Structure-inherent targeting of near-infrared fluorophores for parathyroid and thyroid gland imaging

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The typical method for creating targeted contrast agents requires covalent conjugation of separate targeting and fluorophore domains. In this study, we demonstrate that it is possible to create near-infrared (NIR) fluorophores with different tissue specificities driven by their inherent chemical structures. Thus, a single compact molecule performs both targeting and imaging. We use this strategy to solve a major problem in head and neck surgery: the identification and preservation of parathyroid and thyroid glands.

We synthesized 700-nm and 800-nm halogenated fluorophores that show high uptake into these glands after a single intravenous (IV) injection of 0.06 mg kg\(^{-1}\) in a pig. By using a dual-channel NIR imaging system, we observed—in real time and with high sensitivity—the unambiguous distinction of parathyroid and thyroid glands simultaneously in the context of blood and surrounding soft tissue. This novel technology lays a foundation for performing head and neck surgery with increased precision and efficiency along with potentially lower morbidity, and it provides a general strategy for developing targeted NIR fluorophores.

A fundamental problem in image-guided surgery is the development of contrast agents that target a specific normal or diseased tissue1–5. Recently NIR fluorophores have become the imaging technology of choice because of their relatively low photon tissue attenuation, low autofluorescence and ability to detect targets up to 5 mm in depth (reviewed in ref. 6). Almost all targeted NIR fluorophores described to date require covalent conjugation of a targeting domain (i.e., a small molecule, peptidomimetic, peptide or antibody) to an NIR fluorophore. Although this strategy can work well, NIR fluorophores are relatively large molecules (500–1,200 Da), and chemical synthesis of conjugates can be time consuming. An alternative, albeit difficult, approach is to create NIR fluorophores whose inherent chemical structure provides specific targeting to a tissue of interest.

The endocrine glands are tissues of substantial clinical importance, yet they are difficult to locate during surgery7. 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 parathyroidectomies8,9. 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 and accessory thyroid tissue with the naked eye is still challenging in high-risk procedures, such as those required for re-operative central neck surgery, thyroid cancer and Graves disease9,10. Not only are these tissues small, but their location also varies widely from person to person. Because of this, it takes microscopy to reliably tell the difference between parathyroid tissue, thyroid tissue and the surrounding lymph nodes10. However, surgical biopsy of the parathyroid for identification can lead to devascularization and destruction of its function. Consequently, surgeons must now rely on visual inspection to identify the different tissues, which can be subjective and inconclusive11.

The goal of this study was to create families of halogenated 700-nm and 800-nm NIR fluorophores that exhibit specific uptake in parathyroid glands, thyroid glands or both, by virtue of the molecules’ inherent chemical structures. Such molecules could provide surgeons with unambiguous guidance during head and neck surgery after a simple IV injection.

RESULTS

Synthesis and characterization of T700 and T800 fluorophores

An initial screen of an NIR fluorophore library containing 280 novel compounds, injected IV into CD-1 mice, identified two potential pharmacophores for thyroid uptake: one at an emission of 700 nm and the other at an emission of 800 nm (data not shown). We then prepared a series of new compounds built around these pharmacophores to optimize endocrine gland uptake. The synthetic methods for the preparation of T700 (compounds 13–17) and T800 (compounds 18–22) are shown in Figure 1 and Supplementary Methods.

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T700 and T800 NIR fluorophores, emitting at 700 nm and 800 nm, respectively, were composed of pentamethine or heptamethine cores and decorated with various side chains, including hydrogen, methoxy, fluoro, chloro and bromo groups. Before measuring its optical properties and in vivo performance, each NIR fluorophore was purified to ≥95% according to measurements of absorbance at 210 nm (Supplementary Fig. 1).

The physicochemical and optical properties of T700 and T800 NIR fluorophores are summarized in Table 1. By varying the side chains of the polymethine core, it was possible to systematically modify hydrophilicity, hydrophobicity, polarity and electron resonance. All targeted NIR fluorophores exhibited maximum fluorescence in the wavelength range of 650–780 nm, high extinction coefficients and quantum yields, which together minimize tissue autofluorescence and maximize fluorescence signal (Supplementary Fig. 2).

