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Rational chemical design of the next generation of molecular imaging probes based on physics and biology: mixing modalities, colors and signals

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Abstract

In recent years, numerous *in vivo* molecular imaging probes have been developed. As a consequence, much has been published on the design and synthesis of molecular imaging probes focusing on each modality, each type of material, or each target disease. More recently, second generation molecular imaging probes with unique, multi-functional, or multiplexed characteristics have been designed. This *critical review* focuses on (i) molecular imaging using combinations of modalities and signals that employ the full range of the electromagnetic spectra, (ii) optimized chemical design of molecular imaging probes for *in vivo* kinetics based on biology and physiology across a range of physical sizes, (iii) practical examples of second generation molecular imaging probes designed to extract complementary data from targets using multiple modalities, color, and comprehensive signals (277 references).

A. Introduction

Conventional diagnostic imaging methods employing contrast agents, such as angiography, computed tomography, and magnetic resonance (MR) imaging have had a profound impact on medicine.¹ Yet, improvements are needed in sensitivity, resolution and specificity. Radionuclide imaging provides a good example of current limitations. While radionuclide imaging provides physiologic information (blood flow, perfusion, lymphatic flow) or metabolic information (phosphate, sugar, nucleic acid, and some amino acids), it does so with low spatial resolution. Only a few molecularly targeted radioactive probes (somatostatin analog and radiolabeled antibodies) have been approved for use in clinical practice. Molecular imaging, seen as one of the next important advances in imaging sciences, offers the possibility of obtaining *in vivo* target-specific information with high sensitivity and specificity.^{2–6} To achieve this goal it may be necessary to design probes that mix different modalities, probes, or signal processing.

Molecular imaging has had few clinical successes to date but there have been considerable preclinical advances in oncology,^{5,7–11} cardiology,^{2,12–16} and neuroscience.^{10,17–22} Thus, the field is widely considered still to be in its infancy. One factor limiting molecular imaging is that most currently used probes are monochromatic (yielding only one signal type per molecule) and emit signal continuously (so called “always on”). Monochromatic imaging, although quite useful in current clinical practice, yields linear, uniparametric data. This

results in a “one test–one answer” paradigm that provides only a unidimensional piece of the puzzle resulting in “many tests–many answers”. The problem with “always on” probes is that they diminish target to background ratios secondary to non-specific background signal. This dramatically reduces sensitivity even while the probe may be highly specific. The ability to generate polychromatic, multi-parametric data, and activatable imaging probes will provide more rich and complex data sets with higher sensitivity and specificity.^{23–27} Innovations in chemistry and nanotechnology will lead to develop highly targeted probes^{28–30} that are optimized for the high affinity binding to specific molecular targets and their signaling properties, and eventually yield multiparametric data. Such probes may not only be more informative but may also result in improved efficiencies with regard to delivering medical care, thus lowering costs (one test–many answers)^{31,32}

Each modality offers specific capabilities such as high spatial resolution, high temporal resolution or high sensitivity; and each utilizes electromagnetic waves from different parts of the spectrum for data acquisition followed by data reconstruction. With each technology, there are several approaches to obtaining multi-parametric data: (1) detect two or more photons from different parts of the electromagnetic spectrum using two or more different imaging modalities (multi-modality);^{33,34} (2) detect two or more photons from the same part of the spectrum, and use spectral separation techniques to differentiate the signals (multi-color) and (3) detect photons of the same energy but extract additional information (*e.g.* time domain information) by signal processing (multiple signaling) (Fig. 1).

An important underlying principle of probe design is to optimize tumor to background ratio (TBR). This can be achieved either by maximizing signal from the target, minimizing signal from the background, or both. Improving TBR increases the sensitivity and specificity for detecting target molecules *in vivo*. Strategies for optimizing TBR can be considered at several scales, from the whole organism level to the atomic level. At the organism level, the size of the animal (*e.g.* mouse *vs.* human) should be considered as the depth of signal penetration varies between probe types, most notably for optical imaging, and therefore the depth of target tissue from the surface of the organism is an important component of detected signal strength. At the organ level, physiology should be considered, given that probe pharmacokinetics, including the uptake of the agent, its breakdown, clearance, and excretion all affect TBR. On a cellular level target expression, binding affinity, on and off rates, intracellular processing and catabolism can affect probe signal.^{9,35,36} On a molecular level, the physical interaction with the target and chemical or enzymatic processing alter probe signal and therefore TBR. Finally, at the atomic level, inter- or intra-molecular energy transfer, photon-induced electron transfer, and caging are significant means of achieving signal activation and/or signal amplification and therefore warrant consideration (Fig. 2).

In this article, we discuss design strategies for molecular imaging probes emphasizing multi-modal, multi-color and multi-signal strategies.

B. Multiplexed data acquisition

Each modality has strengths and limitations. Therefore, the simultaneous use of two or more modalities, imaging reagents or signaling methods, to achieve “multiplexed imaging,” could overcome the limitations of each modality and improve the data obtained during a single imaging session. An excellent current example is positron emission tomography/computerized tomography (PET/CT) wherein the PET provides a high sensitivity metabolic image while the CT provides anatomic information on which the metabolic information can be superimposed. An example of multi-color imaging includes the simultaneous detection of different targets, using single-photon emission computerized tomography (SPECT) imaging cameras which can resolve multiple tracers based on different emission energies (*i.e.*

Tc-99m (140 keV) and Tl-201 (71 keV)) using two different energy detection windows to gather multi-parametric information. A current example of multiple signaling is MR imaging in which T1-, T2-, and diffusion-weighted images are obtained in one session, each reflecting a different tissue signal. Thus, the template for the future is already in place. The design of the next generation of molecular imaging probes should take into account whether the agent will provide multiplexed imaging data. In this section, we review rational designs and practical examples of these different types of probes not yet in the clinic.

B.1 Multi-modality imaging probes

As shown in Fig. 1, nuclear imaging employs photons which emit gamma rays at wavelengths less than 1 nm. Among these photons, those with energies greater than 70 keV can generally penetrate through the human body without significant attenuation. From a practical perspective, radionuclides, which emit photons with energies ranging from 80 keV to 350 keV, are appropriate for molecular imaging but require extensive collimation for accurate reconstruction. This results in inefficient photon collection because many photons are absorbed by the collimator. Positron emitting radionuclides produce two 511 keV photons (in opposite directions) that can be detected with co-incident detectors and because the line of response can be determined, PET scanners do not require a collimator, resulting in improved resolution compared with gamma emitters. Radionuclide cameras are sensitive enough to detect pico-molar concentrations of radionuclides even deep in the body. Nuclear imaging is a well-developed area of medicine, which has evolved over the past fifty years. As a result, newly developed agents can be readily tested in humans using a micro-dosing processing. However, given that gamma- or X-ray exposes the subject to ionizing radiation, radionuclide imaging must always take into consideration radiation dose based on the radioactivity of the agent and its physical and biological half life. Thus, an imaging modality with comparable sensitivity but without ionizing radiation would be desirable.

Optical imaging relies on photons ranging in wavelength from the visible to the near infrared (NIR) (500 to 1500 nm wavelength). Charge Coupled Device (CCD) detectors, which are used for optical imaging, generally show better sensitivity for photons at shorter wavelengths. Within this range, NIR light, ranging from 650 to 800 nm, provides the best visualization of deeper structures, up to several centimeters, from the surface, as this range is less readily absorbed by living tissue (Fig. 3). Shorter wavelength photons are limited to imaging the surface or immediately subsurface phenomena because of near complete absorbance of light by oxy- and deoxyhemoglobin. Of course, depth of penetration is partly related to the strength of the light source, therefore, the signal from fluorophores with very strong emission can penetrate tissue more deeply. Numerous visible-to-NIR wavelength emitting agents are used for optical imaging and such fluorophores can be detected with pico-molar sensitivity without ionizing radiation. Optical imaging alone (without exogenous probes) has been used in clinical practice for detecting blood flow or blood oxygen concentration in relatively superficial organs including the breasts, brain, and eyes. Only two optical imaging reagents; fluorescein and indocyanine green (ICG), have been approved for use in clinical practice. Small numbers of target specific fluorescent probes have been introduced for use during surgery- or endoscopy, but none is in common clinical use.

