R E V I E W

# Molecular Imaging of Proteases in Cancer

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**Abstract:** Proteases play important roles during tumor angiogenesis, invasion, and metastasis. Various molecular imaging techniques have been employed for protease imaging: optical (both fluorescence and bioluminescence), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET). In this review, we will summarize the current status of imaging proteases in cancer with these techniques. Optical imaging of proteases, in particular with fluorescence, is the most intensively validated and many of the imaging probes are already commercially available. It is generally agreed that the use of activatable probes is the most accurate and appropriate means for measuring protease activity. Molecular imaging of proteases with other techniques (i.e. MRI, SPECT, and PET) has not been well-documented in the literature which certainly deserves much future effort. Optical imaging and molecular MRI of protease activity has very limited potential for clinical investigation. PET/SPECT imaging is suitable for clinical investigation; however the optimal probes for PET/SPECT imaging of proteases in cancer have yet to be developed. Successful development of protease imaging probes with optimal in vivo stability, tumor targeting efficacy, and desirable pharmacokinetics for clinical translation will eventually improve cancer patient management. Not limited to cancer, these protease-targeted imaging probes will also have broad applications in other diseases such as arthritis, atherosclerosis, and myocardial infarction.

**Keywords:** protease, cancer, molecular imaging, activatable probe, optical imaging, positron emission tomography

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# Introduction

Over the last decade, considerable advances have been made to further our understanding of cancer invasion and metastasis. Although surgery and radiation therapy can effectively control many cancers at their primary sites, metastatic diseases still have very poor prognosis.[1] Metastasis, the spread of cancer from its primary location to a distant organ, is the main cause of death in cancer patients. Cancer metastasis occurs as a series of sequential and interrelated processes which include dissociation of cancer cells in the primary tumor, local invasion, angiogenesis, intravasation of cancer cells into the vasculature or lymphatic systems, survival in these channels, extravasation, and proliferation at a distant site.[1,2] Successful inhibition of tumor metastases is a promising approach for cancer therapy and/or cancer control.

Proteases belong to a group of evolutionarily conserved enzymes which are activated in response to the stimuli in normal cells. They can regulate a variety of different cellular processes such as gene expression, differentiation, and cell death.[3] Briefly, proteases can be classified into six groups by their mechanisms of action: serine, cysteine, threonine, aspartate, glutamic acid proteases, as well as metalloproteases. The threonine and glutamic acid proteases were not discovered until 1995 and 2004, respectively, and glutamic acid protease is the only subtype not found in mammals so far.[4] The mechanism for cleaving a peptide bond with a protease typically involves a serine, cysteine, or threonine residue (typically a histidine is close-by which can activate these residues) or a water molecule (in aspartate, metallo- and glutamic acid proteases) as the nucleophile in the active site.[5]

Tumor progression and metastases are highly dependent on oxygen and nutrient supply, which are stimulated by multiple proteases in the tumor and/or surrounding tissues. Many tumors have been shown to have elevated levels of proteases at an early stage and these proteases are thought to be crucial for tumor angiogenesis, invasion, and metastasis.[6] Further understanding of the roles of proteases in tumor progression will guide the development of novel therapeutic strategies against cancer.

Although the clinical significance of detecting protease activity in vivo has been well recognized, it is quite difficult to achieve. Early detection of small primary tumors is crucial for successful cancer therapy and patient management. Molecular imaging, defined as the in vivo characterization and measurement of biologic processes at the cellular and molecular level,[7,8] can fulfill this goal. Different from traditional diagnostic imaging techniques such as computed tomography (CT), ultrasound, and magnetic resonance imaging (MRI), molecular imaging aims to detect the molecular abnormalities that are the basis of the disease.

Molecular imaging of cancer can be a highly useful tool in clinical medical practice.[9] Systematic efforts are under way to detect both primary tumors and metastatic diseases at their early stages. The insights gained into the biological or molecular events in living subjects will accelerate the development of novel therapeutics agents and provide more accurate means to monitor the effects of these agents.[10] Various molecular imaging modalities have been employed for protease imaging: optical (both fluorescence and bioluminescence), MRI, single-photon emission computed tomography (SPECT), and positron emission tomography (PET). The probes used in these studies can be briefly categorized into two major types: substrate-based and activity-based.[11–13] In this review, we will summarize the current state-of-the-art of protease imaging in cancer.

## Fluorescence Imaging of Protease Activity

Optical imaging is less expensive and more convenient than the other imaging modalities such as MRI and PET.[14] The most widely used optical imaging techniques are fluorescence imaging and bioluminescence imaging (BLI). Many other optical imaging techniques are also under active development, such as Raman spectroscopy and photoacoustic imaging.[15,16] Fluorescence imaging is by far the most widely used technique for imaging protease activity in vivo and the proteases that have been imaged include the cathepsin family, the matrix metalloprotease (MMP) family, and the urokinase plasminogen activator (uPA).

Most of the probes used in these studies are called "smart probes" or "activatable probes", which can change their optical properties after protease cleavage. Typically, fluorescently labeled substrates are designed to be maximally quenched by a quencher

(in some cases the fluorescent dye itself) in close proximity because of fluorescence resonance energy transfer (FRET).[17,18] Upon cleavage, the fluorophore and the quencher are separated which resulted in an enhanced fluorescence signal.

## Imaging of cathepsins

During tumor metastasis, proteases play an important role in mediating the passage of tumor cells between tissue and vessels via degradation of the basement membrane. Cathepsin is a lysosomal cysteine protease family involved in cellular protein degradation.[19] It is over-expressed in many tumor cells as well as the host cells associated with the tumor.[20] The ubiquity of cathepsins makes them very attractive targets for tumor detection.

There are five major types of cathepsins: cathepsin B, D, S, K, and L. Cathepsins B and L are major cysteine proteases involved in protein degradation within the lysosome.[21] Cathepsin D is a proteolytic enzyme that has been implicated in the degradation of basement membranes.[22,23] Cathepsin S belongs to the papain superfamily and a difference in cathepsin S level was found between the tumor and adjacent control tissue in lung cancer patients, which suggested that cathepsin S may be involved in cancer progression.[24] Cathepsin K has strong collagenolytic activity and mediates matrix degradation, a pivotal step in tumor invasion and metastasis.[25] To date, optical imaging studies have been reported for cathepsins B, D, and S.

The first in vivo optical imaging of protease activity was demonstrated in a xenograft lung carcinoma model a decade ago (Fig. 1).[26] Near-infrared fluorescent (NIRF) fluorophores were bound to a long circulating graft copolymer consisting of poly-L-lysine and methoxy-polyethylene glycol (PEG) succinate. Following intravenous injection, the NIRF probe accumulated in solid tumors due to its long circulation life-time and leakage through the tumor neovasculature, termed the "enhanced permeability and retention (EPR)" effect.[27,28] Intratumoral NIRF signal was generated by proteolytic cleavage of the macromolecule which released the fluorescence signal of the previously quenched fluorochrome.
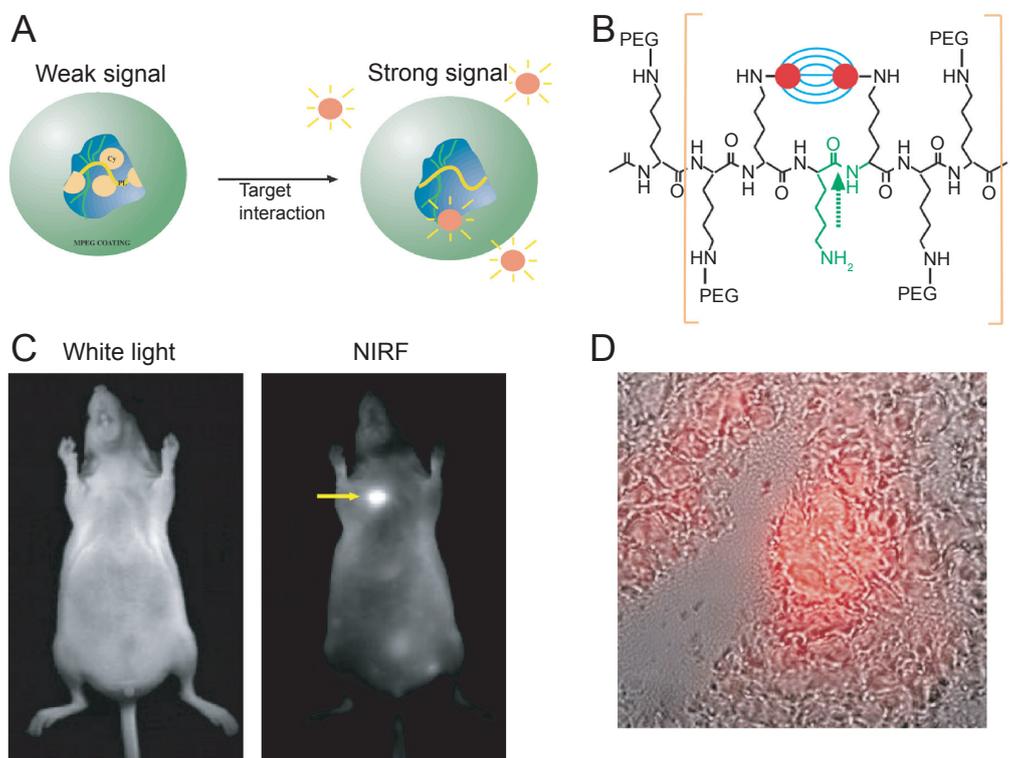


