T2W images, as demonstrated in figure 3a. Most prostate examinations are currently carried out using a pelvic coil in combination with an endorectal coil, at a field strength of 1.5T [95]. 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 [96]. MRI shows a high staging accuracy for PCa [52].

Tumours are often confused with BPH in the central gland [26, 52]. 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% [26]. The estimated sensitivity of MRI to detect PCa varies widely in the literature, figures from 50–96% have been demonstrated during the 1990s and 2000s using endorectal coils and whole-mount prostatectomy specimens as reference [26]. The discrepancies probably arise mainly because different criteria for a positive result have been applied, whether incidental (many studies exclude tumours < 0.5 ml) and/or TZ tumours are included or not, and how many segments the prostate is divided into for the analysis [26]. Interobserver variability is also likely to affect the outcomes [52], and the employment of different techniques [97]. Accordingly, studies that directly compare SB and MRI are particularly valuable [26]. Wefer et al. [98] 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

¹In human tissue it is predominantly the hydrogen nucleus, abundant in water and lipids, that yields a signal [94].
Figure 3: Gland-confined PCa. (a) Axial MRI of the prostate using an endorectal coil demonstrates a tumor (white arrow) presenting as focal low signal in the PZ on T2W imaging (a) and intermediate signal on the T1W imaging (b). The tumor 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.

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the patients had their biopsy performed elsewhere. In a retrospective study including 106 men with PCa, Mullerad et al. [99] showed that MRI added significant incremental value to DRE and sextant SB ($p < 0.0001$).

A study published 2006 [100] 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 [100].

6.2 Magnetic resonance spectroscopic imaging (MRSI)

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 [101]. PCa is also distinguished by lower levels of PA [102]. The peak of PA lies just between the Cho and Cr peaks in the MRSI spectrum and therefore usually cannot be clearly resolved unless state of the art equipment is used [101]. However, a relatively deep dip between the Cho and Cr peaks is indicative of PCa [102]. Figure 4 shows how MRSI is used in conjunction with MRI to detect PCa.

Figure 4: Axial T2W MRI combined with MRSI of healthy and cancerous prostate tissue. (a) The MRI was acquired from the whole gland (heavy white box) showing a large low signal intensity lesion (arrows) in the left PZ. (b) The voxel from the right PZ exhibits a metabolic profile in accordance with healthy prostatic tissue, high level of Cit and lower concentrations of Cho and Cr. (c) The corresponding spectrum from the left voxel shows increased Cho and reduced Cit, indicative of PCa.

(With kind permission from Springer Science+Business Media: Abdominal Imaging, Prostate cancer detection: magnetic resonance (MR) spectroscopic imaging, 32, 2007, page 255, Joan C Vilanova and Joaquim Barceló, Figure 4.)
The most commonly used method to detect PCa due to changes in the metabolic spectrum is to calculate the ratio $\frac{\text{Cho+Cr}}{\text{Cit}}$, where the amount of each metabolite is determined from the area of the corresponding peak [101]. The Cr peak is included for ease of quantification because it is adjacent to the Cho peak [103]. Jung et al. [104] suggested a standardized interpretation system for metabolic spectra. They used a 5-point scale based on the $\frac{\text{Cho+Cr}}{\text{Cit}}$ ratio. 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 interobserver agreement was excellent. Fütterer et al. [105] used the standardized interpretation system presented by Jung et al. [104], 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 interobserver agreement was observed.

Wang et al. [106] performed a meta-analysis of the literature to assess the accuracy of MRSI for PCa detection, based on the $\frac{\text{Cho+Cr}}{\text{Cit}}$ ratio. Prospective, independent and blind studies using prostatectomy histology, SB or surgery as reference were included. The inclusion criteria left only 7 studies. For the diagnostic criterium $\frac{\text{Cho+Cr}}{\text{Cit}} > 0.86$, 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 [107, 108]. Scheidler et al. [109] 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 [110]. The study population was essentially the same as in Scheidler et al. [109].

MRSI may also be valuable for assessing the aggressiveness of PCa. It has been shown that the $\frac{\text{Cho+Cr}}{\text{Cit}}$ ratio correlates with the GS [105, 111, 112]. Zakian et al. [112] 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 [113] and indolent [114] 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 [115]. Thus, unnecessary repeated biopsies may be avoided.
6.3 Dynamic contrast-enhanced MRI (DCE-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, 107]. 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 [26]. There is no standardized way to analyse the enhancement curves, but many studies indicate that tumours are best depicted 30–90 s after injection [26]. 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 [26, 116–119]. Sensitivities up to 90% were reported for detecting PZ PCa. In addition, Hara et al. [120] 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). 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 [26]. A study by Vos et al. [121] 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 [122, 123].

