Summary, Conclusions and Future Perspectives

This chapter was edited from the following articles:

Successful Translation of Fluorescence Navigation During Oncologic Surgery: A Consensus Report

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SUMMARY

In current clinical intraoperative practice surgeons continue to rely on the subjective visual and tactile cues to differentiate diseased versus healthy tissues. Consequently, the ability to adequately assess the extent of tumor spread and the status of resection margins is difficult and objectively challenging to estimate. Conventional anatomical imaging modalities such as ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) are currently neither real-time nor tumor specific, and therefore of limited value for intraoperative surgical use. Although frozen section analysis remains the most common method of intraoperative margin assessment this technique is highly subjective to ‘interpretation errors’ by the pathologist and ‘samplings errors’ by the surgeon, since it only provides information on the small area of the wound bed of which the sample was taken. Given that the primary treatment modality for most solid tumors is surgery and complete tumor removal with tumor free margins is of eminent importance for curative intent, real-time intraoperative strategies to distinguish between tumor and normal tissue would most certainly result in a significant improvement in surgical outcomes, as well as in terms of disease-free survival as overall survival.

The thesis is subdivided into two sections. Section I addresses the development of using therapeutic antibodies for optical imaging to improve detection of subclinical disease. Section II of this thesis is focused on antibody based photodynamic therapy (i.e. photoimmunotherapy (PIT)). For PIT, antibodies are conjugated to a fluorescent photosensitizer and serve as targeting vectors that specifically deliver the photosensitizer to the tumor. A relatively brief exposure from an external light source permits fluorescence imaging for diagnostic tumor localization and cell death by prolonged light activation of the cytotoxic photosensitizer.

A general introduction on cancer surgery using optical imaging and PIT as an effective combination of image-guided surgery and intraoperative treatment of residual disease is provided together with the outline of the thesis in Chapter 1. Chapter 2 provides an overview of the various techniques and contrast agents that are currently used for image-guided cancer surgery. Also its future perspectives are described. As well as in Europe and in the US, the first steps of translation of optical imaging to the clinical are made successfully by collaborating scientific teams. It is anticipated that image-guided surgery has the potential to be a very powerful tool in guiding future patient care in surgical oncology.

Chapter 3 describes a feasibility study of systemically administered cetuximab-IRDye800CW to evaluate whether fluorescence contrast could aid the surgeon to discriminate cancerous
from normal surrounding tissue in a surgical setting for treatment of Glioblastoma Multiforme (GBM). Retrospective studies have provided substantial evidence that completeness of resection is an important predictor of survival. Here, we evaluated the ability of a systemically administered antibody-dye probe conjugate (cetuximab-IRDye800CW) to provide sufficient fluorescent contrast for surgical resection of disease in both subcutaneous and orthotopic animal models of GBM using luciferase positive D-54MG, U-87MG and U-251MG (n=5). To assess whether cetuximab-IRDye800CW would provide sufficient fluorescence contrast to differentiate between tumorous and normal tissue, the subcutaneous model was elaborated due to its permitted longer study duration for optimal characterization of fluorescence changes over time. We showed that fluorescence intensity within the tumors peaked on day-1 post cetuximab-IRDye800CW administration and the tumor-to-background-ratio (TBR) increased over time in two of the three cell lines. Daily imaging of tumors revealed an average TBR of 4.5 for U-87MG, 4.1 for D-54MG, and 3.7 for U-251MG. For the orthotopic model, TBRs on the day of surgery ranged from 19 – 23 during wide-field intraoperative imaging. Utilizing this dual-modality approach we demonstrated that the reduction of luciferase signal was found to correlate well with reduction in fluorescence intensity (R²=0.99, p-value). More importantly, fluorescence-guided resection using wide-field NIR imaging resulted in an 87% greater reduction in luciferase signal (P=0.001) when compared to conventional surgical resection under white-light (i.e. 41%).