**Figure 1.** Fluorescence imaging of tumors with a protease-activated NIRF probe. **A)** A schematic diagram of probe activation. The initial proximity of the fluorochrome to each other results in signal quenching. **B)** Chemical structure of the probe. Green arrow indicates the enzymatic degradation site. **C)** Images of a LX-1 tumor implanted into the mammary fat pad of a nude mouse after probe injection. The arrow indicates the tumor. **D)** NIRF image (in red) superimposed onto the correlative phase contrast microscopy image of the excised tumor. Adapted from.[26]

In vivo imaging revealed a 12-fold increase in the NIRF signal, allowing the detection of tumors with sub-millimeter diameter. Although this probe was not specific for a single protease (it can be activated by many cysteine/serine proteases), the generation of enhanced NIRF signal as a result of lysosomal activity did open up a new research area that has been vibrant over the last decade.

Similar probes were tested in a breast cancer xenograft model in mice,[29] demonstrating that this strategy can be used to detect early stage tumors, probe for specific enzyme activity, and evaluate the therapeutic effect in vivo. Subsequently, the same type of NIRF probe was used to determine that cathepsin B activity in the tumor correlated with the aggressiveness of breast tumor phenotypes.[30] These probes have later been adopted to reveal colonic neoplasms in mice,[31] in which the signal intensity of the tumor was more than 30-fold greater than the control animals. This study demonstrated that the use of NIRF imaging microcatheters, in combination with protease-activatable probes, could delineate tumors with good contrast, which may be a potentially useful adjunct to white light colonoscopy in the clinic.

With the development of a fluorescence-mediated tomography (FMT) system, the up-regulation of cathepsin B was imaged semi-quantitatively in a HT-1080 fibrosarcoma tumor model.[32] The key feature of the FMT system is that the fluorescence signal in deep tissues can be reconstructed to give a three-dimensional image of the probe distribution in vivo.[33,34] This study demonstrated that fluorescence reflectance imaging and FMT using these protease-activatable probes can allow the detection of experimental spontaneous breast tumors.[32] Because the activity levels of various proteases may correlate with the clinical outcome, this technique may help not only to detect, but also to differentiate breast cancer phenotypes non-invasively in the future. To date, the research on FMT has been limited to small animal models due to the limited light penetration even in the NIR range. It is likely that clinical FMT systems will be developed in the future which can help breast cancer patient management, as an adjunct to the other modalities such as CT and PET.

Besides in vivo imaging applications, some probes were designed to visualize the dynamic alteration of cathepsin B with multichannel microscopy.[35]

A series of quenched activity-based probes (qABPs) become fluorescent upon activity-dependent, covalent modification by proteases (Figs. 2A and B). These reagents were able to freely penetrate cells and allow direct imaging of protease activity in living cells, which have been used to monitor real-time protease activity in live human cells with fluorescence microscopy. Subsequently, these qABPs were applied to determine the role of cathepsin B in invasive growth and angiogenesis during multi-stage tumorigenesis.[36] Recently, these qABPs were further optimized with NIRF fluorophores which were able to give a whole-body readout in mice (Fig. 2C).[37]

Other subtypes of cathepsin have also been investigated for tumor detection. A NIRF probe for cathepsin D imaging was reported a decade ago.[38] A NIRF fluorochrome was attached to the amino terminal of a peptide sequence with 11 amino acid residues (GPICFFRLGKC), which was specific for cathepsin D. The peptide was then linked to a synthetic graft copolymer. A striking 350-fold signal amplification was observed upon protease cleavage during in vitro testing with a rodent tumor cell line stably transfected with human cathepsin D. However, in vivo imaging was not achieved, likely due to the low expression level of cathepsin D in physiological conditions. A fluorescent probe for cathepsin S imaging has also been designed which incorporated its substrate, the Leu-Arg dipeptide.[39] Again, no in vivo studies have been reported.

## Imaging of MMPs

Besides cathepsins, MMPs are also attractive targets for cancer imaging. Tumor-associated MMP expression and activity originated from not only tumor cells, but also the surrounding stromal cells. In fact, studies have shown that in certain epithelial cancers, most of the upregulated MMPs are expressed by the host stromal cells.[40] Multiple MMPs are induced in connective tissue cells, such as fibroblasts and inflammatory cells in response to tumor formation, which can provoke invasion of the malignant epithelial cells.[41] MMPs are also expressed in tumor cells. For example, MMP-7 is commonly expressed in the epithelial component of adenocarcinomas and several MMPs are expressed in the malignant epithelium of tumors that have undergone an epithelial-to-mesenchymal transformation.[41]
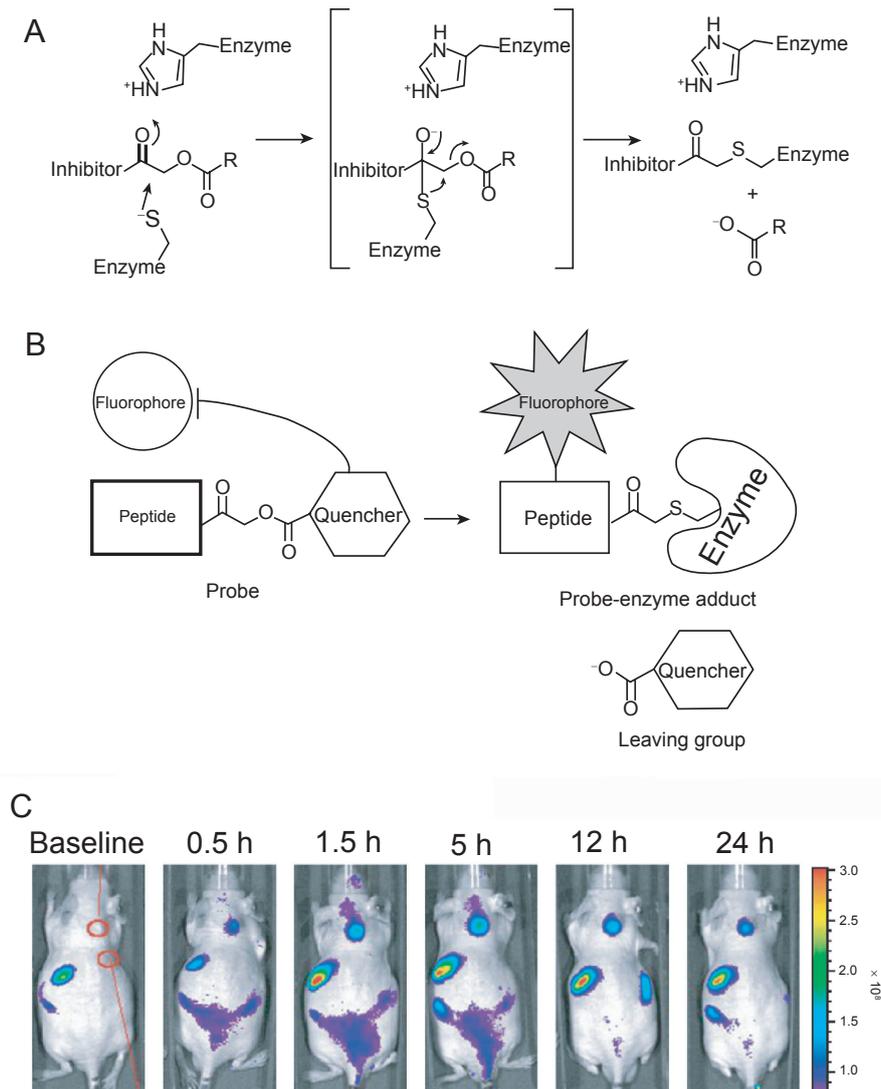
**Figure 2.** Fluorescence imaging of cathepsin activity using quenched activity-based probes (qABPs). **A**) Mechanism of covalent inhibition of a protease by an acyloxymethyl ketone. **B**) Activity-dependent labeling of a protease target by a qABP. Covalent modification of the target results in loss of the quenching group, resulting in production of a fluorescently labeled enzyme. **C**) Optical imaging of tumors in live mice. The red circles indicate the tumor (top) and an area (bottom) used for the measurement of background signal. Adapted from.[35,37]

