Optical Methods

Christoph Bremer

Abstract Molecular imaging requires the highest possible signal-to-noise ratios (SNRs) at the target of interest. In order to maximize the SNR for optical imaging techniques, various strategies have been developed to design fluorescent probes that can be activated, for example, by proteolytic degradation. Generally speaking, these probes are quenched in their native state—e.g., by fluorescence resonance energy transfer (FRET)—and dequenched after cleavage or hybridization, which is associated with a strong fluorescence signal increase.

Different strategies of fluorescence signal amplification ranging from large and small protease-sensing molecules to oligonucleotide-sensing and nanoparticle-based probes are presented in this chapter.

1 Introduction

Molecular imaging techniques require maximal signal-to-noise yields in order to noninvasively resolve specific molecular targets in vivo. Different, mainly enzyme-based signal amplification strategies have been described, which aim at (1)

Christoph Bremer
Department of Clinical Radiology, University of Münster, University Hospital Münster, Germany

Interdisciplinary Center for Clinical Research (IZKF Münster, FG3), University of Münster, Albert-Schweitzer-Str. 33, 48129 Münster, Germany
bremerc@uni-muenster.de

W. Semmler and M. Schwaiger (eds.), *Molecular Imaging II.*
Handbook of Experimental Pharmacology 185/II.

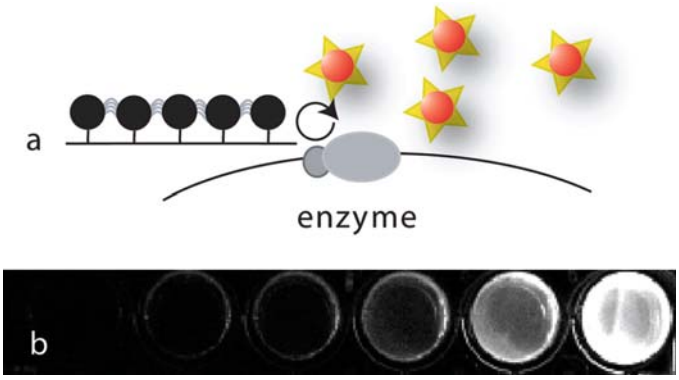

Fig. 1 a, b First generation of large protease sensing probes. The first class of proteolytically activatable optical probes is depicted. These probes consist of a poly-L-lysine backbone shielded by multiple methoxy-polyethylene glycol sidechains. Approximately 12–14 fluorochromes are attached to the backbone, resulting in a FRET-based signal quench in the native state of the molecule. Proteolytic cleavage of the backbone results in **a** a release of the fluorochromes, followed by **b** a strong fluorescence signal increase. (Modified from Bremer et al. 2003)

maximizing the fluorescence signal yield after target interaction and (2) reducing the unspecific background signal of circulating probes. In recent years, various activatable or 'smart' probes have been developed for molecular imaging. Typically, they show a strong fluorescence signal increase after interaction with an enzyme (e.g., a protease). The underlying principle is that the native probe is 'quenched', a phenomenon which has been known for a long time; e.g., in fluorescence microscopy. Enzymatic conversion results in dequenching of the probe accompanied by a strong increase in the fluorescence signal (Fig. 1). Quenching can result from the transfer of energy to other acceptor molecules residing physically close to the excited fluorochromes (e.g., a second acceptor fluorochrome), a phenomenon known as fluorescence resonance energy transfer (FRET). Quenching can, moreover, occur by competing processes such as temperature, high oxygen concentrations, molecular aggregation in the presence of salts and halogen compounds or interaction with metals.

