Whole-Body Optical Imaging in Animal Models to Assess Cancer Development and Progression

Eric L. Kaijzel, Gabrie van der Pluijm, and Clemens W.G.M. Lowik

Abstract

Different optical-based imaging models were used to investigate tumor progression and metastasis with particular emphasis on metastasis to bone and bone marrow. We describe how optical imaging can be used to follow important processes in tumor development and treatment response, including angiogenesis, apoptosis, and proteolysis. Finally, we discuss the translation of one optical imaging modality, near-IR fluorescence, from animal validation studies to applications in the clinic related to cancer management.

Imaging in Cancer

Molecular imaging in cancer. Recent advances in molecular and cell biology and the increased knowledge of the human and mammalian genome have advanced our understanding of the molecular and cellular mechanisms that control biological processes and underlie many diseases, including cancer. Molecular imaging is a new science that exploits this recently acquired information and provides further advances by adding spatial and temporal dimensions to biological processes in vivo. The methods developed in this scientific field permit noninvasive detection of cellular and molecular events by using highly specific probes and gene reporters in living animals, some of which can be directly translated to patient studies.

Small animal imaging in cancer. Animal models of human cancer and metastasis have been developed to aid understanding of disease progression and development of treatment. The need for suitable model systems has resulted in the development of a variety of small animal imaging technologies, such as microcomputed tomography (CT), micropositron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), ultrasound imaging and optical imaging, such as bioluminescence, near-infrared, and fluorescence imaging. (1), and intravital microscopy (2, 3). Certain imaging approaches are better suited for specific applications than others. For example, CT and MRI provide a high degree of spatial resolution that is well suited for tumor phenotyping and anatomic detail, whereas highly sensitive approaches such as PET, bioluminescence, and fluorescence imaging are preferable for monitoring tumor cell biology, as well as tumor burden, progression, and metastasis (4), through the use of direct-targeting probes and reporter systems.

Optical imaging. Whole-body optical imaging, either bioluminescent or fluorescent, is a very versatile, sensitive, and powerful tool for molecular imaging in small animals. Compared with PET/SPECT and MRI, optical imaging is relatively inexpensive without the need of an extensive infrastructure. Optical imaging suffers from limited spatial resolution and depth sensitivity, however, and therefore is primarily used for whole-body imaging of small laboratory animals. Optical-based small animal in vivo imaging approaches are dependent on the detection of photon emissions from within living tissues. Photons can be generated by either fluorescent sources that emit light at one wavelength in response to excitation by light of a different wavelength (5) or by bioluminescent sources that produce light as a result of a chemical reaction (6). Unlike fluorescence imaging, bioluminescence imaging relies on the activity of enzymes (luciferases) that convert unique substrates (like luciferin and coelenterazine) into light (7). Commonly used luciferase genes, such as the luciferases from the sea pansy (Renilla reniformis) and the North American firefly (Photinus pyralis) are currently used as transgene reporters. Firefly luciferase catalyzes the transformation of its substrate β-luciferin into oxyluciferin in an oxygen and ATP-dependent process, leading to the emission of photons at wavelengths from 500 to 620 nm at a sufficient intensity to penetrate small animal tissues. Importantly, the nonimmunogenic substrate luciferin, either i.v. or i.p. injected, diffuses within minutes throughout the entire animal body and is rapidly taken up by cells (8, 9).

Recent technical advances for imaging weak visible light sources using cooled charged coupled device cameras, Peltier cooled detectors, and microplate channel intensifiers allow the detection of photon emission from inside the tissues of small animals.

Whole-body bioluminescence. Until recently, bioluminescence was the most commonly used technology for whole-body optical imaging because there are several important advantages of using luciferase as a reporter. Light is collected from the animal in the absence of external illumination sources and the low background bioluminescence yields high imaging
Miniscence may serve as a qualitative measure of the number of living and dead tumor cells as well as infiltrating host cells, tumor cell debris, and peripheral tumor edema. In these cases, metabolism is derived only from metabolically active transformed cells, whereas living and dead tumor cells as well as infiltrating host cells, tumor cell debris, and peripheral tumor edema are allowed to stably express luciferase can be transplanted at any orthotopic site within a mouse and subsequent tumor development, progression, and possible metastasis can be monitored in a very rapid and time-sensitive manner (7, 33). Apart from transplantable luciferase-expressing cell lines, a growing number of luciferase-based transgenic animal models of spontaneous cancer development have been generated. This has been accomplished by crossing transgenic mice that express tissue-specific expression of luciferase [i.e., in pituitary (POMC-luc) or prostate (PSA-luc)], with mice that spontaneously develop tissue-specific cancers like Cre-inducible Rb knockout mice that develop pituitary cancer and TRAMP mice that develop prostate cancer (see Figs. 1 and 2; refs. 34, 35). In these transgenics, a basa expression level of luciferase in the targeted tissues will strongly and steadily increase when tumor formation and progression starts. Similarly, the development of metastases in distant organs can be detected by bioluminescence.

