Chapter 1

General introduction and thesis outline
INTRODUCTION

Despite many recent improvements in the medical treatment of cancer, surgical removal remains the single most important curative treatment option for solid tumors. For a curative resection, complete resection of the tumor, as confirmed by histopathological evaluation, is essential. To minimize comorbidity it is important to spare vital structures (e.g. nerves, ureters, bile ducts). While there are several imaging modalities available that can visualize tumor size, location and relation to vital structures up to a certain extent in the preoperative setting, during surgery, other than incidental use of intraoperative ultrasonography, surgeons still mainly rely on visual inspection and palpation to determine what should be resected and what should be spared.

Unfortunately, it can be challenging to discriminate tumor tissue from normal tissue by visual inspection and palpation and as a result, irradical resections and damage to vital structures still occur relatively frequently. Therefore, there is a need for an imaging modality that can provide the surgeon with real-time information during surgery on the exact location of tissues to be resected and tissues to be avoided.

OPTICAL IMAGING

Optical imaging using near-infrared (NIR) fluorescence is a technique that has the potential to fulfill this need. Advantages of NIR fluorescent light (700-900 nm, Figure 1) include high tissue penetration (millimeters to centimeters deep) and low autofluorescence, thereby providing sufficient contrast. Because the human eye is insensitive to NIR wavelengths, the use of NIR light does not alter the surgical field. Recently developed intraoperative imaging systems are able to provide simultaneous acquisition of surgical anatomy (white light, color video) and NIR fluorescence signal. Furthermore, systems are available that can simultaneously acquire and display multiple separate fluorescence wavelengths, enabling labeling of tumors on one channel and vital structures on a second channel. Therefore, the use of NIR fluorescence imaging could potentially be of great value in the intraoperative detection of critical anatomical structures and oncologic targets.

In addition to NIR fluorescence imaging systems, exogenous NIR fluorescent contrast agents are necessary to visualize specific tissues. Ideally, tumor cells are labeled by targeted contrast agents. However, the only NIR fluorescent contrast agents currently registered by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) for clinical applications are the non-targeted indocyanine green (ICG; peak emission ≈ 820 nm) and methylene blue (peak emission ≈ 700 nm).
SeNtINeL LyMPh Node deteCtIoN

Sentinel lymph node (SLN) mapping, as introduced in the treatment of cutaneous melanoma by Donald Morton \(^9\) is now considered part of the standard of care in cutaneous melanoma, breast cancer and vulvar cancer. The SLN is the lymph node that drains directly from the tumor and is therefore most likely to be the node to which tumor cells will first metastasize. By injecting a tracer around the primary tumor, the SLN can be identified and resected by following the drainage of that tracer. If the SLN contains no tumor cells, it is unlikely that the remaining lymph nodes contain metastases and the resection of these nodes and the associated comorbidity can be avoided.

Currently, SLN mapping typically involves the injection of a radiotracer preoperatively and injection of a blue dye shortly prior to surgery. Using this combined technique, high sensitivity and low false negative rates are achieved. However, these methods have their disadvantages. The use of a radiotracer exposes caregivers and patients to ionizing radiation and may not be possible in all clinics due to regulatory issues. Local injection of a blue dye stains the surgical field in an unnatural color that persists for several months after surgery and can not be visualized when it is covered by overlying tissue. The use of NIR fluorescence has the potential to overcome these limitations and simplify the use of SLN mapping in various cancer types. Indeed, the

**Figure 1.** Absorption of light by various components varies over the wavelength spectrum, resulting in an optimal window for fluorescence imaging in the NIR light region between 650 and 900 nm. OxyHb, oxygenated hemoglobin; DeoxyHb, deoxygenated hemoglobin. Figure reprinted from Chance.\(^{32}\) With permission, Copyright Clearance Center Rightslink.
first studies have been described that use the currently available ICG in combination with an intraoperative NIR fluorescence imaging system for SLN mapping in breast cancer,\(^6, 10\) gynecologic cancer\(^7, 11\) and gastrointestinal malignances\(^12\).

**TUMOR MARGIN DETECTION AND IMAGE-GUIDED RESECTION**

Different strategies can be followed to detect malignant cells or tissues during surgery. The various hallmarks of cancer can be used as a target for imaging strategies: increased growth and growth factor signaling receptors, limitless replicative potential, sustained angiogenesis, and increased proteolytic activity resulting in tissue invasion and metastasis.\(^13\) Development of these probes is the focus of intensive research and many have been tested in a preclinical setting. Enzyme activatable probes allow detection of proteases that are relatively abundant in malignant tissue, which can be associated with specific characteristics of the tumor, e.g. invasive, aggressive or metastatic tendency. These agents are injected in a quenched (i.e. non-fluorescent) state, resulting in minimal fluorescence at the time of administration. After cleavage by the specific enzyme, the agent becomes dequenched (i.e., fluorescent), resulting in a high signal-to-background (SBR). Examples of activatable agents that have been used in animal cancer models are activatable polyarginine-based cell-penetrating peptides that detect matrix metalloproteinases,\(^14\) activity-based probes that target cysteine cathepsins,\(^15\) and several commercially available probes that have been developed by Weissleder et al., which are activated by cathepsins or matrix metalloproteinases (PerkinElmer, Waltham, USA).\(^16, 17\)

