Structure-Inherent Targeting of Near-Infrared Fluorophores for Image-Guided Surgery

Danbi Jo and Hoon Hyun[*]

Department of Biomedical Sciences, Chonnam National University Medical School, Gwangju, Korea

Although various clinical imaging modalities have been developed to visualize internal body structures and detect abnormal tissues prior to surgical procedures, most medical imaging modalities do not provide disease-specific images in real-time. Optical imaging can provide the surgeon with real-time visualization of the surgical field for intraoperative image-guided surgery. Imaging in the near-infrared (NIR) window (650-900 nm), also known as the "therapeutic window" has high potential by offering low absorbance and scattering in tissues resulting in minimized background autofluorescence. Clinically, optical fluorescence imaging with the targeted contrast agents provides opportunities for significant advances in intraoperative image-guided surgery. There are only two clinically available NIR fluorophores, indocyanine green (ICG) and methylene blue (MB), that support the image-guided surgery. However, neither of them perform *in vivo* by providing optimum specificity and stability for targeted image guidance. Therefore, it is of paramount importance to develop targeted NIR fluorophores for unmet clinical needs. Using the right combination of an NIR fluorescence imaging system and a targeted fluorophore, the desired target tissues can be imaged to provide real-time fluorescence guidance without changing the field-of-view during surgery. Thus, in a clinical discipline, the development of NIR fluorophores for 'structure-inherent targeting' is an unmet need for early phase diagnostics with accurate targeting.

Key Words: *Fluorescent Dyes; Fluorescence; Optical Imaging; Surgery*

INTRODUCTION

The primary and most successful approach for the treatment of cancer is still surgery, even though radiotherapy and chemotherapy techniques are widely used. However, most human surgeries are performed without any image guidance meaning that the practice depends completely on the visual ability of the surgeon. As a result, one of the fundamental limitations of cancer surgery, and in particular of minimally invasive surgery, is that the tumor tissue needs to be visually distinguished from the healthy tissue because most clinical imaging modalities cannot provide the desired target images in real-time.[1-6]

Additionally, biomedical imaging modalities including ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and single photon emission computed tomography (SPECT) are typically used to noninvasively access the disease tissue prior to the surgical procedures, but they are not available in the operating room. Furthermore, the ionizing radiation associated with these techniques limits the overall scope of their real-time translation.[7-11]

In response to this unmet, clinical need, optical imaging provides real-time visualization of the surgical field especially when combined with near-infrared (NIR) fluorophores, allowing intraoperative image-guided surgery.[12-17] Imaging in the NIR window (650-900 nm), known as the "therapeutic window", has shown clinical potential by offering low absorbance and scattering in tissues as a black background while providing maximum depth of light penetration. The advantages of NIR fluorescence have been recognized and many types of fluorescence image-guided surgery systems are continuously developing in both the preclinical and clinical phases.[18,19] Thus, with

the right combination of an NIR fluorescence imaging system and targeted NIR fluorophores, the desired target tissues can be imaged to support real-time fluorescence guidance with unchanging field of view during surgery (Fig. 1).[20-25]

To provide the targeted visualization for more precise surgery, NIR contrast agent development and clinical translation are necessary. However, the development of ideal NIR fluorophores has been a longstanding problem in the field of fluorescence image-guided surgery.[26-30] It is important to note that the visualization of the target tissue mainly depends on the efficacy of fluorescent agents, resulting from the difference in the amount of molecular uptake into the target and neighboring normal tissues. However, it is difficult to achieve a high quality, targeted image due to the limited number of biomarkers and targeting ligands. Currently, there are only two FDA approved NIR fluorophores, indocyanine green (ICG) and methylene blue (MB). Both ICG and MB are blood pool agents that have no inherently specificity for tumor or normal tissues, and thus are not ideal fluorophores for fluorescence image-guided surgery.[31-39] Therefore, in a clinical discipline, the development of NIR fluorophores for target-specific imaging is an unmet need for early phase diagnosis with accurate targeting.
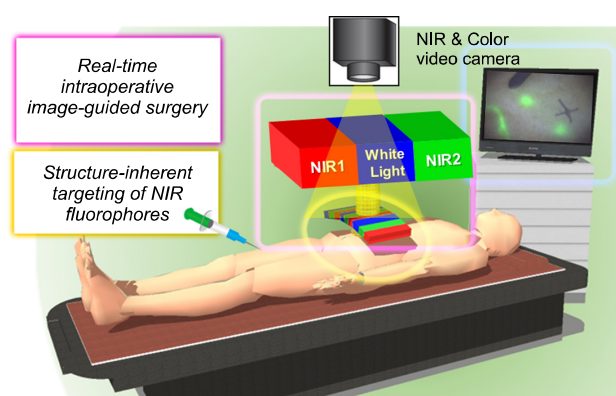

