Abstract  In recent years, molecular imaging gained significant importance in biomedical research. Optical imaging developed into a modality which enables the visualization and quantification of all kinds of cellular processes and cancerous cell growth in small animals. Novel gene reporter mice and cell lines and the development of targeted and cleavable fluorescent “smart” probes form a powerful imaging toolbox. The development of systems collecting tomographic bioluminescence and fluorescence data enabled even more spatial accuracy and more quantitative measurements. Here we describe various bioluminescent and fluorescent gene reporter models and probes that can be used to specifically image and quantify neovascularization or the angiogenic process itself.

Keywords  Angiogenesis · Bioluminescence · Fluorescence · Molecular imaging · Optical imaging

Introduction

Optical imaging

Optical imaging is a very versatile, sensitive and powerful tool for molecular imaging in small animals. It is based on the detection of photons in the visible light spectrum emitted from living cells, tissues or animals and can be divided in two main modalities, bioluminescence imaging (BLI) and fluorescence imaging (FLI). Optical imaging of angiogenesis and existing vasculature is currently dominated by FLI-based imaging models, but there are several interesting BLI models as well. This review provides an overview of the available optical imaging models that can be used within angiogenesis related research.

Bioluminescence imaging

Bioluminescence imaging has been developed over the last decade as a tool for molecular imaging of small laboratory animals, enabling the study of ongoing biological processes such as tumor growth and metastasis and gene expression in vivo [1]. All bioluminescent reporter systems are based on the detection of photons produced in an enzymatic reaction in which a substrate, like luciferin or coelenterazine, is oxidated by an enzyme, luciferase. There are many different kinds of luciferases occurring in nature and being adapted for research. The most commonly used luciferase for molecular imaging purposes is the one extracted from the North American firefly (Photinus pyralis; FLuc) emitting light with a broad emission spectrum and a peak around 560 nm but other useful luciferases have also been cloned from corals (Tenilla), jellyfish (Aequorea), several bacterial species (Vibrio fischeri, V. harveyi) and red or green click beetle (Pyrophorus plagiophthalmus) which have been optimized to produce green–orange (544 nm) or red (611 nm) light after oxidizing luciferin.

Luciferases from the anthozoaan sea pansy (Renilla reniformis) and the marine copepod (Gaussia princeps) react with coelenterazine and are ATP-independent to produce blue light with peak emission at approximately 480 nm. Despite the blue emission wavelength of these enzymes, limited biodistribution and rapid kinetics of coelenterazine in small animals, these luciferases have been proven very useful for in vivo applications for
molecular imaging \[2–6\]. Because the substrates luciferin and coelentrazin for firefly luciferase (F\(\text{Fluc}\)) and Gaussia luciferase (Gluc), respectively, show no crossreactivity, concomitantly imaging of distinct cell populations that either express F\(\text{Fluc}\) or Gluc can be performed within the same animal.

BLI reporter systems are especially suitable for biomedical research purposes due to the extremely low background signal, high signal to noise ratio, non-invasive character, short acquisition time (seconds to minutes) and the possibility to measure more animals at once (high throughput).

Fluorescence imaging

In contrast to BLI, fluorescence imaging is not based on the production of photons by an enzymatic reaction; instead, a fluorescent compound (fluorophore) can be exited by photons of a certain wavelength from an external light source. Upon relaxation to its ground state, the fluorophore emits photons at a different wavelength. These emitted photons are the signal which is used for imaging. Similar to BLI, whole body FLI enables tracking of tumor growth and metastasis, gene expression, angiogenesis and bacterial infection, quantitatively (reviewed in Hoffman \[7\]).

There is a wide range of fluorophores available, ranging from fluorescent dyes and nanoparticles, like quantum dots, to fluorescent proteins which can be expressed in transgenic models. Each fluorophore has several characteristics amongst which the excitation wavelength, emission wavelength and quantum yield or brightness. The excitation wavelength is the wavelength of external light to bring the fluorophore to its exited state. The emission wavelength is the wavelength of the photons emitted upon relaxation. Usually, the emitted photons have a longer wavelength than the photons used for excitation and thus, the emitted photons have a lower energetic value. The brightness or quantum yield of a fluorophore is defined by the fraction of molecules that emit a photon after direct excitation by the excitation light source. In most cases this value is nearly the same value as the ratio between the number of photons emitted from a bulk sample and the total number of absorbed photons \[8\]. All three of these properties, excitation wavelength, emission wavelength and quantum yield, have important implications for the use of a certain fluorophore for imaging \[9\].

