# Raman spectroscopy in bladder cancer diagnosis

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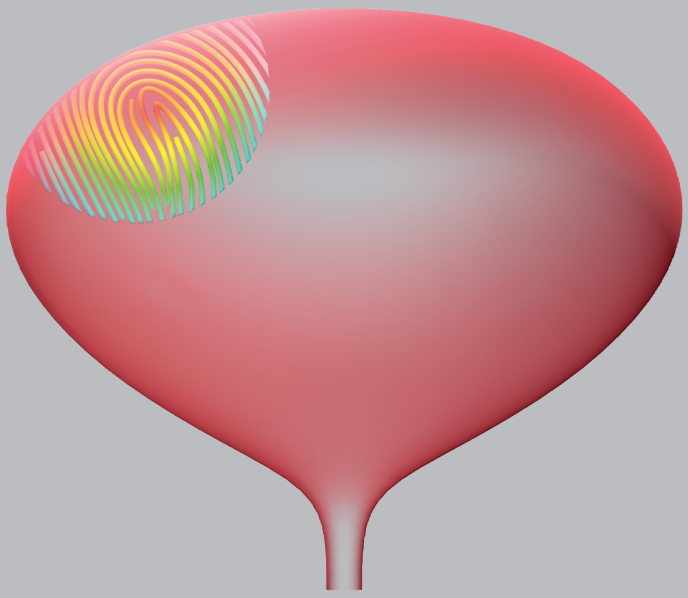

# CHAPTER 6

## SUMMARY AND GENERAL CONCLUSION

# RAMAN SPECTROSCOPY IN BLADDER CANCER DIAGNOSIS

## SUMMARY AND GENERAL CONCLUSION

The main aim of this thesis is to understand the value of Raman spectroscopy for bladder cancer diagnosis and to find ways to improve its application. We evaluated a newly developed Raman probe in a phantom model and in vivo. We also used this probe to evaluate tissue heterogeneity in cystectomy specimens. As a spin-off to enable repeat Raman measurements of specific locations, a bladder registration and navigation tool was developed and we evaluated this in a phantom model.

In the introduction **(Chapter 1)** the standard diagnostic and treatment practice of bladder cancer (mostly urothelial carcinoma (UC)) is described. The background of Raman spectroscopy is explained and publications on the use of Raman spectroscopy in bladder cancer diagnosis are reviewed. In **Chapter 2** two different fiber optic probes are compared to each other in a phantom model. A superficial and non-superficial Raman probe are evaluated regarding depth response function and signal-to-noise ratio. As the urothelial layer is only 3-7 cell layers thick, which is about 200 micron, a probe should be aimed at this superficial layer in order to reduce the irrelevant response of deeper layers. The sampling range of the superficial probe was 0-200 micron and of the non-superficial probe 0-300 micron. In addition, the superficial probe had a two-times increased signal-to-noise ratio at a measurement depth of 200 micron. Therefore, this newly developed superficial Raman probe is expected to improve the urothelial cancer detection in vivo.

In **Chapter 3**, the superficial probe was compared to the non-superficial probe for in vivo measurements. In vivo Raman measurements were acquired during transurethral resection of a bladder tumor (TURBT) before resection and pathological evaluation. Newly acquired superficial probe measurements were compared to previous in vivo measurements obtained with the non-superficial probe [1]. The performance of the superficial probe for urothelial carcinoma detection was improved compared to the non-superficial probe; the area under the curve of the receiver operating characteristic curve increases from 0.88 to 0.95, the sensitivity from 80% to 90% and the specificity from 85% to 87%. This may partly be due to the improved signal-to-noise ratio of the superficial probe with a factor of 1.92 compared to the non-superficial probe, also described in chapter 2. Another explanation for this superior performance could be the limited measuring depth of the superficial probe in which the spectrum is not 'contaminated'

by Raman signals from deeper tissue layers. Although we observed clearly a qualitative improvement, we were not able to quantify this; further research is required to confirm this hypothesis. In chapter 3, the superficial probe was also evaluated in its capacity to distinguish different grades of UC. The ability to distinguish high-grade from low-grade UC was limited. We think that this might be due to tissue heterogeneity (sampling error), or due to a continuum of biochemical tumor characteristics that gradually increase when becoming more malignant in the development of UC.