2 Large Protease-sensing Probes

For molecular imaging applications, activatable probes ideally undergo a status of virtually zero signal in their native state to a strong fluorescence signal after target interaction. A class of 'smart' optical contrast agents, which undergoes conformational changes after cleavage by various enzymes, was first described by Weissleder et al. (1999) (Fig. 1). The first autoquenched fluorescent probe was developed in 1999. This was converted from a non-fluorescence to fluorescence state by proteolytic activation (Weissleder et al. 1999). This type of molecular contrast agent consists of a long circulating carrier molecule (poly-lysine backbone) shielded

by multiple methoxy-polythylene-glycol side chains (PLL-MPEG). The molecular weight of these probes ranges around 450–500 kDa. Between 12 and 14 cyanine dyes (Cy 5.5) are loaded onto this carrier molecule in close proximity to each other, resulting in a FRET-based signal quench (see above; Weissleder et al. 1999). Thus, in its native state, the molecule exhibits very little to no fluorescence, whereas after enzymatic cleavage a strong fluorescence signal increase can be detected (dequenching; Fig. 1). Inhibition experiments revealed that this first generation of protease-sensing optical probe is activated mainly by lysosomal cysteine or serine proteases, such as cathepsin-B (Weissleder et al. 1999). However, the selectivity of this smart optical probe can be tailored to other enzymes by insertion of specific peptide stalks between the carrier and the fluorochromes. Using this approach, smart optical probes have been developed for targeting–e.g., matrix-metalloproteinase-2, cathepsin-D, thrombin or caspases (Tung et al. 2000, 2004; Bremer et al. 2002; Jaffer et al. 2004; Messerli et al. 2004; Kim et al. 2005). In order to impart MMP-2 selectivity, for example, a peptide stalk with the sequence -Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys- was inserted between the backbone and the fluorochrome. This peptide sequence is recognized by MMP-2 with a high affinity, resulting in an efficient dequenching of the completely assembled MMP-2 probe by the purified enzyme (Fig. 2). A control probe, which was synthesized using a scrambled peptide sequence (-Gly-Val-Arg-Leu-Gly-Pro-Gly-Lys-), remained quenched after incubation with the purified enzyme (Fig. 2).

Proteases are known to be key players in a whole variety of pathologies, ranging from carcinogenesis to inflammatory and cardiovascular diseases (Edwards and Murphy 1998). From the oncological literature it is known that various proteases, such as cathepsins and matrix-metalloproteinases, are involved in a cascade of enzymes, which finally leads to digestion of the extracellular matrix and, thus, local as well as metastatic tumor cell infiltration (Edwards and Murphy 1998; Aparicio et al. 1999; Folkman 1999; Herszenyi et al. 1999; Fang et al. 2000; Koblinski et al. 2000). Indeed, clinical data suggest that the tumoral protease burden correlates with clinical outcome. Thus, the activatable probes outlined above have been applied for a variety of different oncological models, including xenograft and spontaneous tumor models (Figs. 2, 3). A cathepsin-sensing probe could be applied successfully to detect micronodules of tumor xenografts and spontaneous tumors using fluorescence reflectance imaging (FRI) or fluorescence-mediated tomography (FMT). The response to protease inhibitor treatment could be monitored early and noninvasively using a MMP sensitive probe (Bremer et al. 2005). Other experimental data suggest that a noninvasive tumor grading (aggressive versus nonaggressive phenotype) may be facilitated using these probes (Bremer et al. 2002).

Since proteases are ubiquitously expressed, the aforementioned probes could also be successfully applied for imaging of inflammatory responses; e.g., in an experimental arthritis model. Interestingly, treatment effects (e.g., methotrexate application) could be monitored sensitively using this approach (Wunder et al. 2004; Fig. 4). Successful treatment of arthritis resulted in a clear reduction of the joint associated fluorescence (Wunder et al. 2004). In a cardiovascular plaque model (ApoE mice), strong probe activation within the atherosclerotic plaques most likely