MR imaging relies on radio waves which have wavelengths on the order of 1 centimetre. Radiofrequency waves can penetrate deep into the body. MR imaging generally measures the magnetic relaxation properties of protons but this requires excitation by radiofrequency. Paramagnetic metal ions or nano-sized metal particles are used as contrast agents as they alter the relaxation rate of adjacent water protons and disrupt the magnetic field around particles. Detectable relaxation change can only be achieved when the paramagnetic concentration of the imaging agent is greater than 10 micro-molar concentration of metal ions or atoms, thus limiting sensitivity. Radio waves do not expose the subject to ionizing

radiation. With regard to contrast agents, gadolinium chelates and iron oxide particles have been approved for use in the clinical practice. However, there is mounting concern regarding the use of gadolinium contrast agents in patients with renal impairment due to the risk of nephrogenic systemic sclerosis, a potentially fatal disorder that results from heavy metal toxicity of gadolinium ions that are released from chelates. With this said, MRI signal can be processed in different ways yielding methods that have a great potential for obtaining multiplexed information beyond anatomy and without the need for contrast material.

By keeping these characteristics in mind, appropriate combinations of two or more signaling moieties, also called “hybrid imaging”, can be effective in providing more complete information about a physiologic process. In the next sections, new molecular probe development, mostly presented as preclinical examples, is discussed.

B.1.1 Nuclear/optical multi-modality probes—Multimodal nuclear and optical imaging probes are promising agents for molecular imaging and drug (theranostic) development.^{37–42} Within this strategy, the role of the radioactive component is to provide quantitative, whole body data. The radioactive component provides a continuously emitting signal that can be utilized to determine probe biodistribution and clearance and provide physiologic data such as glomerular filtration rate and probe concentrations in specific sites (such as in a tumor or organ).^{43–45} The optical component of the probe provides the ability to qualitatively visualize target tissue in real time to assist with interventional, surgical, or endoscopic procedures, or for specific detection of molecular profiles using multi-color “always on” or tumor-cell specific “activatable” optical probes (discussed later). These properties make nuclear/optical probes useful for drug development, physiologic imaging,⁴⁶ enzyme-specific imaging,³⁷ cardiac imaging, and for effective tumor targeting.^{38,39,47,48}

B.1.1.1 Nuclear/optical dual labeled probes: The use of a positron emitting isotope as the radioactive component, provides even better quantitative data for dual nuclear/optical agents.⁴⁹ PET has high penetration and sensitivity and is more quantitative.^{50,51} With this in mind, several combinations of PET/optical agents have been developed. For example, an optical quantum dot (QD) with NIR fluorescence can be attached to a chelating agent to bind ⁶⁴Cu for PET imaging, and to a vascular endothelial growth factor (VEGF) protein analog for site-specific targeting of the VEGF receptor. The pharmacokinetic properties and efficacy of this dual labeled probe were evaluated for tumor-specific imaging in an early clinical trial.⁵⁰ The PET agent provided an overall view of VEGF receptor positive tumors whereas the optical imaging provided real time localization of the tumor for resection.

Another example of a dual radionuclide–optical probe is a trastuzumab (anti HER2 antibody) labeled with a positron-emitting radionuclide and a NIR fluorophore which was tested in a mouse model of breast cancer.⁵² Bimodal nuclear/optical agents possess promise for providing sensitive disease detection over the whole body (nuclear), quantitative data regarding pharmacokinetics (nuclear), as well as the ability to achieve real time, multi-color, highly specific molecular targeting and cellular-level imaging (optical). Practically speaking, the similar sensitivities of the two modalities make it easier to design and apply these probes together (Fig. 4).

B.1.1.2 Cerenkov luminescence imaging (CLI): A recent development in bi-modal optical/nuclear imaging is the use of Cerenkov Radiation (CR) for optical imaging.^{53–58} CR occurs when a charged particle travels faster than the speed of light through an insulating medium.^{53,59–61} The relevance to molecular and optical imaging is that positron-emitting radionuclides used in biomedical imaging have sufficient energy to result in CR that can be detected *in vivo* using optical imaging equipment.^{59,60} Currently used positron emitting radionuclides include: fluorine-18, oxygen-15, gallium-68 and iodine-124; and other

possible beta-ray emitters include: iodine-131, strontium-89, yttrium-90 and copper-67.^{58,59} These agents are mostly used for radionuclide therapy but they may be used in the future for theranostic imaging as well. Unlike typical fluorescence, which results in a peaked spectra, CR spectra are continuous with higher frequencies related to higher energy conditions.^{57,61} The photons produced by this process have been detected in mice using CCD optical imaging systems.⁵³ However, the light produced with CR is mostly in the ultraviolet and blue range where it is highly absorbed, thus limiting its usefulness for *in vivo* optical imaging. However, this limitation has been overcome using a multimodal strategy in which Cerenkov radiation serves as an energy donor for an adjacent fluorophore in a process termed Cerenkov radiation energy transfer (CRET).⁶¹ By coupling PET isotopes and quantum dots which can be excited by a broad range of wavelengths and produce fluorescent light that has a high Stokes-shift (*i.e.* is much higher in wavelength than the excitation light), Cerenkov radiation can induce light which is relatively easy to detect.⁶¹

B.1.2 MR/optical multi-modality probes—Another approach to multimodal imaging is to combine MR and optical imaging capabilities.⁶² In this combination, MR offers detailed anatomic imaging, while the optical component offers the option of real-time molecular targeting that can be used for image guided procedures. Potential clinical applications include preoperative MR imaging followed by optical image-guided surgical intervention during the treatment of cancer. One general challenge in the development of these probes is the >1000 fold discrepancy in detection sensitivity that exists between MR (low sensitivity) and optical imaging (high sensitivity). A much higher concentration of the MR agent is needed to achieve successful imaging compared to the optical agent. Another challenge is that MR is inherently a three dimensional imaging method whereas optical imaging is generally a projection imaging method. Therefore the optical image is often superimposed on the surface of the MR image. Creating a bimodal MR/optical agent that allows for superimposed images and that incorporates enough paramagnetic signal to generate an image yet does not overwhelm the optical signal can be challenging.

B.1.2.1 MR/optical dual-labeled probes: Numerous dual function MR/optical probes have been developed.^{63–68} Three primary approaches are currently used to synthesize such probes: (1) iron oxide nanoparticles functionalized with fluorescent molecules, (2) biocompatible molecules that are inherently paramagnetic yet also have optical properties, (3) nanomaterials specifically designed for both MR and optical imaging.