6.4 Diffusion-weighted imaging (DWI)

An interesting MRI technique is diffusion-weighted imaging (DWI) that 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 [124]. 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 [125–131]. Only three of the studies [125, 126, 131] used prostatectomy histology correlation. In the study by Morgan et al. [128], an inexperienced reader did not obtain better results by adding DWI to T2W MRI, raising concerns about interobserver disagreement. Mazaheri et al. [132] 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 [133, 134]. DWI shows high spatial resolution and high contrast between cancerous and healthy prostate tissue, and enables short acquisition time [107, 135]. However, considerable individual variations of the apparent diffusion coefficient (ADC) have been reported [136, 137]. Another limitation is susceptibility-induced distortions [138].
7 Vibrational spectroscopy

Raman spectroscopy (RS) and infrared (IR) spectroscopy, jointly termed vibrational spectroscopy, utilize the fact that a molecule can start vibrating at a specific frequency in response to light irradiation [139]. 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 [140].

Many in vitro studies indicate that IR spectroscopy can differentiate PCa and healthy tissue [141–146]. Gazi et al. [147] 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 $\geq 70\%$ and $\geq 81\%$, respectively, were obtained. Furthermore, the method performed similarly to Gleason grading in predicting metastatic tumours according to the TNM system. That has also been shown by Malins et al. [148]. A study conducted by Bogomolny et al. [149] 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. Fernandez et al. [142] and Bhargava et al. [141] developed a setup capable of high throughput assessment of cells and tissues, reaching extremely efficient classification of different tissue types and pathologies. In vivo applications are impeded by the very short penetration depth of IR fibreoptic probes, due to the fact that water very strongly absorbs IR radiation [140].

Crow, Stone et al. [150–154] 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% [151]. 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 biological aggressiveness [153]. Taleb et al. [155] attained a 100% accurate classification of benign and malignant (derived from metastases) prostate cells using two different diagnostic algorithms. 30 cells were measured with a Raman microscope set in confocal mode and equipped with a 633 nm laser. Another study by Crow et al. [152] measured 38 prostate samples from 37 patients and showed that a portable fibreoptic system could distinguish PCa from BPH and prostatitis with an overall accuracy of 86%. The group has also recently examined the biochemical basis of different pathologies within the prostate with RS [154]. 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.
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 [15]. 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 [15]. Computerized methods for identifying cancerous tissue in images obtained by ultrasound and MRI are being investigated intensively [15,73,121,156–160]. For example, Mohamed et al. [53] 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 different texture features, and selects the most important ones. Bertaccini et al. [156] performed a blind prospective study on 105 patients with suspected PCa using preprocessing of greyscale ultrasound data, and showed that 26/32 tumours evident at biopsy could be detected. Computer-assisted detection using CE ultrasound is being developed, and could be based on contrast enhancement as a function of time, since contrast agents typically flow faster through malignant than benign areas [73]. Objective image interpretation may reduce interobserver variation [15].

9 Discussion

Table 1 provides an overview and comparison of the methods included in this review. There 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 [73,161,162]. However, the image reading is subjective and the interobserver agreement is not satisfactory [13,71,73]. Work is ongoing to develop computer-assisted detection for CE techniques [73,163]. Contrast agents bring high costs per core, but CE techniques may be cost-effective due to a reduction of the number of cores [65]. Frequent false-positive findings have been reported. Premedication that reduces blood flow in benign lesions can potentially reduce the number of false positives [69]. A report on a multicentre European coordination project [74] provides good evidence of the safety of CE ultrasound. Unfortunately, the project did not succeed to 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 [85]. No data have been presented that evaluate intra- and interobserver variability. The main limitation of elastography seems to be that benign lesions, primarily prostatitis and BPH, can be hard to distinguish from tumours [83,85].

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 to advise its use in screening [2, 26, 57]. 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 [164, 165]. Furthermore, interpretation of the metabolic profile can be standardized and computerized to eliminate interobserver disagreement. Drawbacks of MRSI are the long acquisition time, the high frequency of spectral artefacts and low spatial resolution at 1.5T [107, 135, 166]. The addition of DCE-MRI to T2W sequences significantly improves the detection rate [26]. DCE-MRI shows limitations in differentiating PCa from prostatitis and BPH [138]. There are concerns about the safety of gadolinium-based contrast agents [167]. Moreover, DCE-MRI has difficulties discerning TZ tumours [135]. DWI shows good potential for improving MRI detection, but more studies are needed to assess its accuracy [107, 166]. Individual variations of the ADC may impair diagnostic performance [135]. The diagnostic performance of MR techniques may be further improved at 3T [122].