**Autofluorescence of thyroid and parathyroid glands**

Before testing targeted compounds in thyroid and parathyroid glands, we imaged the autofluorescence of both glands in humans and pigs (Supplementary Fig. 3). For the human cohort, subjects undergoing neck dissection as part of a separate, ongoing study\(^\text{12}\) were imaged consecutively with channel 1 (700-nm NIR) and channel 2 (800 nm-NIR) of the mini-FLARE imaging system\(^\text{13,14}\) after undergoing neck dissection as part of a separate, ongoing study\(^\text{12}\). Both T700-F and T800-F identified the pig parathyroid with high sensitivity and specificity. We believe that this is the first demonstration of pig parathyroid imaging in vivo. Notably, T800-F showed higher fluorescence in pig parathyroid than in thyroid 1 h after injection, and this signal was maintained for over 5 h, whereas T700-F showed lower parathyroid fluorescence and this decreased substantially over time (Fig. 3). The parathyroid-to-thyroid signal ratio (PTR) curves indicate that T800-F can be used for specific identification of parathyroid glands even when T700-F shows equal signal in both thyroid and parathyroid glands (Fig. 3).

Because T700-F and T800-F showed the highest thyroid SBR, we selected these F-appended NIR fluorophores for further in vivo study. To determine the optimal dose of T700-F and T800-F for thyroid imaging, doses ranging from 10 to 100 nmol (0.2 to 2.0 mg kg\(^{-1}\)) were injected IV into mice and the SBR was measured over time. A dose of 50 nmol (1.0 mg kg\(^{-1}\)) was optimal for single IV injection in a CD-1 mouse (Supplementary Fig. 4). With respect to clearance and elimination, T700-F primarily exhibited renal clearance, whereas T800-F accumulated in both the liver and kidneys 4 h after injection (Supplementary Fig. 5). However, the fluorescent absolute amounts of fluorophore excreted into the urine and feces were lower than expected and difficult to detect at 4 h after injection (Supplementary Fig. 5).

**In vivo parathyroid and thyroid imaging in pigs**

We used pigs as a large animal model for parathyroid identification because they represent a worst-case scenario. Unlike humans, pig parathyroid glands are located in the thorax, far from the thyroid glands, and without anatomical landmarks\(^\text{15}\). It is difficult to distinguish pig parathyroid glands with the naked eye because of their small size (a few millimeters) and location embedded within the surrounding thymus. For our experiment, T700-F and T800-F were IV injected into six separate Yorkshire pigs (n = 3 per dose) at a dose of 5 μmol (0.06 mg kg\(^{-1}\)) and imaged over a period of 5 h (Fig. 3). Both T700-F and T800-F identified the pig parathyroid with high sensitivity and specificity. In vivo thyroid imaging of T700 and T800 in mice

As a preliminary in vivo test for thyroid targeting, T700 and T800 NIR fluorophores were injected IV into CD-1 mice (10 nmol; 0.2 mg kg\(^{-1}\)) and imaged after 1 and 4 h (Fig. 2). T700-F and T800-F showed the highest signal-to-background ratio (SBR, calculated by fluorescence intensities between thyroid and neighboring muscle) values compared to the other molecules. Signal intensity of thyroid tissue in mice decreased only 17% between 1 and 4 h after injection (Fig. 2). The salivary glands in humans are anatomically distinct from the thyroid gland; however, in rodents they are more co-localized.

**Table 1** Physicochemical and optical properties of T700 and T800 NIR fluorophores in 100% serum, pH 7.4. In silico calculations of logD at pH 7.4 and total polar surface area were calculated using Marvin and JChem calculator plug-ins (ChemAxon).

| Fluorophore   | Molecular weight (Da) | logD at pH 7.4 | Total polar surface area (Å\(^2\)) | Extinction coefficient (M\(^{-1}\)cm\(^{-1}\)) | Emission maximum (nm) | Quantum yield (%) |
|---------------|-----------------------|----------------|-----------------------------------|---------------------------------|----------------------|------------------|
| T700-H        | 383.55                | 3.56           | 6.25                              | 147,000                        | 670                  | 30.4             |
| T700-OMe      | 443.60                | 3.24           | 24.71                             | 109,000                        | 674                  | 26               |
| T700-F        | 419.53                | 3.84           | 6.25                              | 123,000                        | 651                  | 14               |
| T700-Cl       | 452.44                | 4.77           | 6.25                              | 130,000                        | 660                  | 14               |
| T700-Br       | 541.34                | 5.09           | 6.25                              | 147,000                        | 662                  | 27.6             |

