HHS Public Access

Author manuscript

Acc Chem Res. Author manuscript; available in PMC 2018 January 22.

Published in final edited form as:

Acc Chem Res. 2016 September 20; 49(9): 1731–1740. doi:10.1021/acs.accounts.6b00239.

Tissue-Specific Near-Infrared Fluorescence Imaging

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CONSPECTUS

Near-infrared (NIR) fluorescence light has been widely utilized in clinical imaging by providing surgeons highly specific images of target tissue. The “NIR window” from 650 to 900 nm is especially useful due to several special features such as minimal autofluorescence and absorption of biomolecules in tissue, as well as low light scattering. Compared with visible wavelengths, NIR fluorescence light is invisible, thus allowing highly sensitivity real-time image guidance in human surgery without changing the surgical field. The benefit of using NIR fluorescence light as a clinical imaging technology can be attributed to its molecular fluorescence as an exogenous contrast agent. Indeed, whole body preoperative imaging of single-photon emission computed tomography (SPECT) and positron emission tomography (PET) remains important in diagnostic utility, but they lack the efficacy of innocuous and targeted NIR fluorophores to simultaneously facilitate the real-time delineation of diseased tissue while preserving vital tissues. Admittedly, NIR imaging technology has been slow to enter clinical use mostly due to the late-coming development of truly breakthrough contrast agents for use with current imaging systems. Therefore, clearly defining the physical margins of tumorous tissue remains of paramount importance in bioimaging and targeted therapy. An equally noteworthy yet less researched goal is the ability to outline healthy vital tissues that should be carefully navigated without transection during the intraoperative surgery. Both of these paths require optimizing a gauntlet of design considerations to obtain not only an effective imaging agent in the NIR window but also high molecular brightness, water solubility, biocompatibility, and tissue-specific targetability. The imaging community recognizes three strategic approaches which include (1) passive targeting via the EPR effect, (2) active targeting using the innate overall biodistribution of known molecules, and (3) activatable targeting through an internal stimulus, which turns on fluorescence from an off state. Recent advances in nanomedicine and bioimaging offer much needed promise toward fulfilling these stringent requirements as we develop a successful catalog of targeted contrast agents for illuminating both tumors and vital tissues in the same surgical space by employing spectrally distinct fluorophores in real time. These tissue-specific contrast agents can be versatile arsenals to physicians for real-time intraoperative navigation as well as image-guided targeted

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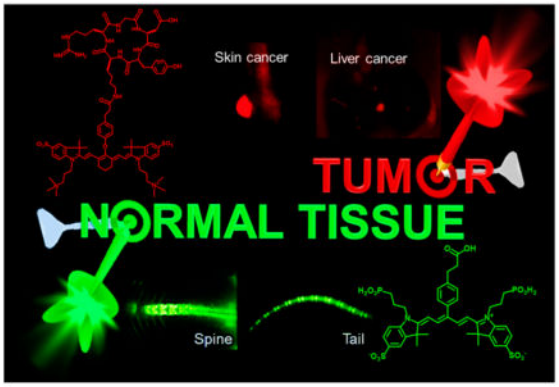
Notes

The authors declare no competing financial interest.

therapy. There is a versatile library of tissue-specific fluorophores available in the literature, with many discussed herein, which offers clinicians an array of possibilities that will undoubtedly improve intraoperative success and long-term postoperation prognosis.

Graphical Abstract



1. INTRODUCTION

Even with the improvement of medications toward the treatment of human diseases, surgery remains the mainstay for most complicated indications.[1,2] Long-term survival of cancer patients depends on the ability to fully resect tumorous tissue while avoiding vital tissue present on the surgical field. Currently, surgeons are limited to their eyesight and physical analyses to determine intraoperative intricacies, even in the performance of complicated resections. Toward offering surgeons a real-time method of visualization, the imaging community has pursued various avenues by translating spectral imaging modalities from extant preoperative techniques that include single photon emission computed tomography (SPECT) or positron emission tomography (PET).[3] Both imaging modalities have been explored with success; however, nonspecific uptake leads to elevated background, which makes deciphering the surgical field challenging, thus obviating potential benefits. Furthermore, ionizing radiation associated with these techniques limits the overall scope of their real-time translation.

In response to this unmet clinical need, alternate possibilities have been established, and they rely on the emission of light, usually through fluorescence (i.e., the absorption of a photon and emission of a lower energy photon) for illumination (Figure 1).[4,5] Because fluorescence is only one pathway that may occur after an incident photon promotes a chemical species from ground state S_0 to an excited S_1, S_2, and beyond,[6] the chemical structure must be modified such that fluorescence dominates the alternate pathways resulting in a high quantum yield of photon emission. This has been a paramount, yet basic, design principle for engineered fluorophores with optimum photophysical characteristics. Herein, we focus on the parameters required for developing tissue-specific probes and the biological infrastructure to be overcome or harnessed to achieve selective tissue imaging.

1.1. Near-Infrared Window

Fluorescence relies on the emission of a photon relaxing from an excited singlet state. Fortunately, there exists a region where tissue features minimum absorption and fluorescence characteristics thus allowing engineered contrast agents to operate effectively by avoiding the disruptive background signal present at lower wavelengths. It has been well-defined and established that near-infrared (NIR, 650–900 nm) light exhibits optimum characteristics for imaging applications *in vivo,* owing to the low tissue attenuation resulting in high penetration depth and minimum background autofluorescence.[7] These characteristics afford high signal-to-background ratio (SBR), which is recognized as the paramount descriptor for successful contrast agents. The opportunity for high SBR paired with cost-effective lasers and detectors and the inherent innocuous nature of NIR light makes it a promising technology for development.[8]

The dual-wavelength "holy grail" of intraoperative imaging exploits the large NIR wavelength window and offers two distinct imaging channels, which would theoretically allow a single disease-targeted fluorophore to be spectrally distinct from another contrast agent directed to vital tissues. The clinical platform that harnesses NIR irradiation is depicted in Figure 2. A broad spectrum excitation light source brightens the surgical field and excites two spectrally distinct targeted fluorophores that have been injected. Optical filters facilitate the isolated detection of 700 nm, 800 nm, and visible light, which can be viewed independently or as an overlay (Figure 2). The combination of highly specific targeting and establishing the wavelength for each targeted tissue (i.e., one channel for diseased tissues and the other for native tissue) affords a clear visualization of the surgical field. However, in our quest toward obtaining the dual-channel pinnacle of NIR imaging, we must design contrast agents within the requisite boundaries for developing any medically relevant agent.