**Whole-body fluorescence imaging.** Unlike bioluminescence, fluorescence imaging does not require the addition of a substrate. Limitations of fluorescent green fluorescent protein reporter imaging include the requirement of an external source of light, autofluorescence, and the exponentially decreasing intensity of light with increasing depth of the target. Endogenous autofluorescence of tissues at the excitation wavelength of green fluorescent protein frequently results in substantial background emissions that until recently has limited the sensitivity and specificity of this imaging technique. The use of selective filters or the application of spectral analysis, however, can significantly reduce the contribution of autofluorescence to the acquired images (11). Furthermore, by using red fluorescent protein and its more red shifted variants (i.e., mFluors like mCherry and mPlum; ref. 12) as well as the development of near-infrared dyes and quantum dots that excite and emit at higher wavelengths, the extent of autofluorescence is substantially diminished. More importantly, tissue penetration of light is substantially increased from millimeters to centimeters using these red-shifted probes due to the lower tissue absorption of photons in the near-infrared spectral window (13).

Quantum dots are inorganic fluorescent nanocrystals that contain a few hundred to a few thousand atoms of a semiconductor material (e.g., cadmium mixed with selenium or tellurium), coated with an additional inorganic shell of metal that improves the optical properties of the material. Quantum dot fluorescence emission can be tuned to virtually any discrete wavelength and absorption is broadband. This “tunability” offers the possibility of multiplexing using different colors. Quantum dots are remarkably resistant to photobleaching, have a high quantum yield, and permit conjugation of targeting molecules like proteins and oligonucleotides. Because the core of the quantum dots contains heavy metals, they are potentially toxic and are not allowed for clinical use. They can be coated to diminish toxicity, however, and have been used successfully for small animal fluorescence imaging (14–16).

**Applications in cancer research.** In cancer research, whole-body optical imaging has allowed semiquantitative measurements of tumor progression, metastasis, and treatment response. Due to their high sensitivity, fluorescence imaging (17) and especially bioluminescence are very useful for early detection of micrometastases and minimal residual disease states in animal models (7, 18, 19). One advantage of bioluminescence is that tumor light output is presumed to be derived only from metabolically active transformed cells, whereas living and dead tumor cells as well as infiltrating host cells, tumor cell debris, and peripheral tumor edema may all contribute to MRI-calculated tumor volume. Therefore, bioluminescence may serve as a qualitative measure of the number of metabolically active tumor cells in a particular tumor or metastasis. Bioluminescence can be used to follow the migration and fate of transplanted immune cells, like cytotoxic T cells or cytokine-induced killer cells (20) as well as processes such as angiogenesis or vasculogenesis (21). The use of optical reporter genes in either transplanted cells or in transgenic animals provides the opportunity to study gene expression, regulation, and function.

Furthermore, “inducible” gene reporters for bioluminescence and “smart” injectable near-infrared fluorescence probes (22, 23) or quantum dots linked to ligands, compounds, peptides, antibodies, and the like (16, 24–27) also make it possible to noninvasively follow molecular processes involved in cancer development and treatment, including proteolysis, bone turnover, apoptosis, and angiogenesis.

**Murine Models of Tumor Progression and Metastasis**

Currently available in vivo models of tumor progression and metastasis include transplantable models and genetically engineered mice that develop primary and metastatic cancers. Transplantable tumor models comprise syngeneic models, in which the cancer cell line/tissue transplanted is of the same genetic background as the animal, and xenograft models referring to human cancer cell lines/tissues transplanted into immunocompromised hosts, including nude and severe combined immunodeficient mice (reviewed in ref. 28). Although s.c. xenograft mouse models are still the standard for cancer drug screening in the pharmaceutical industry, several lines of evidence favor the use of orthotopic xenotransplantation models because it is felt that cancer cell–stromal cell interactions play a crucial role in the biology of cancer progression and metastasis (29). Supportive evidence for this hypothesis has been provided by several experimental metastases models (30–32). It is therefore important to study tumor development and possible metastasis in more biologically relevant environments, like the tissue from which they were derived or the tissue to which they preferentially metastasize.