Instead of detection of tumor-associated proteases, molecular-specific detection of cancer cells can be performed using a specific targeting ligand or monoclonal antibody conjugated to a fluorophore. Tumor detection by exploiting the increased growth factor receptor expression of tumors has been described in all kinds of different tumors. In these studies, fluorophores that were coupled to monoclonal antibodies targeting the epidermal growth factor receptor, Her2/neu receptor, or vascular endothelial growth factor receptor were used.\(^18-21\) For imaging of tumor angiogenesis, targeting of alpha-v-beta-3 (αvβ3) integrin, a critically important adhesion molecule in the regulation of angiogenesis, is a widely used strategy. Targeting of αvβ3 integrin by cyclic arginine-glycine-aspartate conjugated to various non-quenched or quenched fluorophores has been reported.\(^22-24\) Finally, in analogy with PET technology, increased glucose metabolism due to increased expression of membrane glucose transporter proteins in intracranial gliomas has been reported.\(^25\)
IDENTIFICATION OF VITAL STRUCTURES

In order to identify vital structures by using NIR fluorescence contrast agents, a distinction should be made between hollow and solid structures. Hollow structures, such as bile ducts, ureters and blood vessels can be visualized if contrast agents can be delivered intraluminally, whereas solid structures, as for example the nerves, can be visualized by targeting specific cellular markers, as membrane proteins.

Hollow structures can be visualized either by direct injection or by means of excretion from for example the liver or kidneys. ICG is excreted by the liver into the bile and has indeed been shown to identify bile ducts during surgery.\textsuperscript{26-28} MB is cleared both hepatically and renally and has been shown to identify both ureters and bile ducts.\textsuperscript{29}

The identification of nerves requires the development of novel contrast agents. Advances have been made in the development of nerve targeting probes, although many hurdles still exist as the probes are fluorescent in a lower wavelength than the NIR spectrum, thereby lowering tissue penetration and suffering from increased autofluorescence.\textsuperscript{30} Another strategy is to use fluorescent peptides that target the nerve sheath, avoiding the need to cross the blood-nerve-barrier.\textsuperscript{31}

OUTLINE OF THE THESIS

This thesis is divided in two parts: in Part I focus lies on preclinical validation of NIR fluorescence image-guided surgery, Part II describes clinical translation of this technique and first-in-human studies.

Part I, chapter 2 describes the intraoperative identification of colorectal liver metastases using NIR fluorescence and a novel integrin $\alpha_v\beta_3$ targeted probe in an experimental rat tumor model. Chapter 3 explores the use of a protease activatable NIR fluorescent probe for intraoperative identification of tumor margins in a rat model of breast cancer and subsequent image-guided resection of breast tumors. In chapter 4, the clinically available probe indocyanine green (ICG) is used to intraoperatively identify colorectal liver metastases in an experimental rat tumor model.

In part II, chapter 5, NIR fluorescence imaging is used for \textit{ex vivo} SLN mapping in colorectal cancer, to allow the use of novel probes that are not yet approved for clinical \textit{in vivo} injections. First, this technique is validated in a swine model, where \textit{in vivo} injections are compared to \textit{ex vivo} injections. Subsequently, this technique is translated to the clinic in a pilot series of human colorectal cancer specimens. Chapters 6 and 7 are focused on the use of NIR fluorescence imaging for SLN mapping in breast cancer. In chapter 6, the development of an improved imaging system with reduced size and optimization of ICG:HSA dose are described. Whether premixing with HSA is indeed beneficial for SLN mapping in breast cancer patients is then studied in a
randomized trial in chapter 7. The use of intraoperative NIR fluorescence imaging for SLN mapping is reported in cervical cancer patients and vulvar cancer patients in chapter 8 and chapter 9, respectively.

In breast reconstructive surgery using free skin flaps, good oxygenation of the transplanted tissue is essential for flap survival. Imaging of vascular anatomy may help the surgeon in choosing the vessels to be used for transplantation and has the potential to improve outcomes. Chapter 10 describes intraoperative imaging of perforator vessels using NIR fluorescence in patients undergoing diep inferior epigastric perforator (DIEP) flap breast reconstruction after mastectomy.

As no tumor targeted NIR fluorescent probes are yet available for clinical application, the enhanced permeability and retention effect could potentially be used for tumor identification by non-targeted probes. In chapter 11 it is attempted to visualize tumors intraoperatively after ICG injection in pancreatic cancer patients. Furthermore, visualization of bile ducts is studied using NIR fluorescence imaging. Chapter 12 describes the use of NIR fluorescence imaging to intraoperatively visualize colorectal liver metastases in patients who were injected with ICG prior to surgery. In chapter 13, all results are then summarized and discussed and an outlook into the future is provided.
INTRODUCTION

REFERENCES