FIG. 1. Schematic of dual-channel intraoperative NIR fluorescence imaging. A targeted NIR fluorophore is administered intravenously, topically, or intraparenchymally. During surgery, the fluorophore is visualized using a NIR fluorescence imaging system above the surgical field for open surgery or encased within a fiberscope for minimally-invasive and robotic surgery. All systems must have adequate NIR excitation lights (i.e., NIR1 for 700 nm fluorescence and NIR2 for 800 nm fluorescence), collection optics and filtration, and a camera sensitive to NIR fluorescence emission lights. An optimal imaging system includes simultaneous visible (i.e., white) light illumination of the surgical field, which can be merged with NIR fluorescence images. The surgeon display can be one of several forms factors including a standard computer monitor, goggles, or a wall projector (monitor form factor shown). Current imaging systems operate at a sufficient working distance that enables the surgeon to operate and illuminates a sizable surgical field. The monitor image is reproduced with permission from ref 43 (Copyright 2013 Wiley-VCH).

In this review, we focus on the latest achievements in structure-inherent targeting by developing NIR fluorophores for fluorescence image-guided surgery resulting in a high potential to shift the clinical paradigms.

STRUCTURE-INHERENT TARGETING OF NIR FLUOROPHORES

NIR fluorescence imaging techniques can provide target-specific imaging with a high degree sensitivity for target detection because of the ultralow background autofluorescence prior to excitation of the target tissue. The improved detection sensitivity makes it possible for the application to fluorescence-guided surgery. However, a fundamental problem in fluorescence-guided surgery is the development of well-defined fluorophores that target specific normal or diseased tissues. For the targeted fluorescence imaging, the conventional method for preparing targeted imaging agents requires covalent conjugation of separate targeting moieties and fluorescent materials. In this review, we summarize research showing that it is possible to create NIR fluorophores with different tissue specificities driven by their inherent chemical structures (Table 1 and Fig. 2). A single fluorophore could be designed to achieve selective targeting with high efficiency in differentiating specific tissues through modifying the physicochemical properties and incorporating recognition elements into the fluorophore domains. Selective modifications control the pharmacophore to binding sites of the target tissues directed by structure-inherent chemical recognition. Thus, the single compact fluorophore performs both targeting and imaging, simultaneously. This strategy is the paradigm shift in a role of fluorophores from traditional method used for covalent conjugation of targeting ligands and fluorescence imaging agents.
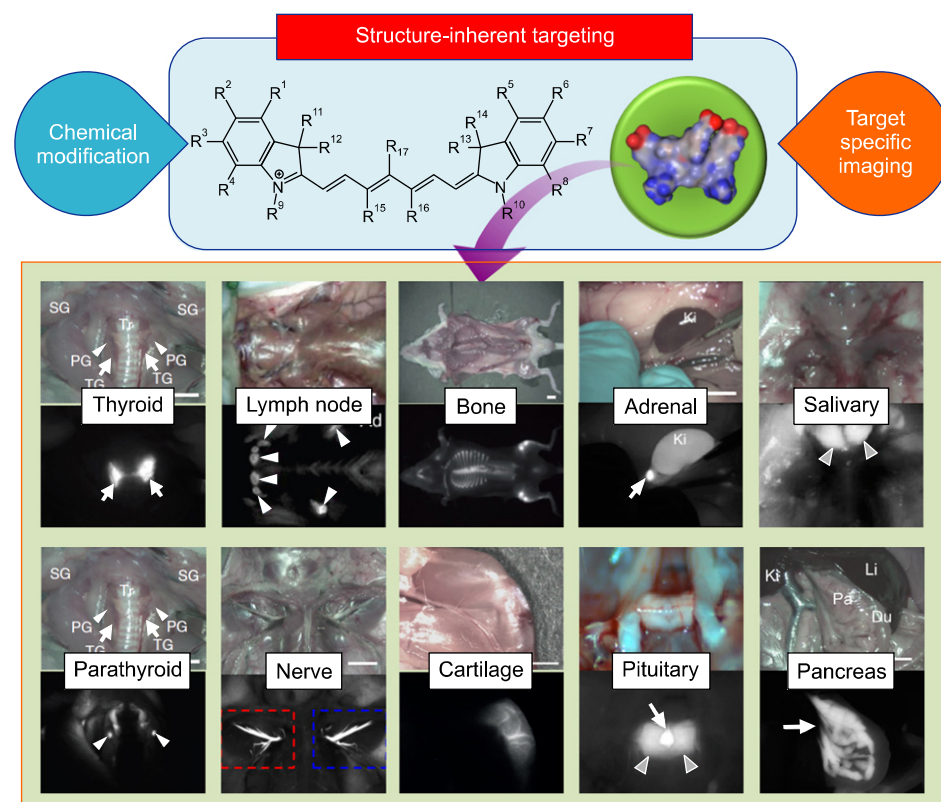
1. Parathyroid and thyroid glands targeting

The endocrine glands are tissues of substantial clinical importance, yet they are difficult to locate during surgery. In particular, a partial or complete resection of the thyroid gland requires accurate identification and preservation of the parathyroid glands. Complications can occur when all functional parathyroid glands are inadvertently damaged or removed during thyroidectomies, or when they are incompletely removed during parathyroidectomies. Damage to these tiny organs, which secrete hormones that regulate blood calcium levels, can have deleterious, lifelong effects on a person's health by causing conditions such as hypocalcemia.