1.2. Requisite Design Parameters

Before NIR imaging successfully emerges in the clinic, contrast agents must be designed to satisfy a very particular set of parameters that are requisite to future successes.[9] Many classes of known fluorescent structures have been used successfully and they encompass three unique classes:[4] (1) the small molecule fluorophores (the most studied class), such as cyanines,[10] porphyrin-based fluorophores,[11] metal complexes,[12] xanthene dyes,[13] squaraine rotaxanes,[14] and phenothiazine-based fluorophores,[15] (2) synthetic nanoparticles such as quantum dots,[16–18] and (3) biologics such as green fluorescent protein.[19] All of these representative agents must be tailored to achieve sufficient stability, specificity, and safety (as listed in Table 1) for human use.

These properties are highly important for future clinical translation and must be maintained throughout the developmental process. The sensitivity, specificity, delivery, pharmacokinetics, and toxicity depend highly on the targeting method used and the overall chemical composition of the contrast agent. Where the stability is also a determining factor to *in vivo* success, the chemical bonds and moieties present only limit the choices available for modification, which does not directly influence the tissue-specific imaging characteristics.

2. METHODS FOR OBTAINING TISSUE-SPECIFIC IMAGING

Tissue specificity is determined as a simple comparison of the signal in targeted tissue to the signal in the surrounding area. This ratio plays a fundamental yet crucial role in the imaging of small and otherwise undetectable tissues. For example, when contrast agents fail to display high tissue specificity resulting in low SBR, small tumors or occult metastases would remain invisible, and the imaging procedure would not afford meaningful guidance. Overcoming this obstacle has proved challenging and has been the focus of a thrust of recent research with various research laboratories engineering contrast agents that exploit biological systems in various ways to achieve optimal SBR.

These different methods of harnessing and manipulating our biological systems to achieve tissue-specific imaging can be grouped into three categories (shown in Figure 3), which all have inherent positive and negative characteristics. These three methods are (1) passive targeting via the enhanced permeability and retention (EPR) effect, (2) active targeting via biodistribution of known molecules, and (3) activatable targeting through an internal stimulus.

Passive targeting through the EPR effect relies on the biodistribution of a contrast agent to achieve tissue-selective imaging. For example, the leaky vasculature of tumors frequently allows larger molecules to enter compared with the more discriminating healthy tissue. In normal tissue, the vasculature remains nonpermeable; only those molecules exhibiting a particular size, hydrophobicity, or molecular recognition moiety may enter. This preferential accumulation in the tumor offers a pathway for obtaining tissue-specific imaging through the EPR effect.

The active approach involves an engineered NIR fluorophore being synthetically tethered to a targeting moiety, which selectively binds the diseased tissue.[20] Surface biomarkers, specifically in cancer cells, have been effectively exploited for homing contrast agents directly to diseased tissues. This is a very rapid process that offers the potential for high SBR and near complete elimination from the background associated with reduced nonspecific binding. However, problems occur when the tumor fails to express large quantities of the surface integrin, which significantly lowers the SBR. Other factors also must be considered when designing active-targeted agents including the molecular weight and size of the contrast domain and the excretion pathway.

The last method takes place when a contrast agent is activatable through a particular internal stimulus. Normally, when this method is used, the contrast agent's fluorescence is quenched using any number of methods (i.e., synthetically attached and biologically labile dinitro compounds, reduction of azides, pH-sensitive moieties, etc.). Once injected, the molecules exhibiting diminished fluorescence intensity travel throughout the body and become activated at the target by a known stimulus commonly associated with the tissue or microenvironment (i.e., pH, concentration of metabolite, enzyme, redox potential in hypoxic cells, etc.)[21] and exhibits fluorescence. This can be a very slow process with nonspecific distribution of the molecules. Ideally, the nonspecifically distributed molecules are never activated; thus this strategy can display very low background signal and an overall high

SBR. Developing distinct tissue-specific fluorophores with complete clearance of unbound contrast agent will afford surgeons a powerful real-time approach to complex surgeries and increased quality of care for surgical patients.

3. TARGETED APPROACH

Numerous targeting ligand–fluorophore tethering approaches have been explored with a varying degree of success. The three factors that are individually requisite in the *in vivo* performance of tethered fluorophores are the targeting ligand, the isolating linker, and the dual-purposed fluorophore with effector and balancing domains (Figure 4). Successful implementation of this approach requires several specific engineering hurdles.

3.1. Targeting Ligand

Perhaps the most crucial aspect for the success of a targeted tissue-specific probe is the choice and overall efficacy of the targeting ligand—the homing beacon to the tissue of choice. Through direct covalent conjugation to the effector domain, this ligand may target and bind surface molecules or overexpressed receptors on the cell surface. Figure 5 depicts a nonexhaustive list of potential small molecule and macromolecule targeting ligands that may direct a contrast agent to a biological target.

3.2. Isolating Linker

Upon first glance, the isolating linker's importance is immediately understated; however, the importance of optimizing the physicochemical, structural, and dynamic properties cannot be underestimated. The isolating linker component of the entire contrast agent should be sufficiently flexible to allow the targeting ligand and fluorophore to act independently but also satisfactorily rigid to keep these two domains physically separate in 3D conformational space. Furthermore, the isolating linker should be chemically inert to avoid unfavorable interactions with either domain but should be robust throughout the procedure.

