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Molecular Imaging: Current Status and Emerging Strategies

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Abstract

In vivo molecular imaging has a great potential to impact medicine by detecting diseases in early stages (screening), identifying extent of disease, selecting disease- and patient-specific therapeutic treatment (personalized medicine), applying a directed or targeted therapy, and measuring molecular-specific effects of treatment. Current clinical molecular imaging approaches primarily use PET- or SPECT-based techniques. In ongoing preclinical research novel molecular targets of different diseases are identified and, sophisticated and multifunctional contrast agents for imaging these molecular targets are developed along with new technologies and instrumentation for multimodality molecular imaging. Contrast-enhanced molecular ultrasound with molecularly-targeted contrast microbubbles is explored as a clinically translatable molecular imaging strategy for screening, diagnosing, and monitoring diseases at the molecular level. Optical imaging with fluorescent molecular probes and ultrasound imaging with molecularly-targeted microbubbles are attractive strategies since they provide real-time imaging, are relatively inexpensive, produce images with high spatial resolution, and do not involve exposure to ionizing irradiation. Raman spectroscopy/microscopy has emerged as a molecular optical imaging strategy for ultrasensitive detection of multiple biomolecules/biochemicals with both *in vivo* and *ex vivo* versatility. Photoacoustic imaging is a hybrid of optical and ultrasound modalities involving optically-excitable molecularly-targeted contrast agents and quantitative detection of resulting oscillatory contrast agent movement with ultrasound. Current preclinical findings and advances in instrumentation such as endoscopes and microcatheters suggest that these molecular imaging modalities have numerous clinical applications and will be translated into clinical use in the near future.

INTRODUCTION

Molecular imaging is defined as the ability to visualize and quantitatively measure the function of biological and cellular processes *in vivo*.[1, 2] While anatomical imaging plays a major role in medical imaging for diagnosis, surgical guidance/followup, and treatment monitoring, the rapidly evolving field of molecular imaging promises improvements in specificity and quantitation for screening and early diagnosis, focused and personalized therapy, and earlier treatment follow-up. The main advantage of *in vivo* molecular imaging is its ability to characterize pathologies of diseased tissues without invasive biopsies or surgical procedures, and with this information in hand, a more personalized treatment planning regimen can be applied. For example, recent strategies for treatment of breast cancer involve combinations of several chemotherapeutic drugs that target epidermal growth

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factor receptor types I and 2 (EGFR and HER2/neu), mammalian target of rapamycin (mTor), estrogen receptor, and/or histone deacetylase, among others; however, the most effective strategy is dependent on the molecular profile of the tumor (e.g., HER2/neu-targeted therapy is only effective in HER2-positive breast cancers).[3] *In vivo* molecular imaging can be used to identify and quantify the molecular marker profile (e.g., EGFR, HER2) of the tumor without the invasiveness of a surgical biopsy and time associated with pathological characterization. The personalized medicine approach is especially important for determining the best care for patients with advanced stage cancers and poor prognosis - in this case, the risk of exposure to unwanted side-effects of therapy may outweigh the quality of remaining life.

Recent preclinical advances in molecular imaging contrast agents have demonstrated the ability to multiplex nano- and/or microparticles with several entities (Figure 1): 1) a molecule for targeting to a specific tissue/disease marker (binding ligand); 2) a molecule that allows detection of the agent with different imaging modalities; and, 3) a direct attachment or system (e.g., Doxel is a liposome encapsulation of doxirubicin, a cytotoxic drug which inhibits DNA replication), for targeted delivery of a therapeutic drug at the site of interest. For example, Blanco *et al.*[4] describe the direct attachment of the chemotherapy drug, Doxirubicin, to a superparamagnetic iron oxide (SPIO) nanoparticle, which is then encapsulated in liposomes coated with RGD-peptides; thus, these particles specifically attach to tumor angiogenic vessels expressing high levels of $\alpha_V\beta_3$-integrins (protein receptors which bind RGD peptides), and the localization of these magnetic particles can be visualized using magnetic resonance imaging (MRI).

In addition, molecular imaging can be used to measure the response to therapy. Current practices in measuring tumor response to chemotherapy are governed primarily by the Response Evaluation Criteria in Solid Tumors (RECIST) approach, which uses anatomical imaging methods such as computed tomography (CT) or magnetic resonance imaging (MRI) to measure changes in tumor size; however, measurable effects of therapy on tumor volume may take considerable time (weeks to months), indicating that tumor volumetric changes are not an accurate reflection of therapeutic efficacy for some therapies.[5] Molecular imaging has the potential to improve therapeutic monitoring by for example measuring the direct effect of a drug at an earlier time point before overt morphological-anatomical changes become visible on imaging. Most chemotherapeutic/anti-cancer drugs are either directed at specific molecular targets such as epidermal growth factor receptor (EGFR; drugs include erlotinib, cetuximab, and gefitnib), VEGFR (drugs include bevacizumab, sunitinib, axitinib, and vatalanib), estrogen receptor (such as tamoxifen), and EGFR type 2 (also known as ErbB2 or HER2/neu; drug such as trastuzamab), or, they are cytotoxic (drugs include paclitaxel/taxol, fluoruracil, or gemcitabine, among others) to promote tumor cell death. Molecular imaging agents have been designed and tested preclinically in rodent models to image all of the aforementioned molecular targets as well as cellular events such as metabolic activity or apoptosis [6] and, therefore, may be used in the future to monitor treatment effect at the molecular level at earlier time points after treatment initiation than with current imaging strategies.

This article reviews current clinical practices of molecular imaging and highlights promising strategies using optical and acoustic techniques that may be translated into clinical applications in the near future.

Current Clinical Molecular Imaging Strategies

Various imaging modalities are used for medical imaging, including positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), ultrasound (US), and

computed tomography (CT) (Table 1). The majority of molecular imaging in the clinic is currently performed only with PET, SPECT, and MRS imaging. Several PET (Table 2) and SPECT (Table 3) radiotracers are used for medical imaging applications, including oncology, cardiology, and neurology, and are discussed in detail elsewhere (YY et al. and ZZ et al. for PET; AA et al. for SPECT in this issue). MRS is a technique of MRI that measures changes in proton/nuclei excitation/relaxation associated with various metabolites, such as choline, pyruvate, lactate, lipids, and polyamines, among others.[7, 8] Several MRS techniques, including ^{1}H, ^{19}F, ^{31}P, and ^{13}C MRS, have been developed and are reviewed elsewhere (see BB et al. in this issue, and reviews[9, 10]). Clinical applications of MRS include oncology,[9] neurology,[8] and musculoskeletal diseases,[11] among others (Table 4).

Current clinical applications of real-time *in vivo* optical imaging techniques are limited to surface (e.g., skin[12, 13]) or ocular[14, 15] imaging since they suffer from limited depth penetration through human tissue (Tables 1 and 4). However, increasing technological advances in endoscopic (e.g., monitoring Barrett's esophagus) and catheter devices (e.g., imaging of atherosclerosis or bladder cancer) for optical coherence tomography(OCT)[15, 16] as well as microscopy hold promise for novel clinical applications (e.g., Wang et al. [17] used a confocal microendoscope with topically-administered fluorescein to image abnormal lesions and colonic pathology THIS IS NOT A CLINICAL TERM – PLEASE WRITE WHAT COLONIC PATHOLGOIES THEY WERE ADDRESSING in patients undergoing colonoscopy). Furthermore, a multi-photon NIRF source, where two or more photons are used to excite the fluorescent dye/nanoparticle, has been integrated in a tomographical scanner and microendoscope; this approach has been used for clinical optical imaging of skin cancer and other dermatological pathologies.[18]