A combination of several MR techniques (MRI/MRSI/DCE-MRI/DWI) is likely to most efficiently detect PCa. According to Kirkham et al. [26] it is reasonable to hope that multiparametric MRI will detect about 90% of significant cancers. Early studies show promising results [168–170]. However, it remains to be solved how such large amounts of data should be optimally analysed [107]. Although MRI could be used for guiding biopsies, the procedure is more complex and time-consuming than TRUS, and the clinical practicability is questionable [26, 171, 172]. 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 [166, 173]. In the future MR-guided biopsy may be performed via robotic systems, and preliminary studies have shown that accurate needle placement is feasible [172, 174]. High costs and limited availability of MR scanners at present impedes the feasibility of MRI [138].

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 PCa. However, RS cannot detect PCa non-invasively due to a shallow penetration depth [175]. Development of techniques that can interrogate deeper into the tissue, such as time-gated RS [175] and spatially offset probes [176], is ongoing. Minimally invasive examinations of the prostate are possible since Raman fibreoptic probes thinner than the diameter of common clinical devices such as needles and endoscopes, typically 1–2 mm thick [177], have been demonstrated [178]. Spectroscopic techniques have great potential for aiding histopathologic examinations [179], which are subjective and time-consuming. A questionnaire indicates that the long waiting time for biopsy results cause the most anxiety in patients undergoing screening for prostate cancer [180]. Novel techniques for PCa detection are needed,
<table>
<thead>
<tr>
<th>Method</th>
<th>Brief description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Evaluated</th>
<th>Concluding remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRUS</td>
<td>Greyscale TRUS resolves blood vessels down to 1 mm in a colour map according to the Doppler signal.</td>
<td>In vivo. Safe. Accurate volume determination. Important for guiding biopsies.</td>
<td>Low sensitivity and specificity. Interobserver disagreement.</td>
<td>Yes</td>
<td>No substantial improvement over CE DU.</td>
</tr>
<tr>
<td>CDU</td>
<td>CDU-like, but colour map is produced 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. Interobserver disagreement.</td>
<td>Yes</td>
<td>No improvement over CE DU.</td>
</tr>
<tr>
<td>PDU</td>
<td>PDU-like, but colour map is produced according to total energy of the Doppler signal.</td>
<td>Low noise. Can detect extracapsular spread of cancer.</td>
<td>Resolution insufficient to visualize neovascularization. Interobserver disagreement.</td>
<td>Yes</td>
<td>No substantial advantage over CDU.</td>
</tr>
<tr>
<td>CEDU</td>
<td>CEDU-like, but colour map is produced according to higher harmonics.</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. Interobserver disagreement.</td>
<td>Yes</td>
<td>Targeted biopsy approach outperforms SB.</td>
</tr>
<tr>
<td>Resonance sensor technology</td>
<td>Resonance sensor technology measures 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>MRI allows high accuracy.</td>
<td>High staging accuracy. MRI/MRSI shows potential to detect indolent disease.</td>
<td>Low specificity. High costs. Interobserver 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>MRSI allows high accuracy.</td>
<td>MRSI/MRA shows high accuracy.</td>
<td>Coarse spatial resolution at 1.5T.</td>
<td>Yes</td>
<td>Due to its high accuracy and potential non-invasive grading capacity, MRSI is a promising MR technique.</td>
</tr>
</tbody>
</table>
**Table 1: Continued**

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Detection Improvement</th>
<th>Safety</th>
<th>Conclusion</th>
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<tbody>
<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>
</tr>
<tr>
<td>Raman Spectroscopy</td>
<td>Monochromatic light changes polarizability of molecules, which start to vibrate - inelastic scattering of light induces wavelength shift.</td>
<td><em>In vitro</em> studies show very high accuracy. Can objectively distinguish different grades of malignancy.</td>
<td>Low penetration depth of current fibreoptic probes excludes non-invasive interrogation. Laser light might affect tissue.</td>
<td>No</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><em>In vitro</em> 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 fibreoptic probes.</td>
<td>No</td>
</tr>
</tbody>
</table>
since both ultrasound and MR techniques show many limitations [181].

Computer-aided detection and diagnosis is likely to play an important role in the future. Intra- and interobserver 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 PCa present the urologist with an ever increasing amount of data to analyse. Computer-aided detection and diagnosis have the potential to objectively, consistently, time- and cost-efficiently evaluate complex data.

To distinguish between indolent and aggressive tumours is of utmost importance for improving the 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 [182,183]. More precise disease characterization is a major objective of prostate cancer imaging [8]. CE ultrasound and elastography data show correlation with the Gleason score. However, in our opinion MR techniques are more promising for grading cancer, since MRSI 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 MRSI reports performed by Wang et al. [106], where only 7 MRSI studies met the inclusion criteria, and merely 2 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 [13]. 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 section 6.1, is a clear example. Studies that correlate the imaging findings with SB do not estimate the sensitivity reliably [106]. 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 sharp picture of the clinical usefulness of different techniques large-scale multi-centre studies of high quality are necessary [74,106]. 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 [184]. 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 aggressiveness. 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. Interobserver disagreement is an issue with both conventional MRI and ultrasound. MRSI 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 vibrational spectroscopy, that have shown promising results in vitro. Appropriate tools for updating the gold standard are available, but large-scale prospective multicentre studies are needed to determine the clinical benefits of these new methods.