Due to the sensitivity of optical imaging and especially bioluminescence, tumor cells that stably express luciferase can be transplanted at any orthotopic site within a mouse and subsequent tumor development, progression, and possible metastasis can be monitored in a very rapid and time-sensitive manner (7, 33). Apart from transplantable luciferase-expressing cell lines, a growing number of luciferase-based transgenic animal models of spontaneous cancer development have been generated. This has been accomplished by crossing transgenic mice that express tissue-specific expression of luciferase [i.e., in pituitary (POMC-luc) or prostate (PSA-luc)], with mice that spontaneously develop tissue-specific cancers like Cre-inducible Rb knockout mice that develop pituitary cancer and TRAMP mice that develop prostate cancer (see Figs. 1 and 2; refs. 34, 35). In these transgenics, a basal expression level of luciferase in the targeted tissues will strongly and steadily increase when tumor formation and progression starts. Similarly, the development of metastases in distant organs can be detected by bioluminescence.
Monitoring Tumor Progression and Bone/Bone Marrow Metastases

Bone and bone marrow are preferential sites of metastasis in a variety of cancers, especially breast and prostate cancer. Direct injection of tumor cells to the systemic circulation leads to the development of site-specific distant metastases determined largely by the injection position. Intracardiac injection introduces tumor cells to the arterial circulation leading to the colonization of cells to specific sites of the skeleton (36). After intracardiac injection of luc-expressing human MDA-MB-231 breast cancer cells, very small amounts of photon-emitting tumor cells can be detected in bone marrow or bone within a few days or weeks, mimicking micrometastatic spread. This bioluminescence-based metastasis model allows monitoring of the development and progression of experimental bone metastases in living animals with high sensitivity (18, 37). In addition, bioluminescence monitoring of minimal residual disease and growth of minimal metastatic deposits in bone marrow at a stage largely preceding tumor-induced osteolysis is feasible. This may help to better identify situations at risk for bone metastasis and novel therapeutic strategies that could be extended to the clinic.

Drug Testing and Drug Development

Optical imaging in the cancer model systems described above allows noninvasive, rapid, sensitive testing of new drugs and therapies for the treatment of cancer in relative high throughput compared with conventional drug testing in animal models (38–40). Therefore, apart from speeding up drug development, it will also lead to much quicker optimization of new therapies. Important for animal welfare, it will also lead to a decrease in the number of animals sacrificed that are needed for such studies.

Monitoring Treatment Response in Bone Metastasis

**Bisphosphonates.** Interference with the microenvironmental growth support system is currently being evaluated as a therapeutic strategy for the treatment of metastatic disease. Bone metastasis is a paradigm of the interactions that take place at the tumor-stroma interface (29, 41) and lines of evidence from animal and clinical studies support the notion that bone turnover, particularly bone resorption, contributes substantially to initiation and maintenance of local tumor growth.
growth through the release of growth factors and bone-resorbing cytokines. Differently from other tissues, bone turnover can be reduced by pharmacologic means; thus, animal models of bone metastasis offer the unique opportunity to test in vivo the therapeutic efficacy of the interference with the tumor-stroma interface.

Bisphosphonates are nonhydrolysable pyrophosphate analogues that exert a strong inhibitory effect on osteoclastic resorption and show a high affinity to bone mineral. Consequently, they exclusively accumulate in bone in vivo (42, 43). Currently, bisphosphonates are used as bone-specific palliative treatments to reduce skeletal complications from bone-metastasizing tumors and have been shown useful in treating prostate, breast, and lung cancer that metastasize to the skeleton (44–46). We reported recently on the action of bisphosphonates on development and growth progression of experimental bone metastasis (47). Whole-body bioluminescence was used for the detection, monitoring, and quantification in vivo of the growth progression of bone metastases induced by intracardiac or intraosseous injection of luciferase-transfected breast cancer cells (MDA-231-B/luc+) in nude mice. Treatment with the bisphosphonate olpadronate strongly inhibited tumor-induced osteolysis. Suppression of bone turnover by the olpadronate, before bone colonization by cancer cells, significantly inhibited the number of developing bone metastases. Reduction of bone turnover, however, had no effect on the growth and progression of established bone metastases. Compelling results were described by Yang et al. (48) who showed a dramatic reduction in the severity of bone lesions and an inhibition of the growth of the prostate tumors by olpadronate treatment, using a green fluorescent protein fluorescent model of prostate cancer.