Most of these devices operate by applying photons for excitation, and measuring reflected light. Alternatively, detection can occur by measuring light scattering effects, as in change of energy before and after the photon collides with a molecule – known commonly as the Raman effect[19] (described in detail below). Since the change in energy is dependent upon the strength of the molecular bond which is colliding with the photon, the Raman signal is a series of peaks representing a specific molecular bond.[19, 20] Thus, Raman spectrophotometry is an emerging molecular imaging technique that can acquire multiple molecular signatures with a single image. Raman spectroscopy and other optical imaging techniques have been used in a few clinical applications; however, they are limited in number since fluorescent-based and Raman-spectra contrast agents, including near-infrared fluorescent (NIRF) (advantageous for deeper penetration and low background fluorescence[12]) dyes, quantum dots (NIRF nanoparticles that are very bright and have long life-span[12]), and nanoparticles with surface enhanced Raman scattering (SERS) properties,[20] have not yet been fully evaluated for human use. So far, Raman spectroscopy for analysis of different molecular signatures has been used in the clinic for identifying atherosclerosis [21] as well as for cancer imaging (e.g., breast,[22] colon[23]). Clinical applications of optical imaging are summarized in Table 4 and include: 1) monitoring atherosclerosis-associated inflammation with protease-activated fluorescent probes (representing capthesin-B and matrix metalloprotease (MMP)-2/9 expression);[24] and, 2) imaging of porphyrin (fluoresces blue light) accumulation in highly-proliferating cancer cells. Therefore, agents that can be chemically converted to porphyrin in cells can be added to "highlight" neoplastic cells. For preclinical applications, optical imaging is frequently used for assessment of many molecular contrast agents, drug testing, and for better understanding basic biological processes. However, clinical translation of the large array of preclinical optical molecular imaging strategies (discussed below) will require significant improvements in instrumentation, contrast agent evaluation, and data analysis for molecular quantification. Contrast-enhanced molecular ultrasound is a very attractive molecular imaging strategy since ultrasound imaging 1) is already a clinical imaging modality; 2) is relatively

inexpensive and portable; 3) offers real-time, high resolution imaging; 4) can separate contrast and morphological imaging (with use of harmonics); and, 5) does not involve ionizing irradiation (Table 1). Contrast agents for current use with ultrasound are microspheres – gas-filled (e.g., perfluorobutane), lipid-shelled bubbles that are 1–4 μm in diameter (see Deshpande et al. in this issue). Microbubbles have been used for imaging primarily the micro- and macrovasculature,[25] since their micron size limits them to vascular compartments. Several commercially available microbubbles include: Luminity®/Definity® (Bristol Myers Squibb), Optison™ (GE Healthcare), Sonovue® (Bracco), and Sonozoid™ (Amersham Health).[26] A common clinical application of contrast-enhanced ultrasound imaging with microbubbles involve characterization of focal lesions (e.g. in the liver) based on vascular enhancement patterns using non-targeted microbubbles.[27, 28] Currently, contrast-enhanced ultrasound is not yet advanced to imaging molecular markers in the clinical realm, although ongoing research is directed towards clinical translation of molecularly targeted ultrasound imaging.[29, 30]

In summary, current clinical molecular imaging is mostly performed with PET and SPECT, and several targeted radiopharmaceuticals for both imaging and dual-imaging/therapy are available. Optical, ultrasound, and other hybrid acoustic imaging strategies (e.g., photoacoustic imaging) offer real-time and inexpensive approaches, which may be well suited for routine clinical applications such as early disease detection and screening protocols involving frequent imaging. The following section reviews emerging preclinical developments in optical, ultrasound, and hybrid acoustic imaging.

PRECLINICAL DEVELOPMENTS IN MOLECULAR IMAGING AND POTENTIAL CLINICAL APPLICATIONS

Preclinical molecular imaging in small animals is an invaluable part of evaluating new molecular targets and contrast agents, as well as developing drugs prior to clinical translation.[31, 32] Figure 2 shows the time intensive and expensive preclinical steps involved in molecular target identification, validation, chemical synthesis, and characterization (*in vitro* and *in vivo* testing for activity, specificity, biodistribution, pharmacokinetics, off-target effects, toxicity, etc) for new molecular imaging agents. In fact, the majority of current molecular imaging agents used in the clinic were discovered through these exhaustive preclinical experiments at academic institutions.[32] It is estimated that a molecular imaging agent costs about $150 million over 10 years to create, test, and move to the clinic, ending with a $200–400 million per year revenue for successful contrast agents.[32] The first step(s) for identifying a molecular target begins with understanding and characterizing the biology to find the differences between a healthy and diseased state. For example, since there is an intricate relationship between inflammation and cancer (i.e., chronic inflammation can often promote cancer and cancer onset can promote an inflammatory response), the differences between inflamed and cancer states must be characterized. In general, much focus is directed towards cancer imaging, and several preclinical studies have identified new molecular targets for imaging cancer (Table 5). In addition to imaging the cancer phenotype such as increases in metabolism, proliferation, angiogenesis, hypoxia, and apoptosis, agents have been developed to target specific protein markers expressed on cancer cells or cancer-associated cells (e.g., tumor angiogenic vessels, stroma). These include tumor cell receptors (EGFR, HER2/neu, ER, folate receptor, somatostatin receptor, VEGFR2, urokinase-plasminogen activator (uPA)/receptor (uPAR), among others), integrins, proteases, and prostate-specific membrane antigen (PSMA) (Table 5 and see BB et al. in this issue), among others. Notably, many chemotherapeutic drugs also target these markers, and have been radiolabelled for assessment of biodistribution and pharmacokinetics using non-invasive molecular imaging (Table 5).[31] Continuing preclinical research has not only exploded in molecular target discovery and imaging probe developments, but also in new strategies for

imaging methodologies, especially in the areas of optical and acoustic imaging. With the advent of new, smaller instruments/devices for insertion into the body, molecular imaging strategies with optical and acoustic devices and specific molecular-targeted contrast agents have great potential for translation into the clinic (Figure 3), which is reviewed in the following sections.

Optical Molecular Imaging

A plethora of optical-based molecular imaging techniques are used in preclinical research for evaluating molecular targets of contrast agents (Table 5) and/or of therapeutics as well as characterizing and understanding biology.[16] Although optical imaging currently is not used in many clinical applications, there are several emerging technologies that foster clinical translation.[15] In terms of novel contrast agent development for optical imaging, Weissleder and colleagues pioneered NIRF-protease-activatible probes for imaging cancer and inflammation (e.g., in colon[33, 34] and lung cancer,[35] as well as in atherosclerosis[36]). These "smart" probes involve a dye-quencher system where the fluorescent dye is connected to a quenching molecule by a short linker peptide. When proteases cleave the peptide, the dye and quencher molecules separate by a distance > 100 Å, and the dye transfers energy to the quencher resulting in a release of light.[37] The "smart" probe strategy has great potential for clinical translation since light is only released when the probe has reached its target and is activated; thus, high signal-to-noise ratios can be obtained due to low background signal and minimal non-specific enzymatic cleavage, which is of paramount importance for imaging weak optical imaging signals in the human body.[38] On the microscale device end, optical imaging is progressing towards integrating confocal microscopy with endoscopes and catheters for real-time "biopsies".[16] Contag and colleagues have developed techniques for early detection of colon cancer using a confocal endomicroscope and a fluorescein-conjugated heptapeptide (VRPMPLQ), which bound to dysplastic colonocytes with high affinity, resulting in a sensitivity of 81% and specificity of 82% for detection of colonic neoplasia in patients undergoing colonoscopy.[39] These confocal microscopy techniques with cancer-specific optical probes can be used for *in vivo* early detection of various cancers in the clinic, including, skin, colon, bladder, prostate, and esophageal cancer, among others.[16] Real-time confocal microscopes can also assist in *ex vivo* analyses of tissue biopsies, such as instantaneous quantification of HER2/neu expression in human breast tumors with 3D microscopy and fluorophore-conjugated anti-HER2/neu antibodies.[40]