The identification of normal parathyroid glands with the naked eye is still challenging in high-risk procedures. Not only are these tissues small, but their location also varies widely from person to person. Moreover, surgical biopsy of the parathyroid for identification can lead to devascularization and destruction of its function. Consequently, surgeons must rely on visual inspection to identify the different tissues, which can be subjective and inconclusive.

TABLE 1. Engineered NIR fluorophores for structure-inherent targeting *in vivo*

Chemical structure	Name	Targeting tissue	Ref.	Chemical structure	Name	Targeting tissue	Ref.
	T700-F	Thyroid glands	40		C700-OMe C700-H C800-OMe C800-H	Cartilage	57
	T800-F	Parathyroid glands	40		N/A	Adrenal glands	58
	ZW800-3a	Lymph node	48		N/A	Pituitary gland	59
	Oxazine 4	Nerve	51		N/A	Salivary glands	59
	P700H P700SO3 P800H P800SO3	Bone	56		T700-F	Pancreas	62

FIG. 2. Examples of structure-inherent targeting of the engineered NIR fluorophores. Reproduced with permission from ref 40 (for Thyroid and Parathyroid images, Copyright 2015 Nature Publishing Group), ref 48 (for Lymph Node image, Copyright 2014 Ivyspring International Publisher), ref 51 (for Nerve image, Copyright 2014 Ivyspring International Publisher), ref 56 (for Bone image, Copyright 2014 Wiley-VCH), ref 57 (for Catilage image, Copyright 2015 Wiley-VCH), ref 58 (for Adrenal image, Copyright 2015 ACS Publications), ref 59 (for Pituitary and Salivery images, Copyright 2016 ACS Publications), and ref 62 (for Pancreas image, Copyright 2015 Ivyspring International Publisher).

Recently, Hyun et al. developed two NIR fluorophores that are efficiently taken up by the thyroid and parathyroid glands after intravenous injection.[40] They tested the NIR fluorophores in rats and pigs and showed that they could be used to distinguish the thyroid and parathyroid glands from surrounding tissue and from each other. The mechanism of uptake and retention of T700-F (thyroid targeting NIR fluorophore) and T800-F (parathyroid targeting NIR fluorophore) in specific cell types of the thyroid and parathyroid glands is still unknown. However, it appears that site-specific halogenation of the polymethine core is crucial for targeting, and it is possible that iodine-processing cellular machinery (such as transporters and enzymes) may be mistaking these molecules as endogenous substrates.

This technique has shown excellent potential application for increased precision in head and neck surgeries and thus fewer surgical accidents and lower mortality. This approach also provides design considerations for developing disease-specific contrast agents with a strategy of 'structure-inherent targeting'.

2. Lymph nodes targeting

The sentinel lymph node (SLN) is the first lymph node receiving lymphatic drainage from a primary tumor. SLN biopsy is essential for staging many cancers and avoiding unnecessary LN dissection, which may cause adverse effects such as lymphedema. However, there is currently no technology available to surgeons to help them identify all LNs within a basin quickly and with high sensitivity.

Previous reports demonstrated that the use of invisible NIR light has several advantages for SLN biopsy, including relatively high penetration (up to 5-8 mm) into living tissue. When compared to blue dye MB, invisible NIR light has a relatively high signal-to-background ratio (SBR) due to low absorption, scattering, and tissue autofluorescence. Because of these advantages, Josephson et al. employed Cy5.5-grafted polymers (PGC) to demonstrate NIR imaging of pan lymph nodes (PLN), and the Vahrmeijer group demonstrated preclinical evidence by using the premixing of ICG with human serum albumin, resulting in better retention in the SLN.[41-47] However, none of these studies used the NIR fluorophores for 'structure-inherent targeting' of LNs, because Cy5.5 and the currently available clinical NIR fluorophores, MB and ICG, cannot visualize PLNs after intravenous injection.