The use of superparamagnetic iron oxide (IO) nanoparticles such as small particle of IO, ultra-small particle of IO, magnetic IO nanoparticle and cross-linked IO is commonly employed as a platform for dual MR/optical probes. IO nanoparticles have the advantage of being clinically available, with favorable toxicity profiles and are metabolized using well known iron salvage pathways that have been conserved through evolution.^{69–71} Additionally, IO nanoparticles (NPs) demonstrate different pharmacokinetic behaviors *in vivo* based on their size, and the size can be tuned for specific applications.^{72–77} For instance, IOs which are over 100 nm in diameter are quickly recognized by the reticuloendothelial system (RES) and are quickly cleared from the plasma to the liver, spleen, and bone-marrow.⁷² These organs are therefore, logical targets for such agents. In contrast, macromolecules smaller than 30 nm in diameter are taken up less rapidly by the RES and therefore, have longer circulating half lives and accumulate with enhanced permeation and retention (EPR) within tumors.^{72,78} In addition, cell surface chemistry modification also affects biodistribution and specific alterations can be made as needed.^{73,79} Applications of bimodal MR/optical probes have been numerous and varied.⁶² In one paradigm, preoperative MR imaging is used to confirm the diagnosis of an implanted brain tumor in a rat and then intraoperative real time NIR fluorescence imaging is used to discriminate cancer tissue from normal brain tissue during resection.⁸⁰

An important drawback of iron oxide nanoparticles is that they produce a negative signal on proton-based MR imaging and this is more difficult to detect than a positive signal. Another design strategy for MR/optical probes is to functionalize molecules or particles (through encapsulation or linking) with traditional small molecule gadolinium chelates, thus making the molecule paramagnetic, so that it produces a positive signal. Although little toxicity data are currently available, organic molecules may prove to be more biocompatible and therefore may be more relevant clinically. Organic molecules capable of linking to paramagnetic chelates include dendrimers,^{63,81–83} nanomicelles^{64,84–88} liposomes^{89–93} viral capsids,^{94–97} and even natural products such as functionalized low density lipoprotein particles.^{69,98} Chelating the gadolinium with a strong macrocyclic chelate greatly reduces the chance of toxicity due to the release of free gadolinium ion. This strategy has been the basis of a number of dual MR/optical agents thought to be potentially translatable to the clinic.⁶⁹ For instance, dendrimers with multiple Gd–DTPA chelates have been proposed as viable MR agents. Substituting one of the amine bound Gd–chelates on the dendrimer for a fluorophore creates a dual MR/optical imaging agent, with the virtue that the sensitivity differences between MR and optical are compensated by the relative number of attached molecules (Gd–chelates > optical fluorophores by >100 : 1). Additionally, toxicity may be further reduced with the use of nano-sized agents, which are small enough to be excreted by the urinary system and thus, theoretically, have better safety profiles^{99–102} (Fig. 5).

Much work is being done in nanomaterial science regarding the development of organic and non-organic nanoparticles for *in vivo* molecular imaging.^{6,103} Among the various types of nanomaterials, dendrimers and fluorescent quantum dots have considerable promise due to the wide range of synthetic possibilities that can be achieved with these agents.^{87,88,101,104,105} The branched structure of dendrimers presents an ideal platform for conjugation and can be useful for overcoming the difference in sensitivities between MR and optical agents, as numerous MR chelates can be attached to a single dendrimer.^{106–108} Additionally, *in vivo* behavior of dendrimer-based MR agents is, in part, determined by dendrimer diameter and particles can be optimized for their desired pharmacokinetic parameters, such as their ability to leak from angiogenic vessels, their ability to be excreted either *via* the kidneys or the liver, or for their ability to be recognized by the RES.^{109–113} Moreover, the larger size of these molecules improves the magnetic relaxivity by factors of 2–3 resulting in a lower required concentration of gadolinium to achieve the same change in signal.

Quantum dots are also a potential platform for dual MR/optical imaging agents and numerous MR-QD applications have been explored.^{6,27,104,114–124} Quantum dots can be conjugated to MR active molecules or doped with these agents during synthesis. A standard conjugation approach is to attach amine-reactive chelators such as tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or diethylenetriaminepentaacetic acid (DTPA) to the amine-rich surface of a QD and then transmetalate with MR-active ions.⁶⁹ The use of dendrimers or QDs can overcome difference in sensitivity between optical and MR.⁶⁹ One general limitation to the clinical use of quantum dots is that they contain heavy metals such as cadmium and selenium which in high doses are toxic.^{125–129} However, toxicity may be reduced with the adoption of size parameters that are specifically designed to permit renal excretion, thus dramatically reducing the exposure time in patients with normal renal function.^{99,100,102}

B.1.2.2 Lanthanide metal MR/optical imaging: A challenge limiting TBR in optical imaging is differentiating target fluorescence from the naturally occurring background fluorescence of native fluorophores (so called autofluorescence). The standard strategy employed to overcome this challenge is to use probes that have emission spectra far from the expected range of typical autofluorescence, which is maximal in the green-red part of the

visible spectrum. A relatively new strategy is the use of upconversion luminescence (UCL), a process whereby low-energy light of high wavelength, usually in the near-infrared (NIR) range, is converted to higher-energy (lower wavelength) visible spectrum light through sequential absorption of multiple photons or energy transfers.^{130,131} This differs from traditional fluorescence where higher energy excitation light is absorbed by the fluorophore but is emitted at lower energy (or higher wavelength resulting in the well known Stokes shift). UCL has been demonstrated in lanthanide metals using excitation wavelengths well above those responsible for autofluorescence.^{130,132,133} For example, rare-earth nanophosphors exhibit unique UCL properties under continuous wave excitation at 980 nm, an energy at which neither endogenous nor conventional fluorescent probes are excited.¹³⁰

With this in mind, lanthanide doped nanocrystal complexes have unique properties for bi-modal MR/optical imaging.^{134–139} These nanocrystals exhibit paramagnetic properties thought to be due to the non-interacting localized nature of the magnetic moment of Gd^{3+} .¹⁴⁰ Additionally, unlike traditional gadolinium ion complexes, which relax protons non-specifically, recent reports suggest that lanthanides have potential as “smart” MR imaging contrast agents by demonstrating enhanced relaxation in the presence of specific biological molecules.¹⁴⁰ One group demonstrated tumor-specific UCL multicolor imaging *in vivo* after targeting an integrin receptor.¹³⁰ The probe was imaged without any background autofluorescence, yielding a high TBR.¹³⁰ Lanthanide doped nanocrystals are potentially important as bimodal nanoprobe because they are capable of exciting fluorophores deep in the body yet, demonstrate excellent photostability, and minimal autofluorescence.^{132,134–138,141,142}

B.1.3 Nuclear MR multi-modality probes—PET has emerged as an extremely useful tool for whole-body imaging with detection sensitivity below the picomolar range.^{143–145} A logical next step after PET-CT success^{146,147} is to combine nuclear imaging, both PET and SPECT with MR imaging.^{148–151} However, numerous hurdles remain.^{150,152–155} For instance, conventional PET detectors do not function well in strong magnetic fields required for MR imaging. Early prototypes have been developed using novel solid state PET detectors which are less susceptible to magnetic fields¹⁴⁸ and this has prompted investigators to develop PET-MR imaging probes. One group developed a bimodal target-specific PET/MR imaging nanoprobe and demonstrated *in vivo* efficacy.¹⁴⁹ Targeting the tumor/endothelial integrin, $\alpha_v\beta_3$, a superparamagnetic iron oxide core was functionalized with DOTA for PET isotope chelation enabling a probe with dual modality imaging capabilities.¹⁴⁹ Another bimodal PET/MR imaging agent was developed using MnEIO as the MR imaging agent and ^{124}I for PET.¹⁵⁶ Of note, continued progress in the realm of multisignal MR, as discussed below, may reduce the need for bi-modal PET/MR probes.

B.2 Multi-color imaging probes

Multicolor imaging provides rapid discrimination of two different targets, enabling multiparametric imaging. The advantages of multicolor imaging are that it is more efficient than sequentially administering probes and that it allows for merged imaging of two different targets. For these reasons, multicolor imaging is a highly desirable strategy and broadly includes the use of multiple nuclear probes distinguished by energy, the use of optical agents, which use different parts of the optical spectra, and even multiparametric MR imaging.