Another area of optical imaging that is emerging as a promising tool for clinical optical imaging is Raman spectroscopy/microscopy, which measures inelastic light scattering effects to determine molecular signatures.[20] Optical energy in the form of lasers (typically near-infrared range for increased penetration depth) is applied in pulses resulting in an excitation of molecules to an elevated energy level (laser "on" – absorbing a photon) and a relaxation to a new energy level (laser "off" – releasing a photon); this shift in energy is related to the vibrational energy of a molecular bond, and "fingerprints" of molecules can be created by measuring Raman shifts.[19] The vibrational component is a measure of the changing shape of the electron cloud during the shift ("on"/"off" or molecule excitement/ relaxation). For an example of molecular Raman imaging, the specific Raman shift or "fingerprint" of carbon nanotubes is known as G-band ($\sim$1593 cm^{-1}), and carbon nanotubes conjugated to RGD peptides have been used for *in vivo* molecular Raman spectrographic imaging of human glioma tumor xenografts in mice.[41] The most commonly used methods of Raman spectroscopy/microscopy are Surface-enhanced Raman Scattering (SERS) and Coherence Anti-Stokes Raman Scattering (CARS); both SERS and CARS involve methods to obtain a higher Raman signal. [20, 42] The SERS technique involves adding metal nanoparticles (e.g., gold[43]), which absorb the optical energy and create an enhanced Raman signal whereby the metal surface can transfer energy to nearby molecules, and also exhibit

an excited energy (due to metal surface ions absorbing and releasing photons). Like carbon nanotubes, SERS nanoparticles have unique Raman "fingerprints"; however, the Raman signal enhancement due to the localized interactions with the metal surface of the nanoparticle enable picomolar sensitivity, which is ideal for *in vivo* imaging applications. Furthermore, labeling several different SERS nanoparticles with molecular ligands (Figure 1) can provide a measurement of multiple molecules with a single Raman image.[43] CARS is a nonlinear method which involves applying two or more photons to 1) excite the molecule from the ground state to an excited state (higher energy level), and then 2) the second photon can excite the molecule from its relaxed state (i.e., energy level after releasing photon during "off" pulse after first photon absorption) to a new higher energy level; this second tier of vibrational energy is typically ~5 times stronger than Raman signal after only one photon pulse. The CARS technique is often used for high resolution, 3-dimensional microscopy; the advantages are that there is no need for fluorescent markers to label molecules and images can be acquired relatively quickly.[44] Several preclinical studies (Table 5) have utilized SERS (Figure 4) or CARS techniques for *in vivo* molecular imaging of cell receptors (e.g., RGD-carbon nanotubes that bind to $\alpha_V\beta_3$ integrin-expressing tumors/tumor microvessels[45]), enzyme activity,[46] changes in pH,[20, 47] lipid composition,[48] and myelin composition.[49]

Acoustic Molecular Imaging

While contrast-enhanced ultrasound is gaining popularity and support for a variety of clinical applications in both cardiology and radiology [28, 50–55] (Table 1), preclinical research is focused on improving this technology to a molecular-based approach. Microbubbles can be molecularly targeted to disease-specific markers expressed on tissue vasculature (Deshpande et al. in this issue and [56]), such as microvessels in tumors or inflamed tissues. Most preclinical molecular ultrasound imaging studies utilize microbubbles that have a streptavidin, avidin, or biotin moiety incorporated into the lipid shell via a polyethylene glycol (PEG) arm for conjugation of an antibody via a strept(avidin)-biotin chemistry (Figure 5). However, strept(avidin) is immunogenic, and therefore, these microbubbles cannot be used in humans.[50, 57] Nonetheless, the wide availability of antibodies and ease of conjugation to microbubbles provide a basis for proof-of-principle pre-clinical studies. Several studies have utilized antibody- or peptide-biotin-strept(avidin)-conjugated microbubbles to image tumor angiogenic markers, including VEGFR2,[58–64] integrins,[59, 65], and endoglin[61] (see Deshpande et al. article in this issue; Table 5). Other studies have used molecular-targeted microbubbles to image molecular adhesion molecules overexpressed in microvessels of inflamed tissues; these include mucosal addressin cellular adhesion molecule (MadCAM1),[66] vascular cellular adhesion molecule (VCAM1),[54, 67] intracellular adhesion molecule (ICAM1)[68–70] and P-selectin[71–73] (also see Deshpande et al. in this issue). Clinical translation of molecular ultrasound imaging with microbubbles requires several important steps, including development of molecular-targeted microbubbles without the toxic strept(avidin)-biotin chemistry, implementation of quantitative software on clinical ultrasound machines, and a standardized technique for quantification of attached microbubbles (Figure 5). A novel microbubble targeted to kinase insert domain receptor (KDR), the human analog of VEGFR2, was recently developed by fusing a heteropeptide (found to bind KDR with high affinity) to a hydrophilic spacer and lipid to form a heterolipopeptide for attachment to the PEG arm of the microbubble shell (Figure 5).[29, 30] Preclinical evaluation of this KDR-targeted microbubble demonstrated cross-reactivity with mouse VEGFR2, and the ability to monitor anti-angiogenic therapy in human colon tumor-bearing mice,[30] providing the groundwork for a translation of this contrast microbubble into future clinical applications, such as monitoring anti-angiogenic cancer therapy.

Hybrid Molecular Acoustic Imaging: Energy in, and sound out

The photoacoustic (PA) effect was first described in 1880 by Alexander Graham Bell,[74, 75] who noted that illuminated objects emit sound waves; however, the application of the PA effect in biomedicine has not taken shape until the last decade.[75, 76] The main advantage of this molecular imaging technique is that a wide variety of contrast agents - from small molecules (e.g., dyes[77]) to nanoparticles (e.g, SWNT[45]) - can be utilized for attachment to molecular ligands (Figure 1) provided that they are highly light absorbent (Figure 6).[75] Nanoparticles and small molecules are very stable in the body; have the ability to extravasate leaky vessels; bind to targets in high densities due to their small size; and, the unbound fraction can clear from circulation relatively rapidly.[78] Furthermore, recent developments have shown that various forms of energy, including optical sources (e.g., lasers; conventional PA; also called optoacoustics),[75] radiofrequency waves (RF; also called thermoacoustics),[75] microwaves,[75, 79] and magnetic field pulses (for detection of magnetic nanoparticles with ultrasound; also called magnetoacoustics),[80, 81] can be used to result in contrast agent "activation" – meaning, the energy input heats the contrast agent causing thermal expansion and increased acoustic pressure.[75] Short pulses of energy are applied to result in time variant pressure changes, and higher signal to noise ratios.[75]

PA sensing instruments include microscopy (PAM), tomography (PAT, or thermoacoustic tomography (TAT)), spectroscopy (PAS), and flow cytometry (PAFC)[82] for high-resolution molecular imaging at surfaces, in circulation (e.g., contrast agents in blood flow (PA Doppler)[83, 84] or cells labeled with contrast agents in blood[82]), and in deep-tissues;[75] thus, PA molecular imaging techniques are promising for a wide range of micro- and macro-scaled applications (Figure 6). Strategies for detection of sound waves can be acoustic-based (e.g., use of ultrasound transducers[75]) or optical-based (e.g., a device that measures changes in thickness of an optical film as a result of acoustic pressures generated by PA[85]) and are reviewed elsewhere.[75] In addition to measuring signal from activated contrast agents, PA imaging can also be used to measure local temperature (by relating PA pressure to temperature with high, sub-degree sensitivity[86, 87]) and chemical environments, such as oxygenation[88] and pH.[89] Wang et al.[76] describes several potential applications of PA molecular imaging for clinical translation, including: 1) real-time PAM imaging as complimentary to optical-based microscopy methods (mentioned above); 2) real-time PAM imaging and/or PAM-Doppler (blood flow imaging – see Fang et al.[83]) imaging of melanoma; 3) PA endoscopy for more sensitive and early detection of gastrointestinal cancer; 4) cancer detection at the molecular level (e.g., RGD-targeted SWNTs for detection of vascularized gliomas[45]); 5) high-resolution PAT imaging of reporter genes; 6) PA Doppler flow measurements of blood velocity (more sensitive than conventional Doppler US, which suffers from noise artifacts generated by red blood cells); 7) PAT imaging of blood oxygenation; 8) real-time PAT staging of breast cancer with sentinel lymph node mapping; 9) Multiscale (i.e., both microscopic and macroscopic; Figure 6) *in vivo* imaging (compared with PET, SPECT, MRI, CT, and US, which are only used for macroscopic imaging); 10) PAT and/or TAT for high-resolution and high-specificity breast cancer diagnosis/screening to replace x-ray mammography; 11) PAT and/or TAT for functional brain imaging; and, 12) RF-TAT molecular imaging once specific RF-activated contrast agents are developed. Infinitely more applications are possible with PAM, PAT, and TAT, as more sophisticated device technology and contrast agents are developed. As with ultrasound transducers for non-invasive (abdominal, transcranial, breast) and more invasive (transvaginal, endoscopic, intravascular) imaging strategies, PAT/TAT imaging devices equipped with both contrast agent activation (e.g., optical or electromagnetic source) and acoustic pressure measurement can access most tissue. Various types of nanoparticles, including nano-rods, -cages, -spheres, -tubes, -shells, and other nanoparticles consisting of gold,[78] iron oxide,[80, 81], cobalt,[90] silica (e.g., photonic explorers for bioanalysis with

biologically localized embedding (PEBBLES)[91, 92] filled with near-infrared-red absorbing dyes, such as the FDA-approved dye, iodocyanine green (ICG); see Yang et al.[93] for review) or carbon,[45] have been tested preclinically as PA imaging contrast agents (Table 5). Furthermore, they can be easily conjugated to targeting moieties (e.g., antibodies, peptides, small molecules) for molecular imaging as well as drugs for targeted therapy.[75, 93, 94]