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cancer studies which use magnetic resonance spectroscopy as a diagnostic tool”, *Korean J Radiol*, vol. 9, no. 5, pp. 432–438, 2008.


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

Stefan Candefjord, Kerstin Ramser and Olof A Lindahl

Abstract

Most Raman spectroscopic studies on tissue are performed in vitro. To assure that the results are applicable to in vivo examinations, 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 a 830 nm laser at an irradiance of $\sim 3 \cdot 10^{10} \text{W/m}^2$. The second was to investigate the effects of snap-freezing of porcine prostate tissue in liquid nitrogen and subsequent storage at $-80^\circ\text{C}$, 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 $-80^\circ\text{C}$ 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 [2,4]. 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 require 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 [5], 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 [6–8]. 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 [9] 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 [4, 9]. 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 a 830 nm laser at an irradiance of \( \sim 3 \cdot 10^{10} \) W/m². Furthermore, the aim was to investigate the effects of snap-freezing of porcine prostate tissue in liquid nitrogen and subsequent storage at \(-80^\circ\)C, by means of multivariate analysis of Raman spectra.

2 Material & 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 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. 15 × 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. 5 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 \(-80^\circ\)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×/1.0W), 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 a 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 $\sim 500 \text{ cm}^{-1}$. The irradiance onto the samples was $\sim 3 \cdot 10^{10} \text{ W/m}^2$. The spectrometer was calibrated for wavelength shift daily by using a single Raman band (520 cm$^{-1}$) from a silicon wafer as reference.

2.3 Measurement procedure

The samples were immersed in PBS during the measurements to prevent dehydration of the tissue, which could disrupt the protein vibrational modes [9].

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, thereafter less frequently during an additional 4 min. To guarantee good signal quality, the microscope focus was moved 20 $\mu$m into the sample with a motorized z-stage. The samples were immobilized with needles. A brightfield 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.

2.3.2 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 $\mu$m into the sample. 5 samples were measured 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 prolonged storage at $-80^\circ\text{C}$ affected the tissue. 5 samples each were measured on day 5, 26 and 81. Each sample was measured at 5 randomly chosen spots.

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 [11]. The background was automatically subtracted using the algorithm by Cao et al. [12], 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,$^1$ were written in-house and implemented in Matlab (version R2007b including Statistics Toolbox version 6.1).

$^1$Electronic Supplementary Information (ESI) available: [Eilers has implemented the algorithm in Matlab and made it available via the Internet]. See DOI: 10.1021/ac034173t
2.5 Multivariate analysis on snap-freezing data

The preprocessed Raman spectra of fresh and snap-frozen tissue were compared by means of multivariate analysis [13]. Three analogous analyses were carried out, fresh tissue was compared to tissue that had been preserved at \(-80^\circ\text{C}\) for 5, 26, and 81 days. Principal component analysis [13] (PCA) on unstandardized\(^2\) 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 tissue. An adapted version of the original test proposed by Kim [14], described by Lix et al. [15], was used. In the modified version, trimmed means and Winsorized variance-covariance replace the standard formulas. Trimming between 10 and 20\% is recommended [15], 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 [16] was used for this purpose.

Matlab was used for the multivariate analysis, and most of the tools were written in-house. \(p < 0.05\) 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 [17] 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 \(\sim 400\text{–}1800\ \text{cm}^{-1}\), requiring three measurement series using the static mode. Fig. 1 shows the first spectrum of a measurement series from 459–961 cm\(^{-1}\). Fig. 2 shows difference spectra for this series. No residual Raman peaks were seen in difference spectra (\(n = 5\)).\(^3\)

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.

\(^2\)The variables were not scaled by dividing them with their standard deviation.
Figure 1: The first Raman spectrum of one of the measurement series evaluating 459–961 cm$^{-1}$.

### 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 are 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 $1 \cdot 10^{-7}$, $2 \cdot 10^{-9}$ and $2 \cdot 10^{-6}$ for snap-frozen tissue stored at $-80^\circ$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 (tentative assignments from [18,19]):

- 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 $\sim 1690$ cm$^{-1}$, whereas snap-frozen tissue shows higher intensity towards $\sim 1660$ cm$^{-1}$.
- Increased intensity at $\sim 1441$ and $\sim 1299$ cm$^{-1}$, which may be assigned to various CH$_2$/CH vibrations of lipids and/or proteins.
- The amide III peak at $\sim 1244$ cm$^{-1}$ is decreased.
Figure 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.
Figure 3a: The total intensity of the spectral background decreased with the laser illumination time.