B.2.1 Optical imaging with multi-color probes—Optical imaging is naturally adapted for multicolor imaging. This strategy is achieved using multicolor optical imaging with numerous different colored probes designed so that each binds a different receptor. Multiple

optical probes can be used simultaneously, each targeting a different cell surface receptor.^{33,157–166} Multicolor strategies can be used to increase the specificity and sensitivity of imaging.¹⁵⁸ For example, although cancers often express specific cell-surface receptors, these same receptors are often also expressed (to a lesser degree) on normal tissue. However, by imaging a combination of cell surface receptors, sensitivity and specificity of the target tissue detection may be increased. Tissues that bind multiple probes are then identified by their merged color (*e.g.* red and green probes binding the same cell may produce a yellow color).^{158,167} Multicolor optical imaging may also be utilized to image other physiologic processes, such as the tumor microenvironment or lymphatic drainage (see also Fig. 4).⁴⁶ One goal in advancing the surgical removal of cancer is to better identify microscopic foci of disease in the vasculature and stromal cells that might otherwise be missed. Using multiple color optical probes it may be possible to detect residual tumor to assist in image-guided tissue removal.^{158,159,167,168} Multiple color optical agents can be combined with multiple multimodality imaging.^{33,169–172}

B.2.2 Nuclear “multi-color” imaging—Using energy resolution features of gamma cameras and different types of gamma emitters, multiple nuclear probes can be detected simultaneously, each targeting a different process. Different photon energies emitted by different radioisotopes can then be used to assign “colors” to a nuclear image. Clinical applications include SPECT for thyroid and parathyroid imaging in which radioactive iodine and thallium are used together^{173,174} as well as in cardiac scintigraphy where radioactive technetium and thallium may be combined.^{169–171} For instance, in the assessment of acute myocardial ischemia ²⁰¹Tl is used to identify areas with patent blood flow and ^{99m}Tc-labelled myosin antibody-fragment is used to identify areas of damaged myocardium by detection of exposed myosin fibers.^{170,171} Although multiplexed nuclear imaging provides useful insights, broader application has been limited by the poor spatial resolution of SPECT and the double dose of ionizing radiation required. Additionally, current strategies for imaging with multiple radiolabelled antibodies require several days from the time of administration for sufficient TBR. Moreover, each type of emitter has a distinct half-life and determining the right combination of isotopes such that the clearance pharmacokinetics and physical half-life of the radionuclides are optimized for a single imaging session is challenging.²³ Therefore, nuclear multi-color imaging is used primarily in applications, in which images can be acquired a short time after injection of reagents to minimize the effects due to different half-lives of radioisotopes.

B.2.3 MR “multi-color” imaging—Contrast enhancement in MR imaging is determined by two factors: the proton density of the tissue under examination, and the relaxation properties (*e.g.* T1 and T2) of protons.¹⁷⁵ Conventional MR contrast agents work by shortening the longitudinal and/or transverse relaxation times of nearby protons, mostly within water molecules.¹⁷⁵ As a result, MR contrast has several important limitations: (1) pre-contrast images are generally needed to serve as a baseline for the identification of enhanced regions; (2) the measured effect is a function of agent microenvironment (pH, temperature) and agent concentration, which are unknown *in vivo*; (3) targeted MR imaging agents allow imaging of only one target per exam.¹⁷⁵ Recently a new contrast strategy has been developed that does not rely on water protons but instead relies on chemical exchange saturation transfer (CEST).^{175–180} These contrast agents work by selectively reducing the magnetization of the water signal with the use of CEST agents with exchangeable protons, while minimally affecting longitudinal relaxation rates.¹⁷⁵ This technology has been used to achieve multicolor MR imaging in which a series of peptide sequences are distinguished by the resonant frequencies of their exchangeable protons.¹⁸¹ In gliomas, CEST has been used to differentiate between radiation necrosis and malignant tissue by detecting higher than normal concentrations of amide protons of endogenous mobile proteins and peptides that

exist in high concentrations in malignant gliomas compared to normal tissue.¹⁸² CEST and combination CEST/conventional MR imaging is a powerful new imaging technology with many potential applications.^{183,184}

B.3 Multi-signal imaging

Multiplexed imaging utilizing multiple signal collection relies on capturing two or more distinct signals from the same acquisition. For example, clinical dual-energy CT relies on the different absorption properties of tissue at two separate X-ray energies¹⁸⁵ to characterize processes such as iodine–calcium separation (*e.g.*, tumors, arterial plaque), renal-stone composition,^{186,187} gout,^{188,189} and perfusion and local blood volume in organs^{190–192} and tumors.¹⁸⁵ Multi-signal imaging can also be achieved with MR and optical imaging technologies.

B.3.1 Multi-signal MR imaging—MR imaging is intrinsically a multi-signal phenomenon. Multiparametric MR imaging, which combines two or more MR imaging techniques is an example of a multi-signal strategy. Given that this can be achieved with most modern MR imaging scanners, without ionizing radiation and the strategy does not require any contrast agent administration, it is in common clinical use. Diffusion-weighted imaging (DWI) relies on the diffusivity of water protons and can provide information about cell density.¹⁹³ Whole-body DWI provides global functional information and is able to highlight both oncological and non-oncological lesions throughout the entire body in a time efficient manner.^{193–196} Multi-signal MR imaging is achieved by using whole-body DWI along with traditional MR imaging (T1 and T2 imaging) providing both functional and anatomical data.¹⁹³ Clinical images acquired in this manner have been reported to compete with PET/CT in their ability to highlight tumor sites.¹⁹⁶ Multi-parametric imaging combining DWI with anatomical MR imaging dramatically increased specificity and accuracy of bone metastases detection in patients with lung cancer compared to DWI alone.¹⁹⁷ Similarly, the combination of DWI/T2-weighted imaging dramatically improved the sensitivity and specificity of DWI in the detection of abdominal malignancies including prostate cancer.^{193,198} Further, multi-parametric MR imaging, combining DWI with perfusion weighted imaging is useful for the identification of acute stroke, and its penumbra and has been used in the selection of patients for thrombolytic therapy treatment after stroke.^{199,200}

B.3.2 Multi-signal optical imaging—Multi-signal molecular imaging relies on extracting multiple types of data from a single source. This same principle can be applied to optical imaging. Signal intensity can serve as a measure of local fluorophore concentration and therefore can provide semi-quantitative data regarding the distribution of the probe. Fluorescence lifetime measurements (the time constant describing the loss of fluorescence after initial emission) serve as a separate parameter which is dependent on the microenvironmental factors, such as temperature and pH.^{201,202} These two distinct parameters can be measured to ascertain probe concentration while simultaneously gaining insight into physical factors pertaining to the tumor microenvironment. (Fig. 6) Clinical applications include tumor-specific optical probes that efficiently localize to the cancer tissue. In such an application, tumor imaging can be achieved by measuring fluorescence intensity, while fluorescence lifetime provides an estimation of tissue oxygenation and degree of hypoxia within the tumor microenvironment.²⁰¹

C. Target-specific molecular imaging probes: chemistry and biology

Thus far, we have discussed technological approaches to optimizing molecular imaging *via* multiplexing (combining multiple imaging strategies). Another approach to improving

molecular imaging is to optimize the imaging probe to provide maximum target-to-background ratio (TBR). In order to maximize TBR, the imaging probe should accumulate signal at the target tissue/cells while clearing rapidly from the background resulting in minimal signal in non-target organs and tissues. However, this is difficult to achieve as the *in vivo* pharmacokinetics of many targeted macromolecular moieties, such as monoclonal antibodies, is dictated by the prolonged clearance from the vascular compartment, which leads to high background signal. On the other hand, small molecules, while less specific, are more rapidly cleared, thus improving the target-to-background ratio but reducing the absolute amount of accumulation at the target. Additionally, imaging of organs within the excretory route of the agent (liver, kidney, gastrointestinal tract) is challenging as clearance can obscure pathology in and around these organs. Conventional imaging probes used for CT, MR imaging and radioisotope imaging, are “always on”, and therefore, the signal emanating from the probes reflects their biodistribution but this results in high background signal. One method to reduce background noise is to employ signal activation mechanisms, which turn the probe “on” only at the target tissue.^{203,204} Signal activation strategies combine biology (target binding), pharmacology (*in vivo* pharmacokinetic and targeting delivery), and chemistry (signal activation)²⁰⁵ (Fig. 7).