When using fluorescence for in vivo imaging, tissue absorbance, scattering and auto-fluorescence can become a problem especially when imaging structures deeper in the animal. Most auto-fluorescence occurs in the green part of the spectrum \[9, 10\]. The amount of auto-fluorescence rapidly decreases when shifting towards a longer excitation wavelength, the red parts of the spectrum. Near infrared (IR) light causes hardly any auto-fluorescence \[10–12\]. Moreover, light penetration, tissue absorption and scattering are greatly reduced at the near infrared end of the spectrum compared to green light.

Recently fluorescent proteins with increasingly longer emission maxima (up to 649 nm), like the series of red shifted proteins obtained by mutating dsRed, i.e., mFruits like mCherry, mTomato and mPlum \[13, 14\], and a series of recently developed very bright, red-shifted proteins derived from the anemone Entacmaea quadricolor like Katushka and mKate \[12\], have been developed. The longer emission wavelengths lead to a reduction of background auto-fluorescence and substantially increase tissue penetration. Moreover, the newest generation of mammalian expressed fluorescent proteins, based on bacterial phytochromes, enter the near infrared with emission spectra exceeding wavelengths of 700 nm \[15\].

Both luciferase for BLI and various fluorescent proteins for FLI can be expressed in cells or animals. Using specific promoters to drive reporter gene expression, these imaging modalities can be used not only for localization but can also provide functional information.

Whole body optical imaging instruments

In the last decade there has been a rapid growth of optical imaging applications in small animal models driven by creative approaches to apply these techniques in biomedical research and also by the availability of innovative instruments. Most of the BLI imaging systems provide 2-dimensional planar information in small animals, showing the locations and intensity of light emitted from the animal in pseudo-color scaling. Nowadays, there are many commercial whole-body pre-clinical BLI systems on the market.

However, in its planar projection form, BLI is non-quantitative and its spatial resolution is relatively poor. Interestingly, recent developments in bioluminescence tomography promise to provide three dimensional quantitative source information with improved spatial resolution \[16–18\].

BLI imaging systems that are able to image moving objects have been developed for experiments in which anesthesia is a problem. Examples of such real time imaging systems are the IVIS Kinetic (Caliper Life Sciences Inc.) and the Photon Imager (Biospace Lab) \[19, 20\].

In analogy with BLI technology, the vast majority of applications of in vivo small animal fluorescence imaging are based on planar epi-illumination imaging. However, an important part of the research conducted in whole-body small animal imaging is concerned with the development of novel fluorescence tomography approaches pushing...
further the limits of the modality in terms of sensitivity, quantification and spatial resolution [21].

New FLI systems are able to capture spectral information of each pixel of an image. The spectral information can be unmixed to reveal all the different spectra that, taken together, form the actual image. By doing so, it is possible to identify multiple fluorescent labels within an image and to remove background (auto-)fluorescence. This way of analyzing spectral data within fluorescence datasets is called spectral unmixing [22, 23].

**Gene reporter systems**

Whole body imaging of VEGF signaling

VEGF is one of the key factors regulating angiogenesis. Most of the vasculogenic and angiogenic effects of VEGF are mediated through the VEGF receptor 1 (VEGFR1, FLT-1) and VEGF receptor 2 (VEGFR2, Flk-1) expressed on endothelial cells [24]. During angiogenesis, including tumor angiogenesis, both VEGF and VEGFR2 expression are locally upregulated [25–28]. VEGF receptors and VEGF itself are attractive targets for molecular imaging given their important role and upregulated expression during (tumor) angiogenesis. As a result, several models have been developed to image and quantify VEGF receptor and VEGF expression.