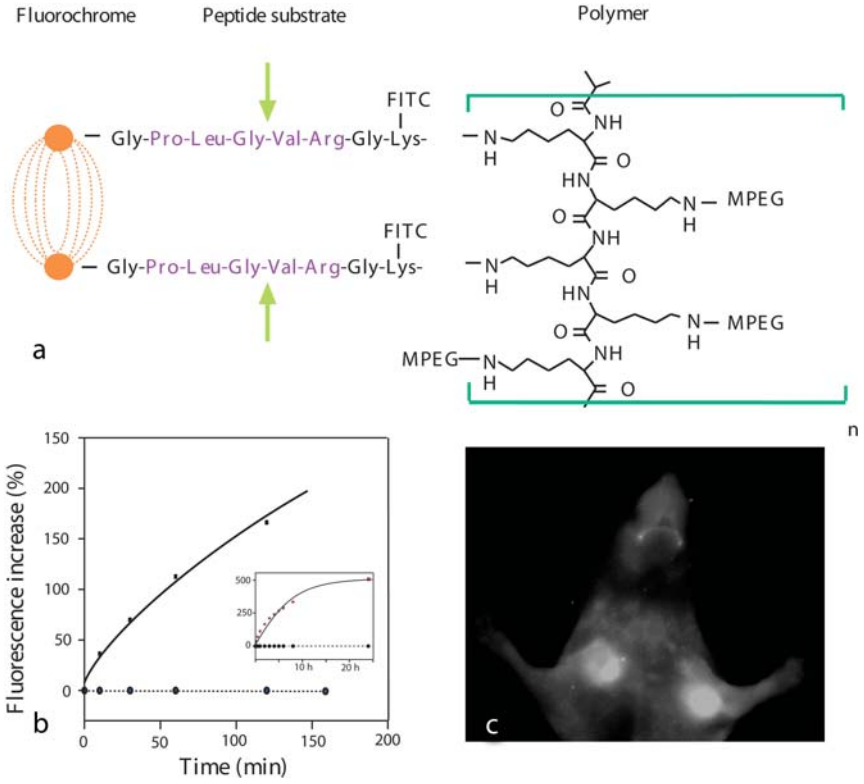

Fig. 2 **a-c** Second generation of large protease sensing probes: MMP imaging. A modification of the first generation of 'smart' optical probes is shown. **a** In order to impart specificity of the probe for matrix metalloproteinases, the fluorochromes were conjugated to the backbone through peptide stalks, which are cleaved with a high affinity by MMP-2. **b** Incubation with the purified enzymes showed a strong fluorescence signal increase for the MMP probe, while the probe containing a scrambled peptide sequence remained quenched. **c** Tumor xenografts overexpressing MMP-2 could clearly be visualized using this approach. (From Bremer et al. 2002)

representing inflammatory plaque reactions could be successfully visualized using this approach (Chen et al. 2002).

3 Small Protease-sensing Probes

Smaller molecules that also undergo an enzymatic conversion have more recently been described. They can be designed by flanking an enzyme substrate with two fluorophores or a fluorophore and a spectrally matched quencher molecule, which absorbs the energy of the fluorochrome via FRET without the emission of photons (Fig. 5).