C.1 Biodistribution of multi-signal agents

Probe pharmacokinetics and biodistribution play an important role in the TBR of a given probe. In general, imaging probes are delivered to target tissues from the vascular compartment and final tumor signal, depends primarily upon tissue perfusion, time spent in circulation, as well as endothelial permeability at the target; factors collectively referred to as the “input function.” On the other hand, unbound circulating probe and its catabolites produces non-specific background signal and, therefore, prolonged circulatory times decrease TBR. With this in mind, optimizing TBR with conventional “always on” signaling agents poses the dilemma of requiring high blood concentrations to maximize target tissue accumulation but low blood concentrations to minimize background signal. Briefly, macromolecular targeting moieties such as monoclonal antibodies are highly specific and can be delivered in high concentrations to the target. Unfortunately, these large molecules have the undesirable feature of prolonged circulatory retention times of unbound molecules, leading to high background signal. Yet, small molecules are rapidly cleared, which improves the target-to-background ratio, but reduces the absolute amount of accumulation within the target and increases accumulation in the excretion pathways—typically the hepato-biliary system or the urinary tract. One different pharmacokinetic approach to reduce background is to “chase” unbound probe from the circulation with an injection of an agent that binds and sequesters the unbound probe.^{206–210} However, this approach requires the injection of a second agent. Local activation was developed to address this.^{35,211–213} New generation “activatable” agents, which have recently been developed and tested in small animals, yield signal only after binding to specific molecules in the target tissue and therefore no signal is produced from the unbound circulating fraction.^{9,35,214–216} Optimizing TBR differs between conventional and activatable probes as the design of activatable imaging agents focuses on achieving a high input function primarily through high blood probe concentrations, while optimizing the bound *versus* unbound probe fraction is paramount for conventional agents. The differences in desired pharmacokinetic properties for conventional “always on” *versus* “activatable” probes, are discussed separately in the following section.

C.1.1 Pharmacokinetics and tissue distribution—Size is a primary determinant of molecular retention time within the vascular and extravascular compartments. Large molecules, such as antibodies, remain in circulation for days to weeks, unless they are recognized and trapped by the reticuloendothelial system in the liver or spleen. Small molecules, such as polysaccharides or amino acids/peptides, are generally cleared from the

circulation over the course of minutes. Therefore, large molecules demonstrate high input function but also high background signal from prolonged circulatory retention. In contrast, small molecules demonstrate low input function and low background signal. As a result, for “always on” agents, there is little difference in TBR between large *versus* small molecular imaging agents. Interestingly, intermediate sized agents demonstrate the highest TBR with acceptable target accumulation for imaging (Fig. 8).²¹⁷ For “activatable” probes large molecules are clearly desirable because these probes achieve the highest target accumulation but background signal is negligible. However, among macromolecules, larger molecules tend to have poorer penetration into deep tissues, resulting in slow target accumulation.^{218,219} Molecules with approximate sizes of an IgG immunoglobulin (150 kD; 12 × 9 nm) are optimal for target accumulation within a reasonable amount of time.

C.1.2 Clearance/excretion—Injected molecules are mostly excreted into the urine or the bile. Kidneys quickly excrete small molecules through two separate processes: (1) glomerular filtration or (2) tubular secretion. Glomerular filtration is the ideal excretion route as it does not involve probe passage through host cells and is therefore felt to be the safest excretory route. Although small molecules such as amino acids and ions might be absorbed and then excreted by tubular secretion, most imaging agents are removed *via* glomerular filtration. Hard molecules/crystals smaller than 5.5 nm in diameter and slightly larger proteins/polymers with flexible shapes can undergo excretion *via* glomerular filtration.^{102,220,221} Both glomerular filtration and tubular excretion are rapid processes, therefore, imaging agents excreted by the kidneys are quickly cleared from the circulation leading to decreased background signal and improved TBR. Again, decreased circulatory retention times compromise the input function and therefore decrease target tissue probe accumulation. However, despite this, renal excretion is almost always considered advantageous for “always on” signaling agents. A disadvantage of renal excretion is that pathology within the genitourinary tract; the kidneys, ureters, and the bladder, may be more difficult to diagnose. In comparison, hepatic excretion is generally a slower process. Most imaging agents, which are not excreted through the kidney, will stay in the circulation for hours, days, and even weeks. Therefore, hepatic excretion is generally advantageous for “activatable” imaging agents as prolonged retention times lead to very high input functions. Of note, the excretory pathway signal is not an issue for “activatable” agents, because they are not activated during excretion.

C.2 Cell binding and activation

In most cases, *in vivo* imaging aims to visualize specific populations of cells or cellular functions. The outer layer comprised of the lipid bilayer provides a semi-permeable protective envelope around the cell. Most large molecules and hydrophilic small molecules cannot penetrate the cell membrane. However, certain non-permeable molecules can be transported across the lipid bilayer through transmembrane pumps, transporters or receptors. Some small and/or hydrophobic molecules, such as steroids, can directly penetrate the cell membrane and then bind specific receptors in the cytoplasm or in the nucleus. Although this strategy has had relatively limited clinical translation thus far, recently, targeted PET imaging of estrogen receptor positive-tissue in the hormone-therapy responsive breast cancer with radiolabeled estrogen derivatives was reported.^{222–224} Thus, probes can be designed with specific cell membrane targets in mind for molecular imaging. Further, upon binding, probes may undergo internalization and further enzymatic or lysosomal processing enabling activation of signal production.

C.2.1 Target accumulation—Millions of different molecules, and distinct combinations of these molecules, are expressed on the cell surface. However, few are truly specific for a particular population of cells. In order to achieve target-specific molecular imaging, TBR is

an important consideration at the cellular level. To achieve this, target cells should either express unique molecules or overexpress a common molecule by at least 100-fold compared to normal cells. For example, in some cancer cells altered receptors such as the EGF receptors^{34,225–227} or glycosylation products such as sialyl-Tn,^{228,229} TAG72^{230,231} and Louis-Y^{232,233} antigens can be unique targets. Epidermal growth factor (EGF) receptors, are over expressed in a large proportion of lung, breast, gastrointestinal cancers, and can therefore be used as targets for tumor-specific imaging.^{227,234–239} In general, molecules such as growth factors or cytokine receptors are expressed during carcinogenesis and may contribute to the cancer cell growth and therefore tend to be reasonable targets for molecular imaging of cancers.