In Chapter 4 we report the results of a first in-human clinical trial using a fluorescently labeled antibody cetuximab-IRDye800CW in patients with HNSCC for the purpose of fluorescence-guided resection of cancer. Twelve patients with biopsy proven HNSCC were included in our study. The first three patients (cohort 1) were given cetuximab-IRDye800CW intravenously at a microdose (1% of therapeutic dose of cetuximab), cohort 2 received 10% of therapeutic dose of cetuximab, and cohort 3 received 25% of therapeutic dose of cetuximab, 2.5 mg m⁻², 25 mg m⁻² and 62.5 mg m⁻² cetuximab-IRDye800CW, respectively. Multi-instrument fluorescence imaging was performed in the operating room and in surgical pathology examination. We demonstrated that the overexpression of EGFR could be safely exploited for diagnostic imaging. Wide-field NIR imaging intraoperatively showed a significantly greater fluorescence intensity in the tumor compared to surrounding normal tissue at each imaging time point in cohort 2 and cohort 3 (P<0.05). Pathological processing of the primary specimen was mapped to histology to evaluate the relationship between fluorescence intensity and tumor deposition. Fluorescence was shown to correlate well with disease areas using hematoxylin and eosin staining. Additionally, fluorescence intensity correlated well with EGFR expression independently from tumor density (P<0.001). Overall, this study demonstrated
that cetuximab-IRDye800CW can be safely administered as a tumor-specific contrast agent, which may be helpful for surgical navigation to identify subclinical disease in EGFR-expressing tumors. Further clinical studies are needed to establish the detection accuracy in terms of sensitivity and specificity and the definitive clinical value for intraoperative decision making.

As a therapeutic modality, anti-EGFR antibodies have been successfully implemented in the treatment of locally advanced HNSCC. However, predicting therapeutic response remains a challenge on a per-patient basis. Although significant efforts have been invested in elucidating EGFR-mediated cell signaling pathways in relation to treatment efficacy, the delivery and histologic localization of antibody based therapeutics in human tumors is poorly understood nor ever made visible. Appropriate patient selection would minimize the considerable costs and significant morbidity associated with unnecessary administration of antibody based therapeutics. In Chapter 5 we present the first in-human study demonstrating that by optical molecular imaging, denominated as in vivo fluorescence immunohistochemistry, it is possible to evaluate the localization of fluorescently labeled cetuximab for reasons of therapeutic efficacy. Correlating morphological (peri-)tumoral characteristics to levels of (fluorescently labeled) therapeutic antibody delivery, may improve our understanding on true antibody delivery. We demonstrated that fluorescence intensity is highly dependent upon EGFR expression levels, as might be expected. Further analysis of well-differentiated tumors, however, demonstrated high levels of EGFR with near absent fluorescence suggesting poor uptake of the antibody in this histological subtype, indicating that less sensitivity to cetuximab of this histological subtype might be expected. Moreover, we provided insight in the distribution of the fluorescently labeled therapeutic antibody in normal healthy tissues, which might help explain some of the known toxicities associated with anti-EGFR therapy. Moderate to strong fluorescence intensities in the sebaceous gland was observed, which might explain the adverse skin reaction commonly seen in patients treated with EGFR inhibitors. Moreover, significant elevated fluorescence levels were also found in the submandibular and sublingual glands. It is unclear, however, if this results in clinically significant xerostomia since cetuximab is commonly given in conjunction with radiotherapy and as such hard to differentiate from radiotherapy induced xerostomia.

In conclusion, this study demonstrates how in vivo fluorescence immunohistochemistry can be used for drug development of cancer immunotherapy based on understanding true tumoral antibody delivery.
The use of fluorescence to guided surgical resection of cancer is rapidly advancing towards routine clinical use. However, the subjective nature of reporting levels of fluorescence contained within tissues has remained largely qualitative, lacking a more standardized approach for reporting. Obviously, there is an urgent need for establishment of diagnostic accuracy and definition of clinical value for intraoperative decision making.

In Chapter 6 a ratiometric threshold is introduced as a potential solution to provide a method for objective assessment of tumor fluorescence during fluorescence-guided surgery. The developed formula produced a highly sensitive and specific threshold for predicting presence of cancer in the tissues tested, when normalized for inter-patient and intra-tumoral variances by using muscle or skin as an internal anatomical control.