To monitor VEGFR2 gene expression in vivo as a read-out for angiogenesis, in real time, Zhang et al. [29] developed a VEGFR2-luciferase transgenic mouse model (FVB/N-Tg(VEGFR2-luc)-Xen). In this mouse, luciferase expression is under control of the VEGFR2 promoter region [30] causing endothelial cells lining the angiogenic blood vessels to express luciferase. The VEGFR2-luc mouse has been validated using cutaneous wound healing models in which VEGFR2 expression was upregulated at the site of a punch wound marked by a clear localized increase of the luciferase signal. The increase in VEGFR2 gene activation reached a maximum after 7–10 days after the wound was inflicted [29]. These findings were consistent with later studies which used the same mouse model [31, 32].

Co-registration of BLI and FLI makes it possible to follow multiple processes in one mouse, for instance tumor growth and angiogenesis. As an example, we used the murine breast cancer cell line SMF [33] and the VEGFR2-luc knock-in mouse [29]. A VEGFR2-luc knock-in mouse received an orthotopic inoculation with red fluorescent SMF-mCherry breast cancer cells (right side of the animal) and wild type SMF cells (left side of the animal). The fluorescent signal from the SMF-mCherry cells and bioluminescent signal from the locally upregulated VEGFR2 expression enabled the simultaneous imaging and quantification of angiogenesis and tumor growth real time in vivo. We show the increase in tumor size from week 2 to week 4 using FLI. The FLI signal is only detected at the site of the fluorescent tumor, not at the site with the wild type tumor. The pro-angiogenic response is visualized with BLI both at the site of the fluorescent tumor as well as the site with the wild type tumor (Fig. 1). This way, both tumor growth and pro-angiogenic signaling can be quantified simultaneously, the ratio of which can be used as a measure for angiogenic characteristics of certain tumors.

Other in vivo approaches to study VEGF signaling and angiogenesis in real time are transgenic mouse models which express green fluorescent protein (GFP) or luciferase driven by the VEGF promoter, namely the fluorescent VEGF/GFP [34] and the bioluminescent pVEGF-TSTA-/f transgenic models [35]. The latter approach makes use of the GAL4-VP16 two step transcriptional amplification (TSTA) system [36] to amplify luciferase expression. In this system, the full length human VEGF promoter is placed upstream of the gene encoding a GAL4-VP16 fusion protein. This fusion protein binds GAL4 binding sites that are placed upstream of an adenovirus E4 TATA minimal promoter. This promoter drives luciferase gene expression resulting in GAL4-induced luciferase expression [35]. The extra step via GAL4-VP16 leads to an amplification in luciferase expression. The TSTA system has been used previously shown to amplify prostate specific luciferase expression leading to a 50-fold increase compared to the direct, one step system [36].

Wang et al. [35] demonstrated the correlation between VEGF expression and BLI signal both in vitro and in vivo. In addition, they showed that this transgenic animal can be used to study the VEGF response in both wound healing assays as well as tumor growth models (Fig. 2).

In vivo imaging of vasculature

The VEGF and VEGFR2 reporter mice make it possible to image the areas in which there is an increased pro-angiogenic and vasculogenic signaling. Other transgenic animal models have been developed to image the actual existing vasculature. These models include several transgenic animals with endothelial specific expression of fluorescent proteins like GFP, e.g., the Tie2-GFP mice [37, 38] and the eNOS-GFP mouse [39]. The GFP models are well suited for in vivo microscopic and confocal approaches in contrast to the whole body optical imaging models discussed above.

The Tie2-GFP nude mouse expresses GFP under direction of the endothelial specific receptor tyrosine kinase (Tie2). This mouse is well suited to study angiogenesis and interactions between various (human) tumor
xenografts and tumor vasculature in vivo due to its athymic nude background. The non-invasive character of fluorescence imaging allows follow up over time and assessment of anti-angiogenic treatment efficacy in vivo (Fig. 3) [37].

The eNOS-GFP mouse expresses GFP driven under the endothelial specific promoter of endothelial nitric oxide synthase (eNOS) [39]. A dorsal skinfold chamber can be used to visualize the interaction between tumor cells and growing vasculature. In such a setup, the skin on the back of a mouse is stretched and fitted between two glass slides allowing in vivo microscopy. When SMF-mCherry cells are inoculated between these glass slides, confocal microscopy allows the fluorescent imaging of both tumor growth (mCherry) and developing tumor vasculature (GFP) in such a dorsal skin-fold chamber (Fig. 4).