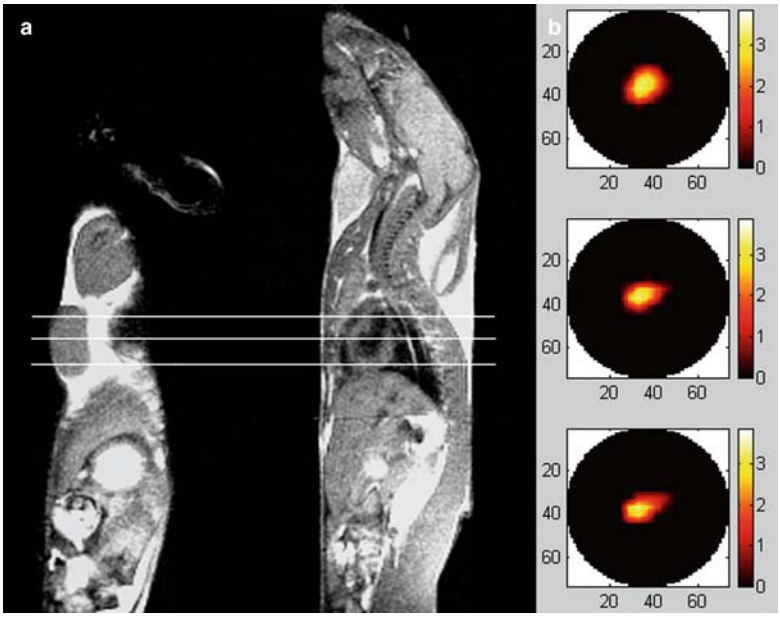

Fig. 3 **a, b** Application of a cathepsin-sensing probe for in vivo tumor detection. Fluorescence mediated tomography (FMT) of spontaneous mammary cancer after injection of a cathepsin sensing optical probe. **a** FMT images were acquired at the levels illustrated in the corresponding sagittal MR images. **b** After injection of the optical probe strong tissue fluorescence could be reconstructed in the tumor region as seen in the corresponding axial FMT slice. (From Bremer et al. 2005)

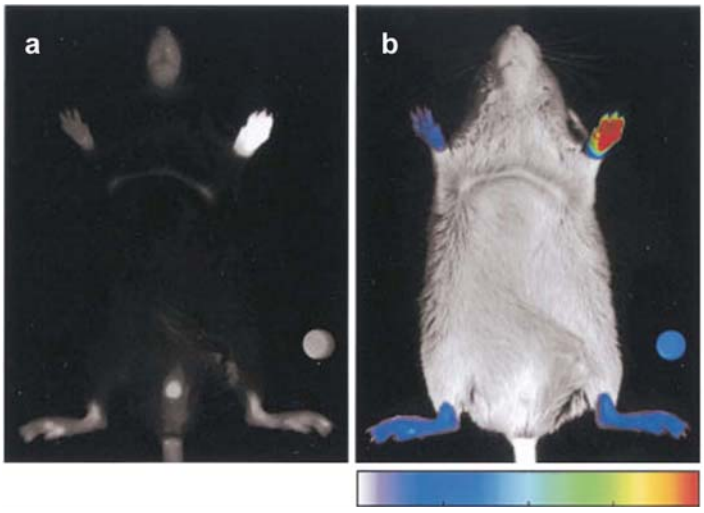

Fig. 4 **a, b** Application of a cathepsin-sensing probe for in vivo imaging of arthritis. **a** Raw NIRF image of a mouse with collagen-induced arthritis in the right for paw, obtained 24 h after probe injection. Note the high fluorescence intensity in the affected extremity. **b** Color-coded NIRF image of **a** superimposed on white-light image. Cy 5.5 dye (16 nmol/ml), seen above the right hind paw, was used for standardization. (Wunder et al. 2004)

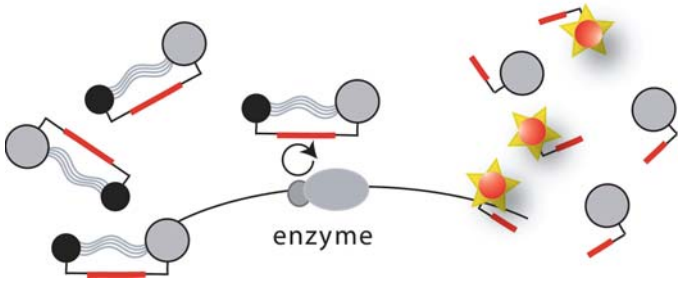

Fig. 5 Small protease-sensing probes. Small protease-sensing probes are designed by flanking an enzyme substrate (*red bar*) with two fluorophores or a fluorophore and a spectrally matched quencher molecule (*gray dot*), which absorbs the energy of the fluorochrome via FRET without the emission of photons. Enzymatic cleavage of the probes results in a significant dequenching effect, followed by a fluorescence signal increase