Chapter 7 assesses the potential of fluorescent-guided histopathology to shift tumor margin assessment from surgical pathology to the operating room to provide a more accurate method for disease localization in frozen room and permanent pathology. A preclinical model of luciferase-positive tumor resection, using bioluminescence as the gold standard, was created. Fluorescence assessment determined by closed-field fluorescence imaging (Pearl, LI-COR Biosciences) of fresh resected margins accurately predicted the presence of disease in 33/39 positive margins yielding an overall sensitivity of 85%, specificity of 95%, positive predictive value (PPV) of 94%, and a negative predictive value (NPV) of 87%, which was superior to both surgical assessment (54%, 61%, 57%, and 58%) and pathological assessment (49%, 95%, 91%, and 66%), respectively. The report provides strong evidence that tumor-specific fluorescence can be used by the surgeon or pathologist to guide sampling.

Among the array of modalities currently being investigated to localize subclinical islands of tumor during cancer surgery, intraoperative fluorescence imaging seems the most promising for real-time image-guided surgery. Often, however, fluorescence images display various intensities due to the complex tissue geometry and densities of tissues being imaged. Since light travelling through tissues is subject to various amounts of absorption, scattering and reflection, detection of fluorescence signals in a heterogeneous optical absorption environment is challenging. Photoimmunotherapy (PIT) comprising antibody-based photodynamic therapy (PDT), the subject of part II of this thesis, is a promising approach as photoimmunodetection in combination with PDT can be achieved for treatment of undetectable and/or irresectable disease.
Over the past few years the use of near-infrared high-power light-emitting diodes (LEDs) for PIT applications has become desirable because of the inexpensive and safe nature of the modality. Recent findings however, have shown that performance can be compromised by illumination variations caused by ineffective heat dissipation. Chapter 8 describes the development and manufacturing of a LED device for PIT application. In this work we validated the technical feasibility, applicability, safety and consistency of the system for cancer treatment. Moreover we provided a framework for standardization for future studies in which other newly developed photosensitizers will be evaluated.

In Chapter 9, the use of PIT to assist in surgical resection of HNSCC is assessed. We demonstrated that PIT significantly delayed occurrence of tumor regrowth after subtotal surgical resection, and therefore should be considered as an efficient adjuvant treatment modality. Since murine models often do not reflect the complex stromal elements found in human tumors and cell line data has significant limitations, we assessed therapeutic efficacy of PIT in ex vivo freshly excised human head and neck specimens. In order to determine cell viability, an assay based on the presence of adenosine triphosphate (ATP) in metabolically active cells was applied since a rapid decrease may reflect necrosis and apoptosis. Our results demonstrate a significant reduction in ATP levels for twelve patient specimens following PIT. To the best of our knowledge, this was the first study evaluating the treatment efficacy of PIT on fresh tissues slices where the multiple tumor components that can influence therapeutic outcomes were maintained.

PIT has been extensively studied in the laboratory setting in preclinical models, but it has not yet been translated to the clinic because the toxicity profile, nor pharmacokinetics and biodistribution of the fluorescently labeled antibody are unknown. In Chapter 10 we evaluated the biodistribution of cetuximab conjugated with IRDye700DX when administered via intravenous infusion to cynomolgus monkeys. In the study, four monkeys (2 each per sex per time point) received systemically cetuximab-IRDye700DX at 40 mg/kg and were terminated 2 days after dosing. The second group received cetuximab-IRDye700DX at 80 mg/kg and were terminated 14 days after dosing, whereas a control group of two animals received cetuximab alone (without conjugated to IRDye700DX) and terminated 14 days after dosing. This group serves as a control for autofluorescence levels for the biodistribution measurements. Fluorescence levels and tissue distribution of the cetuximab-IRDye700DX in intact pieces of tissue samples were measured (n=51) as well as the amount of intact cetuximab-IRDye700DX using gel electrophoresis followed by LDS-PAGE. Finally, fixed and paraffin embedded tissue sections were scanned by using a fluorescence flat bed scanner in order to determine fluo-
rescence intensity levels and tissue distribution of fluorescence. During fluorescence analysis for all three subparts (tissue pieces, gels, and tissue sections) the samples from animals dosed with cetuximab-IRDye700DX were simultaneously compared with identically treated samples from a control group dosed with cetuximab. Our results demonstrate that there was a strong correlation for the results of fluorescence measured in intact tissue pieces, homogenized samples (using gel electrophoresis), and tissue sections; the locations and relatively levels were consistent with expected locations of cynomolgus EGFR. Taken together, these results suggest that IRDye700DX bioconjugates can be translated to the clinical to provide a tumor-specific mechanism to improve detection and treatment of (sub-) clinical disease during oncologic procedures.