**Chapter 4** describes a spatial evaluation of bladder cancer using Raman spectroscopy in cystectomy specimens. A representation consisting of a 2D-map was created of the entire surface of three cystectomy specimens according to both the histopathologic analysis and its Raman spectrum for each measurement location. For tumor detection an AUC of 93%, sensitivity of 86% and specificity of 92% were obtained. The regions surrounding tumorous tissue showed to have a higher prediction uncertainty than the tumor itself and also the tissue further away from the tumor. Therefore, the Raman spectroscopy data suggested a gradual spatial transition from benign to malignant tissue. This may be explained by two theories; first tissue heterogeneity (sampling error), in which multiple cells in one tissue location are evaluated in one Raman spectrum and different biochemical compositions can be measured that belong to different pathologic entities. The second theory is based on the presence of a smooth transition of normal tissue into malignant tissue, meaning no hard cut-off between tumor and healthy tissue. Some (pre-)malignant changes were detected by Raman spectroscopy while not being obvious in the pathologic evaluation (using cut-off values for classification). Therefore, Raman spectroscopy may be used as an early indicator for progression or recurrence, and also as a more accurate way to describe the tumor boundary. If Raman spectroscopy could detect these (pre-) malignant changes, more accurate tumor resections could be performed, that could result in less residuals and recurrences.



A spin-off of this Raman spectroscopic research is presented in **Chapter 5**. Current diagnostic tools such as photodynamic diagnosis (PDD) can be used for screening an entire bladder for bladder malignancies. Techniques like this have a high sensitivity but lack specificity [2]. This means that many lesions are detected, and false positives are frequent, resulting in unnecessary resections of benign tissue in current standard practice. Raman spectroscopy can be used in combination with such a diagnostic screening tool to acquire optical biopsies at specific suspect locations, with a high specificity. To follow up specific lesions by Raman spectroscopy, we developed a real-time registration and navigation system. A repeat measurement at the exact location could possibly indicate a transition into malignancy in time and its need for resection at the right time. In this chapter, we present a phantom study of a real-time

bladder registration and navigation process. The newly developed system showed an acceptable accuracy for bladder lesion registration and navigation. The advantage of the developed system is that detection is not limited to lesions of >5 mm as in CT, MRI and/or sonography [3–13]. Also, no pre-operative imaging or artificial landmarks (fiducials) are required. This system can also be used in patients with severe hematuria that limits vision during cystoscopy or when the light is reduced as in PDD. We found some limitations such as limited accuracy due to volume changes of the model. In vivo studies are required to measure the feasibility of navigation with different bladder volumes and with different rectal filling statuses.

## DISCUSSION, FUTURE PERSPECTIVES AND CONCLUSION

**Instant optical biopsies in bladder cancer diagnosis**

Diagnosing bladder cancer takes time. When a patient suffers from hematuria, a cystoscopy is performed in the outpatient clinic. A suspect lesion is detected, and then the patient is planned for a transurethral resection of a bladder tumor (TURBT). After approval of the anesthesiologist, the operation is performed, mostly within a few weeks. The resected tissue or biopsies are evaluated by the pathologist, which takes about 10 days. Eventually the patient has a doctor's appointment and starts the appropriate follow up and/or treatment scheme after about a month or more. When an instant biopsy is performed at the outpatient clinic, a lot of time and costs are saved and possibly unnecessary treatments are avoided. Obtaining a direct diagnosis at the outpatient clinic enables decisions such as following up lesions instead of direct resections, planning postoperative chemotherapeutic instillations or direct upstaging to cystectomy without TURBT. Such an instant biopsy should be highly specific and easily tolerated by the patient in that specific office setting.

Endoscopic Raman spectroscopy is such a technique, that is both non-invasive and highly specific by revealing the biochemical substrate of lesions. The biggest obstacle in the utilization of Raman spectroscopy in clinical practice is that the signal from Raman scattering is very weak, one in $10^{\wedge 6}$-$10^{\wedge 8}$ scattering photons. This inefficient scattering requires a high laser power and long acquisition times [14]. Translation and implementation into the clinic is consequently hampered. Compromises to reduce acquisition times can decrease the diagnostic accuracy. Nevertheless, new strategies are being developed to address this limitation. Hardware developments to improve collection of the Raman signal but also software developments such as different analytical methods are being evaluated to improve the diagnostic ability of Raman spectroscopy. Imaging modalities based on non-linear Raman scattering, multimodal

integration of Raman spectroscopy and selective-sampling Raman Microscopy (RM), are tools to empower Raman spectroscopy. In addition, the use of nanomaterials and photonic structures to enhance the Raman signals are being evaluated. New analysis methods that are being explored include Artificial Intelligence techniques such as Deep Convolutional Neural Networks and Spatial bagging [15,16]. All these accomplishments are important steps toward maximizing the diagnostic accuracy and speed of Raman spectroscopy. Further improvement will lead to more cost-effective solutions that are likely to be adopted into clinical practice [17].