MMP-2 has been recognized as one of the key MMPs involved in tumor angiogenesis, invasion, and metastasis. It is capable of degrading type IV collagen, the major component of basement membranes.[42] The first molecular probe capable of imaging MMP activity was a slight modification from the original protease-sensing probes, which incorporated a peptide linker that can be cleaved by MMP-2 and certain other proteases.[43] The peptide substrate (GPLGVRGKC) was labeled with a Cy5 dye, which can self-quench when in close proximity. This probe allowed for the assessment of tumor-associated MMP activity in response to treatment with a MMP inhibitor (MMPI),

Prinomastat. After demonstrating its capability to sense and image MMP response directly in vivo, this probe was also tested in other tumor models and high signal-to-background ratio was achieved in MMP-2 positive tumors.[44]

In one study, a protease sensitive probe was developed which incorporated cell penetrating peptides.[45] The fluorescein- or Cy5-labeled probe can penetrate into the cytosol of mammalian cells, upon cleavage of the peptide sequence (PLGLAG) by MMP-2/9. This new strategy could selectively deliver molecules to tumor cells, allowing for accumulation and concentration of imaging or therapeutic agents within the tumor

cells or within the vicinity of MMP-2/9. In another report, a self-assembling homotrimeric triple helical peptide, incorporating segments of type V collagen, with high specificity to MMP-2/9 has been reported.[46] This agent utilizes a pair of fluorophores conjugated to the ε-amino groups of lysine on both sides of the cleavage site, which can efficiently quench the signal and fluoresce after hydrolysis by MMPs.

Nanotechnology has also been applied for MMP-2/9 imaging in vivo. A gold nanoparticle- and Cy5.5-based NIRF probe for MMP imaging in vivo was designed (the substrate sequence: PLGVR).[47] This probe exhibited satisfactory imaging results in a SCC7 squamous cell carcinoma model and could also be useful in drug screening. Recently, a protein interaction-based fluorescence imaging system was designed to image MMP-2 activity and its reaction to MMPIs.[48] This study provided a direct method of molecular target assessment which may benefit MMP-targeted drug screening.

Aside from MMP-2/9, MMP-7 is another important target for tumor imaging. In one study, dendrimer-based fluorogenic substrates were developed to image MMP-7 activity.[49] The substrate used in this study, (Ahx)RPLALWRS(Ahx)C where Ahx represents aminohexanoic acid, was shown to be more selective for MMP-7 than the other MMPs within the tumor microenvironment. Recently, a similar construct using NIR FRET pairs was developed to reduce the absorption and scattering of the fluorescence signal, thereby improving the response of a proteolytic beacon.[50] This approach was shown to be highly sensitive and tumors as small as 0.11 cm in diameter could be detected.

An alternative approach to image MMP-7 activity has been developed using activatable quantum dots (QDs). Comparing to conventional fluorophores such as Cy5, QDs offer brighter signal, better photostability, and the enormous potential for multiplexed imaging.[15,51] In one report, the QD fluorescence was quenched (based on FRET) by a quencher molecule (BHQ-1) with an absorption band that overlaps the QD emission.[52] The BHQ-1 was linked to the QD through a MMP-7-cleavable peptide (GPLGLARK). Adjusting the QD/BHQ-1 ratio and the peptide conformation was able to modify the sensitivity and signal-to-background ratio of the reagent, as well as controlling the MMP-7-mediated photodynamic cytotoxicity in cancer cells. In another study, MMP-2/7-mediated uptake (substrate sequence: PLGVRG) of QDs into tumor cells was reported.[53] Such protease-modulated cellular uptake of QDs may also be applied to other nanoparticles for biological imaging and selective drug delivery into tumor cells.

## Imaging of uPA

A significant body of in vitro and in vivo data has established the uPA system as a promising target for cancer drug development.[54,55] It has been demonstrated to participate in multiple processes during tumor development, tumor progression, and angiogenesis. In 2004, the development of a uPA activatable NIRF probe was reported.[56] This probe consists of a uPA-cleaving motif (GGSGRSANAKA), terminally capped with different NIR fluorochromes (Cy5.5 or Cy7), and a PEGylated poly-L-lysine graft copolymer. Upon addition of recombinant human uPA to the probe, significant fluorescence signal amplification (up to 680%) was observed while no activation was detected with the negative control compounds or in the presence of uPA inhibitors. Subsequently, this uPA-sensitive probe was tested in two distinct tumor models (human colon adenocarcinoma HT-29 and human fibrosarcoma HT-1080).[57] The observed changes in the fluorescence signal, about three-fold higher in animals with probe injection, correlated well with tumor-associated uPA activity.

Generally speaking, there are several advantages of using proteases as the targets for optical imaging probe development. These enzymes are secreted and activated in the extracellular environment, which partially avoid the need of delivering the probe to intracellular compartments. Proteases are generally active at the physiological pH, and perhaps most importantly, such activity is catalytic which provides an opportunity for signal amplification that can not be achieved for probes based on direct binding to their targets. However, despite all these advantages, optical imaging has very limited potential for clinical application, even with NIRF probes, because of the limited tissue penetration of the fluorescence signal (typical less than 1–2 cm).[58,59] In the clinical setting, optical imaging is only suitable for imaging lesions close to the skin (e.g. skin and breast cancer), tissues accessible by endoscopy (e.g. colon cancer), or during surgery (e.g. visualizing the tumor margins). Overall, fluorescence imaging is more suitable for small animal

## BLI of Protease Activity

BLI is another optical imaging technique that has virtually non-existent background signal, a major limitation for fluorescence imaging even in the NIR range.[60] Most BLI studies use D-luciferin as the firefly luciferase substrate, which is tolerant to very limited chemical modification.[61,62] Therefore, the biggest limitation of using BLI to detect MMP activity is the requirement for S' residues in the recognition site, since the downstream residues (luciferin in this case) can dramatically interfere with MMP activity. The Promega Corporation recently presented a novel strategy to overcome this limitation.[60] Using a circularly-permuted firefly luciferase, they joined the original termini of the firefly luciferase with a protease cleavable peptide sequence, thereby locking the luciferase in an inactive state. Cleave of the protease substrate results in a conformational change that activates the luciferase, which neatly provides the opportunity for in vivo imaging of MMP (or other proteases if other peptide sequences are used) activity with BLI. In vivo imaging with this technology has not been reported yet.

Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins.[63,64] BLI of caspase-3 has been reported for the evaluation of tumor apoptosis or the responses to drugs.[65] In this pioneering report, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and resulting changes in tumor burden was imaged in the same animal by dual-substrate BLI, i.e. with D-luciferin (the natural substrate for firefly luciferase) and DEVD-luciferin (active only after caspase cleavage) (Fig. 3). Surprisingly, no follow-up study has been reported since. Another group has also reported the use of the same probe in a cell-based study.[66]

BLI is superior to fluorescence imaging for many applications since it does not require any external light excitation and there is minimal background signal. A few proof-of-principle studies have been reported for BLI of proteases as discussed above. Although it can serve as an invaluable platform for in vitro and cell-based assays, there is still a long way to go before these agents can be widely used in animal studies. Much further effort will be needed in the future yet the high demand for protein engineering expertise in BLI of MMPs makes such techniques not readily generalizable. Lastly, one has to bear in mind that BLI is not applicable for clinical studies therefore it can only be used in cell and animal studies. To date, a variety of proteases have been studied with optical imaging techniques. However, studies on MRI, SPECT, and PET imaging of proteases have so far exclusively focused on the most intensively studied proteases, the MMPs.[60,67]

## MRI of MMPs

MRI has become widely available in hospitals and clinical centers throughout the world and it is a commonly utilized modality for cancer imaging.[68] It detects the interaction of protons (or certain other nuclei) with each other and with the surrounding molecules in a tissue of interest.[69] Different tissues have different relaxation times that can result in endogenous MR contrast. Exogenous contrast agents (CAs) can further enhance this by selectively shortening either the T1 (longitudinal) or T2 (transverse) relaxation time.[70,71] The MR image can be weighted to detect the differences in either T1 or T2 by adjusting certain parameters during data acquisition. Traditionally, $Gd^{3+}$-chelates have been used to enhance the T1 contrast[72] and iron oxide nanoparticles have been used to increase the T2 contrast.[73] The advent of activatable fluorescent probes also accelerated the development of probes for MRI of enzyme activity.[74]