Recently, Ashitate et al. reported that engineered novel NIR fluorophores have fluorescence emission maxima in two distinct ranges; one NIR color for visualization of PLNs and the other for mapping SLN in the same subject.[48] They achieved the successful mapping of PLNs and SLN by using the single NIR fluorophore different from previous studies by using nanoparticles and polymers. They also emphasized that the proper statistical assessment of SLN biopsies, which includes sensitivity, specificity, positive predictive value, negative predictive value, and accuracy, is dependent on finding all LNs within a basin. Thus, this technique will permit improved quantitative evaluation of SLN biopsy for cancers other than breast cancer and melanoma.

3. Nerve tissue targeting

Nerve damage during surgery results in significant morbidity and mortality. However, nerves are still identified via gross appearance and anatomical location, without any intraoperative image guidance. To minimize high morbidity and mortality from nerve injury, intraoperative visual identification during complex surgeries is of paramount importance.

Although only 3 main classes of nerve-specific molecules have been reported to date, including stilbene derivatives, distyrylbenzene (DSB) fluorophores, and styryl pyridi-

nium (FM) fluorophores, none of these compounds were optimal because of high adsorption and scattering in tissues from the ultraviolet and visible wavelengths required.[49] In addition, Tsien and colleagues reported a significant improvement in nerve-specific targeting and imaging, where an engineered peptide FAM-NP41 was used to label *in vivo* animal nerves and resected human recurrent laryngeal nerves with relatively high affinity.[50]

Unlike this approach using macromolecules, Park et al. recently investigated an ideal, nerve-specific NIR fluorophore named Oxazine 4 for image-guided surgery.[51] Based on their criteria to design the nerve-specific small molecule, it should have logD at pH 7.4 between 0.5 to 3 and a molecular weight < 500 Da to maximize blood-nerve-barrier (BNB) penetration, excitation and emission wavelengths in the NIR window, and prolonged retention in the nerve tissue.

Importantly, Oxazine 4 does not stain the central nervous system, staining only the peripheral nervous system, and did not show apparent impairment of motor function and sensory integrity during a set time period. Therefore, Oxazine 4 will be a good reference to develop the optimal nerve-specific NIR fluorophore providing real-time image-guidance to surgeons about nerve locations during surgery.

4. Bone tissue targeting

All targeted NIR fluorophores described to date for bone imaging require covalent conjugation of a targeting domain (i.e., divalent cation chelating agents such as calcein and tetracycline, bone targeting drugs such as pamidronate, etc.) to a NIR fluorophore.[52-55]

For the structure-inherent targeting of bone tissue, Hyun et al. recently reported a new strategy based on the incorporation of targeting moieties into the nonresonant structures of polymethine indocyanines emitting at NIR wavelength range.[56] Using the known affinity of phosphonates for bone minerals as a model system, they developed two families of bifunctional molecules that target bone without the need for a traditional bisphosphonate.

Additionally, longitudinal NIR imaging studies in mice demonstrated that phosphonated NIR fluorophores remain stable in bone tissue for over 5 weeks, and histological analysis demonstrated incorporation into the bone matrix without interfering with normal bone deposition. Therefore, the bifunctional NIR fluorophore could be used to attach drugs that modulate osteoblasts or osteoclasts, creating trifunctional theranostic agents. They emphasized that non-hormonal phosphonates increase bone strength, and repeated injections of this theranostic agent will improve bone density by preventing the loss of minerals. Taken together, this approach will be a new strategy for bone-specific imaging by using the targeted NIR fluorophores.

5. Cartilage tissue targeting

The major techniques for visualization of cartilage tissue

are MRI and CT, but neither technique provides optimum image quality. A new method recently reported by Hyun et al. is based on fluorescence imaging agents that can be detected in the NIR wavelength range.[57] Injection of the cartilage-specific NIR fluorophores allows high-quality visualization of cartilage tissue in real-time. The most important advantage of using NIR fluorophores for cartilage imaging is the integrated method of performing 'structure-inherent targeting' (i.e. molecular targeting and fluorescent imaging) using a single small molecule. The high affinity of cationic NIR fluorophores for cartilage provides high-quality tissue visualization, ultralow background signal, and low injected doses.

Although it is not clear yet what the actual binding target of the NIR fluorophore is, they assume that the highly negatively charged components of the cartilage tissue are the targets that are recognized and specifically bound by the positively charged fluorophores. Typically, it is thought that cationic materials including nanoparticles and polymers show high nonspecific binding in the body. The cationic NIR fluorophores, however, are engineered by modulating the nonresonant side chains of the polymethine core; they can be systematically modified to tune physicochemical properties such as hydrophobicity, polarity, and electron-resonance properties without affecting binding affinity. In addition, the new NIR fluorophores are highly stable and do not degrade in the body, and a strong cartilage signal was obtained 1-4 hours after the injection of a single dose. Moreover, the cartilage-specific NIR fluorophores make it possible to detect cartilage and bone tissue simultaneously in real-time by combining with bone-specific NIR fluorophores.

