Chapter 13

Summary and future perspectives
SUMMARY

Intraoperative imaging using NIR fluorescence has the potential to be of major impact on surgical practice, and cancer surgery in particular. The ability to visualize tumors and lymph nodes that need to be resected, simultaneously with nerves, bile ducts and other structures that need to be spared, can improve outcomes and reduce complications in a wide variety of surgical procedures (chapter 1). Since NIR fluorescence imaging involves no ionizing radiation, requires only relatively low-cost camera systems and can be used for real-time visualization during surgery, the technique can be disseminated all around the world, potentially also to areas were less healthcare budget is available.

Multiple NIR fluorescence imaging systems have recently become available, two NIR fluorescent probes are already clinically available (indocyanine green, ICG, and methylene blue, MB) and many novel probes are being developed. This thesis focuses on preclinical validation of this technique for intraoperative tumor identification and image-guided resection (Part I), and a clinical translation of this technique using clinically available probes (Part II).

Part I, chapter 2 describes the use of an integrin $\alpha_v\beta_3$-targeted NIR fluorescent probe for the intraoperative detection of colorectal liver metastases in a syngeneic rat tumor model. In this study, all of the induced colorectal liver metastases could be identified intraoperatively using a NIR fluorescent imaging system. Furthermore, several intra-abdominal metastases, were detected by NIR fluorescence. The study shows proof-of-principle of the application of novel tumor-targeted probes and NIR fluorescence for intraoperative tumor detection.

Positive resection margins are a major problem in the treatment of breast cancer, with reported rates of up to 40 percent. The possibility of visualizing breast tumors during surgery could reduce the number of irradical resections and therefore be of great impact on patient care. In chapter 3, a protease-activatable NIR fluorescent probe was used to intraoperatively detect breast tumors in a syngeneic rat model of breast cancer. Subsequently, tumors were resected under direct image-guidance, with minimal excision of healthy tissue. NIR fluorescent image-guidance allowed for radical resection of all tumors, with minimal margins of healthy tissue (mean minimum and a mean maximum tumor-free margin of $0.2 \pm 0.2$ mm and $1.3 \pm 0.6$ mm, respectively). When these tumor specific probes become clinically available, patient benefit and the effect on the number of radical resections can be assessed.

The clinically available probe ICG is cleared by the liver and has been shown to passively accumulate around colorectal liver metastases. Chapter 4 focuses on preclinical optimization of the use of indocyanine green for intraoperative detection of colorectal liver metastases, in a syngeneic rat model. All liver metastases could be
identified intraoperatively after ICG injection. The optimal interval between ICG injection and intraoperative imaging was 72 h.

In part II, focus lies on clinical translation of intraoperative NIR fluorescence imaging. Analysis of the sentinel lymph node (SLN), the first node that drains from a tumor, is an important procedure in the staging and treatment of breast cancer, cutaneous melanoma and vulvar cancer. This procedure has been studied extensively in colorectal cancer patients, but it has yet to show clear patient benefit. This could be caused by a suboptimal technical procedure for SLN mapping in colorectal cancer. As an oncologic resection involves the *en bloc* resection of regional lymph nodes, *ex vivo* tracer injection and SLN mapping are possible. In chapter 5, the use of NIR fluorescence imaging for *ex vivo* SLN mapping in combination with an experimental, more optimized probe are studied in colorectal cancer patients. This technique was optimized in a swine model and subsequently tested in a pilot series of colorectal cancer patients. NIR fluorescence imaging enabled the detection of SLNs in all cases. In one case, a mesenteric metastasis was encountered that was not NIR fluorescent, however, this was a tumor mass without any remaining lymph node tissue, preventing lymphatic flow.

SLN mapping in breast cancer typically involves the use of a radiotracer and a blue dye. NIR fluorescence imaging has the potential to improve SLN mapping in breast cancer, by possibly replacing one of the current modalities, or even both, or functioning as an adjunct. In chapter 6, optimization of imaging system and injected ICG dose are performed. In this study, ICG is premixed with human serum albumin (HSA), to increase the retention in the SLN and increase fluorescence brightness of ICG. SLN mapping using NIR fluorescence was uneventful in all patients and allowed the detection of on average 1.45 SLNs. Optimal ICG:HSA dose was between 400 and 800 µM.