Yang et al. [40] used wild type mice and GFP positive tumor xenografts to follow tumor angiogenesis in real time. The blood vessels appear as dark contrast against the bright fluorescent tumor. This way they were able to image, quantify and follow the vessel density in orthotopically transplanted tumors over time either by whole body imaging or intravital examination. In a similar approach, dual-color imaging of red fluorescent tumor xenografts in a mouse expressing GFP driven by the chicken β-actin promoter and cytomegalovirus enhancer [41] was demonstrated [42]. In this model the tumor vasculature and other host-derived stromal cell types such as fibroblasts appear in green within the red fluorescent tumor [41].

Another transgenic mouse model to study angiogenesis and nascent vasculature is the GFP mouse in which GFP expression is driven by the regulatory elements of the nestin gene. Nestin is a marker for neural stem cells, neural progenitor cells and neuro-epithelial stem cells [43]. This so-called nestin regulatory element–driven-GFP (ND-GFP) mouse was originally developed in order to study the self renewal capabilities of neural stem cells and progenitor cells [43–45]. In addition to neural cell types, nestin is expressed in the hair follicular bulge as well [46]. During the murine hair cycle, pronounced vascular remodeling occurs with more than a fourfold increase in perifollicular vessel size [47]. It has been found in the ND-GFP mouse that besides the follicular bulge, the perifollicular nascent blood vessels were GFP and thus nestin positive [48, 49]. By transplanting follicles from the ND-GFP mice into nude mice it became clear that follicle derived cells gave rise to these newly formed perifollicular blood vessels. The perifollicular blood vessels connected with the existing vasculature of the receiving animal. Moreover, the newly formed GFP positive vessels expressed the endothelial cell specific marker CD31 confirming that these vessels were indeed blood vessels [48]. In addition, it has been shown that follicle derived vessel growth was increased near wounded skin and that the GFP positive vessels grew from the transplanted follicle towards the wound [48]. Later studies have confirmed the ND-GFP mouse can be used as a model to image nascent tumor vasculature in the skin, mammary fat pad, pancreas, liver, lung, bone and colon [50–55].

One of the most recent mouse models, the Tie-2-Biotag mouse, makes use of the highly specific and strong binding

![Fig. 1 Multi-modality imaging of tumor growth and angiogenesis. A VEGFR2-luc knock-in mouse was orthotopically, i.e., in the mammary fat pad, inoculated with $1 \times 10^6$ SMF-mCherry cells (marked with asterisk) and SMF wild-type cells (marked with filled diamond), a murine breast cancer cell line. SMF-mCherry cells constitutively express the far red fluorescent protein mCherry, which accumulates in the cytoplasm. Mice were imaged at week 2 and week 4 both FLI (a) and BLI (b) using the IVIS Spectrum (Caliper LifeSciences, USA) camera system. a Tumor growth of the mCherry positive tumor could be followed over time using FLI. b The BLI signal was present at the site of both the mCherry positive and negative tumors indicating a local upregulation of VEGFR2 expression and tumor angiogenesis. (Snoeks et al. unpubl. results)
Fig. 2 Induction of 
expression during wound healing in a pVEGF-TSTA-fl transgenic mouse. a Mouse was imaged before wound creation in the CCD camera (day 0) and imaged again subsequent to wound creation every 4–5 days using a-luciferin (150 mg/kg ip). b Optical CCD images of four pVEGF-TSTA-fl transgenic mice (mice 1–4) on days 19, 20, 21, and 22 after creation of the wound. Arrows indicate the position of the wounds. c Correlation plot of maximum bioluminescence signal (p s⁻¹ cm⁻² sr⁻¹) vs. endogenous VEGF levels in the wound tissue (r² = 0.70). Error bars represent the standard error for triplicate samples in the ELISA assay. d pVEGF-TSTA-fl transgenic mice were injected subcutaneously with 5 × 10⁶ NK2 (FVB/N mouse mammary tumor cell line) cells in the lower right flank. Mice were imaged on day 4 and subsequently every few days until day 29 using a-luciferin as the reporter substrate (150 mg/kg, injected ip). CCD camera imaging revealed a detectable bioluminescence signal in the area around the tumor on day 4. Signal intensity continued to increase until day 17, after which a considerable decline in signal was observed (days 22–29). (Adapted with permission from Wang et al. Physiol Genomics. 2006 Jan 12; 24(2):173–80. [35])
of avidin to biotin, commonly used in immunohistochemical staining techniques. In order to do so, this mouse expresses Biotin Ligase (BirA) and a cluster of BirA substrate sequences (Biotags) fused to a transmembrane domain under control of the endothelial cell specific Tie2 promoter. Cells expressing this Biotag-BirA cassette can be targeted in vivo with avidinated probes of choice. These transgenic mice, in which vascular endothelial cells are biotinylated already during angiogenesis, are highly useful for targeted in vivo imaging of angiogenesis and established vasculature using avidinated agents suitable for any desired imaging modality [56].