Protease-modulated CAs were developed to detect the activity of MMPs in vivo with MRI.[75] The CAs were based on the concept of a solubility switch, from hydrophilic to hydrophobic, which significantly modifies the pharmacokinetic properties of the agents as evidenced by the slow efflux kinetics from the activity site. In live tumor-bearing mice, these CAs were demonstrated to be effective in non-invasive imaging of MMP-7 activity. Recently, a cleavage motif of MMP-9 (GGPRQITAG) was incorporated into a nanosensor for in vitro detection of protease activity by MRI (Fig. 4).[76] The experimental results suggested that upon protease cleavage, the nanoparticles rapidly switch from a stable low-relaxivity stealth state to become
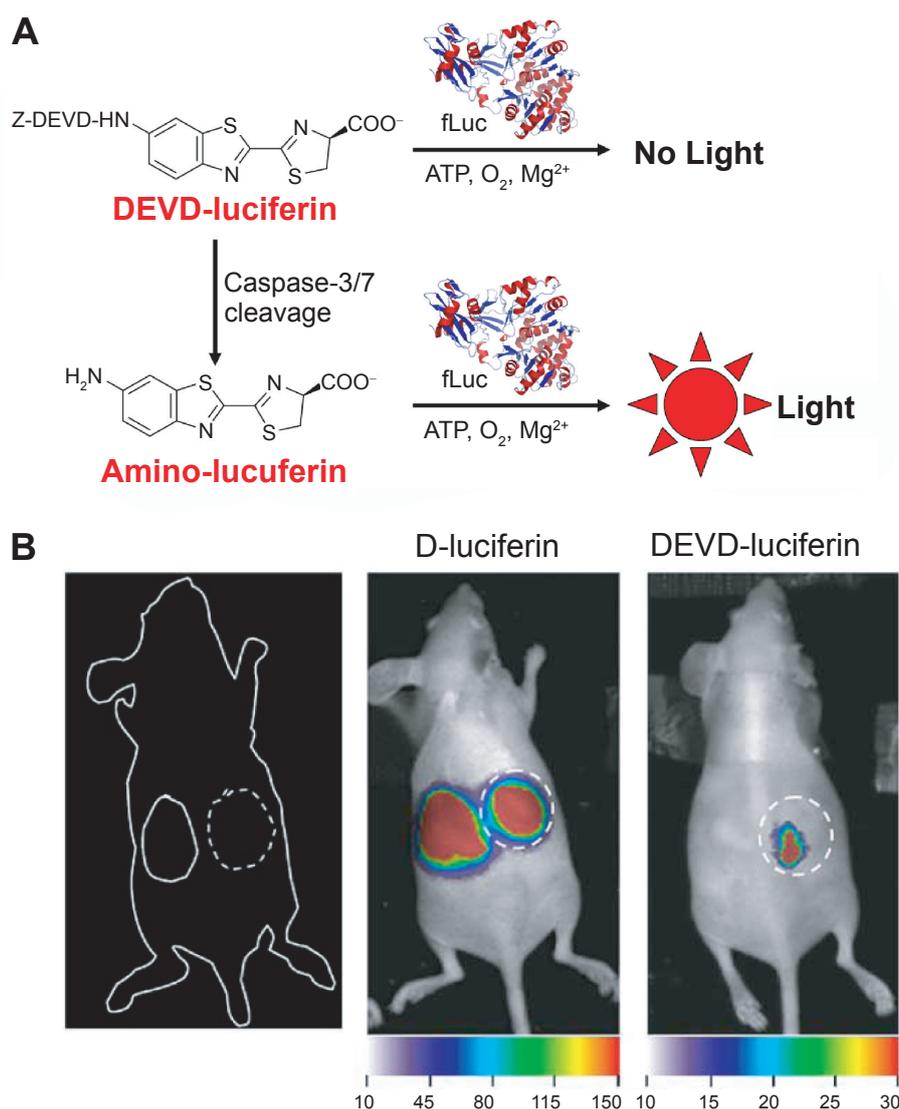
**Figure 3.** Bioluminescence imaging of protease activity in vivo. **A**) DEVD-luciferin, which is not a substrate for firefly luciferase (fLuc), becomes a fLuc substrate upon caspase-3/7 cleavage of the peptide. **B**) Left: A cartoon of the tumor implantation sites in mice. Dotted circle: a caspase-positive tumor. Center: The mouse was imaged for fLuc activity and tumor progression with D-luciferin injection. Right: Twenty-four hours later, the same mouse was injected with DEVD-luciferin and imaged. Note the different scale for the two images. Adapted from.[65]

adhesive, aggregating high-relaxivity particles. However, this agent has not been investigated in animals yet.

Protease-modulated CA designed to detect MMP-2 activity was also reported and the accumulation of protease-cleaved CAs in a MMP-2-positive tumor was achieved in a mouse model.[77] This agent used a peptide substrate (SGESPAYYTA) that can be cleaved by MMP-2. When a scrambled peptide sequence was used, the cleavage efficiency of MMP-2 was markedly reduced. Recently, the same group designed new CAs for imaging tumor MMP-2 activity with a different strategy,[78] which contains a paramagnetic gadolinium chelate (i.e. Gd-DOTA) attached to the N-terminus of the same MMP-2 cleavable peptide sequence via a hydrophobic chain. Since the aqueous solubility of the CA depends on the presence of a PEG chain on the C-terminus of the peptide, MMP-2 cleavage of the peptide detaches the PEG chain from the CA and makes it less water-soluble. Although this CA and control compounds were tested in an animal model bearing two tumors with different levels of MMP-2 activity, strangely, no imaging data was reported.[78]

Molecular MRI is in its infancy. The major disadvantage of MRI is the inherent low sensitivity, which can only be partially compensated by working

at higher magnetic fields (4.7–14 T in animal studies), acquiring data for much longer time periods, and using exogenous contrast agents. Although proof-of-principle studies have been reported for molecular MRI of a few targets,[9,79] whether molecular MRI can significantly improve cancer patient management remains to be elucidated. To overcome the intrinsic low sensitivity of MRI and poor tissue penetration capability of optical imaging, radioisotope-based imaging techniques (i.e. SPECT and PET) should be used if protease imaging is needed in the clinical setting.

## SPECT Imaging of MMPs

SPECT imaging detects gamma rays. Internal radiation is administered through inhaling, ingesting, or injecting a low mass amount of radiolabeled pharmaceuticals. A collimator is used to only allow the emitted gamma photon to travel along certain directions to reach the detector, which ensures that the position on the detector accurately represents the source of the gamma ray. The gamma camera can be used in planar imaging to obtain two-dimensional images, or in SPECT imaging to obtain three-dimensional images.[80,81] Because of the use of lead collimators to define the angle of incidence, SPECT imaging has a very low detection efficiency ($<10^{-4}$ times the emitted number of gamma rays).[8,82] Common radioisotopes used for SPECT imaging are $^{99m}$Tc ($t_{1/2}$: 6.0 h), $^{111}$In ($t_{1/2}$: 2.8 d), $^{123}$I ($t_{1/2}$: 13.2 h), and $^{131}$I ($t_{1/2}$: 8.0 d).