In previous preclinical studies, premixing of ICG with HSA showed clear advantages: it improved the retention of the dye in the SLN and increased the fluorescence brightness. Chapter 7 aims to test this advantage in a clinical, randomized setting, as injection into a human breast might induce coupling with the physiologically available albumin and eliminate the need of premixing. Patient groups injected with or without premixed ICG showed no difference in fluorescence contrast (P = 0.18), or in number of identified nodes (P = 0.74), indicating that premixing of ICG with HSA can be omitted in case of breast cancer. This simplifies the clinical procedure and can facilitate the introduction of this technique in clinical practice.

Chapter 8 describes the use of NIR fluorescence for the intraoperative detection of SLNs in cervical cancer patients. Shortly prior to surgical scrub, patients received peritumoral injections of ICG:HSA. After exposure of lymph node basins, NIR fluorescence imaging enabled successful detection of SLNs in all patients. No false negatives were observed. In chapter 9, the use of NIR fluorescence imaging was
described in SLN mapping in vulvar cancer patients, who also received standard-of-care injections of radiotracer and patent blue dye. In all patients, SLNs (N = 11) could be detected by NIR fluorescence and radiotracer, 3 nodes, however, were not blue. These pilot studies show the successful use of NIR fluorescence imaging in the detection of SLNs in gynecologic malignancies.

Nowadays, the majority of breast cancer resections are breast-conserving surgeries, where only the tumor itself and a safety margin around it are resected. When a mastectomy is performed, several reconstructive techniques are available. The use of free skin flaps is associated with good cosmetic results and high patient satisfaction. The surgical procedure, however, can be challenging and creation of skin flaps for autotransplantation requires careful planning to select the right blood vessels for optimal flap perfusion. In chapter 10, the use of NIR fluorescence imaging for visualizing flap vascularization is assessed in a clinical trial of breast cancer patients undergoing deep inferior epigastric perforator flap reconstructive surgery after mastectomy. ICG was injected at 3 dose levels and NIR fluorescent angiography was performed at fixed moments during surgery. NIR fluorescence permitted visualization of flap vascularization in all patients and a dose of 4 mg ICG was found to be optimal.

Intraoperative visualization of pancreatic tumors could help reduce the number of irradical resections. As no tumor specific probes are clinically available, in the study described in chapter 11, ICG was injected in order to test if tumors could be identified by passive accumulation (the enhanced permeability and retention effect). Furthermore, as ICG is excreted into bile, it was evaluated if bile ducts could be visualized intraoperatively. Unfortunately, no useful tumor contrast could be observed in all but one patient. NIR fluorescence did, however, enable the identification of extrahepatic bile ducts during surgery. Chapter 12 then describes a study in which patients suffering from colorectal liver metastases were injected intravenously with ICG, prior to surgery. During surgery, superficially located metastases could clearly be identified by a fluorescent rim around the tumor. This could be caused by hampered excretion of ICG into bile, by compression of liver tissue by the expanding tumor. Importantly, other than tumors identified preoperatively by CT or MRI, and intraoperatively by visual inspection and palpation, NIR fluorescence enabled the detection of 4 hotspots that were not found by other modalities. These were histologically confirmed to be metastases. Tumor-to-liver ratios of 7.4 (range 1.9 – 18.7) were observed, which is higher than any of the preclinically tested tumor-targeted probes.
FUTURE PERSPECTIVES

Future perspectives

The availability of already clinically approved NIR fluorescent probes has been essential for the first clinical trials. However, these probes, indocyanine green and methylene blue were not designed as contrast agents for image-guided surgery and are not optimal, but can be used off-label for imaging applications. When the first intraoperative imaging systems became available in the course of the last decade, research groups all around the world have used these probes for many applications.\textsuperscript{4-9} For NIR fluorescence imaging to perform up to its full potential and have a significant impact on patient care, several new developments are necessary.