3.3. Effector Domain

Satisfactory molecular brightness *in vivo* and clearance properties (preligand attachment) are the major obstacles to perfecting characteristics for future implementation in the clinic (reviewed in ref 4). The optical profile, specifically regarding Stokes shift, extinction coefficient, and quantum yield, is highly dependent on the rigidity of the core fluorophore structure, specific modifications to the conjugated system, and solvent-fluorophore effects. It is important that this effector domain does not influence the biological recognition of the targeting ligand. Indeed these are design considerations that must be overcome in the pursuit of an efficient effector domain; however, when navigating this area, it is also necessary to structurally engineer a balancing domain that reduces the biological interactions that result in off target imaging.

3.4. Balancing Domain

The balancing domain refers to moieties that balance physicochemical properties (i.e., molecular weight, total polar surface area, hydrogen bond donors and acceptors, acidic or basic pK_a, distribution and partition coefficient, and stability) and heavily influences *in vivo*

fate with slight structural modifications posing significant biological perturbation. If correctly selected, these physicochemical descriptors may provide a shielding characteristic to the fluorophore allowing the targeting ligand to act independently of the optical contrast agent. In the most simplistic structural design, the targeting moieties and balancing domain must be well integrated into the overall molecular structure such that both properties are retained without jeopardizing tissue-selective targeting.

4. NIR FLUORESCENT CONTRAST AGENTS FOR TARGETED IMAGING

4.1. Nanoparticle-Based Bioconjugates

The molecular design must feature four major components: targeting ligand, isolating linker, effector domain, and balancing domain (Figure 4). This modular schematic shows the effector domain, in this case a nanoparticle-based contrast agent, being attached to a targeting ligand through an isolating linker.[22] These approaches have been utilized extensively in the design of nanoparticle-based contrast agents for optical image-guided surgery, of which *in vivo* performance depends strongly on their molecular design and physiochemical and optical properties (reviewed comprehensively in ref 5).

Recent advancements in nanoparticle-based imaging suggest high promise in the future; currently, however, the intrinsic character of nanoparticles does not readily lend itself to biological compatibility.[23] Owing to this principle, rapid clinical translation is not common in the nanoparticle space. Though nanoparticles are not preferred by regulation agencies, in the meantime, small molecule imaging agents offer a unique and appealing alternative with respect to the ability to synthesize a single chemical entity with high reproducibility and purity. These characteristics lend themselves to Food and Drug Administration (FDA)-approved clinical translation and predictably reproducible *in vivo* success. With the FDA-approved indocyanine green (ICG),[5] it is likely these nano- and molecular imaging agents will be the first clinically relevant contrast agents in tissue-specific imaging; therefore, we tailored this Account to exclusively cover robust and cutting edge research in small molecule contrast agents.

4.2. Small Molecule-Based Bioconjugates

Compared with the nanoparticle blueprint, a more simplistic modular design for disease or tissue specific imaging agents is shown in Figure 6A. Similar to the first schematic, there is a targeting ligand that serves as the homing beacon that delivers the imaging agent to the tissue of interest; however, the effector domain must remain either comparatively small (against the targeting ligand) or biologically silent through the synthetic incorporation of a balancing domain within the structure of the fluorophore. These two design approaches are not equal since reducing the effector domain size effectively limits the aromatic system resulting in non-NIR absorbance and fluorescence wavelengths; therefore, we are synthetically bound by these molecular constraints. Even more challenging, the dual channel imaging capability requires the targeting of healthy tissue, which necessitates the engineering of additional fluorophores that exhibit native tissue selectivity. Cellular surface receptors and subcellular targeting domains for native healthy tissue remain scarcely known

within the literature; therefore, a tethered approach would not be an obvious choice for obtaining tissue-specific contrast agents for these tissues.

Many of the fluorophores described to date have one or more detrimental fallbacks ranging from limited chemical and optical stability to insufficient fluorescence quantum yield in serum or high background signal *in vivo* arising from nonspecific binding to extracellular proteins. These chemical structures have been extensively modified for decades with only minor improvements to tissue affinity and background reduction. Choi et al.[24,25] explored the importance of judiciously incorporating charge in the engineering of fluorophores for maximizing targeting efficacy by carefully incorporating zwitterionic character into a heptamethine cyanine chromophore, which yielded the final fluorophore, ZW800-1, resulting in minimal fluorophore–serum protein interactions. This engineered characteristic imparts ZW800-1 with the unique ability to allow any functional biological ligand to act independently thus effectively targeting the corresponding receptor.

These data indicate that the zwitterionic balancing domain on the fluorophore is highly effective at preventing interactions with biological tissues and proteins. In theory, once this fluorophore is conjugated with a targeting ligand, the biodistribution would rely solely on the targeting ligand as the fluorophore has to be engineered to exhibit minimal *in vivo* binding.[24] In fact, when the commercial alternative, Cy5.5, and the ZW800-1 analogs are modified with cRGD, Choi et al. observed unparalleled tumor targeting with low nonspecific background as seen in Figure 6B.

4.3. Structure-Inherent Targeting

Reducing the complexity in modular design even further, the isolating linker may be removed, leaving a single fluorophore with all three of the requisite characteristics expressed on the one fluorophore domain as shown in Figure 7, known as structure inherent targeting. Both molecular blueprints have been exploited to assume excellent properties *in vivo*, and the design and structural requirements vary greatly depending on the targeted tissue (i.e., disease/tumor for resection or sensitive tissue for avoidance). While active targeting is an attractive and heavily researched field, there are also improvements that would come from reducing the size of the overall complex: this concept is embodied by structure-inherent targeting. EPR effect is preferred for passive targeting because of the ability to localize within one particular tissue versus others, usually based on the general biodistribution and long-term circulation phenomenon. Recently, it has been postulated that the parent structures of various classes of fluorescent small molecules that have innate biodistribution pathways may be manipulated through the addition or removal of certain isolated functional groups to achieve target-selective imaging. Our previous publications suggest that subtle variations in chemical structure result in large variations in the trafficking, biodistribution, and clearance of a contrast agent, which directly affect its targeting and imaging of the specific tissue and organ.[26–29]