Importantly, NIR fluorophores provide high spatial resolution in real-time to distinguish the boundary and thickness of cartilage tissues from the adjacent bones, where other contrast agents with nanoparticle formulations have limited penetration and delineation. Thus, the new cartilage-specific NIR fluorophores have many beneficial applications in terms of cartilage degeneration, new drug design, and image-guided surgery.

6. Adrenal, pituitary and salivary glands targeting

During endocrine surgeries, surgeons depend on their naked eye and experience during often-lengthy procedures to avoid sensitive glands and tissues. The important task of avoiding sensitive tissues is difficult as the small glands are obscured by blood and surrounding tissues. Surgeons need to visualize these tissues during cancer surgery to improve the surgical success rate and advance patient prognosis.

Among the endocrine glands, the adrenal glands located above the kidney produce essential and nonessential hormones that primarily control the body's metabolism, blood pressure, and stress response. The pituitary gland is also responsible for controlling the hormonal release of signaling chemicals throughout the body. In addition, imaging of exocrine salivary glands is useful in identifying their masses and also in distinguishing them from the masses/pathologies of adjacent cervical spaces.

Significant advancements have been reported in the development of NIR fluorophores that target tissues and help surgeons avoid these hormonal glands without changing the surgical field of view in real-time. This class of NIR fluorophores has shown excellent promise in the field of NIR fluorescence-guided surgery for targeting of tumor- and/or normal tissues.

Recently, Owens et al. systematically developed various NIR fluorophores that focused on the effects on biodistribution based on modulating electronic influencing moieties from donating to withdrawing moieties at both the heterocyclic site and through *meso*-substitution of pentamethine cyanine fluorophores.[58-60] The selective modifications controlled innate biodistribution routes through 'structure-inherent targeting' resulting in efficient imaging of the adrenal glands, pituitary gland and salivary glands. These tissues are important for the regulation of human hormone levels and should be carefully avoided during surgical resection in surrounding areas. Thus, these native-tissue specific NIR fluorophores will help surgeons by providing a powerful guidance tool for intraoperative NIR imaging in real-time.

7. Pancreas targeting

Recent technical advances in abdominal surgery have assisted in making complex surgical procedures safer and shorter. However, pancreas-related complications after gastrectomy with radical lymphadenectomy including peripancreatic lymph nodes resection, splenectomy, adrenalectomy, and nephrectomy are still high. Intraoperative pancreatic injuries are typically caused by the proximity of the pancreas to surrounding organs and the difficulty in distinguishing the edge of the pancreas from surrounding fat tissue, especially in obese patients.

Intraoperative identification of the pancreas with high sensitivity in real-time could help avoid such injuries. Previously, Winer et al. reported that NIR fluorescent light could be used to identify both the pancreas and insulinoma by employing the clinically available NIR fluorophore MB.[61] Although MB provided contrast between the pancreas and surrounding organs, it is not an ideal NIR contrast agent because of low fluorescence intensity, nonspecific uptake, and short retention time in the pancreas.

More recently, Wada et al. reported pancreas-specific NIR fluorophore T700-F that showed significantly high uptake in the pancreas and low background uptake in the neighboring tissue. Furthermore, its retention period in the pancreas was over 8 h, making this fluorophore potentially useful in complex abdominal surgeries including laparoscopic operations.[62] Although the mechanism of specific pancreas uptake and retention of T700-F is still unknown, it shows that this approach could be used to identify the pancreas tissue and the novel NIR fluorophore could outperform MB. Thus, it will be a great candidate for real-time intraoperative pancreas imaging during complex abdomi-

nal surgeries.

CONCLUSION AND FUTURE PERSPECTIVES

Real-time image-guided surgery is gaining attention in oncologic surgery because it has a high potential to improve clinical outcomes. Not only can this approach direct intra-operative image guidance for surgical margin assessments, the approach can help surgeons detect microscopic tumors or residual lesions that are unconsciously missed during surgery. If surgeons are able to find tumors completely or avoid neighboring normal tissues more easily then operating time could be shortened, resulting in reduced anesthesia time and its associated risks. The improved surgical system for a successful surgery lowers recurrences and complication rates so it is an excellent way to save on healthcare costs.