Figure 3b: The total intensity of the Raman signal did not change with the laser illumination time, only random fluctuations were seen.
Figure 4: A Pareto chart showing the amount of variance explained by each PC, and the accumulated explained variance, for fresh tissue and snap frozen tissue stored for 5 days.

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.

<table>
<thead>
<tr>
<th>PC nr</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>
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<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><strong>0.0074</strong></td>
<td>0.12</td>
<td>0.26</td>
<td>0.34</td>
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<tr>
<td>26 days</td>
<td>0.56</td>
<td>0.31</td>
<td>0.056</td>
<td>0.47</td>
<td><strong>0.0004</strong></td>
<td>0.17</td>
<td>0.36</td>
<td><strong>0.037</strong></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><strong>0.024</strong></td>
<td>0.82</td>
<td>0.22</td>
<td>0.22</td>
<td><strong>0.033</strong></td>
</tr>
</tbody>
</table>

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 $10^2$–$10^6$ larger than the p-values obtained comparing fresh and snap-frozen tissue.
Figure 5: The calculated PCs whose scores were proven to be significantly different for fresh and snap-frozen tissue. 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.
Figure 6: A spectrum on fresh (blue) as compared to snap-frozen prostate tissue (red) for day 26. Note, e.g. that the phenylalanine (1004 cm$^{-1}$) and tryptophan (758 cm$^{-1}$) peaks are increased for fresh tissue. These peaks are prominent in PC5, see Fig. 5.

4 Discussion

4.1 Photoinduced effects

No photoinduced effects on the Raman signal were observed at an irradiance of $\sim 3 \cdot 10^{10}$ W/m$^2$ 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, 7, 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. [6] who used 785 nm illumination at an irradiance in the same order of magnitude for studying individual cells. Shim et al. [9] studied excised tissue using 1064 nm at an irradiance of $\sim 2 \cdot 10^{7}$ W/m$^2$ 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 [20]. Which molecular species that are involved depend on the excitation wavelength, as shown in the ultraviolet and the visible wavelength...
range [21,22]. 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 [23]. Photobleaching of tissue fluorophores in general and porphyrins in particular is a well known phenomenon [24,25]. 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 tissue should be handled as similar as possible, and tissue degradation should be minimized. Prior to snap-freezing, the prostate was protected by the fascia and adjacent tissue that surrounds 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). A 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 tissue. 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 tissue in all comparisons. For example, the $p$-value obtained for comparing snap-frozen tissue stored at $-80^\circ$C to fresh tissue was $3 \cdot 10^{-10}$ when including 20 PCs, explaining about 94% of the total variance, as compared to $1 \cdot 10^{-7}$ when 10 PCs were included.

This study examined macroscopically thick samples, hence changes due to the freezing process should preferably be investigated using homogenous tissues. O Faolain et al. [4] chose to examine tissue from the placenta due to its homogenous nature to study the effects of freezing. However, it cannot be excluded that different tissue types react differently to the snap-freezing process. Furthermore, the use of thin-section microscopy would have permitted histopathologic, and probably also spectroscopic [26], 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 heterogenous tissues, such as prostate tissue [26], necessitates the use of statistical methods that can extract the changes due to the freezing process in a heterogenous set of spectra. The heterogeneity of the tissue explains why the first 3–5 PCs, which made up a large percentage of the total variance as exemplified in Fig. 4, did not show a statistical difference between fresh and snap-frozen tissue, as seen in Table 1. It is plausible that these PCs reflect the variance due to different tissue types, which indicates that the spectral variance due to the heterogenous prostate tissue is greater than the relatively small but nevertheless detectable variance between fresh and snap-frozen tissue.

The standard test for comparing multivariate means, Hotelling’s $T^2$, exhibits a high type I error rate when the assumptions of multivariate normality and equal variance-covariance matrices are violated [15, 27]. Univariate Anderson-Darling tests [28] and normal probability plots showed that it was not reasonable to assume a multivariate normal distribution, since some of the PCs were not normally distributed. In the aggregate it might be argued that the departures from normality were not so severe, but a stringent analysis cannot rely on tests that are sensitive to non-normality. Fortunately, alternative robust tests that outperform the $T^2$ statistic are available [15, 27]. A procedure that shows good control of the type I error rate under a wide variety of conditions [15, 27], and does not assume equality of the variance-covariance matrices, is the approach suggested by Kim [14] in 1992. To further improve its robustness to non-normality, it is recommended that robust estimators of mean value and variance-covariance are used [15]. Following Lix et al. [15], Kim’s test was adapted using trimmed means and Winsorized variance-covariance. The modified test is very robust to departures from normality and variance-covariance heterogeneity [15].