A structure-inherent targeting strategy would help overcome the obstacles of targeting ligand-based contrast agent to native tissues and sensitive glands, which remains a longstanding clinical problem in contrast agent development. Hyun et al.[26] and Owens et al.[29] were two of the first suggestions in the literature that the molecular character may be

modified and finely tuned to achieve selective uptake with high SBR in particular tissues using a structure-inherent approach that exploits chemical recognition to biological tissue for targeting and biodistribution. Selective modifications can direct the pharmacophore to distinct regions utilizing the structure-inherent chemical recognition to the targeted tissue. We observed that tuning the lipophilicity and noncharged structures of NIR fluorophores allows for high uptake in the endocrine system.[29]

Another example of structure-inherent targeting is parathyroid- and thyroid-targeted agents.[27] It was found that the pentamethine compound (700 nm fluorescence) localized effectively in the thyroid gland, while the heptamethine cyanine (800 nm fluorescence) became flushed from the thyroid to the parathyroid gland (Figure 8). Both of them have the same chemical structure except for the different length of functional methine groups, the physicochemical properties of which drove them to the specific target. Dual channel imaging of these glands afforded a very clear delineation of these two glands for biopsy or surgical excision.

Similar results have been achieved through tuning the physicochemical properties and rationally incorporating recognition elements into the compounds. Cartilage and bone imaging plays a crucial role toward more fully understanding the disease progression within these particular tissues. Hyun et al. rationalized that the negatively charged glycosaminoglycans (GAG) present in cartilage would be an effective target for cartilage-selective imaging (Figure 9A).[26] Selecting quaternary ammonium moieties for chondrocyte and GAG targeting in cartilage afforded a core pharmacophore that highlighted all types of cartilage tissues. Figure 9B shows the 700 nm channel, which highlights the cartilage imaging contrast agent seen in red in the merged image. On the other hand, phosphonate groups in the nonresonant side chain of the fluorophore serve as the structure-inherent homing beacon to bone.[28,30] The phosphonates interact favorably with the hydroxyapatite and calcium phosphate in bone tissue. These two contrast agents epitomize the desired success in dual-channel NIR imaging, and these two tissue-specific fluorophores have great potential in the monitoring of cartilage tissue engineering and therapeutics and also in the study of osteoclast formation and maturation in bone.

5. ACTIVATABLE NIR IMAGING AGENTS

The development of successful paradigm shifting activatable NIR imaging probes has been slow compared with alternate approaches. This owes strongly to the complexity in design and increased requirements for engineering of these highly intelligent molecules. In this high-risk, high-reward situation, a successful imaging agent could dominate the optical imaging space; however, the tissue specificity of healthy tissues remains an unsolved shortcoming of this approach, though tumor targeting is well established.

These well-established methods for targeting tumors exploit unique attributes of cancer cells, for example, the Urano and Kobayashi groups described activatable NIR fluorophores that are covalently linked to monoclonal antibody trastuzumab, which binds to human epidermal grown factor receptor type 2 (HER2).[21] After binding, the probe–antibody complex is internalized within cancer cells through a lysosomal degradation pathway (Figure 10A). This acidic environment causes protonation of the tertiary amine installed on

the fluorophore core resulting in increased fluorescence compared with the nonprotonated counterpart. Figure 10B shows the fluorescence images of pH activatable contrast agent and the always on version as a function of pH. Using these probes, we can see cellular turn-on of fluorescence in panel C in Figure 10 when the diethylamine is present; however, the always on probe retains fluorescence signal over time. The *in vivo* images (Figure 10D) show excellent tumor targeting, but the always on probe shows high background in the tissue surrounding the tumors, while the activatable probe lowers the background signal that allows high SBR and a clear depiction of diseased tissue for surgical removal.

The use of pH activation is well described with this report embodying an excellent implementation of this approach.[31–35] Despite this success, we require an arsenal of contrast agents for universal implementation of NIR imaging technology. Increasing evidence shows that folate receptors are overexpressed on the surface of many cancer cells, and targeting these receptors through covalently tethering a folic acid moiety to an effector domain has been a longstanding concept. Using this folic acid dually as a quenching moiety and homing beacon, Lee et al.[31] explored the targeting of ovarian cancer using an activatable probe that remains quenched through intramolecular charge transfer between the effector domain, dye ATTO655, and folic acid (Figure 11). The isolating linker in this case is uniquely constructed to cleave in the presence of cathepsin B. After the folic acid domain recognizes the cellular target and the probe enters the cell, the linker is recognized and cleaved by lysosomal cathepsin B resulting in enhanced fluorescence. Cellular data that shows fluorescence turn-on in the presence of tumor cells and no fluorescence when a folate receptor inhibitor is added confirms the suspected mechanism of action. This enhanced fluorescence results in SBR imaging of tumors with small signal in the liver. The peptide and folic acid incorporation is critical in the targeting ability of this contrast agent because the dye alone is excreted rapidly from the body and does not preferentially accumulate in any particular organ or tissue based on the NIR images.

6. SUMMARY AND OUTLOOK

The technological advancements in this field of sub-nanometer NIR fluorophores in imaging has exploded resulting in tremendous potential to shift clinical paradigms. With the sustained efficacious progress, as we have seen over the past five years, more in-human clinical trials will be seen, and NIR imaging systems will become a more common technology in operating rooms. These advancements are, in turn, expected to solidify NIR fluorescence imaging technology and allow for the expanse into many surgical procedures.

7. FUTURE DIRECTIONS

Though the influx of new tissue-specific NIR fluorescent contrast agents has great potential to revolutionize image guided surgery, the NIR fluorescence imaging modality is limited to only intraoperative environments due to the signal loss through tissue. A single molecule that allows for both whole-body imaging (i.e., PET/SPECT, MRI, etc.) and a disease-specific fluorescent domain would allow one chemical entity to be used during an entire surgical operation from identification to resection. Also, the ability to provide a structure-inherent imaging approach for site-selectivity opens the doors to new possibilities of

therapeutic imaging agents in the quest for new theranostic agents using a single chemical entity thus obviating the need for separate therapeutic and imaging domains.