Fig. 3  GFP vessels in the athymic Tie2-GFP transgenic adult mouse. a–g Images from heart, kidney, liver, spleen, lung, brain, and calf muscle, respectively. Scale bar represents 20 μm. h–j Intravital fluorescence microscopy images of GFP vessels in the muscle (h–i) and in the ear skin (j). (Reprinted from Hillen F, Kaijzel EL, Castermans K, oude Egbrink MG, Lowik CW, Griffioen AW. A transgenic Tie2-GFP athymic mouse model; a tool for vascular biology in xenograft tumors. Biochem Biophys Res Commun 2008; 368:364–7. [37])

Fig. 4  In vivo fluorescent microscopy of tumor vasculature. An eNOS-GFP mouse received a subcutaneous inoculation with $1 \times 10^6$ SMF-mCherry cells in a dorsal skinfold chamber. Images were taken using a Zeiss CLSM 510 Meta microscope with a 10× objective. The following excitation lasers and emission filters were used, green: laser 488 nm, filter BP 505–550 nm, red: laser 543 nm, filter BP 560–615 nm. a Green fluorescent vasculature. b Red fluorescent tumor-cells. c Superimposed images of the vasculature and tumor-cells (Kaijzel, Ten Hagen et al. unpubl. results)
Hyoxia and the subsequent stabilization of hyoxia inducible factor-1 (HIF-1) is one of the key initiators of (tumor-)angiogenesis as reviewed by Liao and Jonson [57]. In short, HIF-1 has an oxygen dependent degradation domain (ODD) which is hydroxylated in an oxygen dependent manner by prolyl hydroxylases. As a result, the ODD binds the Von Hippel-Lindau factor (VHL) under normoxic conditions, targeting HIF-1 for proteasomal degradation. VHL cannot bind HIF-1 under hypoxic conditions, leading to stabilization of HIF-1 and downstream signaling [58].

The promoter regions of most genes upregulated under hypoxic conditions, amongst which VEGF, contain a hyoxia responsive element (HRE). This HRE is the HIF-1 target binding site [59]. To enable real time measurement of both in vivo as well as in vitro HIF-1 activity several reporter constructs have been developed in which reporter gene expression such as luciferase [60–62] or GFP [63, 64], is driven by HREs. The presence of HIF-1 leads to a significant upregulation of the reporter gene expression. HRE-Luciferase reporter constructs have been shown to be valuable tools to evaluate tumor hyoxia and the efficacy of hyoxia directed therapies in vivo (Fig. 5) [61, 62]. Bioluminescence, a process requiring ATP and O2, did not seem to be hampered by the low oxygen tension in the tissue.

In another approach to visualize hyoxic signaling and HIF-1 stabilization, reporter cell lines, constitutively expressing a fusion protein consisting of the ODD coupled to luciferase, have been generated [65]. Slightly different models are reporter cell lines in which express a HIF-1:luciferase fusion protein [61, 66]. In both reporter cell lines, luciferase will be degraded immediately under normoxic conditions, so no luciferase signal can be detected. Under hyoxic conditions, luciferase will be stabilized in a similar manner as HIF-1 which will result in a detectable signal. These imaging tools are useful for studying the biology of tumor hyoxia and mechanisms of response to experimental antiangiogenic therapy.