An analogous line of argument applies to the univariate testing of the equality of means of the individual PC scores. Because some PCs were nonnormally distributed and heteroscedastic, as evaluated by the Levene [29] test in Matlab, was sometimes evident, a robust test had to be used. The choice fell upon the two-sided trimmed $t$ statistic for unequal population variances proposed by Yuen in 1974 [16], which is very robust to heteroscedasticity and non-normality [30].
4.2.4 Observed changes

Subtle significant alterations, mainly related to protein bands, as seen in Fig. 5, were found when snap-frozen tissue was compared to fresh tissue. A possible explanation is that the snap-freezing/thawing process alters the protein conformations. The fact that the amide I peak is shifted towards lower wavenumbers for snap-frozen tissue indicates changes in the secondary protein structure, mainly unraveling of $\beta$-sheets [18]. This might also explain the lowered intensity at 1244 cm$^{-1}$, since the amide III peak is located at 1227–1247 cm$^{-1}$ for the $\beta$-sheet formation [18]. Furthermore, proteins in $\beta$-sheet conformation have a peak at 1002 cm$^{-1}$, which was clearly reduced in snap-frozen tissue. There were also changes in the region 500–550 cm$^{-1}$, which can be assigned to disulfide bridges and which are related to a change in the tertiary protein structure [18].

O Faolain et al. [4] evaluated freezing of tissue from the placenta using OCT medium. They noted that freezing induced substantial changes at 1002 cm$^{-1}$, 1447 cm$^{-1}$ and 1637 cm$^{-1}$. They related these changes to depolymerization of the cellular cytoskeleton, which would result in unravelling of the secondary protein structure, and that agrees with our explanation. However, they observed substantial changes of spectra, and in contrast to our findings, they found a decrease of intensity at 1441 cm$^{-1}$. They used a 514.5 nm laser at an irradiance of approx. $3 \cdot 10^9$ W/m², which could have induced photoinduced effects. Shim et al. 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 $I_{1590}/I_{1655}$ 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 $-80^\circ$C for different time-spans showed statistically significant differences between these groups. However, the difference between fresh and snap-frozen tissue 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 applies 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

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 \textit{in vitro} measurements, as compared to the \textit{in vivo} situation. This is especially important to consider for studies finding diagnostic information in the Raman peaks related to protein conformation.

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References


Evaluating the use of a Raman fiberoptic probe in conjunction with a resonance sensor for measuring porcine tissue in vitro

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Evaluating the use of a Raman fiberoptic probe in conjunction with a resonance sensor for measuring porcine tissue in vitro

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Abstract

Prostate cancer is the most common form of cancer and the third leading cause of cancer-related death in European men. There is a need for new methods that can accurately localize and diagnose prostate cancer. In this study a new approach is presented: a combination of resonance sensor technology and Raman spectroscopy. Both methods have shown promising results for prostate cancer detection in vitro. The aim of this study was to evaluate the combined information from measurements with a Raman fiberoptic probe and a resonance sensor system. Pork belly tissue was used as a model system. A three-dimensional translation table was equipped with an in-house developed software, allowing measurements to be performed at the same point using two separate instruments. The Raman data was analyzed using principal component analysis and hierarchical clustering analysis. The spectra were divided into 5 distinct groups. The mean stiffness of each group was calculated from the resonance sensor measurements. One of the groups differed significantly ($p < 0.05$) from the others. A regression analysis, with the stiffness parameter as response variable and the principal component scores of the Raman data as the predictor variables, explained 67% of the total variability. The use of a smaller resonance sensor tip would probably increase the degree of correlation. In conclusion, Raman spectroscopy provides additional discriminatory power to the resonance sensor.

Keywords: Raman spectroscopy, resonance sensor, combination, porcine tissue, cluster analysis

1 Introduction

Prostate cancer has the highest incidence of all cancers and is the third leading cause of cancer-related death after lung and colorectal cancer in European men [1]. Current clinical methods cannot reliably localize and diagnose prostate cancer [2]. New tools are needed that can aid in making an accurate diagnosis at an early stage [3].

Resonance sensor technology can objectively sense hardness of a material. The resonance frequency of a piezoelectric ceramic shift as the probe is pressed onto a material, and the shift is related to the hardness. In vitro studies show that healthy epithelial prostate tissue, which is abundant in the posterior region of the prostate where most tu-
mors develop, can be differentiated from cancerous tissue using a resonance sensor [4, 5]. Stiff prostate tissue indicates tumor presence although the stiffness is not necessarily due to cancer. The resonance sensor alone cannot distinguish between cancerous tissue and stiff healthy prostate tissue such as stroma and prostate stones [5].

Raman spectroscopy is an optical method where the examined material is irradiated with monochromatic light. The spectrum of the backscattered light reflects the biochemical content of the tissue [6]. Several *in vitro* studies show that the method can differentiate healthy and cancerous prostate tissue with high accuracy [7–9]. The method also holds promise for grading tumors [9]. However, the laser illumination may cause photo-induced degeneration of the tissue [10], and it would be beneficial to constrict the irradiance of tissue.