The coupling of a quencher to a fluorophore via a caspase-cleavable nonapeptide, for example, can be exploited to detect caspase activity (a marker of cellular apoptosis) in vitro (Pham et al. 2002). A similar design was proposed for imaging MMP activity using a different peptide bridge, which is cleaved with a high affinity by MMPs (Pham et al. 2004). Here an absorber molecule (NIRQ820) was linked to Cy 5.5 via a MMP-7 substrate. Incubation of the probe with the purified enzyme resulted in a sevenfold signal increase after dequenching, while MMP-9, for example, did not result in dequenching of the probe, which supports the selectivity of this system. Bullok and co-workers recently presented a small, membrane-permeable probe that is capable of sensing intracellular caspase activity (Bullok and Piwnica-Worms 2005). The molecule consists of a Tat-peptide-based permeation sequence and a caspase recognition sequence (DEVD) flanked by a fluorochrome (Alexa Fluor 647) and a quencher (QSY 21) (Bullok and Piwnica-Worms 2005). Efficient quenching was achieved in the native state of the molecule, while incubation with the effector caspases (especially caspases 3 and 7) resulted in a significant dequenching of the probe. Cell experiments demonstrated a successful permeation of the probe into the cell so that caspase activity could be visualized by a clear fluorescence signal (Bullok and Piwnica-Worms 2005).

Law et al. (2005) recently developed a small FRET-based probe that recognized protein kinase A (PKA). The probe consists of a specific binding peptide sequence (LRRRRFAFC) conjugated with two fluorophores (FAMS, TAMRA). In the absence of PKA, the two fluorophores associate by hydrophobic interactions, forming an intramolecular ground-state dimer; this results in fluorescein quenching (>93%). Upon PKA addition, the reporter reacts with the sulfhydryl functionality at Cys199 through a disulfide-exchange mechanism. FAMS is subsequently released, resulting in significant fluorescence amplification (Law et al. 2005). The remaining peptide sequence, which acts as an inhibitor, is attached covalently to the enzyme.

While the in vitro results of these small protease-sensing probes are promising, in vivo applications may be more difficult since rapid clearance of the probes may counteract sufficient probe accumulation at the target of interest.

4 Oligonucleotide-sensing Probes

A number of different oligonucleotide-based small, activatable optical probes have been described which were designed to monitor gene expression. As outlined in 4.1.2, these probes are quenched in their native state by either dimerization of fluorophores or by interaction with a specific quencher molecule. Tyagi et al. (2000) designed a probe that contains a harvester fluorophore that absorbs strongly in the wavelength range of the monochromatic light source, an emitter fluorophore of the desired emission color, and a nonfluorescent quencher (Fig. 6). In the absence of complementary nucleic acid targets, the probes are dark, whereas in the presence of targets, they fluoresce, though not in the emission range of the harvester fluorophore that absorbs the light, but rather in the emission range of the emitter fluorophore (Tyagi et al. 2000). This shift in emission spectrum is due to the transfer of the absorbed energy from the harvester fluorophore to the emitter fluorophore by fluorescence resonance energy transfer, and it only takes place in probes that are bound to targets (i.e., hybridized to the target oligonucleotides).

Metelev et al. (2004) proposed a similar molecule that consists of a hairpin oligonucleotide flanked by two cyanine dyes (e.g., Cy 5.5), which upon hybridization with the target oligonucleotide sequence (here: NF-κB) shows a strong dequenching effect. These types of probes can be applied for in vitro gene analysis or, ultimately, potentially for in vivo genotyping. However, delivery barriers for in vivo applications are significant so that up to date true in vivo applications have not yet been described.