The advantages of PA imaging are numerous: 1) target-specific signal can be obtained without interference from background tissues (similar in respect to PET modality), and can be overlaid on top of an ultrasound anatomical image for cross-referencing with morphology (similar to PET signal overlaid on CT); 2) does not involve ionizing irradiation; 3) can visualize targeted nanoparticles with high sensitivity and resolution; and, 4) can image both on the macroscopic (with PAT imaging) and microscopic (with PAM imaging and other molecular detection methods including PA flow cytometery and spectrophotometry) levels. Additionally, PAT imaging has similar advantages to US imaging (Table 1) in that it can provide real-time, inexpensive, and quantitative molecular imaging at high resolution. This preclinical molecular imaging approach has a large potential; however, several steps are needed to fully translate PA imaging techniques (not listed in order). First, PA imaging detection hardware and computation software for real-time imaging must be integrated with clinical imaging systems. Most current preclinical devices are home-made and designed for small animal imaging; therefore, future work should focus on integrating this technology on a clinically relevant device and establishing proof-of-principle studies in small animals (for example, as for molecular ultrasound imaging as shown in Figure 5, where a clinical ultrasound machine was used to image breast cancer-bearing mice). Secondly, nanoparticles must be fully characterized for toxicity, biodistribution, and pharmacokinetics[94] to take full advantage of the highly specific and sensitive molecular imaging tool that PA represents. Thirdly, further developments in contrast agent "activation" by RF, microwaves, and magnetic fields can extend the applications limited by the minimal depth penetration of optical sources. Currently a few in-human studies have used PA techniques: 1) PA spectroscopy to measure oxygenation levels in hyper- and hypo-ventilating healthy volunteers;[95] 2) PA flow cytometry to quantify circulating melanoma cells in a stage IV melanoma patient's blood sample;[96] and 3) *in vivo* PAT imaging to examine human breast tumors to visualize tumor vascularity based on optical contrast characteristic of breast tissue.[97, 98] Further developments in instrumentation and evaluation of contrast agents for photoacoustic imaging will move toward a molecular approach in the clinic.

CONCLUSIONS

Molecular imaging can be applied to all avenues of medical imaging: early detection/ screening, diagnosis, therapy delivery/monitoring, and treatment follow-up. The current status of clinical molecular imaging is limited, with most current applications using PET and SPECT imaging, and a small number of highly specific applications for MRI/MRS, optical, and ultrasound. Current demands and trends are calling for new strategies to focus on early disease detection through improved imaging and screening protocols, as well as patient-specific treatment selection (personalized medicine), delivery (possibly through targeted therapy), and therapy-specific (i.e., therapeutic target) monitoring. It is hoped that these new strategies of early diagnosis and immediate treatment monitoring will improve success rates for curing diseases with high mortality rates such as cardiovascular disease and cancer, as well as providing more specific treatment for other diseases (for example, neurological disorders such as Alzheimers and/or Parkinsons). Preclinical research has resulted in the identification of a large number of molecular targets and the development of novel molecular imaging contrast agents as well as device, hardware, and software technologies. It is expected that molecular imaging with imaging modalities other than PET and SPECT,

including MRI/MRS, optical (Raman), molecular ultrasound and photoacoustic tomography, will be integrated into more frequent clinical use in the near future.