**Fluorescent probes**

In addition to the bioluminescent and fluorescent gene reporter systems discussed above, various fluorescent protease-sensing probes and targeted probes have been developed. The first protease-sensing optical probes were autoquench fluorescent probes that convert from a nonfluorescent to a fluorescent state by proteolytic activation of lysosomal cysteine or serine proteases like cathepsin-B [67]. The utility of these probes has also been extended to other enzymes such as thrombin and matrix metalloproteinase-2 by inserting enzyme-specific peptide stalks between the carrier and the fluorochromes [67–70]. Targeted probes consist of a fluorophore coupled to a highly specific antibody or single chain lama-antibodies, affibodies or ligands.

**αvβ3 targeted probes**

The αvβ3 integrin plays a crucial role during tumor angiogenesis. It is highly expressed on endothelial cells of newly formed tumor vasculature and on various tumor cells, whereas quiescent endothelial cells and other non-cancerous cells are largely αvβ3 negative. The amino acid sequence Arg-Gly-Asp (RGD), also present in matrix molecules like...
vibronectin, is a tripeptide that selectively binds to integrin receptors including αvβ3 and αvβ5 [71–73]. The activation of αvβ3 integrin is not limited to tumor biology and thus probes targeting αvβ3 integrin can be a valuable tool to study other processes and diseases as well, for example angiogenesis, atherosclerosis, inflammation, bone remodeling and arthritis.

Based on the binding of RGD to αvβ3 integrin, Haubner et al. [74] developed 125I and later 18F [75] labeled tracers to image αvβ3 integrin expression and tumor angiogenesis. In recent years, many fluorescently labeled cyclic RGD peptides have been developed for near infrared fluorescence imaging like RGD-Cy7, RGD-Cy5.5, RGD-QD705 [76–78] and cyclic RGD coupled to IRDye 800CW-labeled peptide which is even more shifted towards the infrared parts of the spectrum resulting in ideal fluorescent characteristics for in vivo use [79, 80]. Mulder et al. [81, 82] recently described paramagnetic lipid-encapsulated quantum dots with RGD presented at the outside and a green fluorescent quantum dot at the inside of the micellar shell. These targeted particles are both suitable for fluorescence imaging as well as magnetic resonance imaging (MRI), thus providing unique opportunities for the use of multi modality molecular imaging.

Several groups have improved αvβ3 integrin specificity, binding affinity, and possible targeted delivery of therapeutics, by coupling multiple RGD motifs on a backbone molecule [81–85]. Although many types of tumor cells are positive for αvβ3 integrin, RGD-modified proteins mainly localize at the tumor endothelium and not in the tumor itself [86].

![Fig. 6](image)

**Fig. 6** Effect of antiangiogenic Avastin treatment on αvβ3 integrin and cathepsin B signals in A673 tumor-bearing mice. (A) Representative volume renderings of IntegriSense and ProSense. Note the differential localization of fluorescent signal with IntegriSense and ProSense in the same animals (IntegriSense in blue and ProSense in red). (B) Quantification of tumor fluorescence shows that IntegriSense and ProSense signal in tumors is decreased following Avastin treatment (P = 0.0008 and P = 0.013, respectively). (C) Avastin also significantly inhibits tumor growth as determined by tumor volume calipers measurements (by 58.4%, P = 0.022). (Reprinted from Kossodo S, Pickarski M, Lin SA, Gleason A, Gaspar R, Buono C, Ho G, Blusztajn A, Cuneo G, Zhang J, Jensen J, Hargreaves R, Coleman P, Hartman G, Rajopadhye M, Duong LT, Sur C, Yared W, Peterson J, Bednar B. Dual In Vivo Quantification of Integrin-targeted and Protease-activated Agents in Cancer Using Fluorescence Molecular Tomography (FMT). Mol Imaging Biol 2009. [87])
The commercially available near infrared fluorescent (NIRF) probe IntegriSense (Visen Medical) consists of a small molecule nonpeptide $\alpha_v\beta_3$ antagonist fused to the VivoTag-S680 near infrared fluorophore. IntegriSense has a much higher specificity for $\alpha_v\beta_3$ integrin compared to RGD-based probes. Unlike RGD-based probes, extravasation of IntegriSense does occur and the probe localizes at both at the surface of $\alpha_v\beta_3$ integrin positive endothelial cells and the surface of $\alpha_v\beta_3$ integrin positive tumor cells. Furthermore, IntegriSense is internalized by $\alpha_v\beta_3$ integrin positive tumor cells leading to a slower clearance of the probe from tumors compared to the surrounding tissues. A drawback of the slower clearance from a tumor is the practical upper limit on the possible frequency of repeated measurements [87].