**Figure 1** Synthetic schemes for T700 and T800 NIR fluorophores.
Simultaneous in vivo NIR imaging of parathyroid and thyroid

To provide surgeons with unambiguous landmarks during head and neck surgery, we exploited the dual-NIR channel capability of the FLARE imaging system to highlight parathyroid and thyroid glands simultaneously and in real time. For initial experiments we chose rats because they possess a single pair of parathyroid glands located on the anterior and lateral aspects of the thyroid lobes. For dual-channel imaging of parathyroid and thyroid glands, 0.2 μmol (0.35 mg kg⁻¹) of T800-F was IV injected into a 250-g Sprague-Dawley (SD) rat 24 h before imaging, followed by 0.2 μmol of T700-F injected into the same animal 6 h before imaging. The doses and timing used are the optimal ones we found during initial screening tests (data not shown). Under these conditions, T800-F allows the parathyroid to be visualized unambiguously and T700-F simultaneously highlights both glands (Fig. 4a). The identities of resected tissues were confirmed using NIR fluorescence microscopy and consecutive H&E staining (Fig. 4b). As expected, T700-F was observed in both thyroid and parathyroid glands, whereas T800-F remained only in the parathyroid gland (Fig. 4b).

To confirm that these results were species independent, we repeated experiments in the pig model, using 5 μmol (0.06 mg kg⁻¹) of T800-F injected IV followed 2 h later by 5 μmol of T700-F (ref. 17). T700-F targets both follicular and parafollicular cells of the pig thyroid, whereas T800-F targets the chief and oxyphil cells of the pig parathyroid (Fig. 5).

DISCUSSION

Incorporating tissue-targeting moieties into the chemical structure of a fluorophore creates a compact, bifunctional contrast agent. This strategy eliminates the need for covalent conjugation of targeting and fluorophore domains and facilitates subsequent optimization of other in vivo properties, such as biodistribution, clearance and elimination. Of course, finding such contrast agents is a laborious task. In our case, it required the synthesis and screening of a large NIR fluorophore library (≥280) and optimization of the presumed pharmacophore. Nevertheless, the strategy we used is applicable to virtually any desired target, and as the NIR fluorophore library increases in size, pharmacophores for any particular target become more robust.

The only other agent known to target glands in the head and neck is methylene blue, which shows uptake in a tumor of the parathyroid (adenoma), but not in normal parathyroid glands at such a low dose (<0.2 mg kg⁻¹). Moreover, methylene blue has a completely different chemical structure (phenothiazine derivative) from T700-F (pentamethine cyanine) and T800-F (heptamethine cyanine) as well as relatively poor optical properties, especially compared with T800-F. The mechanism of methylene blue's uptake and retention in certain tissues, and its maintenance in the non-leuco (i.e., absorbent and fluorescent) form is currently unknown, but it is undoubtedly related to its chemical structure.

Two recent studies by Mahadevan-Jansen and colleagues suggest that human parathyroid and thyroid glands have a detectable autofluorescence. This may be true at fluence rates more than 5,000-fold higher than those used in our study, or as a result of the local diet of study subjects (e.g., foods containing chlorophyll or other fluorescent compounds can generate autofluorescence during imaging); however, we have not found detectable autofluorescence in either of these glands in five species studied to date, including humans. Moreover, the need
to identify and resect the glands before contact probe–based spectroscopy negates the utility of real-time image guidance.

Judicious choice is required when matching contrast agents to specific procedures. On the basis of first principles of optics, 800-nm NIR light provides SBRs of two- to tenfold higher than 700-nm light owing to the combined effects of absorption, scatter, extinction coefficient and autofluorescence. This is true even though silicon charge-coupled devices (CCDs) have lower quantum efficiency at 800 nm versus 700 nm. Thus, we always employ 800 nm-emitting NIR fluorophores and channel 2 of the FLARE imaging system when working with small targets (i.e., parathyroid glands), dim targets or targets embedded deep in tissue. Conversely, 700-nm-emitting NIR fluorophores and channel 1 are typically used for large targets (i.e., thyroid) or bright targets. The use of two independent NIR fluorescence channels permits complex surgeries to be performed with complete image guidance. Take, for example, Graves disease, where the surgeon has two simultaneous tasks: namely, to find all thyroid tissue (including accessory thyroid tissue) in the neck, as well as to identify, resect without damage and re-implant functional parathyroid glands. The combination of contrast agents and imaging system described in our study makes possible real-time guidance throughout this complex procedure.