Acknowledgments

This study was supported by a Georgia State University dissertation grant (E.A.O.) and the NIH/NIBIB Grant Nos. R01-EB-011523 (H.S.C.) and R01-EB-017699 (H.S.C.). E.A.O. was supported through a predoctoral fellowship from the Center for Diagnostics and Therapeutics.

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Biographies

Eric Owens, Ph.D., is currently a Scientific Advisor working in biotechnology and pharmaceutical patent prosecution and intellectual property litigation and counseling at

Haynes and Boone, LLP, in Washington D.C. After finishing his B.S. in chemistry at The University of North Carolina at Chapel Hill in 2010, Eric completed his Ph.D. in medicinal chemistry at Georgia State University focusing on new NIR contrast agents under the direction of Drs. Henary and Choi.

Maged Henary, Ph.D., is an Assistant Professor of Chemistry at Georgia State University. Since his appointment, Dr. Henary's research focuses on the development of new heterocyclic compounds as anticancer agents, including different classes of fluorescent near-infrared dyes with various substituents for tissue-specific targeting, bioanalytical, and diagnostic applications.

Georges El Fakhri, Ph.D., is a Professor of Radiology at Harvard Medical School, the Director of MGH PET Core, and Co-director of the Division of Nuclear Medicine and Molecular Imaging. Dr. El Fakhri is the founding Director of Gordon Center for Medical Imaging (GCMI), and his laboratory focuses on the development of quantitative molecular imaging devices and algorithms for oncologic, neurologic, and cardiac imaging.

Hak Soo Choi, Ph.D., is an Associate Professor of Radiology at Harvard Medical School, and faculty of Dana Farber/Harvard Cancer Center. Currently Dr. Choi is Director of Bioengineering and Nanomedicine Program at GCMI of Massachusetts General Hospital, and his laboratory focuses on the development of novel targeted contrast agents to solve important problems in oncology and clinical medicine with an emphasis on *in vivo* imaging and tissue-specific targeting.

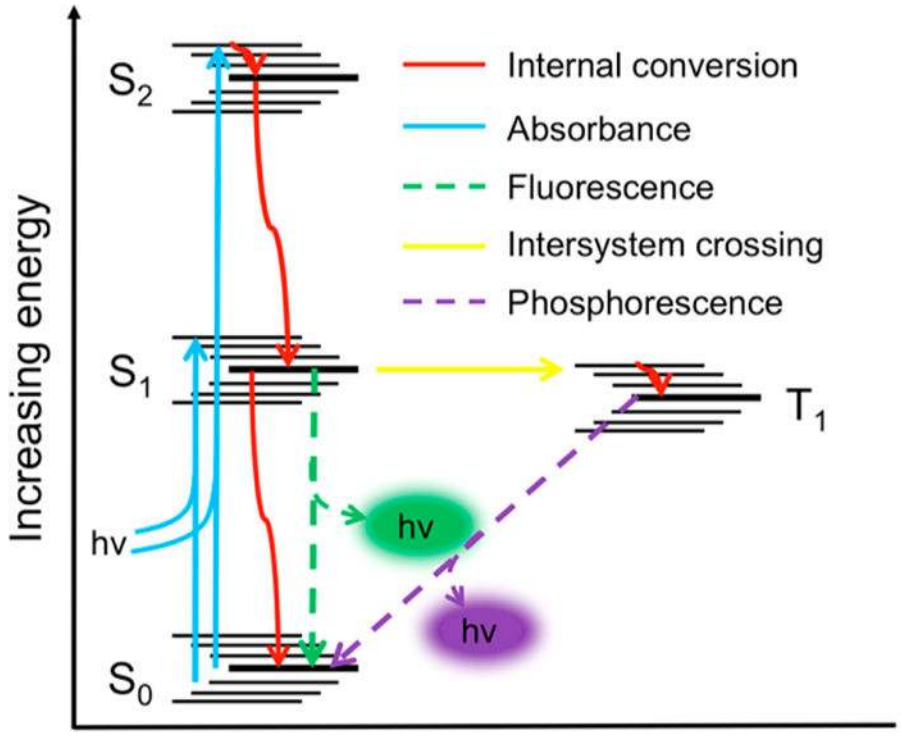

Figure 1.
A simplified Jablonski diagram.

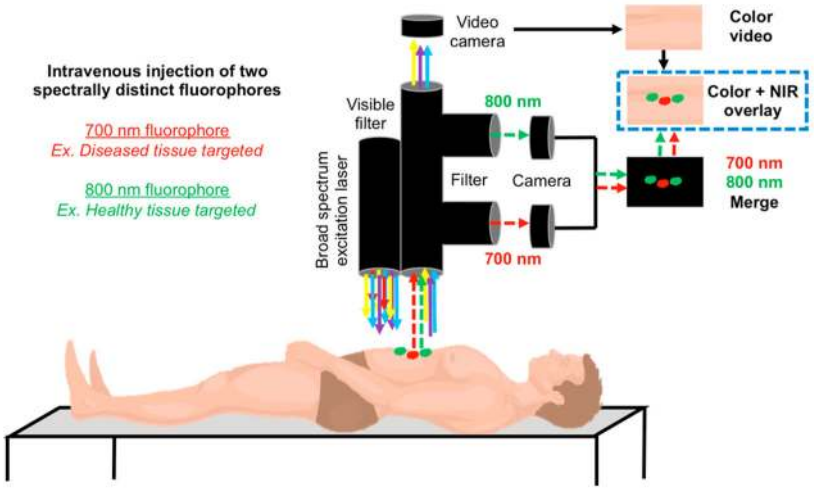

Figure 2.
Schematic of dual channel intraoperative imaging.