Using fluorescence-mediated tomography (FMT), Kossodo et al. [87] measured tumor volume and vascularization in A673 tumor-bearing mice. They used IntegriSense to visualize blood vessels and ProSense, a cathepsin-based protease activated fluorescent probe which only fluoresces after being cleaved by proteases, to visualize the tumor. In addition they were able to quantify the effect of antiangiogenic treatment (Fig. 6).

VEGF-receptor targeted probes

The VEGFR-2 is a very attractive endothelial target for imaging of tumor angiogenesis due to its temporal and local upregulation during (tumor-)angiogenesis [24–28]. To target the VEGFR-2, Backer et al. [88] used single chain VEGF (scVEGF) with an N-terminal cysteine-containing tag (Cys-tag) which can be used for site specific attachment of various agents [89]. The scVEGF-based family of probes has been validated to target the VEGF receptor in vitro and in vivo tumor angiogenesis [90, 91]. More recently, scVEGF labeled with the near infrared dye Cy5.5 has been used for fluorescence imaging of VEGF receptor in angiogenic vasculature, in regions of sterile inflammation and during tumor angiogenesis [92, 93].

Long circulating fluorescent agents

Long circulating blood pool macromolecules labeled with gadolinium have been used as traditional MRI contrast agent for determining vascular volumes [94]. The rapid advancements in fluorescence imaging lead to the need for long circulating fluorescent agents. The first generation of these fluorescent probes was based on long circulating dextrinated iron oxide (LCDIO) preparations coupled to a fluorescent label [95–97]. These probes were used by Bremer et al. [97] to determine the tumoral vascular volume fractions by MRI validated with fluorescence intravital microscopy.

With the development of FMT for small laboratory animals the imaging focus shifted from intravital microscopy to 3D whole body imaging. Using the commercially available near IR fluorochrome labeled long circulating synthetic graft copolymer AngioSense-750 and the fluorescently labeled...

![Fig. 7 Dual target imaging. Mice bearing xenograft tumors (either breast cancers or gliosarcomas) were imaged at two different wavelengths to map angiogenesis (a 748 nm) and HER-2/neu (b 672 nm) in the same animal. The gliosarcoma is largely devoid of HER-2/neu. c a combined three-dimensional map of both processes. (Adapted with permission from Montet et al. Cancer Res. 2005 Jul 15; 65(14):6330–6. Fig. 4 [95])](image-url)
Her2 antibody herceptin (Herceptin-Cy5.5), Montet et al. [95] demonstrated the possibility to co-register angiogenesis and tumor growth with FMT. In their experiment they used a Her2/neu positive breast cancer xenograft model and a glioma model with low expression of Her2/Neu (Fig. 7). In later studies they confirmed that FMT allows quantitative visualization of tumoral vascular volume fractions in mice using Herceptin-Cy5.5, AngioSense-680 and AngioSense-750 [98]. Both AngioSense-680 and AngioSense-750 are fluorescently labeled long circulating synthetic graft copolymers optimized for 680 and 750 nm excitation wavelengths, respectively.

**Conclusion and future directions**

Here we discussed a great number of optical imaging models that can be used to image the angiogenic process on various levels ranging from intravitral microscopy to whole body imaging. The cost effective and non invasive character of optical imaging makes it an ideal imaging modality for repeated measurements and to follow treatment response of anti-angiogenic treatment over time. New imaging systems, that are able to unmix signals of various wavelengths, make it possible to study various fluorescent and luminescent signals simultaneously. This offers a powerful tool to study different processes and interactions, for example tumor growth and angiogenesis, in the same animal. Furthermore, optical imaging can be combined with various other imaging modalities, for example the fluorescent paramagnetic \( \alpha, \beta_3 \) targeted probes with MRI contrast that offer the opportunity to image angiogenesis at high resolution with MRI while confirming the specificity of the MRI contrast with the co-localized fluorescent signal. The development of less toxic near infrared fluorescent probes open up the opportunity to apply fluorescence imaging in various clinical settings in the near future.

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