The proteolytic activities of MMPs are regulated by endogenous tissue inhibitors of MMPs (TIMPs).[83] TIMPs specifically inhibit active forms of MMPs, and in some cases latent MMPs as well, and disturbance in this balance may lead to pathological situations in tissues. Therefore, TIMPs can serve as potential targeting molecules for imaging MMP expression and such a radiopharmaceutical ($^{111}$In-DTPA-N-TIMP-2) has been reported (Fig. 5).[84] DTPA-N-TIMP-2 (DTPA represents diethylenetriamene pentaacetate) was labeled with $^{111}$In in >95% radiochemical yield, which was quite stable in serum. This tracer has been tested in patients with Kaposi's Sarcoma for SPECT imaging applications.[85] Although the probe was found to be safe, the authors concluded that its tumor targeting/imaging capability was very poor and unlikely to be of use for imaging applications.
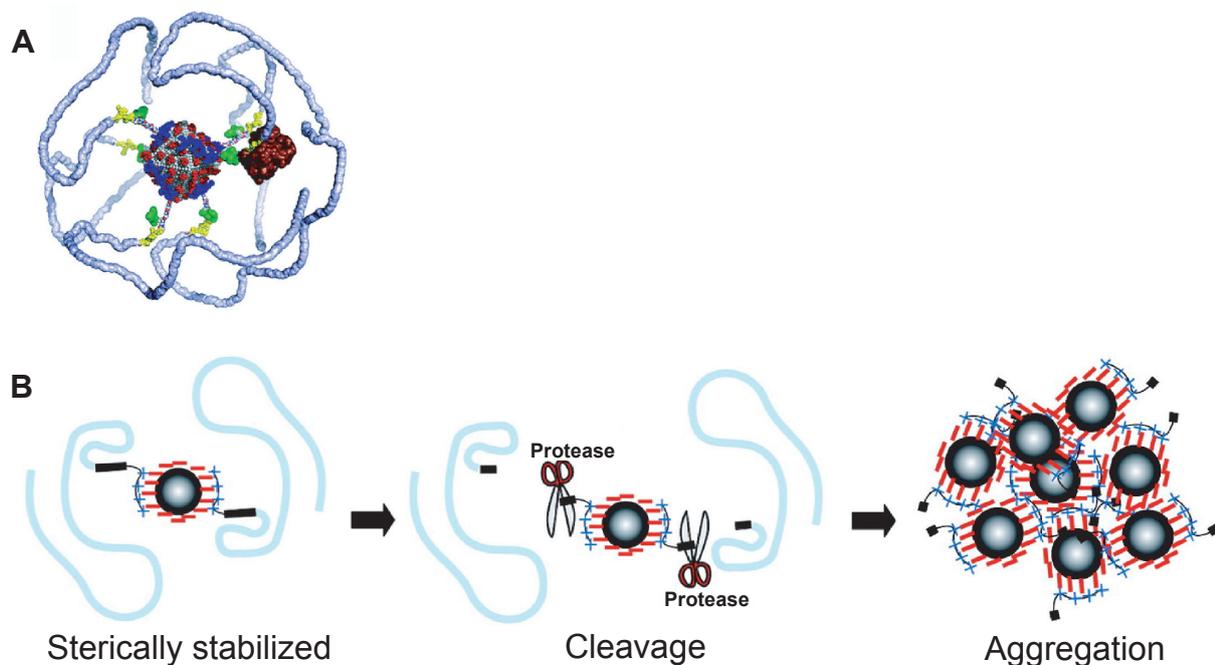


**Figure 4.** Protease-sensitive agents for MRI. **A**) The agent consists of a 5 nm magnetite core (gray) covered by a negatively charged citrate shell (red). The peptide-mPEG copolymers are electrostatically bound by the positively charged coupling domains (blue). The mPEG polymers (light blue) are linked to the coupling domain via the cleavage domain (yellow) and a linker peptide. A model of MMP-9 (brown) is shown for size comparison at the cleavage domain. Green: fluorescein dyes. **B**) When the sterically stabilized agent is exposed to a protease, the peptide-mPEG linkage is cut at the cleavage domain, resulting in a loss of sterical stabilization. This leads to particle aggregation and enhanced MR signal. Adapted from.[76]

A MMP-2/9 inhibitory peptide, CTTHWGFTLC (CTT), was labeled with ^{125}I and [99m]Tc.[86] It was shown that the radiolabeled CTT peptide inhibited MMP-2 activity in vitro and could home to a MMP-2-positive tumor in mice. SPECT imaging of the tumors in mice was achieved with liposomes coated with ^{99m}Tc-labeled CTT. The reason for using liposomes as a carrier can be explained by another report of the ^{125}I-labeled CTT peptide.[87] In this study, it was shown that although the ^{125}I-labeled CTT peptide exhibited interesting properties in vitro for targeting MMP-2/9, its poor solubility and metabolic instability made it unsuitable for in vivo applications.

One intrinsic disadvantage of peptide-based probes is their poor stability in vivo, in particular for linear peptides. Many non-peptidyl MMPIs have been reported in the literature (Fig. 5). Radioiodinated analogs of the non-peptidyl MMPI, CGS 27023A, were synthesized for MMP detection in vivo.[88] In vitro studies revealed that the non-radioactive analogs of the MMPI exhibited affinities against MMP-2 and MMP-9 in the nanomolar range. Biodistribution of the probes in mice showed rapid blood and plasma clearance and low retention in normal tissues. Subsequently, radioiodinated carboxylic and hydroxamic MMPIs were reported.[89] In vitro enzyme assays showed high inhibition capacities of these MMPIs on MMP-2/9. However, the in vivo tumor targeting capability was quite disappointing. Another study of ^{123}I-labeled biphenylsulfonide (a small molecule MMPI) and its analogs in A549 lung carcinoma mice models also revealed that these probes were not suitable as tumor imaging agents.[90] Non-hydroxamate C-5-disubstituted pyrimidine-2,4,6-triones were reported to be subgroup-selective MMP inhibitors.[91] The compound has been labeled with ^{125}I yet in vivo testing has not been reported yet.

To date, SPECT imaging of MMPs in cancer has not been successful. Interestingly, the development of an activatable, MMP-14 targeted SPECT imaging probe was reported recently.[92] Only a modest two-fold reduction of cellular radioactivity was observed in the presence of a broad-spectrum MMPI. In the past, gamma cameras and SPECT imaging systems were much more readily accessible than PET systems.[93] However, PET has significantly higher sensitivity than SPECT and with
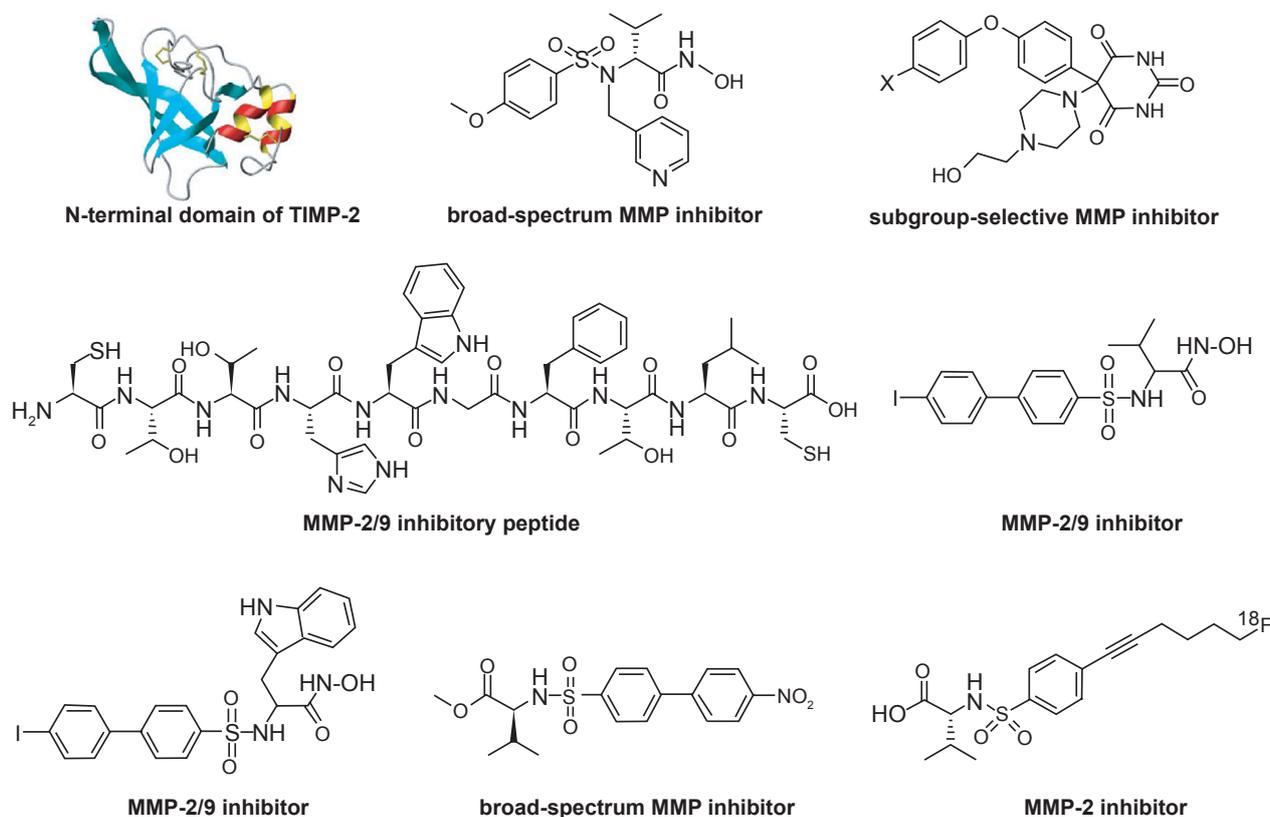


**Figure 5.** Representative MMPIs that have been radiolabeled for SPECT/PET applications.

the continuous developmental effort, state-of-the-art small animal PET scanners can have spatial resolution (<1 mm) comparable to SPECT and they are also becoming increasingly widely available.[94,95] Therefore, PET imaging will become more and more dominant over SPECT imaging in the future.