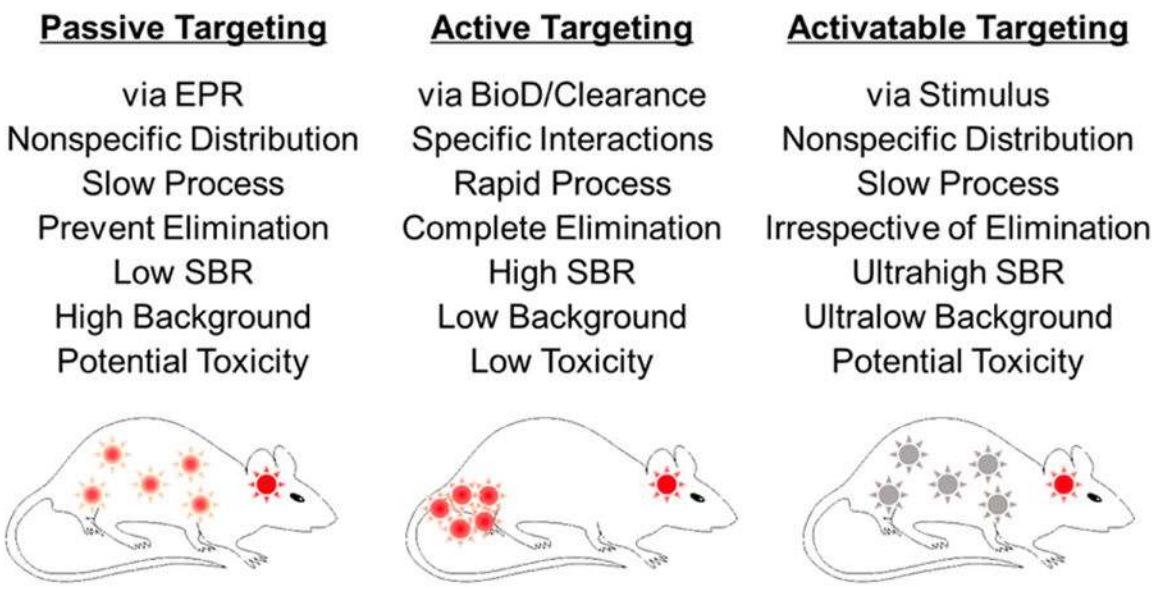

Figure 3.
Major targeting strategies to the particular region of diseased or cancerous tissue.

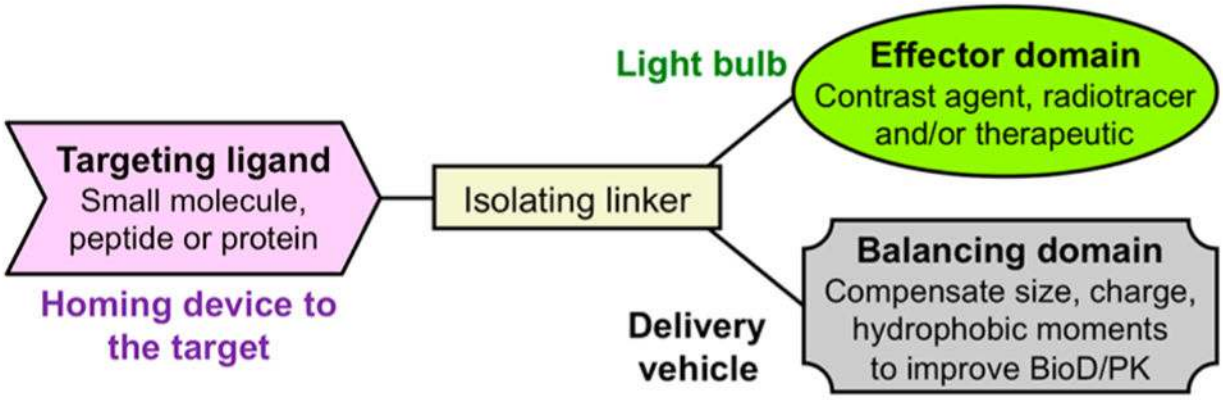

Figure 4.

General overview for the modular approach to the design of targeting contrast agents and therapeutics. The light bulb would be a NIR fluorescent molecule, and in an alternate embodiment, it could also represent a theranostic agent (i.e., molecular bomb). Note, This coloring scheme has been applied throughout the manuscript for facile determination of the various components of the overall imaging schematic.

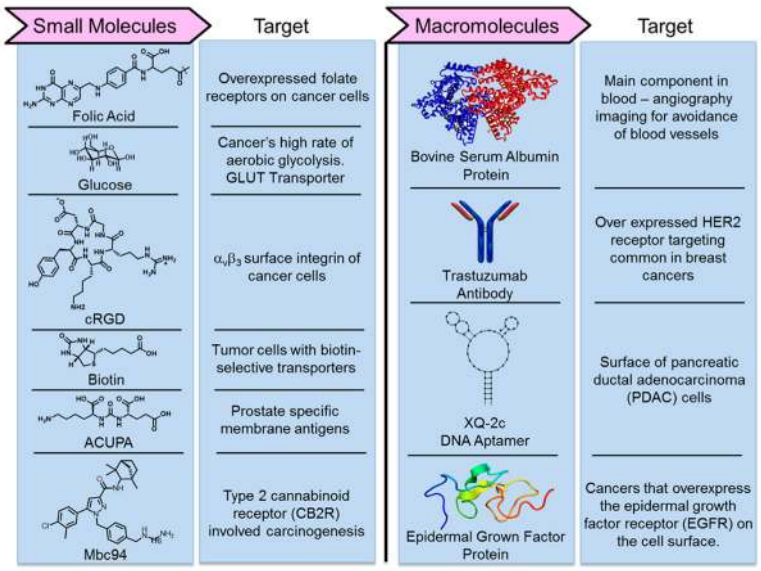

Figure 5.