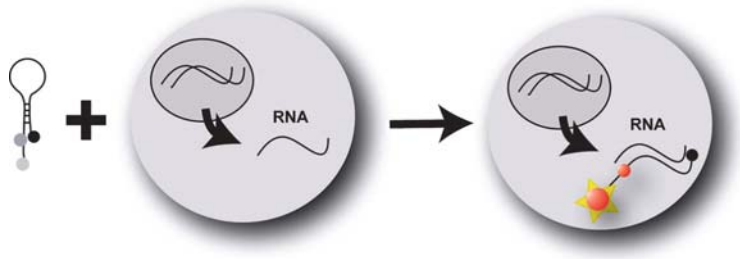

Fig. 6 Oligonucleotide-sensing probes. These consist of an oligonucleotide coupled to two fluorochromes or a harvester fluorochrome, an emitter fluorochrome and a nonfluorescent quencher. In the absence of complementary nucleic acid targets, the probes are dark, due to their hairpin configuration with approximation of the quencher (or the second fluorophor) to the fluorochrome (*left*). In the presence of targets, however, the probe unfolds and hybridizes with the oligonucleotide, resulting in spatial separation of the fluorochromes from the quencher/second fluorophor so that a fluorescent signal can be detected (*right*). (From Bremer et al. 2003)

5 Nanoparticle-based Probes

Fluorophores can interact with nanoparticles, such as superparamagnetic iron oxides, resulting in a signal quench of the probe (Fig. 7). Josephson et al. (2002) recently described a hydrid iron oxide-based nanoparticle that was conjugated with a fluorochrome (Cy 5.5). The surface of the nanoparticles was covered with aminated cross-linked dextran, which allowed covalent binding of Cy 5.5 via protease-sensitive (or protease-resistant) peptides. Interestingly, the authors found that even nanoparticles that were, on average, labeled with only 0.14 Cy 5.5/particle showed significant dequenching effects, suggesting that interaction between the iron oxide nanoparticle and the fluorochrome contributes to the quenching effect. Loading the nanoparticle with multiple fluorochromes (up to 1.19/particle) significantly increased the quenching/dequenching mechanism (Josephson et al. 2002). The quenching of fluorescence in proximity to the magnetic nanoparticle may be due to nonradiative energy transfer between the dye and the iron oxide or due to collisions between Cy 5.5 and the nanoparticle. Josephson et al. (2002) could successfully apply this probe for imaging lymph nodes in a mouse model by both MRI as well as near infrared fluorescence reflectance imaging (FRI). Modifications of this multivalent magneto-optical probe were presented by Schellenberger et al. (2004) who were able to attach Annexin V to the nanoparticle and therefore target apoptotic cells using these probes. A similar phenomenon was also described by Dubertret et al. (2001), who demonstrated that colloidal gold particles can efficiently quench fluorochromes.

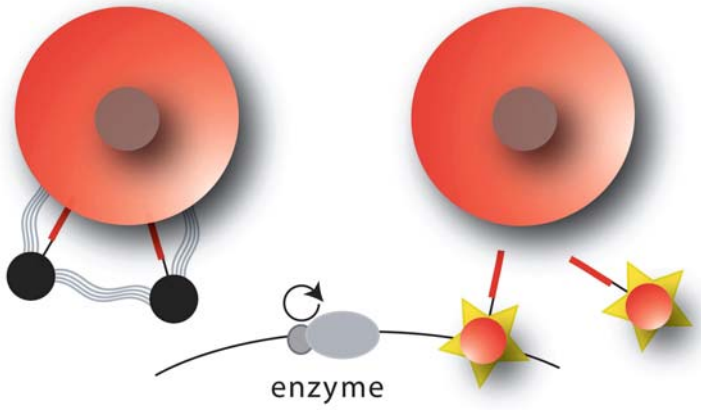

Fig. 7 Nanoparticle-based probes. Functionalized nanoparticles [e.g., aminated superparamagnetic iron oxides (SPIOs)] can be linked to fluorochromes via a peptide spacer. Quenching will occur based on interactions of the fluorochrome with the iron core and/or FRET-based quenching with neighboring fluorochromes. Enzymatic release of the fluorochromes results in a significant increase of the fluorescence signal. These multivalent probes can be applied for multimodal imaging; e.g., with MRI and optical techniques. In a clinical scenario, noninvasive MR-based probe localization could be combined with high-resolution, real-time fluorescence imaging of the probe; e.g., in an intraoperative setting

Multimodal probes may well have a clinical perspective since they may be applied, for example, preoperatively for noninvasive detection of the SPIO distribution by MRI and finally for intraoperative guidance using simple fluorescence reflectance imaging techniques (Kircher et al. 2003).