CONCLUSIONS

In this thesis we have demonstrated that tumor-targeted intraoperative imaging has potential clinical advantages for localization and treatment of head and neck cancer. We have demonstrated that commercially available antibodies can be fluorescently labeled and safely administered to humans after preclinical testing in cynomolgus monkeys. Several important observations could be made based on this first in-human trial. First, microdosing of cetuximab-IRDye800CW provided limited contrast intraoperatively and as such strategies that target EGFR tumor receptors should not be constrained to an exploratory approach (Phase 0 trials). Depending on the affinity for the target and the clearance characteristics for an optical imaging probe, in combination with limited tissue penetration and scattering and absorption properties, the microdose approach may not be useful for optical probes in general. Second, tumor mapping ex vivo identified suspicious areas on the specimen and on peripheral margins, which demonstrated potential for reducing sample error after systemic administration of cetuximab-IRDye800CW. Third, observed adverse events of the labeled antibody in humans were consistent with the known toxicity profile of unlabeled cetuximab\textsuperscript{1}, demonstrating that this antibody based optical labeling technique can be safely applied to other protein-based therapeutics in the future.

Furthermore, we introduce in vivo fluorescence immunohistochemistry, defined as a systemic injection of near-infrared fluorescent monoclonal therapeutic antibodies, as a highly suitable, straightforward and unique methodology to provide insights into (peri-) tumoral antibody delivery. We foresee that as the importance of antibody based therapies continues to grow, that further definition of the platform of “in vivo fluorescence immunohistochemistry” may have great potential to guide future antibody-based treatment strategies for a more pa-
tient specific tailored approach which fits in novel strategies like precision medicine or precision surgery.

Fluorescence imaging can be ‘threshold adjusted’ along a continuum of intensities that must be standardized to an acceptable baseline to fully appreciate its diagnostic value and in order to compare data among different institutions, studies, camera systems and tumor types. Therefore, a widely adopted methodology for immediate objective identification of unknown samples in the operating room is highly recommended. We introduced, to the best of our knowledge, the first standardized method (based on a ratiometric threshold derived from mean fluorescent tissue intensities) to objectively identify disease tissue intraoperatively in real-time. A ratiometric threshold for positive disease can be experimentally developed and integrated into the camera imaging system software to objectively distinguish diseased tissue from normal. However, we anticipate that calibration of the threshold should be performed uniquely for each patient taking into consideration the differences in tumor physiology, tissue properties, timing, molecular target expression, and clearance.

We demonstrate that ‘Fluorescence-guided pathology’ can be used by the surgeon or pathologist to guide sampling for frozen sections, but does not replace the need for histological diagnosis. Although fluorescence guidance may be better than conventional sampling techniques, which include inspection and palpation, it does not allow for histopathological analysis of tissue to confirm margins on frozen sections.
We have designed and manufactured a standardized, validated, and safe LED/photodynamic therapy device for IRDye700DX-based PIT cancer treatment. Moreover, a framework was provided for standardization for future studies in which other newly developed photosensitizers can be evaluated for in vitro and in vivo experimental setups. We have shown that photoimmunotherapy offers significant advantages over more traditional methods of imaging due to the ability for photodetection of the targeted tissue of interest as well as phototoxicity induced by photosensitizers after exposure to near infrared light.

We performed, the first study to assess the pharmacokinetic, biodistribution and toxicity profile of an IRDye700DX-conjugated antibody. We showed a strong correlation for the fluorescence measured in intact tissue pieces, homogenized samples (using gel electrophoresis), and histologic tissue sections in cynomolgus macaques infused with cetuximab-IRDye700DX. Moreover, we demonstrated that the tissue distribution and relative levels of cetuximab-IRDye700DX accumulation were consistent with the expected biodistribution of EGFR indicating target specific binding. Taken together, these results strongly suggest that IRDye700DX may be safely translated to the clinic to provide tumor specific antibody based surgical imaging and phototherapy for cancer to improve detection and treatment of (sub-) clinical disease during oncologic procedures.

**FUTURE PERSPECTIVES**

Successful translation of modalities for medical use typically follows three broad phases ranging from; I) system development and early feasibility studies, to II) regulatory approval and larger clinical trials, and eventually to III) adoption as the standard of care.\(^5\) Progression through the phases is largely dependent upon the demonstration of safety and efficacy. In the following paragraphs, fundamental barriers that may impede clinical translation are expanded and suggestions for future developments are discussed.