Small molecules and macromolecules used for tethering to direct NIR fluorescent domains to particular targets for developing tissue-specific contrast agents.

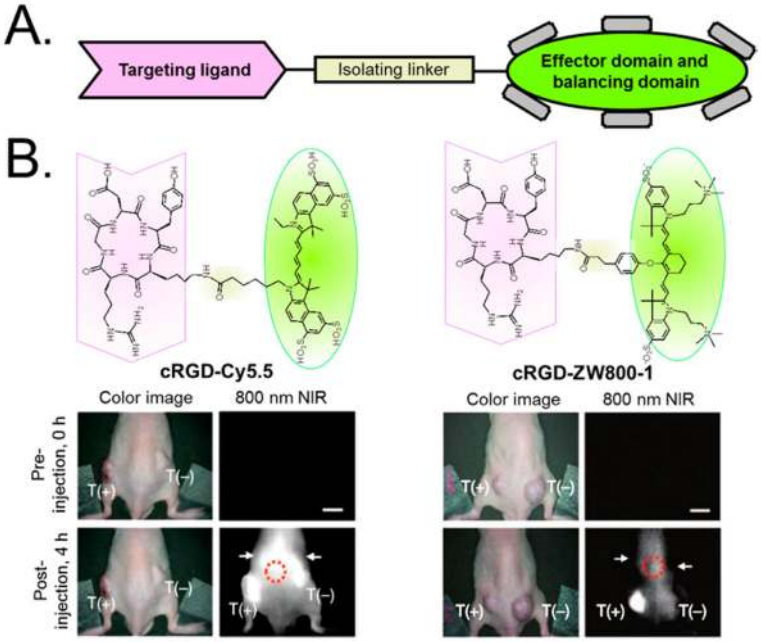

Figure 6.

(A) Compact modular design for compact targeted contrast agents and (B) the *in vivo* performance of cRGD-conjugated Cy5.5 and ZW800-1. T = tumor. Reproduced with permission from ref 24. Copyright 2013 Nature Publishing Group.

Figure 7.
Structure-inherent targeting through the incorporation of targeting and balancing domains into the small molecule fluorophore beacon.

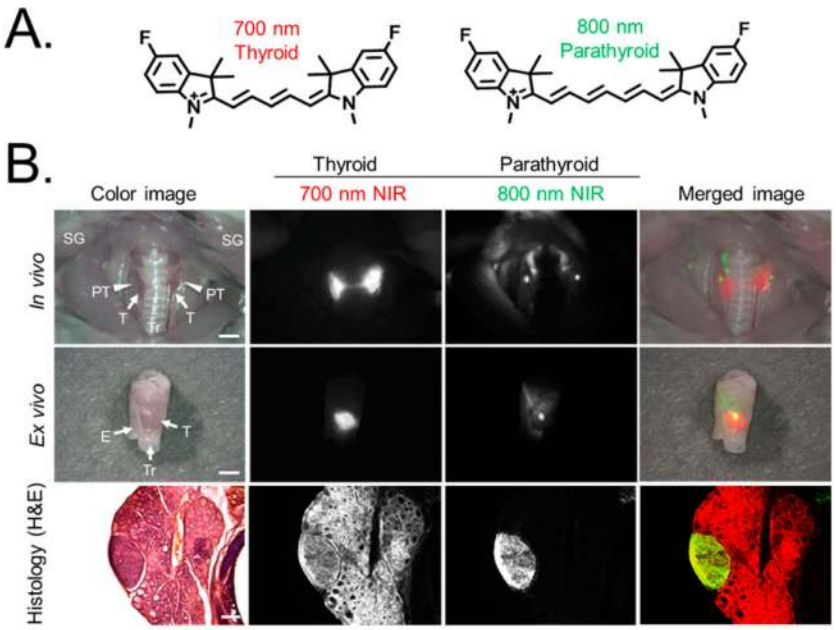

Figure 8.

(A) Molecular structure of NIR fluorescent imaging agents and (B) corresponding *in vivo* NIR images for the small molecule imaging agents that are tailored for imaging of thyroid and parathyroid in the same surgical space. Reproduced with permission from ref 27. Copyright 2015 Nature Publishing Group.

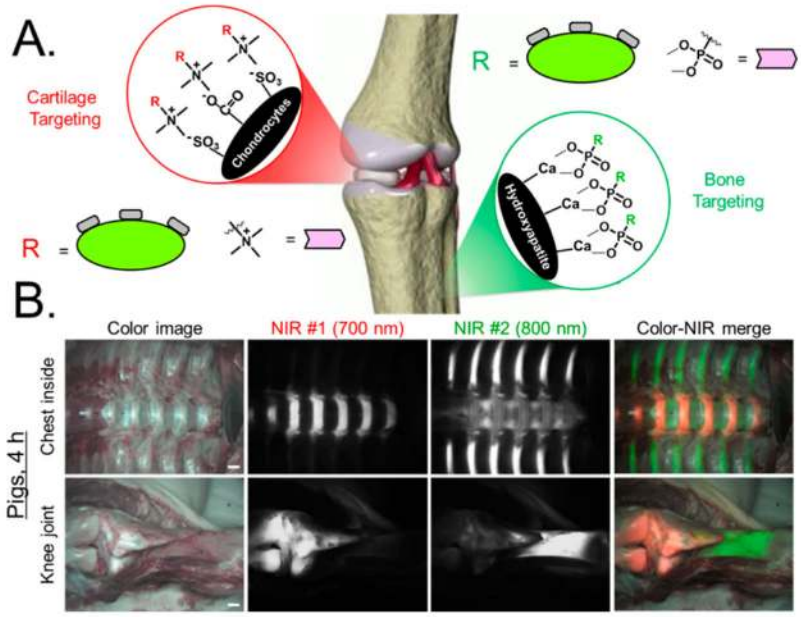

Figure 9.