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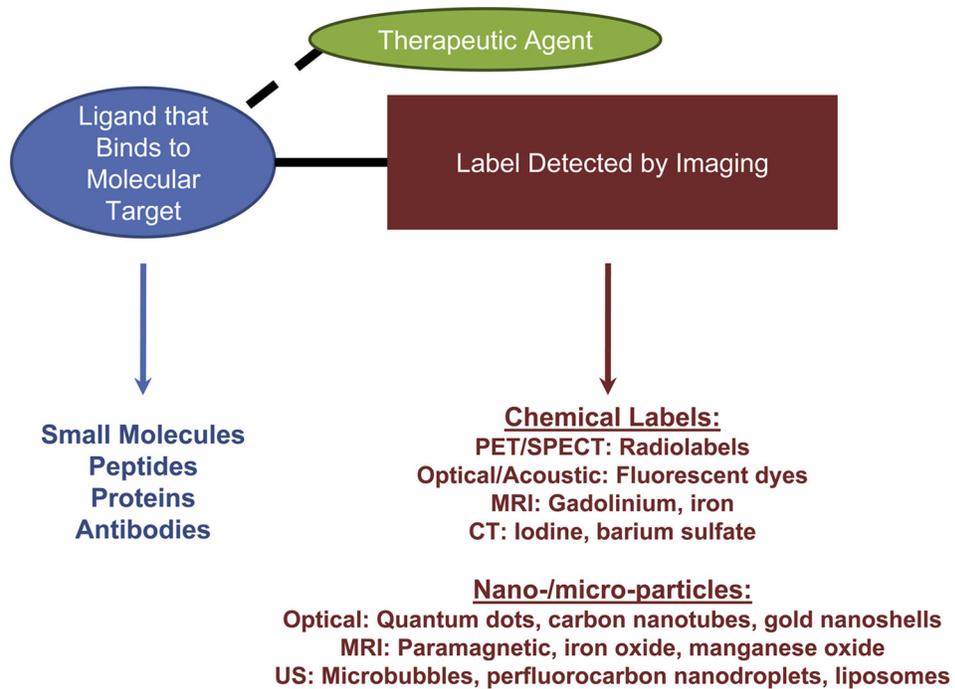
Figure 1.
Contrast agents used for molecular imaging are composed of at least 2 entities: one component such as an antibody, peptide, nucleic acid, or a small molecule for binding to the molecular target, and a label for readout by an imaging modality (see also Table 1). More sophisticated contrast agents can include multiple parts for targeting several molecules at once, as well as, several labels for multimodality imaging. Drugs can also be attached/encapsulated for targeted therapy.
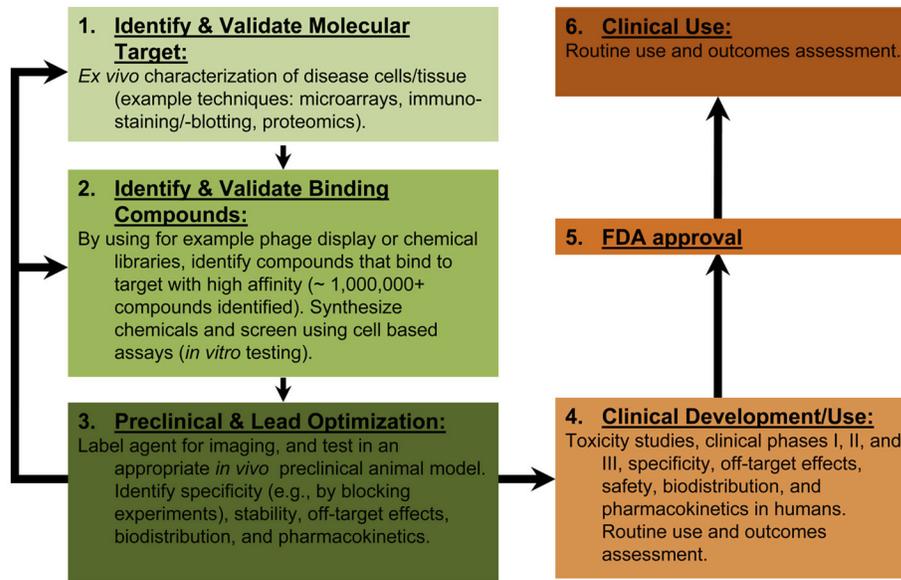
Figure 2.
Molecular contrast agent design and clinical use involve a series of steps similar to those used in drug development (Adapted from Willmann et al.[31]). Preclinical steps (green-shaded boxes) involve target identification, validation, chemical labeling, *in vitro* cellular characterization, and *in vivo* animal testing. After many optimization steps (arrows between boxes 1, 2, and 3), the agent can be tested in humans after FDA applications for investigatory new drug (IND) or exploratory IND (eIND). Clinical testing (orange boxes) involves rigorous testing for agent properties (effectiveness, specificity, toxicity, off-target effects, kinetics, etc.) before potential approval by the FDA for routine clinical use, which is also heavily monitored for outcomes assessment.
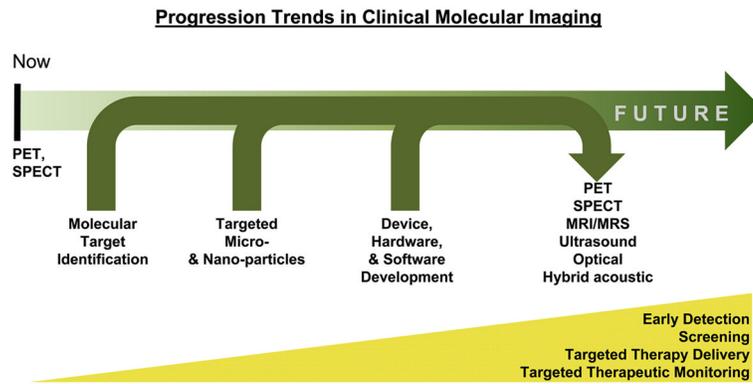
Figure 3.
Timeline representation of current and future utilization of molecular imaging in the clinic. Current approved molecular imaging techniques are mostly PET and SPECT, which are expected to continue for use in diagnosing advanced stage diseases. With a plethora of molecular targets identified preclinically for various diseases (e.g., cancer: Table 5) and advances in material engineering for nano- and micro-particle contrast agents, a large potential exists to expand clinical molecular imaging beyond nuclear medicine approaches. Molecular imaging techniques that do not expose patients to ionizing irradiation, such as MRI/MRS, optical, ultrasound and hybrid acoustic imaging approaches are ideal for early disease detection and screening. Hardware and software implementations for sensitive and quantitative detection of targeted contrast agents will also pave the way for targeted therapeutic delivery (tracking) and response monitoring. Optical, ultrasound, and hybrid acoustic imaging are expected to be forerunners in screening methodology since they can provide real-time, inexpensive, and high-resolution images.
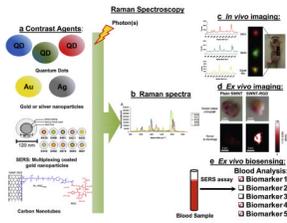
Figure 4.
Raman Spectroscopy/Microscopy techniques are an emerging molecular imaging tool for potential translation to clinical applications with endoscopes or catheters. Left to right: Nanoparticles with specific Raman signatures (*a*), including quantum dots, metal (gold (Au) or silver (Ag)) nanoparticles which can be coated for several different spectra (e.g., SERS nanoparticles such as silica coated gold nanoparticles), and carbon nanotubes/fullerenes, can be used for attaching specific moieties to bind to molecular targets (as in Figure 1 where the label is the nanoparticle). The nanoparticles (when excited with an optical laser) produce specific Raman spectra (*b*), which can be used to generate *in vivo* imaging signal (*c*), *ex vivo* imaging signal (*d*), and for *ex vivo* biosensing assays (*e*). Image in (*c*) represents *in vivo* imaging of SERS nanoparticles (S421 and S441) injected subcutaneously in nude mice, and the spectra obtained for S421, S441, and an equal mix of both S421 and S441. Reprinted with permission from Keren et al.[99] Image in (*d*) represents *ex vivo* Raman imaging of excised subcutaneous tumor xenografts that were pre-injected (in mouse prior to euthanization) with either plain, non-targeted single-walled carbon nanotubes (Plain SWNT) or SWNT's conjugated to RGD peptide (binds to $\alpha_V\beta_3$ integrin); note that RGD-targeted SWNTs show high Raman signal compared to non-targeted, plain SWNTs. SWNT structure and Raman image were reprinted with permission from de la Zerda et al.[45] Image in (*e*) represents a schematic for using Raman Spectroscopy for analyzing blood samples with nanoparticles (*a*) targeted to specific biomarkers (DNA, RNA, or proteins); this technique can be advantageous for ultrasensitive, rapid screening for early detection of cancer. Not shown: Raman imaging can also be used for resolution microscopy (e.g., CARS).
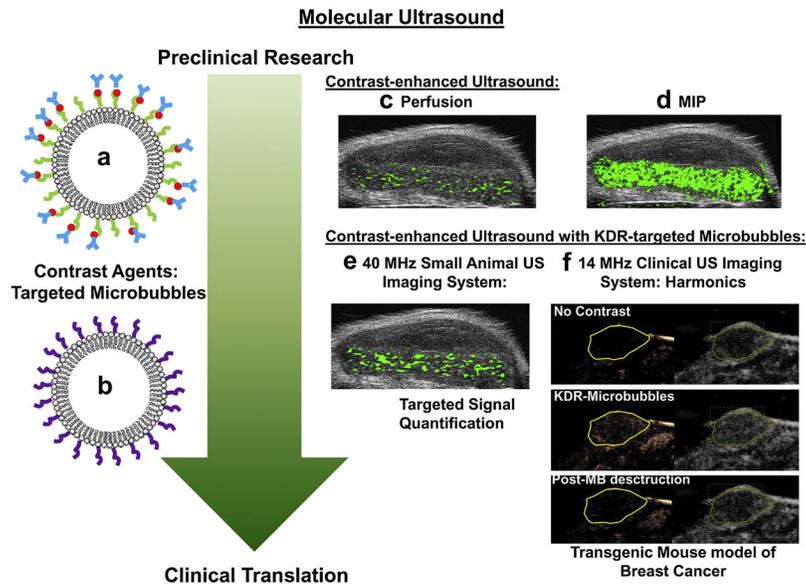
Figure 5.
Contrast-enhanced ultrasound imaging with molecularly-targeted microbubbles (MBs; molecular ultrasound imaging) is moving rapidly towards clinical translation. Left to right: Proof-of-principle preclinical studies include testing targeted MBs constructed by conjugating an antibody (blue "Y" molecule) to a MB using strept(avidin)-biotin (green line-red circle) binding chemistry (*a*) for endothelial cell target specificity and validation. Then, MBs using peptides (purple lines) with high binding affinity for the human targets can be constructed with direct conjugation to lipid MB shell (*b*), and after further rounds of preclinical testing (see Figure 2), peptide-conjugated MBs can progress to clinical testing. Functional ultrasound imaging methods including perfusion analysis with assessment of first-pass time-intensity curves (*c*) and maximum intensity persistence (MIP) (*d*) can provide levels of tissue vascularity. Molecular ultrasound imaging with KDR-targeted MBs [30] can be used to measure KDR expression levels (*e*; for more details on calculating the attached MB signal, refer to Willmann et al.[59]). (*f*) A clinical ultrasound system was used to acquire B-mode images (right side) and contrast images (see Deshpande et al. in this issue for a more detailed review of contrast imaging) of breast cancer in a transgenic mouse model.
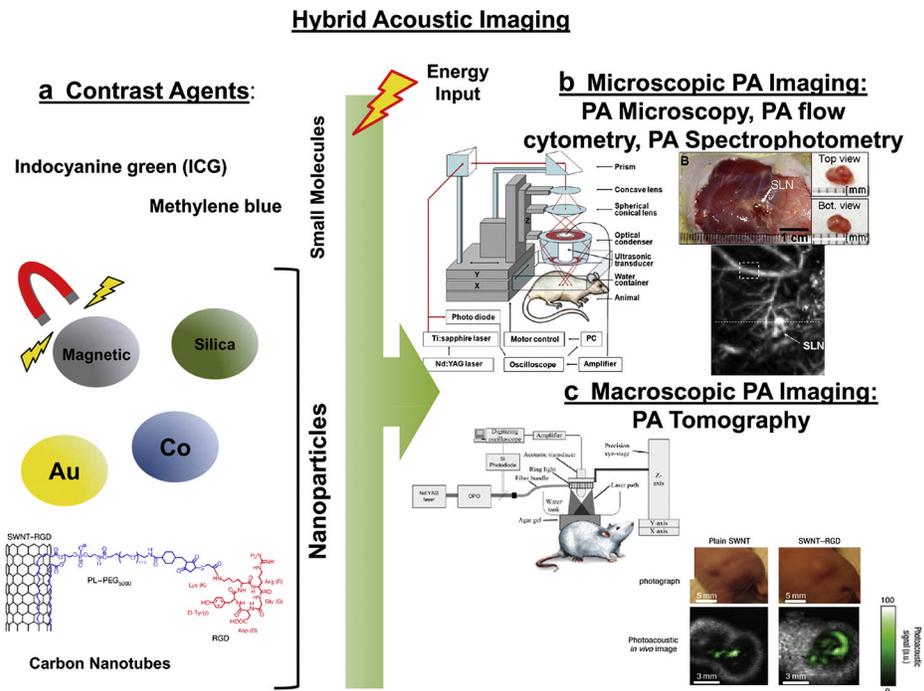
Figure 6.
Photoacoustic (PA), thermoacoustic, and magnetoacoustic imaging techniques are emerging as a highly sensitive and versatile tool for molecular imaging. Left to right: Contrast agents for use with hybrid acoustic imaging methods are optically-absorbing nanoparticles, such as small molecule dyes, nanoparticles made of silica, gold, or cobalt, and carbon nanotubes/fullerenes, for use with photoacoustic imaging; and, magnetic nanoparticles for use with magnetoacoustic imaging. Energy is applied to the nanoparticles in the form of near-infrared light, radiofrequency, microwaves, or magnetic field; this energy causes the contrast agent nanoparticles (*a*) to oscillate and produce sound waves for measurement with ultrasound. PA imaging devices have been developed for imaging at the microscale (*b*), and macroscale (*c*) levels. *In vivo* and *in vitro* micromolecular analyses can be performed with PA imaging devices such as PA microscopy, PA flow cytometry, and PA spectrophotometry (*b*). Image represents an *in vivo* PA spectrophotometry system used to image surrounding vascularity and sentinal lymph node with gold nanorods (activated with 807 nm laser) in male rats (Reprinted with permission from Song et al.[100]). Macromolecular analyses can be performed with PA tomography for *in vivo* imaging applications (*c*). Images (reprinted with permission from de la Zerda et al.[45]) represent the device setup of a PA imaging system for small animals; this system was used to generate the adjacent, lower right image of subcutaneous glioma tumor xenografts in nude mice. Plain (non-targeted)- or RGD- (targeting $\alpha_V\beta_3$ integrin) single-walled carbon nanotubes (*a*) were injected in the mouse tail vein, and PA signal (green) was imaged with B-mode US imaging (grey).