A new approach is to combine Raman spectroscopy and resonance sensor technology. The resonance sensor system would be used to scan the prostate tissue and localize stiff areas, which would be analyzed with the Raman probe to determine if malignant tissue is present. The long-term goal is to develop a united instrument where the Raman fiberoptic probe is molded into a cylindrical resonance sensor element.

The first step towards developing a united instrument is to examine how data obtained using both methods separately correlate. The aim of this study was to perform and evaluate measurements on porcine tissue *in vitro* using a Raman fiberoptic probe in conjunction with a resonance sensor system. Pork belly tissue was used as a model system.

## 2 Material and Methods

### 2.1 Sample preparation

Two pieces of pork belly obtained from the local grocery store was used for this study. The tissue was stored at 6°C until the sample preparation began. It was cut into slices of approx. 50 × 40 × 9 mm. The measurements were performed on freshly cut surfaces. The samples were placed on a 19 mm thick Styrofoam plate, which was fixated to the XYZ-stage by tape. Totally four samples, two from each piece of pork, were measured.

### 2.2 Measurements

**Raman spectroscopy** A Raman spectroscopy (Kaiser Optical Systems, RXN1, USA) with a 400 mW 785 nm laser coupled to a 0.8 mm thin fiberoptic probe (Machida Endoscope Co, Japan) was used. The spectrometer was calibrated for wavelength shift and energy sensitivity of the detector. The laser power onto the samples was approx. 80 mW. The Raman signal was integrated for 30 s and measured over a spectral range of 100–3425 cm$^{-1}$.

**Resonance sensor instrumentation** A Venustron® resonance sensor system (Axiom Co. Ltd., Koriyama Fukushima, Japan) was used [5]. Its resonance frequency was...
59 kHz. During a measurement run the resonance frequency change ($\Delta f$), the force ($F$) exerted on the sensor and the impression depth ($d$) was sampled at 200 Hz. The sensor was set to measure down to an impression depth of 1 mm at a speed of 1 mm s$^{-1}$, and the zero-level detected through frequency change. The radius of the hemispherically shaped sensor tip was 2.5 mm.

**Combined measurements** The experimental setup is shown in Figure 1. The combined measurements were performed using a XYZ-stage (Thorlabs) controlled by a LABVIEW® program written in-house. A picture of the tissue sample was taken (Canon Powershot S3 IS with close-up lens 500D and LAH-DC20 conversion lens adapter) and loaded into the program. A grid with 42 measurement points was defined. Adjacent points were distanced 5 mm.

![Figure 1: The experimental setup. 1) Venustron® resonance sensor 2) Raman fiberoptic probe 3) Tissue sample 4) Styrofoam plate 5) XYZ-stage.](image)

Measurements with the resonance sensor were conducted before the acquisition of Raman spectra, because the laser illumination may cause photo-induced degeneration of the tissue. The order in which measurements were acquired was randomized. To avoid viscoelastic effects to influence the resonance sensor results, adjacent points were not measured shortly after each other.

The tissue was kept moist by brushing the surface with phosphate buffered saline every fifth min. The measurements were performed at room temperature (25 ± 0.8°C).
2.3 Data preprocessing and analysis

Matlab® (version R2008b including Statistics Toolbox version 7.0) was used for all preprocessing and analysis.

Preprocessing and analysis of Raman data  The spectral region 600–1800 cm$^{-1}$ was selected for further analysis. Spectral spikes due to cosmic rays were removed. Each Raman spectrum was filtered by a noise-reduction algorithm [11]. The background was automatically subtracted using an algorithm [12], which fits a piecewise modified polynomial to the spectrum. The spectra were vector normalized so that their integrated areas were equal.

Preprocessed spectra were analyzed using principal component analysis (PCA) and unsupervised hierarchical cluster analysis (HCA) [13]. The data set included all 168 spectra measured on the four samples. 10 principal components (PCs) were selected and input to a HCA algorithm using Ward’s linkage [14]. The spectra were divided into 5 clusters, labeled A–E.

Analysis of resonance sensor data  From $\Delta f$, $F$ and $d$ a stiffness sensitive parameter $\frac{\partial F}{\partial \Delta f}$ [15] was calculated as:

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

(1)

where the derivatives of $\Delta f$ and $F$ with respect to $d$ was estimated numerically by linear regression in an interval of $d \pm 0.1$ mm. $\frac{\partial F}{\partial \Delta f}$ was calculated at $d = 0.6$ mm, which represents the tissue stiffness near the surface [4].

The nonparametric Kruskal-Wallis test followed by Tukey-Kramer’s multiple comparison test, were performed to test for differences between groups of stiffness measurements. A regression analysis was performed with the stiffness parameter as the model response variable and the PC scores of the Raman data as the model predictor variables. A residual analysis of constant variance and normality was done for model validation. The Anderson-Darling test was used to test for normality. The adjusted coefficient of determination ($R^{2}_{adj}$) was used as the model control [16]. A $p$-value < 0.05 was considered as statistically significant.