6 Other Amplification Mechanisms

In order to detect ß-galactosidase activity, Tung et al. (2004) employed a fluorogenic substrate that undergoes a significant wavelength shift after conversion by the enzyme. While the initial substrate (DDAOG) is excited at 465 nm and fluoresces at 608 nm, enzymatic cleavage by ß-galactosidase results in a release of another flourogenic substrate (DDAO), which is excited at 646 nm and fluoresces at 659 nm. Thus, the cleavage product has far-red fluorescence properties that can be imaged by FRI. Moreover, significantly, the wavelength shift (approximately 50 nm) allows detection of the cleaved substrate without background signal from the intact probe (Tung et al. 2004).

Another elegant way to amplify the optical signal in the target tissue was described by Jiang et al. (2004), who designed an imaging agent that consists of polyarginine-based cell-penetrating peptides (CPP), which are fused through a cleavable linker to an inhibitory domain consisting of negatively charged residues. Cleavage of the linker, typically by a protease, releases the CPP portion and its attached cargo (e.g., a fluorochrome) to bind and enter cells. In cell culture and in vivo, protease activities (e.g., MMP-2 and -9) were successfully visualized, showing in vivo contrast ratios of 2–3 and a 3.1-fold increase in standard uptake values for tumors relative to normal tissue or control peptides with scrambled linkers. Thus, these cell-permeating probes may be another suitable way of amplifying the fluorescence signal for molecular optical imaging.

References

Aparicio T, Kermorgant S, Dessirier V, Lewin MJ, Lehy T (1999) Matrix metalloproteinase inhibition prevents colon cancer peritoneal carcinomatosis development and prolongs survival in rats. Carcinogenesis 20:1445–1451

Bremer C, Ntziachristos V, Weissleder R (2003) Optical-based molecular imaging: contrast agents and potential medical applications. Eur Radiol 13:231–243

Bremer C, Ntziachristos V, Weitkamp B, Theilmeier G, Heindel W, Weissleder R (2005) Optical imaging of spontaneous breast tumors using protease sensing 'smart' optical probes. Invest Radiol 40:321–327

Bremer C, Tung CH, Bogdanov A Jr, Weissleder R (2002) Imaging of differential protease expression in breast cancers for detection of aggressive tumor phenotypes. Radiology 222:814–818

Bremer C, Tung CH, Weissleder R (2002) Molecular imaging of MMP expression and therapeutic MMP inhibition. Acad Radiol 9(Suppl 2):S314–S315

Bullok K, Piwnica-Worms D (2005) Synthesis and characterization of a small, membrane-permeant, caspase-activatable far-red fluorescent peptide for imaging apoptosis. J Med Chem 48:5404–5407

Chen J, Tung CH, Mahmood U, Ntziachristos V, Gyurko R, Fishman MC, Huang PL, Weissleder R (2002) In vivo imaging of proteolytic activity in atherosclerosis. Circulation 105:2766–2771

Dubertret B, Calame M, Libchaber AJ (2001) Single-mismatch detection using gold-quenched fluorescent oligonucleotides. Nat Biotechnol 19:365–370

Edwards DR, Murphy G (1998) Cancer. Proteases–invasion and more. Nature 394:527–528

Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, Harper J, Tamvakopoulos G, Moses MA (2000) Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci USA 97:3884–3889