Probe development

The ability to selectively visualize tumor cells and nerves can be a game changer in cancer surgery, and can potentially result in higher radical resection rates and lower complication rates. However, for this to be a reality, tumor and nerve specific probes need to be approved for clinical application.\textsuperscript{10,11} In general, these targeted probes consist of a fluorophore and a targeting ligand. Currently clinically available fluorophores have suboptimal properties and cannot be conjugated to targeting ligands, necessitating the development of novel fluorophores. These fluorophores should be non-toxic, highly fluorescent (high quantum yield) and have the possibility of conjugation to a targeting ligand. IRDye 800CW (LI-COR Biosciences, United States) is a fluorophore that matches these requirements and has recently completed its toxicity tests in rodents.\textsuperscript{12} Choi et al. have shown that quantum dots can be cleared rapidly from the body, if the hydrodynamic diameter is smaller than 5.5 nm and the surface charge is balanced of the molecule.\textsuperscript{13} Following these observations, the Frangioni Lab (Harvard Medical School, United States) has developed a novel organic fluorophore that is zwitterionic (ZW800-1).\textsuperscript{14} Both IRDye 800CW and ZW800-1 are currently manufactured following cGMP guidelines and it is expected that the first clinical studies can start within the next months to years. Future research should be focused on maximizing the fluorescent properties of probes, optimizing rapid excretion and further reduction background uptake.\textsuperscript{15}

To selectively label tumor cells, various distinguishing hallmarks of cancer, as described by Hanahan and Weinberg, can be used as targets.\textsuperscript{16} An optimal target is exclusively and abundantly expressed by tumor cells and can be targeted without causing toxicity. Novel NIR fluorescent probes have been developed that target growth factor receptors,\textsuperscript{17-20} glucose metabolism,\textsuperscript{21} angiogenesis,\textsuperscript{22-24} and enzymatic activity,\textsuperscript{25,26} and these probes have been studied in preclinical tumor models. First-in-human
results of intraoperative fluorescence imaging in debulking surgery for metastatic ovarian cancer have been reported with a folate-receptor targeted probe (van Dam et al., manuscript accepted for publication). Although the probe used in these studies was based on fluorescein, which fluoresces in the non-optimal visible light spectrum, these results are highly promising for clinical application of targeted NIR fluorescent probes in image-guided surgery.

Iatrogenic nerve damage is a major complication in oncologic surgery, which could potentially be avoided by NIR fluorescence imaging. Small molecule, nerve specific agents BMB and its derivative GE3082 pass the blood-nerve-barrier and selectively target nerves (although background uptake in adipose tissue is also observed). These probes, however, do not fluoresce in the NIR spectrum and have therefore limited tissue penetration. Furthermore, these probes show in vivo toxicity. Further research is currently focused on reducing toxicity and shifting the fluorescence excitation and emission wavelengths to the NIR window. Whitney et al. developed a nerve-specific probe by using phage display to select a peptide specific for peripheral nerves and conjugating to a NIR fluorophore. Although the chemical properties of this probe prevent it from penetrating the blood-nerve-barrier, nerve staining was observed in vivo. Future research will have to show what strategy is most optimal to selectively target nerves.

Intraoperative imaging systems

Currently available imaging systems all have their drawbacks, some only show NIR fluorescence signal without displaying anatomical context, others are relatively large and most are not yet unobtrusive and sufficiently user friendly to be used outside of a research setting. Furthermore, laparoscopic systems are not widely available, and currently available systems do not provide anatomical context. Depth penetration of NIR light is limited and novel camera system designs are focused on increasing the depth at which a fluorophore can be detected. Various strategies can be followed to achieve higher detection depth. Detection of tissue autofluorescence will minimize background noise and increase the maximal depth at which a fluorophore can be detected. For this purpose, fluorescence lifetime imaging (FLIM, which measures the decay of fluorescence intensity of a fluorophore) can be utilized. Temporal and spatial frequency domain modulation of the light source can be used to determine depth information of the fluorescent signal (as reviewed by Gioux et al.). Optimized camera systems are being developed by various groups and companies and research is focused on improving performance, and improving the ease of use in the operating room. When these optimized imaging systems become available, NIR fluorescence imaging has a chance to leap from the research setting into general clinical practice.
Conclusions

Intraoperative imaging using NIR fluorescence is a highly promising imaging modality that has the potential to revolutionize cancer surgery. The studies described in this thesis show proof of principle that it is possible to use NIR fluorescence imaging in surgical practice. When targeted contrast agents and optimized camera systems become available, this technique has the opportunity to prove its true benefit to patient care.
REFERENCES

15. Frangioni JV. The problem is background, not signal. Mol Imaging 2009; 8:303-304.