## PET Imaging of MMPs

Several PET probes for imaging protease (almost exclusively MMPs) expression in tumors have been reported. Those probes are generally MMPIs that have been labeled with $^{11}$C[96–98] and $^{18}$F.[99–102] However, in most of these reports, selective binding of the labeled compounds to specific MMPs was not shown, and high non-specific binding was observed possibly due to low in vivo stability of the tracers. Except for one report,[103] none of the abovementioned studies showed any convincing in vivo tumor targeting/ imaging results.

The previously mentioned MMP-2/9 inhibitory peptide, CTT, was conjugated with DOTA and labeled with $^{64}$Cu for PET imaging of MMP expression in xenograft models.[103] Zymography of tumor extracts supported the in vivo PET imaging results. MMP-2 and MMP-9 bands were clearly detectable in the mouse imaged at 7 weeks, which had prominent tumor uptake, whereas the MMP-2 and MMP-9 expression was very weak afterwards, which gave low tumor uptake. However, MMP expression in the MDA-MB-435 tumor model used in this study had quite large individual variance. The low in vivo stability of the probe also limits further investigation of this strategy.

## Conclusion and Future Perspectives

Molecular imaging of proteases in cancer has been investigated with a wide variety of agents (e.g. small molecules, peptides, proteins, polymers, and nanoparticles) labeled with many different imaging tags (e.g. fluorescent dyes, quantum dots, luciferins, Gd-chelates, magnetic nanoparticles, and radioisotopes) (Table 1). Optical techniques, in particular fluorescence imaging, are the most intensively validated and many of the protease imaging probes are already commercially available. It is also generally agreed that imaging with the "activatable probes" is the most accurate and appropriate means for measuring protease activity. Imaging protease activity with other techniques (i.e. MRI, SPECT, and PET) has not been well-documented in the literature which certainly deserves much effort in the future.

MRI of protease activity in cancer is so far limited to cell-based studies and whether molecular MRI of protease activity has a future in the clinic is questionable. PET/SPECT imaging certainly has the best potential for clinical application. However, suitable probes for imaging protease expression in cancer have yet to be developed. The fact that most of the radiolabeled MMPIs are not specific to a single MMP makes the scenario even more complicated as in many cases the experimental results were hard to interpret due to the presence of too many variables. First, the MMPs targeted in these studies are mostly cell secreted and their expression is known to vary extensively, depending on the stage of the tumor and the presence of naturally occurring TIMPs. Given the lack of data on the levels of MMP expression in the tumor tissue at the time of imaging in most studies reported to date, it cannot be excluded that the negative results reported are, in fact, false-negative imaging results.

Second, most of the agents used for imaging studies are intrinsically broad-spectrum agents. Therefore,

**Table 1.** A brief summary of protease imaging in cancer.

| Modality | Strategy | Tissue penetration | Sensitivity | Target proteases | Clinical potential |
|----------|----------|--------------------|-------------|------------------|--------------------|
| Fluorescence | Activatable or Activity-based | 1~2 cm | ++ | Cathepsins, MMPs, uPA | Low |
| Bioluminescence | Activatable | <1 cm | +++ | MMPs and Caspases | No |
| MRI | Activatable | No limit | + | MMPs | Low |
| SPECT | Inhibitor-based | No limit | +++ | MMPs | High |
| PET | Inhibitor-based | No limit | +++ | MMPs | High |

their higher affinity for specific subsets of MMPs does not necessarily imply that a positive imaging result corresponds to over-expression of specific subsets of MMPs, as suggested in some reports. Well-characterized tumor models need to be developed for validating the currently available, as well as future, MMP imaging probes. To develop suitable probes for protease imaging in the clinic, inhibitors with better specificity and higher affinity to a certain MMP should be developed.

Although the proof-of-principle has been demonstrated for protease imaging, there are certain limitations/flaws in many of the reported in vivo imaging studies. For successful in vivo imaging applications, the biochemical and cell biological properties of the probes should be demonstrable ex vivo. However, very few of the probes described, in particular the fluorescent probes, have been shown to be optimal even for live cell imaging. In addition, some control studies in these reports were also not very convincing. In vivo imaging is much more challenging and complex than cell-based imaging. In many cases, the following concepts may be intermingled in the interpretation of signal that was observed in vivo: signal intensity vs. specific protease activity, permeability vs. cleavage, and affinity vs. specificity. In future studies, these issues should be evaluated in both ex vivo and in vivo environments to provide more robust results for in vivo protease imaging.

Until recently, clinical trials targeting MMPs have yielded disappointing results, highlighting the need for better insight into the mechanisms by which enzymes contribute to tumor growth.[104,105] Molecular imaging to monitor protease expression non-invasively in vivo will be critical for early cancer detection, future protease-targeted drug development, and monitoring the therapeutic efficacy of these drugs. First, non-invasive imaging of proteases can help the clinicians to choose the right timing for protease inhibitor-based therapy. The reason why most MMPIs failed in clinical trials is that they are not administered at the right time.[106] Imaging can help determine the protease expression level at different stages, which can maximize the effect of protease inhibitors during treatment. Second, imaging of proteases can enable accurate monitoring of the therapeutic responses to certain treatment, since protease expression/activity is closely related to cancer progression and many proteases can serve as the target of drug treatment themselves. Lastly, protease imaging can also be used to develop in vitro, cell-based, or small animal assays for drug screening, which can benefit the cancer patients in the long run.

Protease imaging is not only applicable to cancer, it can also be applied in a wide variety of diseases such as arthritis,[107] renal diseases,[108] atherosclerosis,[109,110] myocardial infarction,[111] viral infection,[112] among others. In vivo imaging of protease activity, using non-optical techniques (which has good clinical potential), is a field in its infancy but with substantial promise. The next decade is likely to witness dramatic advances in the technologies required to bring novel protease imaging agents to clinical trials.

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## Disclosure

The authors report no conflicts of interest.

## References

1. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med.* 2006;12:895–904.
2. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* 2002;2:563–72.
3. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer.* 2002;2:161–74.
4. Turk B. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov.* 2006;5:785–99.
5. Garcia Boy R, Knapp EM, Eisenhut M, Haberkorn U, Mier W. Enzymes/transporters. *Handb Exp Pharmacol.* 2008;131–43.
6. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell.* 1998;94:353–62.
7. Weissleder R, Mahmood U. Molecular imaging. *Radiology.* 2001;219:316–33.
8. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* 2003;17:545–80.
9. Cai W, Niu G, Chen X. Imaging of integrins as biomarkers for tumor angiogenesis. *Curr Pharm Des.* 2008;14:2943–73.
10. Weissleder R. Scaling down imaging: molecular mapping of cancer in mice. *Nat Rev Cancer.* 2002;2:11–8.
11. Baruch A, Jeffery DA, Bogyo M. Enzyme activity—it's all about image. *Trends Cell Biol.* 2004;14:29–35.

12. Funovics M, Weissleder R, Tung CH. Protease sensors for bioimaging. *Anal Bioanal Chem.* 2003;377:956–63.

13. Blum G. Use of fluorescent imaging to investigate pathological protease activity. *Curr Opin Drug Discov Devel.* 2008;11:708–16.

14. Weissleder R, Pittet MJ. Imaging in the era of molecular oncology. *Nature.* 2008;452:580–9.

15. Cai W, Chen X. Nanoplatforms for targeted molecular imaging in living subjects. *Small.* 2007;3:1840–54.

16. Hong H, Gao T, Cai W. Molecular imaging with single-walled carbon nanotubes. *Nano Today.* 2009;4:252–61.

17. Tsien RY. Building and breeding molecules to spy on cells and tumors. *FEBS Lett.* 2005;579:927–32.

18. McIntyre JO, Matrisian LM. Molecular imaging of proteolytic activity in cancer. *J Cell Biochem.* 2003;90:1087–97.

19. Jedeszko C, Sloane BF. Cysteine cathepsins in human cancer. *Biol Chem.* 2004;385:1017–27.

20. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer.* 2006;6:764–75.

21. Keppler D, Sloane BF. Cathepsin B: multiple enzyme forms from a single gene and their relation to cancer. *Enzyme Protein.* 1996;49:94–105.

22. Benes P, Vetvicka V, Fusek M. Cathepsin D—many functions of one aspartic protease. *Crit Rev Oncol Hematol.* 2008;68:12–28.

23. Zaidi N, Maurer A, Nieke S, Kalbacher H. Cathepsin D: a cellular roadmap. *Biochem Biophys Res Commun.* 2008;376:5–9.