Table 1

Advantages and disadvantages of imaging modalities used with molecularly targeted or non-targeted contrast agents in a clinical setting. Adapted from references:[31, 101]

Modality	Advantages	Disadvantages	Common Contrast agents/Readout	Example Clinical Applications
CT	• Unlimited depth penetration • High spatial resolution • Whole-body imaging possible • Short acquisition time (minutes) • Moderately expensive • Anatomical imaging	• Irradiation exposure • Poor soft tissue contrast • Probably not used for molecular imaging – currently only anatomical and functional imaging	• barium • iodine • krypton • xenon	• Tumor perfusion[102]
PET	• Unlimited depth penetration • Whole-body imaging possible • Quantitative molecular imaging • Can be combined with CT or MRI for anatomical information	• Irradiation exposure • Expensive • Low spatial resolution (1–2 mm; 4–8 mm^3) • Long acquisition times (minutes to hour)	• ^{11}C • ^{18}F • ^{64}Cu • ^{68}Ga	• ^{18}F-FDG-PET for cancer staging [103] • Diagnosis of various diseases (see Table 2)
SPECT	• Unlimited depth penetration • Whole-body imaging possible • Quantitative molecular imaging • Theranostic: Can combine imaging & radiotherapy • Can be combined with CT for anatomical information	• Irradiation exposure • Low spatial resolution (0.3–1 mm; 12–15 mm^3) • Long acquisition time	• ^{99m}Tc • ^{123}I • ^{111}In • ^{177}Lu	• Diagnosis of various diseases (see Table 3) • Radiotherapy for NHL: ^{90}Y-Bexxar or ^{131}I-Zevalin[104] • Radiotherapy of thyroid carcinoma with ^{131}I-iodide[105]
MRI	• Unlimited depth penetration	• Expensive • Long acquisition	• Gadolinium (Gd^{3+}) • iron oxide particles (SPIO, USPIO)	• SPIOs for detection of lymph node

Modality	Advantages	Disadvantages	Common Contrast agents/Readout	Example Clinical Applications
	• Whole-body imaging possible • No ionizing irradiation • Excellent soft tissue contrast • High spatial resolution	time (min-hours) • Limited sensitivity for detection of molecular contrast agents	• manganese oxide • [19]F	metastases of prostate cancer[106] • Characterization of focal hepatic lesions[107] • Perfusion imaging of the heart[108]
MRS	• Whole-body imaging possible • No ionizing irradiation	• Expensive • Long acquisition time (min-hours) • Low sensitivity	• choline • creatine • lactate • lipids • polyamines • N-acetyl-aspartate	• Metabolite levels in brain tumors [109] • Treatment monitoring of Alzheimers[110]
US	• No ionizing irradiation • Real-time imaging/ short acquisition time (min) • High spatial resolution • Can be applied externally or internally (endoscopy) • Inexpensive • Highly sensitive	• Whole-body imaging not possible • Contrast agents currently limited to vasculature • Operator dependency	• Contrast Microbubbles	• Characterization of focal liver lesions[28] • Echocardiography[111] • Tumor perfusion of cancer[27]
Optical	• No ionizing irradiation • Real-time imaging/ short acquisition time (sec-min) • Relatively high spatial resolution • Can be applied externally or internally (endoscopy) • Inexpensive • Highly quantitative & sensitive	• Limited depth penetration (≤ 1 cm) • Whole-body imaging not possible	• Fluorescent molecules & dyes • Light absorbing nanoparticles	• OCT imaging of artherosclerosis[112] • OCT imaging for colonoscopy screening[17] • Raman imaging of skin cancer[113]

Modality	Advantages	Disadvantages	Common Contrast agents/Readout	Example Clinical Applications
	• Multiplexing			

Table 2

Commonly used PET tracers for clinical molecular imaging of diseases. Adapted from references:[114–122]