Figure 3 In vivo parathyroid and thyroid imaging using T700-F and T800-F in pigs. Top, 5 µmol (0.06 mg kg−1) of T700-F and T800-F were injected IV into 35-kg Yorkshire pigs 5 h before imaging. All NIR fluorescence images have identical exposure and normalizations. Parathyroid signal to thyroid signal ratio (PTR) curves were determined from individual SBR curves. (a–c) Shown are magnified images of parathyroid (a), thyroid and parathyroid glands 1 h (b) and 4 h (c) after injection. La, larynx; PG, parathyroid glands (arrowheads); TG, thyroid gland (arrows). Th, thymus. Scale bars, 1 cm. Each data point is the mean ± sd from n = 3 animals.

Figure 4 Simultaneous in vivo NIR imaging of parathyroid and thyroid glands in rats. (a) Dual-channel in vivo fluorescence imaging using both T700-F and T800-F in a rat. 0.2 µmol (0.35 mg kg−1) of T800-F was IV injected into a 250 g SD rat 24 h before imaging, followed by 0.2 µmol of T700-F injected 6 h before imaging. Es, esophagus; PG, parathyroid glands (arrowheads); SG, salivary glands; TG, thyroid glands (arrows); Tr, trachea. Scale bars, 1 cm. (b) H&E and NIR imaging of resected parathyroid and thyroid tissues from (a). Scale bars, 300 µm. All NIR fluorescence images for each condition have identical exposure times and normalizations. Pseudocolored red and green colors were used for 700-nm and 800-nm channel images, respectively, in the color-NIR merged image. Yellow in the merged image indicates colocalization of the T700 and T800 fluorophores.
The mechanism of uptake and retention of T700-F and T800-F in specific cell types of the thyroid and parathyroid glands is presently 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. Future studies could be directed toward identifying the intracellular targets of these contrast agents, although it should be noted that contrast agent optimization can continue even when only the target cell type and preliminary pharmacophore are known.

In summary, the ability to highlight parathyroid and thyroid glands after simple IV injection and to identify each gland simultaneously using the dual-NIR channel capability of the FLARE imaging system, raises the potential for head and neck surgery to be performed with increased precision and thus fewer surgical accidents and lower mortality. This study will also provide design considerations for developing diseas-specific contrast agents with a ‘structure-inherent targeting’ strategy.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

H.H., M.H.P., H.W., E.A.O. and H.J.M.H. performed the experiments. H.H., M.H.P., M.H., A.L.V., J.V.F. and H.S.C. reviewed, analyzed and interpreted the data. H.H., J.V.F. and H.S.C. wrote the paper. All authors discussed the results and commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Synthesis of T700 and T800 NIR fluorophores. All chemicals and solvents were of American Chemical Society grade or HPLC purity. Sigma-Aldrich is the commercial source for the starting materials used in the presented synthesis and the reagents were used without purification. All compounds were obtained in high purity as determined by TLC analyses and high-resolution $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra. Chemical purity was measured by using ultraperformance liquid chromatography (UPLC, Waters) combined with simultaneous evaporative light scatter detection (ELSD), absorbance (photodiode array), fluorescence and electrospray time-of-flight (ESI-TOF) mass spectrometry (MS). See Supplementary Methods for detailed chemical syntheses and analyses.

Optical and physicochemical property analyses. All optical measurements were performed at 37 °C in 100% FBS buffered with 50 mM HEPES, pH 7.4. Absorbance and fluorescence emission spectra of the NIR fluorophore library were measured using fiber-optic HR2000 absorbance (200–1100 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics). NIR excitation was provided by 5 mW of 655-nm red laser pointer (Opcom Inc.) and 8 mW of 765-nm NIR laser diode light source (Electro Optical Components) coupled through a 300-µm-core diameter, numerical aperture (NA)-0.22 fiber (Fiberguide Industries). For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%)$^{24}$ and ICG in DMSO (QY = 13%)$^{25}$ were used as calibration standards, under conditions of matched absorbance at 655 and 765 nm. In silico calculations of the partition coefficient (logD, at pH 7.4) and total polar surface area were calculated using Marvin and JChem calculator plug-ins (ChemAxon).