Folkman J (1999) Angiogenic zip code. Nat Biotechnol 17:749

Herszenyi L, Plebani M, Carraro P, De Paoli M, Roveroni G, Cardin R, Tulassay Z, Naccarato R, Farinati F (1999) The role of cysteine and serine proteases in colorectal carcinoma. Cancer 86:1135–1142

Jaffer FA, Tung CH, Wykrzykowska JJ, Ho NH, Houng AK, Reed GL, Weissleder R (2004) Molecular imaging of factor XIIIa activity in thrombosis using a novel, near-infrared fluorescent contrast agent that covalently links to thrombi. Circulation 110:170–176

Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY (2004) Tumor imaging by means of proteolytic activation of cell-penetrating peptides. Proc Natl Acad Sci USA 101:17867–17872

Josephson L, Kircher MF, Mahmood U, Tang Y, Weissleder R (2002) Near-infrared fluorescent nanoparticles as combined MR/optical imaging probes. Bioconjug Chem 13:554–560

Kim DE, Schellingerhout D, Jaffer FA, Weissleder R, Tung CH (2005) Near-infrared fluorescent imaging of cerebral thrombi and blood-brain barrier disruption in a mouse model of cerebral venous sinus thrombosis. J Cereb Blood Flow Metab 25:226–233

Kircher MF, Mahmood U, King RS, Weissleder R, Josephson L (2003) A multimodal nanoparticle for preoperative magnetic resonance imaging and intraoperative optical brain tumor delineation. Cancer Res 63:8122–8125

Koblinski JE, Ahram M, Sloane BF (2000) Unraveling the role of proteases in cancer. Clin Chim Acta 291:113–135

Law B, Weissleder R, Tung CH (2005) Mechanism-based fluorescent reporter for protein kinase A detection. Chembiochem 6:1361–1367

Messerli SM, Prabhakar S, Tang Y, Shah K, Cortes ML, Murthy V, Weissleder R, Breakefield XO, Tung CH (2004) A novel method for imaging apoptosis using a caspase-1 near-infrared fluorescent probe. Neoplasia 6:95–105

Metelev V, Weissleder R, Bogdanov A, Jr. (2004) Synthesis and properties of fluorescent NF-kappa B-recognizing hairpin oligodeoxyribonucleotide decoys. Bioconjug Chem 15:1481–1487

Pham W, Choi Y, Weissleder R, Tung CH (2004) Developing a peptide-based near-infrared molecular probe for protease sensing. Bioconjug Chem 15:1403–1407

Pham W, Weissleder R, Tung CH (2002) An azulene dimer as a near-infrared quencher. Angew Chem Int Ed Engl 41:3659–3662, 3519

Schellenberger EA, Sosnovik D, Weissleder R, Josephson L (2004) Magneto/optical annexin V, a multimodal protein. Bioconjug Chem 15:1062–1067

Tung CH, Mahmood U, Bredow S, Weissleder R (2000) In vivo imaging of proteolytic enzyme activity using a novel molecular reporter. Cancer Res 60:4953–4958

Tung CH, Zeng Q, Shah K, Kim DE, Schellingerhout D, Weissleder R (2004) In vivo imaging of beta-galactosidase activity using far red fluorescent switch. Cancer Res 64:1579–1583

Tyagi S, Marras SA, Kramer FR (2000) Wavelength-shifting molecular beacons. Nat Biotechnol 18:1191–1196

Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. (1999) In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nat Biotechnol 17:375–378

Wunder A, Tung CH, Muller-Ladner U, Weissleder R, Mahmood U (2004) In vivo imaging of protease activity in arthritis: a novel approach for monitoring treatment response. Arthritis Rheum 50:2459–2465

http://www.springer.com/978-3-540-77449-5

Molecular Imaging II
Semmler, W.; Schwaiger, M. (Eds.)
2008, XXVII, 365 p. 96 illus., 44 illus. in color., Hardcover
ISBN: 978-3-540-77449-5