The current bottleneck preventing the potential benefits of fluorescence image-guided surgery is the lack of FDA approved tumor-, tissue-selective fluorescence imaging agents. Although successful preclinical studies of NIR fluorophores have recently been reported, none of them, except for ICG and MB, are yet available in clinics pending FDA approval. For clinical translation, the NIR fluorophores need to be designed by considering physicochemical and optical stability, selective targeting ability, reasonable pharmacological activity, efficient elimination from the body, and non-toxicity. Among these considerations, toxicity issue is a major hurdle for the FDA approval, because improper biodistribution and incomplete clearance can cause potential toxicity threats such as reproductive risks, immunotoxicity, neurotoxicity, acute/chronic toxicology, and genotoxicity through biological interactions in the body. Therefore, focusing on rapid biodistribution and complete clearance are essential for safety. That is, the target-specific NIR fluorophores should be designed for complete excretion from the body in a certain period time after achieving site-specific targeting during image-guided surgery. The *in vivo* fate of targeted NIR fluorophores is affected by various factors including hydrodynamic diameter, formulations, surface charge distribution, administration route, injection dose, protein binding, blood flow, and blood pH. Thus, retention and clearance of the injected NIR fluorophores in the target tissues/organs is based on the difference between distribution and elimination behaviors that plays a key role in the controlled accumulation period of these agents in the targets. Therefore, we believe that a fuller understanding of the molecular properties, tissue properties, biodistribution, and targeting mechanisms of these NIR fluorophores is the only way to overcome the barriers to their clinical use.

With continued progress, more preclinical trials will be reported and NIR imaging systems will become a more common technology in clinic. Furthermore, the innovative design and optimization of NIR fluorophores has the potential to lay a foundation in the development of other target-specific small molecule contrast agents.

CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

1. Frangioni JV. In vivo near-infrared fluorescence imaging. Curr Opin Chem Biol 2003;7:626-34.
2. Frangioni JV. New technologies for human cancer imaging. J Clin Oncol 2008;26:4012-21.
3. Gioux S, Choi HS, Frangioni JV. Image-guided surgery using invisible near-infrared light: fundamentals of clinical translation. Mol Imaging 2010;9:237-55.
4. Vahrmeijer AL, Hutteman M, van der Vorst JR, van de Velde CJ, Frangioni JV. Image-guided cancer surgery using near-infrared fluorescence. Nat Rev Clin Oncol 2013;10:507-18.
5. van Dam GM, Themelis G, Crane LM, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-α targeting: first in-human results. Nat Med 2011;17:1315-9.
6. Pierce MC, Javier DJ, Richards-Kortum R. Optical contrast agents and imaging systems for detection and diagnosis of cancer. Int J Cancer 2008;123:1979-90.
7. Jang B, Park S, Kang SH, Kim JK, Kim SK, Kim IH, et al. Gold nanorods for target selective SPECT/CT imaging and photothermal therapy in vivo. Quant Imaging Med Surg 2012;2:1-11.
8. Kaneko OF, Willmann JK. Ultrasound for molecular imaging and therapy in cancer. Quant Imaging Med Surg 2012;2:87-97.
9. Li Y, Pang Y, Vigneron D, Glenn O, Xu D, Zhang X. Investigation of multichannel phased array performance for fetal MR imaging on 1.5T clinical MR system. Quant Imaging Med Surg 2011;1:24-30.
10. Pysz MA, Guracar I, Tian L, Willmann JK. Fast microbubble dwell-time based ultrasonic molecular imaging approach for quantification and monitoring of angiogenesis in cancer. Quant Imaging Med Surg 2012;2:68-80.
11. Yuan J, Mei CS, Panych LP, McDannold NJ, Madore B. Towards fast and accurate temperature mapping with proton resonance frequency-based MR thermometry. Quant Imaging Med Surg 2012;2:21-32.
12. Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nat Biotechnol 1999;17:375-8.
13. Hawrysz DJ, Sevick-Muraca EM. Developments toward diagnostic breast cancer imaging using near-infrared optical measurements and fluorescent contrast agents. Neoplasia 2000;2:388-417.
14. Weissleder R. A clearer vision for in vivo imaging. Nat Biotechnol

2001;19:316-7.

15. Kereweer S, Kerrebijn JD, van Driel PB, Xie B, Kaijzel EL, Snoeks TJ, et al. Optical image-guided surgery--where do we stand? Mol Imaging Biol 2011;13:199-207.

16. Gibbs SL. Near infrared fluorescence for image-guided surgery. Quant Imaging Med Surg 2012;2:177-87.

17. Kobayashi H, Ogawa M, Alford R, Choyke PL, Urano Y. New strategies for fluorescent probe design in medical diagnostic imaging. Chem Rev 2010;110:2620-40.

18. Frangioni JV. The problem is background, not signal. Mol Imaging 2009;8:303-4.

19. Lee JH, Park G, Hong GH, Choi J, Choi HS. Design considerations for targeted optical contrast agents. Quant Imaging Med Surg 2012;2:266-73.

20. Ashitate Y, Tanaka E, Stockdale A, Choi HS, Frangioni JV. Near-infrared fluorescence imaging of thoracic duct anatomy and function in open surgery and video-assisted thoracic surgery. J Thorac Cardiovasc Surg 2011;142:31-8.e1-2.

21. Hutteman M, Choi HS, Mieog JS, van der Vorst JR, Ashitate Y, Kuppen PJ, et al. Clinical translation of ex vivo sentinel lymph node mapping for colorectal cancer using invisible near-infrared fluorescence light. Ann Surg Oncol 2011;18:1006-14.