Medical Imaging	Molecular Target	Radiotracer(s)	Clinical Applications
Oncological Imaging	Protein synthesis	^{11}C Methionone,	Protein synthesis in tumors
	Glucose transporter	^{11}C-DG, ^{18}F-FDG	Glucose metabolism in tumors
	Choline transporter	^{11}C-choline	Tumor phospholipid synthesis
	thymidine uptake in DNA/RNA synthesis	^{18}F-FLT, ^{18}F-FMAU; ^{18}F-FU	Tumor cell proliferation
	$\alpha_V\beta_3$ integrin	^{18}F-galacto-RGD	Tumor angiogenesis
	HSV1-tk	^{18}F-FHBG	Suicidal gene therapy
	Hypoxia	^{18}F-FMISO; ^{64}Cu-, ^{60}Cu-ATSM	Tumor hypoxia
	Somatostatin receptor	^{64}Cu-TETA-octreotide	Neuroendocrine tumors
	Estrogen receptor	^{18}F-FES	Breast Cancer
	Androgen receptor	^{18}F-FDHT	Prostate Cancer
Cardiovascular Imaging	Cell metabolism	^{11}C Acetate	Cardiac metabolism
	Fatty acid metabolism	^{11}C Palmitate	Ischemia
	Adrenergic neurotransmission	^{11}C Metahydroxy-ephedrine	Heart failure
	Cardiac Sympathetic Neurons	^{18}F Norephinephrine	Cardiac sympathetic innervation
Neurological Imaging	Dopamine post synaptic receptors	^{11}C Raclopride	Schizophrenia, Addiction
	β-amyloid	^{11}C-PIB	Alzheimer's disease
	NK-1 Receptor	^{18}F SPARQ; ^{11}C-R116301	Depression, Anxiety
	Dopamine transporter	^{18}F FECNT	Schizophrenia, Addiction
	Dopamine metabolism	^{18}F DOPA	Schizophrenia, Addiction

Abbreviations: DG: 2-deoxyglucose; FDG: fluoro-2-deoxy-D-glucose; FLT: fluoro-L-thymidine; FMAU: 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)thymine; FU: fluorouracil; galacto: galactose; RGD is a peptide sequence of arginine-glycine-aspartic acid; HSV1-tk: herpes simplex virus 1 – thymidine kinase; FHBG: fluoro-3-[hydroxymethyl]butyl)guanine; FMISO: fluoromisonidazole; ATSM: N^4-methylthiosemicarbazone; TETA: 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; FES: Fluoro-17-β-Estradiol; FDHT: Fluoro-5α-Dihydrotestosterone; PIB: 2-(4′-(methylamino)phenyl)-6-hydroxybenzothiazole (Pittsburgh Compound-B); NK-1: neurokinin-1; SPARQ: 2-fluoromethoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl]([2S,3S]2-phenylpiperidin-3-yl)-amine); R116301: neurokinin-1 receptor antagonist hydroxybutanedioate; ^{18}F FECNT: 2β-carbomethoxy-3β-(4-chlorophenyl)-8-(2-[(18)F]-fluoroethyl)-nortropane; DOPA: dopamine.

Table 3

Commonly used SPECT tracers for clinical molecular imaging of diseases. Note, many agents image blood vessels and perfusion and/or excretion (labelled N/A for Molecular Target). Adapted from references:[105, 123]

Medical Imaging	Molecular Target/Localization	Radiotracer(s)	Clinical Applications
Oncological Imaging	Carcinoembryonic antigen	^{111}In altumomab pentetate	Colon Cancer
	Prostate-specific membrane antigen (PSMA)	^{111}In capromab pendetide/ProstaScint®	Prostate cancer
	Somatostatin receptor	^{111}In pentetreotide/Octreoscan®	Neuroendocrine cancers, gastroenteropancreatic tumors
	Tumor-associated glycoprotein	^{111}In satumomab pendetide/OncoScint®	Colorectal or Ovarian cancer metastasis
	Carcinoembryonic antigen	^{99m}Tc Arcitumomab/CEA-Scan®	Colorectal cancer
	Somatostatin receptor	^{99m}Tc Depreotide/Neotect®	Pulmonary and lung cancer
	CD20	^{90}Y Ibitumomab tiuxetan/Zevalin®	Non-Hodgkins lymphoma
	CD20	^{131}I Tositumomab/Bexxar®	Non-Hodgkins lymphoma
	NK-1 Receptor	^{90}Y-labelled substance P	Glioma
	Albumin	^{90}Y-MAA	Liver cancer
	N/A – (localizes in adrenergic tissue)	^{123}I-/^{131}I-MIBG	Neuroendocrine tumors
	N/A - Perfusion	^{99m}Tc-sulfur colloid	Sentinel lymph node metastasis/biopsy
	N/A - Perfusion	^{99m}Tc Sestamibi/Cardiolite®, Miraluma®	Breast cancer, lymph node metastasis
Cardiovascular Imaging	N/A - Perfusion	^{99m}Tc Teboroxime/Cardiotec®	Myocardial Perfusion
	N/A - Perfusion	^{99m}Tc Tetrofosmin/Myoview®	Myocardial Perfusion
	N/A - Perfusion	^{99m}Tc Sestamibi/Cardiolite®, Miraluma®	Myocardial Perfusion
Neurological Imaging	N/A - Perfusion	^{99m}Tc Bicisate (ECD)/Neurolite®	Cerebral Perfusion
	N/A - Perfusion	^{99m}Tc Exametazine (HMPAO)/Ceretec®	Cerebral Perfusion
	N/A - Perfusion	^{111}In pentetate/^{111}In DTPA®	CSF Kinetics
	Amphetamine receptor	^{123}I-iodoamphetamine	Neurodegenerative disorders; regional cerebral blood flow
	Phosphatidylserine	^{99m}Tc-HYNIC-annexin V	Dementia[124]
Gastrointestinal and Genitourinary Imaging	N/A – Perfusion/clearance	^{99m}Tc Disofenin (DISIDA)/Hepatolite®	Hepatobiliary
	N/A – Perfusion/clearance	^{99m}Tc Lidofenin (HIDA)/Technescan® HIDA	Hepatobiliary
	N/A – Perfusion/clearance	^{99m}Tc Gluceptate/Glucoscan®	Renal
	N/A – Perfusion/clearance	^{99m}Tc Mertiatide/Technescan® MAG3	Renal
	N/A – Perfusion/clearance	^{99m}Tc Pentetate (DTPA)/Techneplex®, Technescan®	Renal
	N/A – Perfusion/clearance	^{99m}Tc Succimer (DMSA)/DMSA	Renal, brain
	N/A – Perfusion/clearance	^{99m}Tc labelled red blood cells	Gastrointestinal bleeding and associated disorders; splenosis

Medical Imaging	Molecular Target/Localization	Radiotracer(s)	Clinical Applications
	N/A – Perfusion/clearance	^{99m}Tc-sulfur colloid	Splenosis
	Adrenal gland	^{131}I-6-β-iodomethyl-19-norcholesterol or ^{131}I-norcholesterol	Adrenocortical disorders
Musculoskeletal Imaging	Bone	^{153}Sm EDTMP/Quadramet®, or ^{99m}Tc-MDP	Palliative treatment of bone pain and bone metastases
	Hydroxyapatite crystals	^{99m}Tc Oxidronate (HDP)/Osteoscan® HDP	Bone

Abbreviations: CEA: carcinoembryonic antigen; CD20: leukocyte surface antigen; NK-1: neurokinin-1 receptor; MAA: macroaggregated albumin; MIBG: metaiodobenzylguanidine; ECD: ethyl cysteinate dimer; HMPAO: hexamethylpropyleneamine oxime; DTPA: diethylenetriamine-pentaacetic-acid; HYNIC: hydrazinonicotinamide; DISIDA: Di-isopropyliminodiacetic Acid; HIDA: *N*-(2,6 diethylphenylcarbomoylmethyl) iminodiacetic acid; Mag-3: mercaptoacetyltriglycine; DMSA: dimercaptosuccinic acid; EDTMP: ethylene diamine tetramethylene phosphonate; MDP: methylene diphosphonate; HDP: hydroxymethylene diphosphate. AGA: antigranulocyte antibody.

Table 4

Clinical applications with molecular MRI/MRS and Optical/Raman *in vivo* imaging.

Imaging Modality	Molecular Target	Contrast Agent	Example Clinical Application
MRS[8, 125–127]	Metabolites (e.g., lactate, choline, lipids, etc.)	N/A – spectroscopic measurement	Neurological diseases and abnormalities, metabolic disorders, and various cancers
Optical Tomography	Porphyrin	hexaminolevulinate (Hexvix); aminolevulinic acid	Bladder, laryngeal cancer[15]
Optical Tomography	Proteases	capthesin B- or MMP2/9-activated "smart" fluorescent probes	Atherosclerosis[24]
Optical Multiphoton Tomography	NAD(P)H, collagen	N/A – endogenous fluorescence	Skin cancer/diseases[13]
Raman Spectroscopy	N/A – Tissue specific spectra	N/A – Tissue specific spectra	Atherosclerosis;[21] colon cancer;[20, 128] breast cancer;[22] skin cancer;[113]

Abbreviations: MMP: matrix metalloprotease; NAD(P)H: nicotinamide adenine dinucleotide phosphate.