Animal models. Animals were housed in an AAALAC-certified facility and were studied under the supervision of Beth Israel Deaconess Medical Center’s Institutional Animal Care and Use Committee in accordance with approved institutional protocols (no. 101-2011 for rodents and no. 046-2010 for pigs). Male CD-1 mice weighing ~25 g and male Sprague-Dawley (SD) rats weighing ~250 g (Charles River Laboratories) were anesthetized with ketamine and xylazine intraperitoneally (Webster Animal Care, Inc.) and were studied under the supervision of Beth Israel Deaconess Medical Center's Animal Care and Use Committee. Female Yorkshire pigs (E.M. Parsons and Sons) averaging 25 kg were used as calibration standards, under conditions of matched 100 mg kg$^{-1}$ ketamine and 10 mg kg$^{-1}$ xylazine intraperitoneally (Webster Animal Care, Inc.).

Synthesis of T700 and T800 NIR fluorophores. In brief, the mini-FLARE system is composed of 2 NIR fluorescence channels: channel 1 (656–678 nm excitation; 689–725 nm emission; 1.08 mW/cm$^2$ fluence rate) and channel 2 (745–779 nm excitation; 800–848 nm emission; 7.70 mW/cm$^2$ fluence rate). The working distance is at most 13 inches away from the patient, with field-of-view adjustable from 4.7 to 2 inches by moving the device toward or away from the surgical field.

Quantitative analysis. At each time point, the fluorescence and background intensity of a region of interest over each tissue was quantified using custom FLARE software. The SBR was calculated as SBR = fluorescence/background, where background is the signal intensity of neighboring muscle obtained over the imaging period. All NIR fluorescence images for a particular fluorophore were normalized identically in all conditions of a given experiment. At least three animals were analyzed at each time point. Results were presented as mean ± SD and curve fitting was performed using Prism version 4.0a software (GraphPad). Statistical analyses were carried out using one-way analysis of variance followed by Tukey’s multiple comparisons test. One-way ANOVA was used for statistical evaluation. $P$ values less than 0.05 were considered significant: $P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Histology and NIR fluorescence microscopy. Parathyroid and thyroid tissues from rats and pigs were preserved for hematoxylin and eosin (H&E) staining and NIR fluorescence microscopic analysis. Tissues extracted from the animals after intraoperative imaging were placed in 2% paraformaldehyde in PBS for 30 min before mounting in Tissue-Tek OCT compound (Fisher Scientific) and flash-freezing in liquid nitrogen. Frozen samples were cryosectioned (50 µm per slice), observed by fluorescence microscopy and then stained with H&E.

NIR fluorescence microscopy for resected tissues was performed on a Nikon Eclipse TE300 microscope system with four filter sets as previously described$^{15,16}$. The microscope was equipped with a 100-W mercury light source (Chiu Technical Corporation), NIR-compatible optics, a NIR-compatible 10× Plan Fluor objective lens and a 100× Plan Apo oil immersion objective lens (Nikon). Images were acquired on an Orca-AG (Hamamatsu). Image acquisition and analysis was performed using iVision software (BioVision Technologies). Two custom filter sets (Chroma Technology Corporation) composed of 650 ± 20 nm and 750 ± 25 nm excitation filters, 675 nm and 785 nm dichroic mirrors, and 710 ± 25 nm and 810 ± 20 nm emission filters were used, respectively, to detect T700-F and T800-F signals in the frozen tissue samples.

Additional methods. Detailed methodology is described in the Supplementary Methods.

Clinical study and mini-FLARE imaging system. This study was approved by the Medical Ethics Committee of the Leiden University Medical Center and was performed in accordance with the ethical standards of the Helsinki Declaration of 1975. All patients gave informed consent and were anonymized. NIR fluorescence imaging of the neck region was performed using the mini-FLARE imaging system, which has also been previously described$^{15,14}$. In brief, the mini-FLARE system is composed of 2 NIR fluorescence channels: channel 1 (656–678 nm excitation; 689–725 nm emission; 1.08 mW/cm$^2$ fluence rate) and channel 2 (745–779 nm excitation; 800–848 nm emission; 7.70 mW/cm$^2$ fluence rate). The working distance is at most 13 inches away from the patient, with field-of-view adjustable from 4.7 to 2 inches by moving the device toward or away from the surgical field.