22. Ashitate Y, Stockdale A, Choi HS, Laurence RG, Frangioni JV. Real-time simultaneous near-infrared fluorescence imaging of bile duct and arterial anatomy. J Surg Res 2012;176:7-13.

23. Poellinger A. Near-infrared imaging of breast cancer using optical contrast agents. J Biophotonics 2012;5:815-26.

24. Hadjipanayis CG, Jiang H, Roberts DW, Yang L. Current and future clinical applications for optical imaging of cancer: from intra-operative surgical guidance to cancer screening. Semin Oncol 2011;38:109-18.

25. Kovar JL, Simpson MA, Schutz-Geschwender A, Olive DM. A systematic approach to the development of fluorescent contrast agents for optical imaging of mouse cancer models. Anal Biochem 2007;367:1-12.

26. Owens EA, Henary M, El Fakhri G, Choi HS. Tissue-specific near-infrared fluorescence imaging. Acc Chem Res 2016;49:1731-40.

27. Choi HS, Frangioni JV. Nanoparticles for biomedical imaging: fundamentals of clinical translation. Mol Imaging 2010;9:291-310.

28. Choi HS, Liu W, Liu F, Nasr K, Misra P, Bawendi MG, et al. Design considerations for tumour-targeted nanoparticles. Nat Nanotechnol 2010;5:42-7.

29. Hellebust A, Richards-Kortum R. Advances in molecular imaging: targeted optical contrast agents for cancer diagnostics. Nanomedicine (Lond) 2012;7:429-45.

30. Owens EA, Lee S, Choi J, Henary M, Choi HS. NIR fluorescent small molecules for intraoperative imaging. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2015;7:828-38.

31. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, et al. Renal clearance of quantum dots. Nat Biotechnol 2007;25:1165-70.

32. Choi HS, Ipe BI, Misra P, Lee JH, Bawendi MG, Frangioni JV. Tissue- and organ-selective biodistribution of NIR fluorescent quantum dots. Nano Lett 2009;9:2354-9.

33. Choi HS, Ashitate Y, Lee JH, Kim SH, Matsui A, Insin N, et al. Rapid translocation of nanoparticles from the lung airspaces to the body. Nat Biotechnol 2010;28:1300-3.

34. Choi HS, Nasr K, Alyabyev S, Feith D, Lee JH, Kim SH, et al. Synthesis and in vivo fate of zwitterionic near-infrared fluorophores. Angew Chem Int Ed Engl 2011;50:6258-63.

35. Hyun H, Bordo MW, Nasr K, Feith D, Lee JH, Kim SH, et al. cGMP-Compatible preparative scale synthesis of near-infrared fluorophores. Contrast Media Mol Imaging 2012;7:516-24.

36. Choi HS, Gibbs SL, Lee JH, Kim SH, Ashitate Y, Liu F, et al. Targeted zwitterionic near-infrared fluorophores for improved optical imaging. Nat Biotechnol 2013;31:148-53.

37. Ogawa M, Kosaka N, Choyke PL, Kobayashi H. In vivo molecular imaging of cancer with a quenching near-infrared fluorescent probe using conjugates of monoclonal antibodies and indocyanine green. Cancer Res 2009;69:1268-72.

38. Schaafsma BE, Mieog JS, Hutteman M, van der Vorst JR, Kuppen PJ, Löwik CW, et al. The clinical use of indocyanine green as a near-infrared fluorescent contrast agent for image-guided oncologic surgery. J Surg Oncol 2011;104:323-32.

39. van der Vorst JR, Vahrmeijer AL, Hutteman M, Bosse T, Smit VT, van de Velde CJ, et al. Near-infrared fluorescence imaging of a solitary fibrous tumor of the pancreas using methylene blue. World J Gastrointest Surg 2012;4:180-4.

40. Hyun H, Park MH, Owens EA, Wada H, Henary M, Handgraaf HJ, et al. Structure-inherent targeting of near-infrared fluorophores for parathyroid and thyroid gland imaging. Nat Med 2015;21:192-7.

41. Josephson L, Mahmood U, Wunderbaldinger P, Tang Y, Weissleder R. Pan and sentinel lymph node visualization using a near-infrared fluorescent probe. Mol Imaging 2003;2:18-23.

42. Schaafsma BE, Verbeek FP, Elzevier HW, Tummers QR, van der Vorst JR, Frangioni JV, et al. Optimization of sentinel lymph node mapping in bladder cancer using near-infrared fluorescence imaging. J Surg Oncol 2014;110:845-50.

43. van der Vorst JR, Schaafsma BE, Verbeek FP, Swijnenburg RJ, Hutteman M, Liefers GJ, et al. Dose optimization for near-infrared fluorescence sentinel lymph node mapping in patients with melanoma. Br J Dermatol 2013;168:93-8.