In this thesis, a hardware design to improve signal collection is evaluated. A new superficial Raman probe was developed that is biocompatible, complies with the Medical Device Directive requirements and is suitable for endoscopic evaluation. This superficial probe is aimed at the required measurement depth and with an increased signal-to-noise ratio, compared to a non-superficial probe. Also, signals generated from the probe, silica in this case, do not interfere with the Raman signal of urothelium, as opposed to the substance of other beveled or ball lens probes that have a limited field (and depth) of view. The measurement depth and increase in signal-to-noise ratio were confirmed in both the phantom model and the in vivo evaluation (chapter 2 and chapter 3). The use of Raman spectroscopy to discriminate benign from malignant tissue was shown to be adequate in chapter 3; however, discrimination between different grades of UC was limited in the in vivo study. This could be explained by tissue heterogeneity or a transition into malignancy. To adopt this technique into the outpatient clinic, adjustments should be made to enable use of the Raman probe through a flexible cystoscope.



To improve the diagnostic ability of Raman spectroscopy, more than just hardware and software improvements are required. Sufficient data is necessary to improve the Raman spectroscopic knowledge on bladder cancer. In future, a spatial micro-Raman analysis using a more dense measurement grid with automated placement of the probe on a cystectomy specimen, could evaluate the impact of tissue heterogeneity in UC. Generation of a larger multi-center database of Raman measurements and biopsies of different UC entities (more power) could increase the diagnostic accuracy of Raman spectroscopy, because the diagnostic algorithm is based on prior Raman measurements. Knowledge about specific Raman contributions of certain biochemical tumor compositions, linked to their corresponding biochemical alterations, provides information about tumor biology.

Raman characteristics of disagreeing Raman and pathological classification in one tissue location gives insight into the biochemical substrate responsible for a transition into

malignancy. These findings could consecutively lead to more therapeutic targets in bladder cancer treatment or be the basis of research on bladder cancer (recurrence or progression) prevention. Because Raman spectroscopy determines a biological fingerprint of tissue without necessity of human interpretation, this technique could possibly overcome some inter- and intra-observer variability of the pathologist. Raman spectroscopy measures biochemical characteristics as opposed to the histopathologic analysis, which assesses morphologic and histopathologic characteristics. Maybe the poor reproducibility of the histopathological analysis could be resolved by adding Raman spectroscopy to this analysis.

Finally, if Raman spectroscopy generates an instant optical biopsy with high accuracy and is applicable in the outpatient clinic, immediate treatment decisions could be made as described above. When a lesion requires follow up, the exact same location should be evaluated again at follow-up. Bladder registration and navigation would enable such repeat measurements. In combination with deep learning augmented bladder tumor detection, the accuracy of the navigation systems and reproducibility of cystoscopy can be improved. Also, quality verification of cystoscopies could be accomplished by improving the documentation of findings during cystoscopy by such a system. Nevertheless, this registration and navigation system is still in its infancy. Combinations of techniques should be explored in future clinical studies.

**Other Raman spectroscopic applications in bladder cancer**

As described in chapter 1, more implementations of Raman spectroscopy in bladder cancer diagnosis are under development, such as evaluation of urine or serum constituents. Raman spectroscopy on urine samples is being evaluated to detect early recurrences. Alternatives to urine cytology and repeat cystoscopy in the follow up of UC, are being searched for. High sensitivities, specificities and accuracies of Raman spectroscopy on urine have been presented [15,16,18–23]. However, none of these has been implemented in standard care. All these cystoscopies are invasive and not cost-effective and the patient waiting lists are increasing. A paradigm shift is required in favor of the diagnostic costs, time and complications of cystoscopy and cytology that may cause a few more false negative findings which could be registered to avoid unnecessary biopsies.