C.2.2 Biological processing and catabolism in the cell—After binding to a specific molecule or entering target cells through a specific channel, biological processing and/or catabolism can help increase probe accumulation, resulting in improved TBR. For example, ¹⁸F-fluorodeoxy glucose (¹⁸F-FDG) or ¹⁸F-fluorothymidine (¹⁸F-FLT) undergo phosphorylation by specific kinases (hexokinase and thymidine kinase, respectively) following transport into the cytoplasm and are subsequently trapped within the cell. Furthermore, ¹⁸F-FDG-6-phosphate is not hydrolyzed by the glucose-6-phosphatase because it is not recognized as a substrate and accumulates in the cytoplasm unable to go through the glycolytic cascade. Therefore, ¹⁸F-FDG accumulates within the cell. Target-specific ligands, which bind to cell surface molecules with high specificity, can provide similar trapping mechanisms for imaging probes. For example, monoclonal IgG-based imaging agents against EGF receptor families can bind to adjacent receptors on the membrane, leading to dimerization and subsequent phosphorylation at the intercellular domain, which then leads to endocytosis of the antibody–receptor complex and internalization into the endolysosome. Within the endolysosome, imaging probes are exposed to harsh chemical environments leading to catabolism, a process which can be utilized for retaining activated signaling as is described later in Section, 3.3.2. Since target-specificity is secured at the time of antigen–antibody binding, any biological processing mechanism that leads to probe retention or signal activation can improve TBR.

C.3 Molecular level

By taking into account pharmacological and biological *in vivo* processing of imaging probes, agents with superior specificity can be designed. In general, targets are molecules, *e.g.* antigens, receptors, or substrates, that alter the probe after binding, *e.g.* by enzymatic catalysis. Therefore, a central principle in the design of molecular imaging agents is that the probes should contain moieties, which bind the target or are recognized by target molecules. Furthermore, incorporating post-binding molecular processing into probe design can result in enhanced retention or signal activation, and can further improve TBR (see Fig. 7).

C.3.1 Target specificity—Targeting with agents such as antibodies or receptor-specific ligands, results in highly specific molecular imaging probes. In contrast, probes that target catabolic processes require additional mechanisms to achieve specificity at sites containing processing molecules. Most enzyme-targeting probes rely on reactions that occur outside of or on the surface of target cells. However, there are several notable exceptions, *i.e.*, ¹⁸F-FDG, which binds to hexose-6-monophosphate enzymes in the cytoplasm. Therefore, without a localizing mechanism, processed imaging probes can leak out of target tissue, increasing the background signal and decreasing TBR. Thus, an important design criterion is to assure that probes remain at the desired; *i.e.*, making the enzyme catabolite hydrophobic outside of the cell membrane so that it can readily permeate into the cell membrane of adjacent cells,³⁵ and then, again making the enzyme catabolite hydrophilic inside the target cell so that it cannot leak out from the cell. This is similar to the probe cell capture strategy

of ^{18}F -FDG, which undergoes intracellular phosphorylation, effectively trapping the probe in the cell. Utilization of this mechanism results in efficient retention of probes.

C.3.2 Chemical or enzymatic processing after specific binding—After binding to specific molecules on the surface of target cells, large proportions of imaging probes are then internalized into endolysosomes, where specific chemical and biological conditions exist, including low pH (4–5), highly oxidative conditions, and highly reactive acid proteases. An example of enzymatic processing increasing probe retention is the release of radiometals from chelates such as DTPA derivatives in radiopharmaceuticals. Such probes release elemental radioisotopes in acidic conditions. The released radiometals then undergo transchelation by endogenous metal-containing proteins including metallothionein, resulting in prolonged retention in target cells.^{240,241} Some hydrophobic organic fluorophore-labeled imaging probes are catabolized in the lysosome to release fluorophore–amino acid catabolites. These small molecules can penetrate the lipid bilayers and migrate into intracellular organelles including mitochondria, leading to prolongation of probe retention.^{242–246} Such retention mechanisms are advantageous for increasing TBR of both “always on” and “activatable” imaging probes.

Intracellular chemical or enzymatic processing plays a significant role in the design of some “activatable” imaging probes which rely on specific activation mechanisms.^{24,47,247–249} For example, pH-activatable,⁹ oxidation-activatable, enzyme-activatable,^{248,250,251} unfolding or catabolism-activatable²⁵² probes have recently been synthesized by employing inter-molecular, inter-fluorophores, or intra-molecular photochemical reactions as shown in the next section.

If specific probes demonstrate minimal internalization after binding to target molecules, “activatable” imaging strategies can be tailored towards pretargeting mechanisms. One strategy is to first inject target-specific binding reagents conjugated with “activating” moieties such as biotin or enzymes, and follow this with a second injection containing “activatable” imaging agents, which are activated upon reacting with the pretargeted moieties through binding²¹⁴ or enzymatic cleavage.³⁵

C.4 Signal activation *via* energy transfer and dequenching

In the design of “activatable” optical imaging probes, photon-induced mechanisms, including energy or electron transfer and uncaging, can activate fluorescence. The available “activatable” quenching mechanisms can be separated into two categories; (1) inter-fluorophore quenching mechanisms including Förster resonance energy transfer (FRET) and homo- or hetero-dimer formation between two fluorophores, or (2) intra-fluorophore quenching mechanisms including photon-induced electron transfer and so called “caged” fluorophores (Fig. 9).

Non-irradiating FRET for quenching fluorescence signal occurs when two fluorophores are in close proximity thereby exchanging energy between the excited donor and the acceptor within a single molecule or even between two different bound molecules.^{253,254} In addition, when two fluorophores are close enough to physically behave as a single molecule, some molecules form H- or J-type dimers, which are totally non-fluorescent but emit signal upon dissociation.²⁵⁵ The dissociation constant of H-type dimers was reported to be approximately 10^{-4} M in solution.²⁵⁶ However, when two molecules are conjugated to a single protein backbone, the H-dimer can be stabilized at a much lower concentration.^{255,256} In both mechanisms quenching and activation depends upon the distance between the two fluorophores.

Photon-induced electron transfer is based on the energy level difference between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) states, which can be rationally modified by attaching or detaching certain side residues on core xanthenes or benzene rings of the fluorophore.^{257,258} Photon- or enzyme-induced uncaging occurs when chemical changes induced by electron transfer after absorbance of a photon or enzymatic cleavage results in a conformational change releasing the fluorophores from close steric proximity.²⁵⁹ Given that quenching and activation can occur within a single fluorophore, small molecules employing these mechanisms can be incorporated within larger macromolecules with target-specific “activatable” imaging probe complexes.

C.4.1 Inter- and intra-molecular Förster resonance energy transfer—Fluorescent signal quenching based on FRET can be induced when the intermolecular distance equals the inverse of the square of half the distance ($1/r^2$) between the energy donor and acceptor molecules. Thus, energy transfer generally operates within 10 nm. Therefore, this mechanism applies not only to a single macromolecule but also between two different molecules. Protein cleavage by enzymes leading to separation of a single molecule into several pieces is necessary to achieve complete dequenching of “activatable” imaging probes.^{35,215} However, when large numbers of fluorophores were bound to a single molecule, unfolding and linearization of the protein was all that was required to activate the quenched imaging probe, in one case leading to a 30-fold increase in signal compared to baseline.²⁵² When using two of the same fluorophores to achieve Homo-FRET, baseline quenching states are incomplete and will demonstrate low level fluorescence, however, the probes yield strong signal once they are dequenched. In contrast, fluorophore–quencher pairs, have near complete baseline quenching states, yet, the probes cannot yield as strong signal as Homo-FRET “activatable” probes once completely separated from each other (Fig. 9a).