44. Hutteman M, Mieog JS, van der Vorst JR, Liefers GJ, Putter H, Löwik CW, et al. Randomized, double-blind comparison of indocyanine green with or without albumin premixing for near-infrared fluorescence imaging of sentinel lymph nodes in breast cancer patients. Breast Cancer Res Treat 2011;127:163-70.

45. Mieog JS, Troyan SL, Hutteman M, Donohoe KJ, van der Vorst JR, Stockdale A, et al. Toward optimization of imaging system and lymphatic tracer for near-infrared fluorescent sentinel lymph node mapping in breast cancer. Ann Surg Oncol 2011;18:2483-91.

46. Troyan SL, Kianzad V, Gibbs-Strauss SL, Gioux S, Matsui A, Oketokoun R, et al. The FLARE intraoperative near-infrared fluorescence imaging system: a first-in-human clinical trial in breast cancer sentinel lymph node mapping. Ann Surg Oncol 2009;16:2943-52.

47. Hutteman M, van der Vorst JR, Gaarenstroom KN, Peters AA, Mieog JS, Schaafsma BE, et al. Optimization of near-infrared fluorescent sentinel lymph node mapping for vulvar cancer. Am J Obstet Gynecol 2012;206:89.e1-5.

48. Ashitate Y, Hyun H, Kim SH, Lee JH, Henary M, Frangioni JV,

et al. Simultaneous mapping of pan and sentinel lymph nodes for real-time image-guided surgery. Theranostics 2014;4:693-700.

49. Gibbs-Strauss SL, Nasr KA, Fish KM, Khullar O, Ashitate Y, Siclovan TM, et al. Nerve-highlighting fluorescent contrast agents for image-guided surgery. Mol Imaging 2011;10:91-101.

50. Whitney MA, Crisp JL, Nguyen LT, Friedman B, Gross LA, Steinbach P, et al. Fluorescent peptides highlight peripheral nerves during surgery in mice. Nat Biotechnol 2011;29:352-6.

51. Park MH, Hyun H, Ashitate Y, Wada H, Park G, Lee JH, et al. Prototype nerve-specific near-infrared fluorophores. Theranostics 2014;4:823-33.

52. Bao K, Nasr KA, Hyun H, Lee JH, Gravier J, Gibbs SL, et al. Charge and hydrophobicity effects of NIR fluorophores on bone-specific imaging. Theranostics 2015;5:609-17.

53. Zaheer A, Lenkinski RE, Mahmood A, Jones AG, Cantley LC, Frangioni JV. In vivo near-infrared fluorescence imaging of osteoblastic activity. Nat Biotechnol 2001;19:1148-54.

54. Bhushan KR, Tanaka E, Frangioni JV. Synthesis of conjugatable bisphosphonates for molecular imaging of large animals. Angew Chem Int Ed Engl 2007;46:7969-71.

55. Bhushan KR, Misra P, Liu F, Mathur S, Lenkinski RE, Frangioni JV. Detection of breast cancer microcalcifications using a dual-modality SPECT/NIR fluorescent probe. J Am Chem Soc 2008;130:17648-9.

56. Hyun H, Wada H, Bao K, Gravier J, Yadav Y, Laramie M, et al. Phosphonated near-infrared fluorophores for biomedical imaging of bone. Angew Chem Int Ed Engl 2014;53:10668-72.

57. Hyun H, Owens EA, Wada H, Levitz A, Park G, Park MH, et al. Cartilage-specific near-infrared fluorophores for biomedical imaging. Angew Chem Int Ed Engl 2015;54:8648-52.

58. Owens EA, Hyun H, Tawney JG, Choi HS, Henary M. Correlating molecular character of NIR imaging agents with tissue-specific uptake. J Med Chem 2015;58:4348-56.

59. Owens EA, Hyun H, Dost TL, Lee JH, Park G, Pham DH, et al. Near-infrared illumination of native tissues for image-guided surgery. J Med Chem 2016;59:5311-23.

60. Ashitate Y, Levitz A, Park MH, Hyun H, Venugopal V, Park G, et al. Endocrine-specific NIR fluorophores for adrenal gland targeting. Chem Commun (Camb) 2016;52:10305-8.

61. Winer JH, Choi HS, Gibbs-Strauss SL, Ashitate Y, Colson YL, Frangioni JV. Intraoperative localization of insulinoma and normal pancreas using invisible near-infrared fluorescent light. Ann Surg Oncol 2010;17:1094-100.

62. Wada H, Hyun H, Vargas C, Gravier J, Park G, Gioux S, et al. Pancreas-targeted NIR fluorophores for dual-channel image-guided abdominal surgery. Theranostics 2015;5:1-11.