24. Kos J, Sekirnik A, Kopitar G, et al. Cathepsin S in tumours, regional lymph nodes and sera of patients with lung cancer: relation to prognosis. *Br J Cancer.* 2001;85:1193–200.

25. Quintanilla-Dieck MJ, Codriansky K, Keady M, Bhawan J, Runger TM. Cathepsin K in melanoma invasion. *J Invest Dermatol.* 2008;128:2281–8.

26. Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol.* 1999;17:375–8.

27. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics. A review. *J Control Release.* 2000;65:271–84.

28. Tanaka T, Shiramoto S, Miyashita M, Fujishima Y, Kaneo Y. Tumor targeting based on the effect of enhanced permeability and retention (EPR) and the mechanism of receptor-mediated endocytosis (RME). *Int J Pharm.* 2004;277:39–61.

29. Mahmood U, Tung CH, Bogdanov A Jr, Weissleder R. Near-infrared optical imaging of protease activity for tumor detection. *Radiology.* 1999;213:866–70.

30. Bremer C, Tung CH, Bogdanov A Jr, Weissleder R. Imaging of differential protease expression in breast cancers for detection of aggressive tumor phenotypes. *Radiology.* 2002;222:814–8.

31. Alencar H, Funovics MA, Figueiredo J, et al. Colonic adenocarcinomas: near-infrared microcatheter imaging of smart probes for early detection—study in mice. *Radiology.* 2007;244:232–8.

32. Ntziachristos V, Bremer C, Tung C, Weissleder R. Imaging cathepsin B up-regulation in HT-1080 tumor models using fluorescence-mediated molecular tomography (FMT). *Acad Radiol.* 2002;9 Suppl 2:S323–5.

33. Ntziachristos V, Bremer C, Graves EE, Ripoll J, Weissleder R. In vivo tomographic imaging of near-infrared fluorescent probes. *Mol Imaging.* 2002;1:82–8.

34. Bremer C, Ntziachristos V, Weitkamp B, et al. Optical imaging of spontaneous breast tumors using protease sensing 'smart' optical probes. *Invest Radiol.* 2005;40:321–7.

35. Blum G, Mullins SR, Keren K, et al. Dynamic imaging of protease activity with fluorescently quenched activity-based probes. *Nat Chem Biol.* 2005;1:203–9.

36. Joyce JA, Baruch A, Chehade K, et al. Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. *Cancer Cell.* 2004;5:443–53.

37. Blum G, von Degenfeld G, Merchant MJ, Blau HM, Bogyo M. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat Chem Biol.* 2007;3:668–77.

38. Tung CH, Bredow S, Mahmood U, Weissleder R. Preparation of a cathepsin D sensitive near-infrared fluorescence probe for imaging. *Bioconjug Chem.* 1999;10:892–6.

39. Galande AK, Hilderbrand SA, Weissleder R, Tung CH. Enzyme-targeted fluorescent imaging probes on a multiple antigenic peptide core. *J Med Chem.* 2006;49:4715–20.

40. McKerrow JH, Bhargava V, Hansell E, et al. A functional proteomics screen of proteases in colorectal carcinoma. *Mol Med.* 2000;6:450–60.

41. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol.* 2000;18:1135–49.

42. Foda HD, Zucker S. Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. *Drug Discov Today.* 2001;6:478–82.

43. Bremer C, Bredow S, Mahmood U, Weissleder R, Tung CH. Optical imaging of matrix metalloproteinase-2 activity in tumors: feasibility study in a mouse model. *Radiology.* 2001;221:523–9.

44. Bremer C, Tung CH, Weissleder R. In vivo molecular target assessment of matrix metalloproteinase inhibition. *Nat Med.* 2001;7:743–8.

45. Jiang T, Olson ES, Nguyen QT, et al. Tumor imaging by means of proteolytic activation of cell-penetrating peptides. *Proc Natl Acad Sci U S A.* 2004;101:17867–72.

46. Lauer-Fields JL, Minond D, Brew K, Fields GB. Application of topologically constrained mini-proteins as ligands, substrates, and inhibitors. *Methods Mol Biol.* 2007;386:125–66.

47. Lee S, Cha EJ, Park K, et al. A near-infrared-fluorescence-quenched gold-nanoparticle imaging probe for in vivo drug screening and protease activity determination. *Angew Chem Int Ed Engl.* 2008;47:2804–7.

48. Zhang Z, Yang J, Lu J, et al. Fluorescence imaging to assess the matrix metalloproteinase activity and its inhibitor in vivo. *J Biomed Opt.* 2008;13:011006.

49. McIntyre JO, Fingleton B, Wells KS, et al. Development of a novel fluorogenic proteolytic beacon for in vivo detection and imaging of tumour-associated matrix metalloproteinase-7 activity. *Biochem J.* 2004;377:617–28.

50. Scherer RL, VanSaun MN, McIntyre JO, Matrisian LM. Optical imaging of matrix metalloproteinase-7 activity in vivo using a proteolytic nanobeacon. *Mol Imaging.* 2008;7:118–31.

51. Cai W, Hsu AR, Li ZB, Chen X. Are quantum dots ready for *in vivo* imaging in human subjects? *Nanoscale Res Lett.* 2007;2:265–81.

52. Zheng G, Chen J, Stefflova K, et al. Photodynamic molecular beacon as an activatable photosensitizer based on protease-controlled singlet oxygen quenching and activation. *Proc Natl Acad Sci U S A.* 2007;104:8989–94.

53. Zhang Y, So MK, Rao J. Protease-modulated cellular uptake of quantum dots. *Nano Lett.* 2006;6:1988–92.

54. Rabbani SA, Xing RH. Role of urokinase (uPA) and its receptor (uPAR) in invasion and metastasis of hormone-dependent malignancies. *Int J Oncol.* 1998;12:911–20.

55. Mazar AP. The urokinase plasminogen activator receptor (uPAR) as a target for the diagnosis and therapy of cancer. *Anticancer Drugs.* 2001;12:387–400.

56. Law B, Curino A, Bugge TH, Weissleder R, Tung CH. Design, synthesis, and characterization of urokinase plasminogen-activator-sensitive near-infrared reporter. *Chem Biol.* 2004;11:99–106.

57. Hsiao JK, Law B, Weissleder R, Tung CH. In-vivo imaging of tumor associated urokinase-type plasminogen activator activity. *J Biomed Opt.* 2006;11:34013.

58. Luker GD, Luker KE. Optical imaging: current applications and future directions. *J Nucl Med.* 2008;49:1–4.

59. Schulz RB, Semmler W. Fundamentals of optical imaging. *Handb Exp Pharmacol.* 2008;3–22.

60. Scherer RL, McIntyre JO, Matrisian LM. Imaging matrix metalloproteinases in cancer. *Cancer Metastasis Rev.* 2008;27:679–90.

61. O'Brien MA, Daily WJ, Hesselberth PE, et al. Homogeneous, bioluminescent protease assays: caspase-3 as a model. *J Biomol Screen.* 2005;10:137–48.

62. Shinde R, Perkins J, Contag CH. Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays. *Biochemistry.* 2006;45:11103–12.

63. Abu-Qare AW, Abou-Donia MB. Biomarkers of apoptosis: release of cytochrome c, activation of caspase-3, induction of 8-hydroxy-2'-deoxyguanosine, increased 3-nitrotyrosine, and alteration of p53 gene. *J Toxicol Environ Health B Crit Rev.* 2001;4:313–32.

64. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* 1999;6:99–104.

65. Shah K, Tung CH, Breakefield XO, Weissleder R. In vivo imaging of S-TRAIL-mediated tumor regression and apoptosis. *Mol Ther.* 2005;11:926–31.

66. Liu JJ, Wang W, Dicker DT, El-Deiry WS. Bioluminescent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin. *Cancer Biol Ther.* 2005;4:885–92.

67. Van de Wiele C, Oltenfreiter R. Imaging probes targeting matrix metalloproteinases. *Cancer Biother Radiopharm.* 2006;21:409–17.

68. Zahra MA, Hollingsworth KG, Sala E, Lomas DJ, Tan LT. Dynamic contrast-enhanced MRI as a predictor of tumour response to radiotherapy. *Lancet Oncol.* 2007;8:63–74.

69. Pathak AP, Gimi B, Glunde K, et al. Molecular and functional imaging of cancer: Advances in MRI and MRS. *Methods Enzymol.* 2004;386:3–60.

70. Zhang Z, Nair SA, McMurry TJ. Gadolinium meets medicinal chemistry: MRI contrast agent development. *Curr Med Chem.* 2005;12:751–78.