C.4.2 H-dimer formation—H-dimer formation can also occur when two fluorophores bind sufficiently close to each other on another molecule. The distance between the fluorophores must be within a few angstroms to form an H-dimer and, therefore, H-dimer formation can occur on a single macromolecular imaging probe, such as monoclonal antibodies or receptor ligands.²⁵⁶ Additionally, hydrophobic pockets are especially conducive to H-dimer formation. Once two fluorophores form an H-dimer, fluorescent signal is totally quenched. However, given the often random configuration of fluorophores on probes, not all fluorophores form H-dimers. Therefore, baseline states of “activatable” H-dimer imaging probes are not completely quenched, reducing the difference between the signal intensity in the quenched and activated states.^{255,256} The proportion of fluorophores within the probe that form H-dimers depends on the chemical structure of the fluorophore and the molecule to which it is conjugated (Fig. 9b).

C.4.3 Photon-induced electron transfer—Photon-induced electron transfer (PeT) is a widely accepted mechanism for “activating” fluorescence quenching, whereby electron transfer from the PeT donor to the excited fluorophore quenches the fluorescence signal. When the PeT donor is cleaved from the fluorophore or inactivated by changing HOMO or LUMO energy status, full activation of fluorescent signal is achieved.²⁶⁰ This switching can operate within a single small fluorophore by utilizing environmental queues including pH (proton density), oxidation by specific reactive oxygen species, specific metals, or enzymatic cleavage to cleave the PeT donor.^{255,261–265} Therefore, activatable fluorophores based on the PeT mechanism can be conjugated with almost any target-specific macromolecular probe including monoclonal antibodies and receptor ligands (Fig. 9c).

C.4.4 Photon- or enzyme-induced uncaging—Conventional caged fluorophores, which have internal photo-breakable bonds after exposure to UV, form chemically unique structures, and are another widely used activating mechanism, most often for fluorescence microscopy. When irradiated with UV light, this additional bond referred to as a “cage” is broken and the probe then emits a fluorescent signal.^{266–270} Caged fluorophores, which can uncage with irradiation of visible light, have recently been reported and are more applicable to bio-imaging as they do not rely on UV which not only has potential toxicities but also does not penetrate tissue to any degree.. Using a similar mechanism, a spirocyclic cage has recently been reported in the design of activatable fluorophores. The spirocyclic cage is composed of additional bonds formed in the fluorophore that can be broken through enzymatic cleavage of molecules on the side chain of the fluorophore.^{271,272} Similar to the PeT mechanism, this switching mechanism can operate within a single fluorophore. Therefore, activatable fluorophores based on the PeT mechanism can be conjugated with any target-specific macromolecular probe (Fig. 9d).

C.4.5 Combined approaches using more than one mechanism—Inter-fluorophore quenching, FRET and H-dimer, and intra-fluorophore quenching mechanisms, PeT and caging, operate independently. Therefore, by combining mechanisms, synergistic quenching and activation effects can be achieved. For example, a pH-activatable fluorophore based on PeT can also form H-dimers upon conjugation with target-specific macromolecules, resulting in improved activation ratios compared to the baseline quenched state.^{255,256}

D. Practical preclinical applications of the next generation of molecular imaging probes

Effective molecular imaging detects and characterizes tissue. This requires consideration of the desired imaging objectives (real-time image guided intervention, pharmacokinetic assessment, *etc.*), a targeting strategy, and a strategy to obtain optimal TBR. To achieve optimization of these endpoints, multiplexed imaging is often warranted. In this section we demonstrate the thought processes involved in the design of a multiplexed imaging strategy. Creation of an optimal probe and ultimately the most effective clinical application requires thoughtful consideration of the numerous and often interconnected aspects of each modality, molecule and application.

D.1 Highly specific cancer detection

One desired medical application for multimodal molecular imaging is to be able to specifically identify tumors with a low false positive rate. For this medical application there are several important considerations (1) finding a highly specific tumor target and (2) optimizing TBR. For this application, monoclonal antibodies were selected as they offer highly specific binding to tumor cell surface molecules. Additionally, numerous humanized antibodies targeting cancer related epitopes have been approved by the FDA for clinical use. The same sub-class of humanized monoclonal antibodies, which are grafted specific binding site, complementarily determining regions (CDRs), into a human immunoglobulin framework such as IgG1, show >98% homology of protein sequence, therefore, the biodistribution of different humanized antibodies against distinct antigens is similar. However, targeting with humanized antibodies poses an important dilemma for the overall strategy. Due to slow clearance from the vascular compartment, antibody based probes have high background signal and therefore, have a low TBR. With this in mind, a multimodal strategy is warranted. Two ways of approaching the limitations posed by conventional antibody-based imaging using multimodal imaging are (1) multicolor imaging and (2)

activatable imaging; either strategy will improve the TBR for the proposed clinical application.

Multicolor optical imaging uses multiple, differently colored optical probes. This improves TBR since the additional, non specific colors serve as an internal control for unbound antibody within the blood pool (Fig. 10a).^{34,168} Spectral unmixing is used to accurately differentiate multiple probes and this facilitates highly specific cancer imaging without waiting for clearance of unbound antibody.¹⁶⁸ Additionally, multi-excitation spectral fluorescence imaging allows greater flexibility in color selection and permits the simultaneous use of numerous targeting agents. TBR is ultimately increased as the number of independent signals increase, yielding more specific data. The use of multiple colors can depict single lesions with higher TBR and can also detect molecularly heterogeneous lesions, as may occur in naturally occurring tumors over time (Fig. 10b). The combination of multi-excitation and unmixing techniques employing multiple, humanized antibodies allows the performance of multi-target imaging with relatively high TBR.

The other approach for target specific cancer imaging, which overcomes limitations of low TBR of conventional antibody based imaging is the use of “activatable” probes. As discussed above, these probes are activated by cancer-specific factors such as enzymatic reactions or cancer-cell mediated processing initiated by binding specific molecules. In the implementation and design of these probes, special consideration is given to the design of signaling molecules that harness unique aspects of the cancer milieu for probe activation. For instance, we utilized a cancer-cell specific targeting probe to design an activatable fluorescent probe.⁹ Our probe emitted fluorescence only after target binding and subsequent cellular internalization. We designed the probe such that the low pH in the lysosome resulted in the addition of a proton to the fluorophore, which inhibited electron transfer and resulted in dequenching or activation of the fluorescence signal. This strategy uses an activatable approach to overcome the low TBR normally expected with labeled antibodies (Fig. 10c). Furthermore, the design strategy can be further refined to achieve signal activation that is reversible. Relying on the premise that damaged or dead cells cannot effectively acidify the lysosome because the ATP-consuming proton pumps are not functioning, activated fluorescent signal disappears upon cancer cell death thus enabling real-time monitoring of cellular viability (Fig. 10c).

D.2 Molecular binding specific imaging

An ideal application for molecular imaging is drug development.^{273,274} Molecular imaging of the drug provides critical information about agent pharmacokinetics and multi-plexed imaging can provide the added benefit of real-time imaging of probe binding. Thus far, imaging of drug–target binding has not been feasible with conventional strategies, but the implementation of a thoughtful multimodal approach may make this possible in the near future. In order to achieve real-time binding we must know: the absolute amount of probe in the local area and the component of the signal that arises from the bound fraction. Two different approaches are proposed. 1. Bi-modal probes with pharmacokinetic imaging capabilities using “always on” labeling and probe-binding imaging capabilities using “activatable” labeling. 2. Dual-signaling for probe density and binding activation from a single labeled probe.