(A) Physiological targets, molecular character, and representative molecule and (B) corresponding *in vivo* images of small molecule fluorophores that are tailored for imaging of cartilage and bone in pigs 4 h postinjection. Reproduced with permission from ref 26. Copyright 2015 Wiley-VCH.

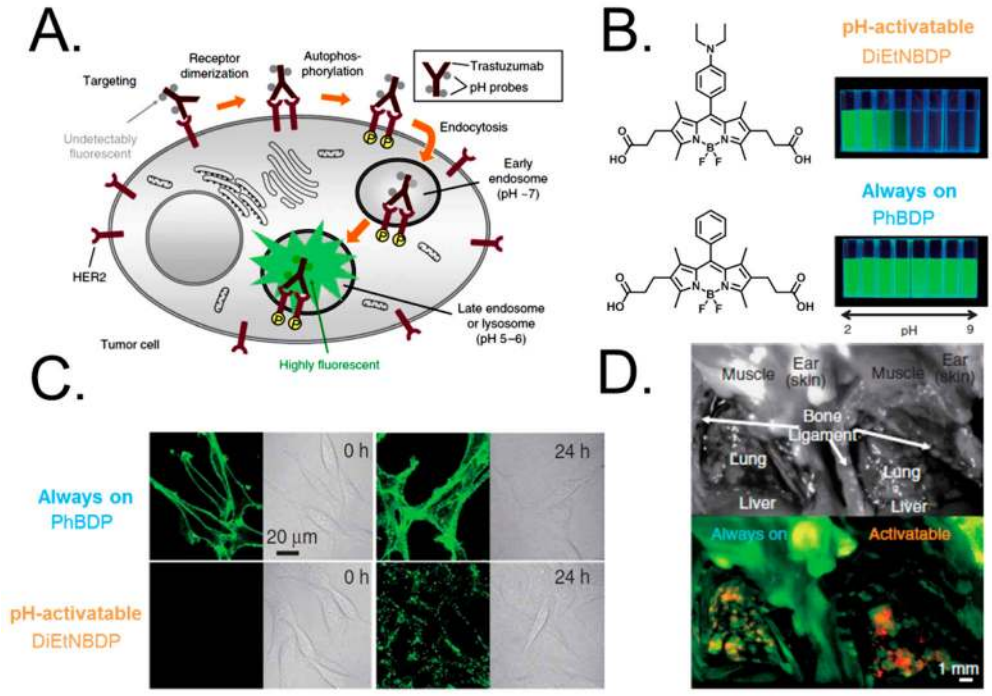

Figure 10.

pH-activatable NIR imaging probes. (A) General strategy for targeting pH probes to tumor cells and activating fluorescence with a pH response, (B) two analogs of pH-activatable and always on (control) compounds and their pH-fluorescence profile, (C) cellular imaging at time 0 and 24 h showing always on fluorescence and cell endocytosis mediated fluorescence activation, and (D) *in vivo* imaging showing effective targeting of tumors in both always on and activatable contrast agents. Reproduced with permission from ref 21. Copyright 2009 Nature Publishing Group.

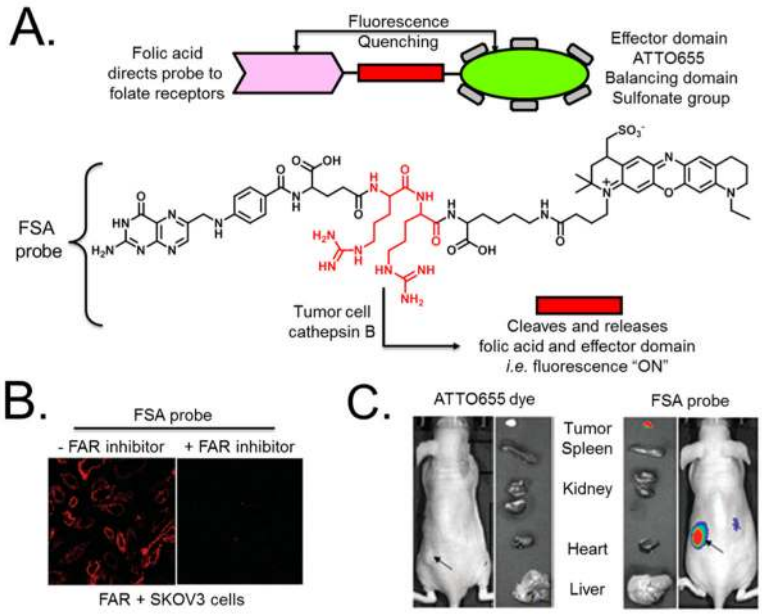

Figure 11.

Folate receptor-specific activatable (FSA) probe. (A) The final probe used in (B) cellular experiments in SKOV3 cells expressing the folic acid receptor without a folic acid receptor inhibitor present and with a folate acid receptor inhibitor present and (C) *in vivo* data showing rapid clearance of the free dye after 30 min and high SBR imaging of the tumor tissue when using the FSA probe. Reproduced with permission from ref 31. Copyright 2014 Royal Society of Chemistry.

Table 1

Requisite Design Parameters for Disease-Specific Probes

1. High Stability
physicochemical stability of targeting ligand
optical stability of probe
biological (in vitro) and physiological (in vivo) stability
2. High Sensitivity
high target concentration
high molecular brightness of optical probe
high performance of imaging modality
3. High Specificity
high signal-to-background (noise) ratio (SBR or SNR)
high affinity of ligand to the target (signal)
low nonspecific binding to normal tissue (background)
4. Favorable Delivery to the Target across Biological Barriers
biodistribution (organ delivery with minimum nonspecific uptake)
delivery across cell membranes (intracellular targets)
5. Favorable Pharmacokinetics and Metabolism
rapid accumulation to the target (bound molecules)
sustained retention on the target (bound molecules)
rapid clearance from nonspecific tissues (unbound molecules)
rapid elimination from the body (unbound molecules)
6. Low Toxicity
chemical, biological, physiological toxicity
acute and chronic toxicity
cytotoxicity and in vivo toxicity