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Table 5

Molecular targets, imaging probes, and drugs identified preclinically for imaging cancer with various modalities.

Molecular Target/Event	PET	SPECT	MRI/MRS	US	Hybrid Acoustic	Optical
			Imaging Modalities			
Apoptosis and/or Cytotoxicity Review:[129]	^{18}F-Paclitaxel (microtubule formation);[130][124] I-Annexin V; ^{18}F-Annexin V	^{99m}Tc-Annexin V	MRI: Annexin V-QDs with paramagnetic-lipid coating;[131] MRS: CH_2/CH_3 ratio			Annexin V-Cy5.5; Cy5.5-Caspase activity; Raman: SERS NPs conjugated to anti-BAX Ab or anti-BAD Ab[132]
Epidermal growth factor receptor (EGFR) Review:[133, 134]	^{11}C-Gefitinib; ^{11}C-Erlotinib/Tarceva; ^{64}Cu-Cetuximab	^{125}I-Anti-EGFR-Fab[135]	Anti-EGFR antibody-IO NPs		anti-EGFR Ab-gold nanorods[136]	Cy5.5-Cetuximab; Raman: anti-EGFR Ab-gold NPs[137]
Epidermal growth factor receptor type II (HER2/neu) Review:[134, 138]	[90]Y, [86]Y, or ^{68}Ga-Trastuzamab	^{111}In-Trastuzamab; [111]In-, [131]I-, or ^{99m}Tc-labeled anti-HER2 antibodies/fragments	Trastuzamab-MnO NP; Avidin-Gd^{3+}-anti-HER2 antibody; Herceptin-IO NPs		anti-HER2 Ab-gold nanorods;[136] Alexa fluor 750-herceptin[139]	Cy5.5-Trastuzamab; Cy5.5-anti-HER2 Ab; Raman: anti-HER2 Ab-SERS NPs[140]
Estrogen receptor Review:[134]	^{18}F-Tamoxifen; [94m]Tc-cyclofenil; ^{18}F-estradiol(FES)	^{131}I-Tamoxifen; tridentate [99m]Tc(I)-estradiol-pyridin-2-yl hydrazine				
Folate receptor Review:[134]	[66]Ga, ^{68}Ga-deferoxamine-folate; ^{18}F-folic acid	^{111}In-DTPA-folate; ^{99m}Tc-folate	PEG-G3-(Gd-DTPA)11-(folate)5		SWNT-folic acid[141]	Pyropheophorbide-peptide-folate
Hypoxia Reviews:[142-144]	[124]I-anti-cG250 (CAIX Ab); ^{18}F-FMISO, [124]I-FIAU; ^{18}F-FAZA, Cu-ATSM		MRI: ^{19}F oximetry, ^{19}F-FMISO; MRS: lactate measurement		oxyhemoglobin:de-oxyhemoglobin[145]	HIF-1α-reporter with GFP; Raman: lactate sensing[146]
Integrins (Tumor angiogenesis) Reviews:[147, 148]	^{64}Cu-RGD (SWNT); ^{64}Cu-RGD (QD); ^{64}Cu-RGD (SPIO); ^{64}Cu-knottin peptides[149]	^{111}In-perfluorocarbon NP-RGD	RGD peptide-Gd containing paramagnetic and fluorescent liposomes; RGD peptide-SPIOs	Knottin-RGD conjugated MBs;[65] RGD MBs; Anti-β3 Ab-MB; Echistatin-coated MBs; β3-targeted perfluoro-carbon NP	SWNT-RGD;[45] αvβ3 peptide-ICG[50]	RGD-QD705; RGD-Rhodamine/PE-liposomes; Cy5.5-knottin peptides; Raman: SWNT-RGD[41]
Metabolism/Proliferation	^{18}F-FDG; ^{18}F-FLT; ^{18}F-FBC[151]					Cy5.5-2DG[152]
Proteases Review:[153]	Radiolabeled small molecule MMP inhibitors[154]	Radiolabeled small molecule MMP inhibitors[154]	MMP2-specific peptide-Gd^{3+}-DOTA "smart probe"[155]			Cyanine-peptide to capsesin; Cy5.5-peptide to MMP
Prostate Specific Membrane Antigen (PSMA)	^{64}Cu-anti PSMA Ab[156]	^{123}I-labelled glutamate-urea-lysine analogues;[157][99m]Tc-chelates[158]	MRI: anti-PSMA Ab-IO NPs[159]			YC-27(3) fluorescent probe;[160] QD-anti PSMA Ab[161]
Reporter gene expression[162, 163]	HSV1-tk: ^{18}F-FHBG, ^{18}F-FHPG, ^{18}F-FMAU, ^{18}F-FEAU	HSV1-tk: ^{123}I-FIAU	βgal (EgadMe); Transferrin receptor (Transferrin-MION); Ferritin (iron); lysine rich protein		βgal[164]	luciferases (bioluminescence); fluorescent proteins (Fluorescence)

	Imaging Modalities					
Molecular Target/Event	PET	SPECT	MRI/MRS	US	Hybrid Acoustic	Optical
Vascular endothelial growth factor (VEGF) receptor (VEGFR) (Tumor angiogenesis) Reviews:[147,148]	[89]Zr-Avastin; ^{64}Cu-DOTA-VEGF; ^{64}Cu-DOTA-VEGF (peptide)	^{111}In-Avastin; ^{125}I-VEGF[165], (^{125}I or ^{99}Tc)-VEGF[121], ^{111}In-hnTf-VEGF		Anti-VEGFR2 Ab-MB;[59] KDR peptide-conjugated MBs[30]		VEGF-Cy5.5; VEGF-QD

Abbreviations: QDs: quantum dots; SERS: surface-enhanced Raman scattering; NPs: nanoparticles; Ab: Antibody; BAX: protein involved in mitochodrial stress-associated apoptosis; BAD: a BCL-2 family member protein that promotes apoptosis; Fab: fragment antibody; IO: iron oxide; DTPA: diethylenetriamine-pentaacetic-acid; PEG: polyethylene glycol; Gd/Gd^{3+}: Gadolinium; MnO: Manganese oxide; FES: fluoroestradiol; SWNT: single-walled nanotubes; CAIX: carbonic anhydrase IX; FMISO: fluoromisonidazole; FAZA: 1-α-D-(2-deoxy-2-fluoroarabinofuranosyl)-2-nitroimidazole; ATSM: N^4-methylthiosemicarbazone; HIF-1α: hypoxia inducible factor type 1α; GFP: green fluorescent protein; RGD: is a peptide sequence of arginine-glycine-aspartic acid; SPIO: superparamagnetic iron oxide; MBs: microbubbles; ICG: iodocyanine green; PE: phycoerythrin; FDG: fluoro-2-deoxy-D-glucose; FLT: fluoro-L-thymidine; FEC: fluoroethylcholine; 2DG: 2-deoxyglucose; MMP: matrix metalloprotease; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; YC-27(3): specific name for a small molecule NIRF-imaging agent synthesized from a N-hydroxysuccinimide portion of PSMA-binding urea and a NIR dye (IRDye 800);160 HSV1-tk: herpes simplex virus 1 thymidine kinase; FHBG: 9-[(3-fluoro-1-hydroxy-2-propoxy) methyl] guanine; FHPG: 9-[(3-fluoro-1-hydroxy-2-propoxy) methyl] guanine; FIAU: 9-[(3-fluoro-1-hydroxy-2-propoxy) methyl] guanine; βgal: β galactosidase; EgadMe: (1-(2-(b-galactopyranosyloxy)propyl)-4,7,10-tris(carboxymethy)-1,4,7,10-tetraazacyclododecane)gadolinium(III); MION: monocrystalline iron oxide; hnTf: n-lobe of human transferrin; VEGFR2: VEGFR type 2; KDR: kinase insert domain receptor (human VEGFR2).