The creation of an effective bi-modal probe with pharmacokinetic imaging capabilities using “always on” labeling and imaging using “activatable” labeling requires a multifaceted design strategy. If we select cancer-specific humanized monoclonal antibodies for target tissue binding (due to high binding affinities) and utilize an imaging modality that is easy to quantitate this method could be used to monitor a drug. To achieve this, the radiometal (¹¹¹In) can be conjugated to the targeting antibody *via* a chelate. To achieve the other

endpoint of detecting the binding of the probe, an “activatable” ICG is an ideal choice as it has the unique property of losing fluorescence when it is covalently bound to an antibody. However, the signal is then activated by cellular processing after target-specific binding and internalization as described above. This quality is useful in designing an antibody-based activatable probe which is irreversible.²⁷⁵ Therefore, *in vivo* multimodality imaging can be used to achieve target-specific imaging with optical probes while also investigating the probe pharmacokinetics and biodistribution with nuclear imaging (Fig. 11a). This bi-modal strategy provides data regarding both the absolute local amount of probe and the bound fraction.²⁷⁶

The other multiplexed approach for imaging probe concentration and molecular specific binding is multi-signaling fluorescence. This strategy relies on targeting with antibodies but utilizes two different aspects of fluorescence signal—intensity and lifetime—to derive imaging information. For instance, when AlexaFluor750 is conjugated to an anti-HER2 antibody, it will yield signal related to the fluorophore concentration. Independently, fluorescence lifetime measurements will depend on microenvironmental conditions of the fluorophore: such as solvent, temperature, pH, and oxygen content. Bearing in mind that target-specific binding and subsequent cellular processing change the microenvironment of the fluorophore, it is not unexpected that there are changes in the fluorescence lifetime signal; thus, this parameter can be used to determine real-time probe binding. Using an AlexaFluor750–anti-HER2 antibody conjugate, fluorescence lifetime measurements could distinguish the bound and unbound fraction *in vitro*.²⁷⁷ Since fluorescence lifetime is unrelated to fluorophore concentration and light scattering, this dual-signal detection imaging technique provides information regarding probe concentration and cell specific binding (Fig. 11b).

E. Conclusion

Conventional imaging methods have revolutionized medicine, yet the next advance will involve overcoming inherent limitations of individual modalities, such as poor spatial resolution or low sensitivity. Multiplexed imaging aims to advance imaging by strategically combining existing technologies with uniquely designed molecular probes. The ways in which imaging can be multiplexed are plentiful. Additionally, there are many aspects and approaches to designing an optimal multimodal imaging probe. Achieving effective molecular imaging, in which tissue is both detected and molecularly characterized, requires consideration of the desired objectives (real-time image guided intervention, pharmacokinetic assessment, *etc.*) as well as a strategy for achieving effective targeting and optimal TBR. To optimally achieve these endpoints, a multi-disciplinary approach that takes into account physics and engineering as well as target-specific delivery and signal production mechanisms that integrate pharmacology, biology, and chemistry is needed. Multiplexed imaging aims to provide greater sensitivity and specificity for earlier and more accurate disease detection. We hope that this review has provided useful insights into achieving these endpoints.

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Abbreviations

MR	Magnetic Resonance
TBR	Tumor to Background Ratio
PET	Positron Emission Tomography
CT	Computerized Tomography
NIR	Near InfraRed
CCD	Charge Coupled Device
QD	Quantum Dot
VEGF	Vascular Endothelial Growth Factor
IO	Iron Oxide
RES	ReticuloEndothelial System
EPR	Enhanced Permeation and Retention
CLI	Cerenkov Luminescence Imaging
CR	Cerenkov Radiation
NP	NanoParticles
UCL	UpConversion Luminescence
SPECT	Single-Photon Emission Computerized Tomography
DOTA	1,4,7,10-tetraazacycloDodecane-1,4,7,10-Tetraacetic Acid
DTPA	DiethyleneTriaminePentaacetic Acid
CEST	Chemical Exchange Saturation Transfer
DWI	Diffusion-Weighted Imaging
FDG	FluoroDeoxyGlucose
FLT	FLuoroThymidine
FRET	Förster Resonance Energy Transfer
EGF	Epidermal Growth Factor

HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Occupied Molecular Orbital
PeT	Photon-induced electron Transfer
CDR	Complimentarily Determining Regions
ICG	IndoCyanine Green
HER2	Human Epidermal growth factor Receptor type-2

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Fig. 1. Schematic explanation of multiplexed imaging technologies based on the electromagnetic waves of different wavelengths.

Fig. 2.

A schema of the rational strategies for target specific imaging in all physical levels.

Fig. 3.

A schema of the *in vivo* NIR window and the extinction coefficient value of water, oxy- and deoxy-hemoglobin are plotted ranging from visible to near infrared wavelength.

Fig. 4.

Multi-color and quantitative lymphatic images using a nuclear and NIR optical dual labeled imaging agent are shown. As depicted in this schema, generation-6 PAMAM dendrimer-based dual labeled contrast agents of approximately 10 nm in diameter are employed. A gamma scintigram camera shows quantitative lymphatic drainage and a 5-color optical image shows distinct drainages from different injection sites of agents.

Fig. 5.

MR and NIR optical sentinel lymph node imaging of breast cancer in a mouse are shown. As depicted in this schema, generation-6 PAMAM dendrimer-based dual labeled contrast agents of approximately 10 nm in diameter containing 172 Gd ions and 2 Cy5.5 fluorophores are employed. Both MRI and NIR optical images define neck (yellow arrow) and axillary (red arrow) lymph nodes as sentinel lymph node of this breast cancer.

Fig. 6.

Fluorescence intensity and lifetime multi-signal images of a HER2-positive (3T3/HER2) and negative (Blab/3T3) tumors' bearing mouse 2 days after injection with trastuzumab–Alexa680 fluorescent antibody are shown. The HER2-negative tumor still shows high fluorescence intensity due to superior EPR effect. In contrast, the HER2-positive tumor shows elongation of fluorescence lifetime due to specific binding of antibodies to antigens on the cell surface.

Target-specific imaging probe design

Fig. 7.

A schema of the molecular design for target-specific activatable imaging probes.

Fig. 8. Schemas explanation of blood clearance and tumor accumulation of antibody and genetically modified antibody fragments of various sizes are shown.

Fig. 9. Schematic explanation of activation mechanisms based on the photo-chemical reactions; a. FRET, b. H-dimer formation, c. photon-induced electron transfer (PeT), d. caged fluorophore.

Fig. 10.

Two practical examples for cancer-cell specific imaging using combinations of proposed technologies; multi-color imaging (a, 2-color; b, 3-color), and reversible pH-activatable imaging using a target-cell activatable imaging probe. (a and b) Multi-color imaging targeting HER1, HER2, and CD25 using Cy5-, Cy7-, and Alexa700-labeled respective specific antibodies; cetuximab (anti-HER1), trastuzumab (anti-HER2), and daclizumab (anti-CD25) clearly shows different specific target molecules, which were expressed by distinct cancers, in different colors shown in pink, yellow, and blue, respectively. (c) A reversible activatable imaging probe of pH-sensitive trastuzumab-dimethyl-phenyl-

BODIPY conjugate cannot only depict receptor-positive tiny target tumor metastasis (green) but also monitor the therapeutic effect of anti-cancer therapy in real time.

Fig. 11.

Two practical examples for visualizing target-binding of imaging probes using combinations of proposed technologies; dual-modality imaging using an always on ^{111}In and activatable ICG optical dual-labeling trastuzumab antibody (a), and Dual-signaling tumor imaging of trastuzumab–Alexa750 conjugate employing fluorescence intensity and lifetime technologies (b). Both methods simultaneously show distribution of imaging probes with always on gamma-ray (^{111}In ; a) or Alexa750 signal (b) as well as binding specific-image with activatable ICG (a) or elongated fluorescence lifetime of Alexa750 (b).