Alternative applications of Raman spectroscopy are being investigated in basic research. Several studies were designed to evaluate bladder tumor specific biomarkers by (Surface Enhanced) Raman spectroscopy (SERS). For example, anti EGFR antibody, circulating cancer-derived small extracellular vesicles, Alfa-1-Antitrypsin antigen and Hyaluronic acid-ase and telomerase activity were investigated to enable tumor detection in urine.

In the future, detection of these biomarkers by Raman spectroscopy could be used as an alternative to urine cytology [24–29]. In search for alternatives to urine cytology, other biomarkers are also being investigated. For example Circulating Tumor Cells, urinary long non-coding RNA's (prognostic marker for Non Muscle Invasive Bladder Carcinoma (NMIBC)), neutrophil-to-lymphocyte ratio (prognostic prediction marker in primary T1HG UC), and systematic inflammatory biomarkers that are related to the oncological outcomes in patients with high-risk NMIBC, are being explored [30–34]. In the future, Raman spectroscopy could be used as a tool to enable detection of such markers.

Raman spectroscopy was also used to evaluate bladder cancer detection in serum, another form of liquid biopsy [35,36]. Interesting is that these groups evaluated whether serum samples could detect NMIBC and Muscle-Invasive Bladder Cancer (MIBC), while logically when the disease is limited to the bladder, no cancerous characteristics would be detectable in serum. In contrast, when (micro) metastasis are present these would be detectable in serum as they can be spread by serum. This indicates another application for Raman spectroscopy; a detection tool of (micro-)metastasis that are not detectable by regular imaging, which is vital for the appropriate treatment. The 5-year overall survival rate after cystectomy is about 50% and has not improved in the last decades [37,38]. Many patients die because of preoperatively undetected micro-metastasis. Therefore, more research should be performed on detection of micro-metastasis in serum pre-cystectomy, and Raman spectroscopy is an adequate tool.

To improve outcomes of this patient group, a response prediction to (neo-)adjuvant therapy (chemo- or immunotherapy) is important. When patients are unresponsive to neo-adjuvant therapy, cystectomy is delayed while the tumor has more time to advance and metastasize. Raman spectroscopy might enable detection of specific tumor characteristics that predict an adequate response to (neo-)adjuvant therapy to choose the correct (neo-)adjuvant therapy or omit this therapy. One point of interest is the amount and kind of inflammation present in the tumor and serum. This might be related to the response of the immune system to tumorigenesis. Maybe the kind and amount of inflammation, detectable by Raman spectroscopy could be prognostic for the progression of the tumor, or the response to immunotherapy.

Raman spectroscopy is also being investigated for detection of biochemical processes in bladder tumor development. Raman spectroscopy enables detection of Green Fluorescent Protein in bladder cancer cell lines. The corresponding gene is used as tool in gene engineering for determining changes in function or expression to evaluate cellular biology, especially of gene silencing [39]. In another study, SERS was used

to evaluate leak-resistance DNA hybridization chain reaction in urine samples. This detection is an important tool in DNA nanotechnology studies to gain information about DNA breakage in tumor development [40]. Also, SERS was used to detect an enhanced surface permeability and retention effect in human bladder cancer tissue [41]. Finally, preliminary results have been published on using Raman spectroscopy for detection of intracellular nano-transporters that could contain therapeutic and/or imaging agents, to enable targeted drug delivery in the field of oncology [42].

## CONCLUSION

In this thesis improvements for the clinical application of Raman spectroscopy are being evaluated. The way is being smoothed for the use of Raman spectroscopy in bladder cancer diagnosis in vivo.

Non-invasive bladder cancer diagnosis is an ongoing challenge, because the gold standard cystoscopy and TURBT are invasive procedures and are timely and costly. Raman spectroscopy is a powerful analytical method that enables measurements of chemical compounds in complex biological samples, such as cells, tissues and biological fluids. The equipment for in vivo measurements has been developed, but the system is not yet applicable in the outpatient clinic with flexible cystoscopes. When discriminating malignant from benign tissue or cells, high accuracy rates are achieved. For liquid biopsies, Raman spectroscopy outperforms the current standard pathologic urine cytology. Furthermore, Raman spectroscopy has an added value in basic research. Thus, Raman spectroscopy has many opportunities and more research is required to increase the ease of clinical use and applicability in order to improve the quality of bladder cancer diagnosis.