*Tumor heterogeneity*

Using antibodies, previously developed for therapeutic reasons, for surgical imaging and phototherapy of cancer has multiple advantages, including the fact that the targeting moiety is already FDA- and EMA approved with a well-known toxicity, pharmacokinetic and biodistribution record and therefore will allow for a more rapid development in a more cost effective manner. However, tumor heterogeneity also creates a challenge for antibody based surgical imaging and phototherapy.\(^6\) Due to genomic instability combined with high cell turnover, tumor cells can show different genotypical and phenotypical profiles, which ultimately lead
to different biological profiles within tumors, and thus antigen expression rates. Even in a
tumor originating from a single initiation clone, these processes can make the genome differ
significantly from one another, highly impairing uniform antigen expression rates, and thus
consistent antibody based imaging and treatment strategies for both detection and treatment
strategies.6

To a certain extend, variability in antigen expression can be assessed by immunohistochem-
istry on a core needle biopsy prior to surgery. However, one should realize that such a biopsy
might be compared with taking a sample of the world population in New Zealand and claim-
ing that the world population consists of Maori’s. Moreover, it may be possible that targeting
a panel of antigens by administering a cocktail consisting of multiple fluorescently labeled
antibodies with different emission wavelengths in the NIR spectrum might yield better results
instead of focusing on a single tumor associated antigen and a single wavelength. Implement-
tation of such an approach is considered an important step towards personalized medical
imaging and precision surgery.

Influence of optical tissue properties
Quantification of fluorescence signals remains a challenging task. Since the targeted fluoro-
phore is embedded within the tissue, the excitation and emission wavelength is subject to
three influencing parameters, which are used to characterize the interaction of photons with
tissue, including: I) light absorption (Fig. 1a), II) reflection (Fig. 1b), and scattering (Fig. 1c).
These all results in the attenuation of the fluorescent signal, ultimately limiting optical imag-
ing by the strong scattering of light in biological tissue, which severely degrades the spatial
resolution from targets situated deeper in the tissues.

Absorption and scattering of light are largely dependent on the wavelength of the excitation
source and tissue properties. Light absorption and scattering increase with decreasing wave-
lengths. Below 750 nm, light is absorbed to a great extent by physiologically abundant mole-
cules such as hemoglobin and lipids, thus reducing the tissue penetration to only a few milli-
meter. As such, future progress in fluorescence imaging should focus on the development of
new fluorescent agents that fluorescence in the near infrared range (750 – 1000 nm), or other
deeper penetrating optical imaging techniques like optoacoustic imaging in cancer applica-
tions.7 Optoacoustic imaging is a new technique on the horizon, that allows for detection of
fluorescence signals up to several centimeters that could be of substantial use in the (peri-) and
intraoperative setting.8 Photoimmunotherapy (PIT) comprising antibody-based photody-
namic therapy (PDT) may be a promising approach as photoimmunodetection in combi-
nation with PDT can be achieved for treatment of undetectable and/or irresectable disease in
a primary or (neo-)adjuvant setting.
Towards standardized reporting and standardized imaging

The current intraoperative imaging systems superimpose the fluorescent signal on a color video of the surgical view, thereby providing the surgeon with anatomical positioning of the fluorescent signal. Due to the fact that nowadays surgeons are used to working with 2D screens during laparoscopic procedures, visualizing the fluorescent signal on an operating room monitor seems reasonable and straightforward. Moving forward, however, there is a high need for a widely adopted more standardized method for fluorescence assessment, since current fluorescence imaging can be threshold adjusted along a continuum of intensities. A ratiometric threshold that is integrated in to the imaging software to objectively separate diseased tissue apart from normal may be highly advantageous to the surgeon. Several institutions are already developing attenuation correction algorithms. Incorporation of these algorithms in the imaging systems may be great way to correct and manage for the effects of optical tissue properties and deliver the tools for exchange and comparison of data derived from separated studies, multiple institutions and camera systems.

In summary, although challenges will continue to arise we anticipate that antibody based imaging and phototherapy for cancer will be important strategies to improve the efficacy of oncologic surgery.
REFERENCES

Addendum