71. Pautler RG, Fraser SE. The year(s) of the contrast agent—micro-MRI in the new millennium. *Curr Opin Immunol.* 2003;15:385–92.

72. de Roos A, Doornbos J, Baleriaux D, Bloem HL, Falke TH. Clinical applications of gadolinium-DTPA in MRI. *Magn Reson Annu.* 1988;113–45.

73. Thorek DL, Chen AK, Czupryna J, Tsourkas A. Superparamagnetic iron oxide nanoparticle probes for molecular imaging. *Ann Biomed Eng.* 2006;34:23–38.

74. Ntziachristos V, Ripoll J, Wang LV, Weissleder R. Looking and listening to light: the evolution of whole-body photonic imaging. *Nat Biotechnol.* 2005;23:313–20.

75. Lepage M, Dow WC, Melchior M, et al. Noninvasive detection of matrix metalloproteinase activity in vivo using a novel magnetic resonance imaging contrast agent with a solubility switch. *Mol Imaging.* 2007;6:393–403.

76. Schellenberger E, Rudloff F, Warmuth C, et al. Protease-specific nanosensors for magnetic resonance imaging. *Bioconjug Chem.* 2008;19:2440–5.

77. Lebel R, Jastrzebska B, Therriault H, et al. Novel solubility-switchable MRI agent allows the noninvasive detection of matrix metalloproteinase-2 activity in vivo in a mouse model. *Magn Reson Med.* 2008;60:1056–61.

78. Jastrzebska B, Lebel R, Therriault H, et al. New enzyme-activated solubility-switchable contrast agent for magnetic resonance imaging: from synthesis to in vivo imaging. *J Med Chem.* 2009;52:1576–81.

79. Cai W, Chen X. Multimodality molecular imaging of tumor angiogenesis. *J Nucl Med.* 2008;49 Suppl 2:113S–28S.

80. Franc BL, Acton PD, Mari C, Hasegawa BH. Small-animal SPECT and SPECT/CT: important tools for preclinical investigation. *J Nucl Med.* 2008;49:1651–63.

81. Spanoudaki VC, Ziegler SI. PET and SPECT instrumentation. *Handb Exp Pharmacol.* 2008;53–74.

82. Gambhir SS. Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer.* 2002;2:683–93.

83. Vincenti MP. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. *Methods Mol Biol.* 2001;151:121–48.

84. Giersing BK, Rae MT, Carballido-Brea M, Williamson RA, Blower PJ. Synthesis and characterization of 111In-DTPA-N-TIMP-2: a radiopharmaceutical for imaging matrix metalloproteinase expression. *Bioconjug Chem.* 2001;12:964–71.

85. Kulasegaram R, Giersing B, Page CJ, et al. In vivo evaluation of 111In-DTPA-N-TIMP-2 in Kaposi sarcoma associated with HIV infection. *Eur J Nucl Med.* 2001;28:756–61.

86. Medina OP, Kairemo K, Valtanen H, et al. Radionuclide imaging of tumor xenografts in mice using a gelatinase-targeting peptide. *Anticancer Res.* 2005;25:33–42.

87. Kuhnast B, Bodenstein C, Haubner R, et al. Targeting of gelatinase activity with a radiolabeled cyclic HWGF peptide. *Nucl Med Biol.* 2004;31:337–44.

88. Kopka K, Breyholz HJ, Wagner S, et al. Synthesis and preliminary biological evaluation of new radioiodinated MMP inhibitors for imaging MMP activity in vivo. *Nucl Med Biol.* 2004;31:257–67.

89. Oltenfreiter R, Staelens L, Lejeune A, et al. New radioiodinated carboxylic and hydroxamic matrix metalloproteinase inhibitor tracers as potential tumor imaging agents. *Nucl Med Biol.* 2004;31:459–68.

90. Oltenfreiter R, Staelens L, Labied S, et al. Tryptophane-based biphenylsulfonamide matrix metalloproteinase inhibitors as tumor imaging agents. *Cancer Biother Radiopharm.* 2005;20:639–47.

91. Breyholz HJ, Schafers M, Wagner S, et al. C-5-disubstituted barbiturates as potential molecular probes for noninvasive matrix metalloproteinase imaging. *J Med Chem.* 2005;48:3400–9.

92. Watkins GA, Jones EF, Scott Shell M, et al. Development of an optimized activatable MMP-14 targeted SPECT imaging probe. *Bioorg Med Chem.* 2009;17:653–9.

93. Blake P, Johnson B, VanMeter JW. Positron emission tomography (PET) and single photon emission computed tomography (SPECT): clinical applications. *J Neuroophthalmol.* 2003;23:34–41.

94. Stickel JR, Qi J, Cherry SR. Fabrication and characterization of a 0.5-mm lutetium oxyorthosilicate detector array for high-resolution PET applications. *J Nucl Med.* 2007;48:115–21.

95. Visser EP, Disselhorst JA, Brom M, et al. Spatial resolution and sensitivity of the Inveon small-animal PET scanner. *J Nucl Med.* 2009;50:139–47.

96. Fei X, Zheng QH, Liu X, et al. Synthesis of radiolabeled biphenylsulfonamide matrix metalloproteinase inhibitors as new potential PET cancer imaging agents. *Bioorg Med Chem Lett.* 2003;13:2217–22.

97. Zheng QH, Fei X, Liu X, et al. Synthesis and preliminary biological evaluation of MMP inhibitor radiotracers [11C]methyl-halo-CGS 27023A analogs, new potential PET breast cancer imaging agents. *Nucl Med Biol.* 2002;29:761–70.

98. Zheng QH, Fei X, DeGrado TR, et al. Synthesis, biodistribution and micro-PET imaging of a potential cancer biomarker carbon-11 labeled MMP inhibitor (2R)-2-[[4-(6-fluorohex-1-ynyl)phenyl]sulfonylamino]-3-methylbutyric acid [11C]methyl ester. *Nucl Med Biol.* 2003;30:753–60.

99. Wagner S, Breyholz HJ, Law MP, et al. Novel fluorinated derivatives of the broad-spectrum MMP inhibitors N-hydroxy-2(R)-[[(4-methoxyphenyl)sulfonyl](benzyl)- and (3-picolyl)-amino]-3-methyl-butanamide as potential tools for the molecular imaging of activated MMPs with PET. *J Med Chem.* 2007;50:5752–64.

100. Wagner S, Breyholz HJ, Holtke C, et al. A new 18F-labelled derivative of the MMP inhibitor CGS 27023A for PET: radiosynthesis and initial small-animal PET studies. *Appl Radiat Isot.* 2009;67:606–10.

101. Breyholz HJ, Wagner S, Levkau B, et al. A 18F-radiolabeled analogue of CGS 27023A as a potential agent for assessment of matrix-metalloproteinase activity in vivo. *Q J Nucl Med Mol Imaging.* 2007;51:24–32.

102. Furumoto S, Takashima K, Kubota K, et al. Tumor detection using 18F-labeled matrix metalloproteinase-2 inhibitor. *Nucl Med Biol.* 2003;30:119–25.

103. Sprague JE, Li WP, Liang K, Achilefu S, Anderson CJ. In vitro and in vivo investigation of matrix metalloproteinase expression in metastatic tumor models. *Nucl Med Biol.* 2006;33:227–37.

104. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer.* 2006;6:227–39.

105. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science.* 2002;295:2387–92.

106. Rothenberg ML, Carbone DP, Johnson DH. Improving the evaluation of new cancer treatments: challenges and opportunities. *Nat Rev Cancer.* 2003;3:303–9.

107. Wunder A, Tung CH, Muller-Ladner U, Weissleder R, Mahmood U. In vivo imaging of protease activity in arthritis: a novel approach for monitoring treatment response. *Arthritis Rheum.* 2004;50:2459–65.

108. Aikawa E, Aikawa M, Libby P, et al. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation.* 2009;119:1785–94.

109. Jaffer FA, Kim DE, Quinti L, et al. Optical visualization of cathepsin K activity in atherosclerosis with a novel, protease-activatable fluorescence sensor. *Circulation.* 2007;115:2292–8.

110. Jaffer FA, Vinegoni C, John MC, et al. Real-time catheter molecular sensing of inflammation in proteolytically active atherosclerosis. *Circulation.* 2008;118:1802–9.

111. Jaffer FA, Sosnovik DE, Nahrendorf M, Weissleder R. Molecular imaging of myocardial infarction. *J Mol Cell Cardiol.* 2006;41:921–33.

112. Shah K, Tung CH, Chang CH, et al. In vivo imaging of HIV protease activity in amplicon vector-transduced gliomas. *Cancer Res.* 2004;64:273–8.