3 Results

The first 10 PCs explained 99.3% of the total variance. The result of the cluster analysis for one sample is shown in Figure 2. Similar results were obtained for the other samples. The mean stiffness for the five groups A–E was calculated from the resonance sensor measurements, see Figure 3. Group E was significantly different from the other groups ($p < 0.05$). The estimated model coefficients ($\beta_i$) from the regression analysis are presented in Table 1. These model coefficients give the relative proportion of the $i$th PC score and its effect on the measured stiffness parameter.
Figure 2: An image of a pork belly sample with a measurement grid overlay showing 42 measurement points on fat (light area) and muscle tissue (dark area). The measurement order is shown by the numbers and the Raman data clusters are represented by: black squares (group B), white squares (C), white circles (E), black circles (A). Group D was not present in this sample.

Figure 3: Mean ± standard deviation of the measured stiffness parameter for the five groups (A–E) determined from Raman spectroscopy measurements. A high value indicates a higher stiffness. The number of measurements (n) in each group is given in the parenthesis. Group E was significantly different from the other groups (p < 0.05).
Table 1: Model coefficients ($\beta_i$) for each of the ten PC scores in the full model. The asterisk (*) denotes the model coefficients in the full model that were statistically significant ($p < 0.05$). In a selected model, only the six statistically significant PC scores were used as predictor variables, and new model coefficients were estimated. The values presented are the standardized model coefficients divided by their sum and given as a percentage. The negative sign indicates that the specific PC score decreased the stiffness parameter.

<table>
<thead>
<tr>
<th>Model coefficient ($\beta_i$) (%)</th>
<th>Full $R^2_{adj} = 0.67$</th>
<th>Selected $R^2_{adj} = 0.67$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$</td>
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<td>33</td>
</tr>
<tr>
<td>$\beta_2$</td>
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<td>-14</td>
</tr>
<tr>
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<td></td>
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<td>32</td>
</tr>
<tr>
<td>$\beta_5$</td>
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<td>-7</td>
</tr>
<tr>
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<td>6*</td>
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<tr>
<td>$\beta_{10}$</td>
<td>6*</td>
<td>7</td>
</tr>
</tbody>
</table>

4 Discussion

In this study we take a step towards combining the information from resonance sensor stiffness measurements with the Raman spectroscopic measurements of the biochemical tissue composition.

Recently, multivariate clustering analysis have been successfully applied to infrared microspectroscopy data in order to create spectral images that correlate well with histopathologic images [14, 17, 18]. Different tissue types are most efficiently distinguished using a multivariate approach [18]. In this study, an unsupervised multivariate analysis based on PCA and cluster analysis was employed for the Raman spectroscopy data. Five clusters (A–E) were used to represent the pork tissue histology. The optimal number of clusters cannot be known beforehand, it must be empirically determined. For example, Bird et al. [17] constructed images with 2–15 clusters that were presented to the collaborating pathologists who reported which images that best replicated the haematoxylin and eosin (H&E) stained histopathologic images.

The measurement sites were grouped into 5 distinct clusters according to the biochemical content as measured by Raman spectroscopy. The Raman spectral data showed that group A was purely muscle tissue, D and E were fat tissue, while B and C contained both fat and muscle tissue of varying degree. The data analysis indicated that the content of unsaturated fatty acids was higher in group D than in E.

The stiffness measurements separated group E, the hard fat tissue, from the soft tissue groups A–D. Thus, the biochemical information obtained from the Raman measurements gave additional information to the stiffness measurements. The fact that the Raman data indicated that group D contained a higher proportion unsaturated fat than group E agrees
with the resonance measurements, since unsaturated fat is expected to make the tissue softer.

A regression analysis (Table 1) showed that there existed a linear relationship between the measured stiffness parameter and the biochemical composition. The PC scores of the Raman spectral data described the molecular composition of the data representing the tissue histology. PC1 was related to the proportion of fat and described 33% of the stiffness parameter when using the selected model (Table 1). PC2 concerned the protein content and described 14% of the stiffness parameter, whereas PC4 described the difference in the molecular composition of the two fat groups D and E and contributed to 32% of the stiffness parameter.

The Raman probe collected data mainly from the outermost surface (of the order of 100 μm) of the measured sample, while the resonance sensor probe had a radius of 2.5 mm and was affected by the stiffness of a much larger volume. If a smaller resonance sensor probe would be used, a better differentiation of the measured stiffness (Figure 3) and a better model agreement (Table 1) might be obtained.

5 Conclusion
This study shows that Raman measurements of the biochemical composition provide additional information to the resonance sensor stiffness measurements on pork belly tissue. The results are promising for further development of an instrument combining these measuring modalities to be used in prostate cancer detection and diagnosis.

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References