These findings suggest that bisphosphonates, via their antiresorptive activity, can reduce breast and prostate cancer metastasis to bone. Most probably, this occurs by reducing bone remodeling leading to a decrease of local factors that are normally released during the resorption process and that are involved in activation of micrometastases. However, our data (47) also suggest that once micrometastases have turned into a macrometastase or small tumor, it becomes independent of local bone turnover for its growth and, therefore, bisphosphonate treatment will not slow down tumor progression of already established tumors in bone.

On the other hand, optical imaging strategies using bisphosphonate analogues have been studied for bone metastases detection, although none are yet in clinical use. Zaheer et al. (49) described nonisotopic imaging of bisphosphonate analogues in living animals by covalently coupled pamidronate to a near-infrared dye. In a follow-up study, they also show the potential usefulness of this labeled compound in the detection of breast cancer micrometastases using near-infrared fluorescence imaging techniques (50). Using a Cy5-labeled bisphosphonate, we show that it can also be used to identify osteolytic metastasis (Fig. 3D).

**Imaging Tumor Angiogenesis**

Molecules regulating angiogenesis include vascular endothelial growth factor (VEGF) and VEGF receptors, tyrosine kinase receptors, integrins, like the αvβ3 integrin, and matrix metalloproteinases (reviewed in ref. 51). Given their critical role in tumor angiogenesis, methods are needed to noninvasively image and quantify VEGF and/or VEGF receptor expression during tumor growth and on antiangiogenic treatment. Transgenic mouse models like the VEGF-green fluorescent protein (52) and VEGF receptor 2-luc mice (21), have been developed to study VEGF (receptor) biology and drug optimization (53). In addition, the expression of αvβ3 integrin is significantly up-regulated on vascular endothelial cells during tumor angiogenesis (54) and correlates well with tumor invasiveness and disease state (55, 56). Near-infrared fluorescence detection approaches offer straightforward, rapid, and cost-effective preclinical evaluation (Fig. 3D and E) that may be translated into the clinic with fluorescence-mediated tomography (5, 57, 58). Fluorescence-labeled or quantum dot–conjugated cyclic RGD peptides that specifically bind to αvβ3 integrins were shown to visualize s.c. and orthotopically inoculated integrin αvβ3–positive xenograft tumors (24, 59, 60). Recent developments of sophisticated techniques such as fluorescence-mediated tomography and life-time fluorescence imaging show the feasibility of whole-body near-infrared fluorescence for tumor localization and its spatial functional status in living small animals, even in deeper lying sections (61, 62).

**Imaging Proteases for Tumor Detection**

Various proteases such as matrix metalloproteinases and cathepsins are involved in the degradation of the basement membranes and digestion of the extracellular matrix leading to local and metastatic tumor cell infiltration (63, 64). In addition, their expression levels have shown to correlate with tumor burden and clinical outcome in a variety of pathologies (65–67). 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 (68). Even small tumor nodules were detectable with these probes, suggesting that protease imaging might be a feasible approach for early detection of cancer (69). 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 (68, 70–72).

**Imaging Apoptosis**

Optical imaging offers the opportunity for sensitive real-time monitoring of apoptosis. Laxman et al. (73) developed an elegant tool to monitor apoptosis with bioluminescence based on the activity of caspases that are specifically activated on initiation of apoptosis. The response of p53 regulatory sequences has been shown in vivo by PET imaging of DNA damage–induced apoptosis (74) and in a bioluminescent xenograft model of human colon carcinoma cells (75). Very early in apoptosis, translocation of membrane phosphatidylinerine to the outer membrane leaflet occurs. Annexin V is able to bind to externalized phosphatidylserine and up to eight Annexin V moieties can bind to one exposed phosphatidylserine, which contributes to its efficiency as a cell label. Most
extensively investigated are the $^{99m}$Tc-labeled Annexin V molecules in \textit{in vivo} models of apoptosis and clinical trials have already been undertaken using this radioligand (76). Several Annexin V–based labeling molecules and derivatives have been developed for optical \textit{in vivo} imaging purposes (reviewed in refs. 75, 77–79).

Analysis of Gene Function in Metastasis Models

To investigate the role of specific genes on tumor development, progression, and/or metastasis, a model system should be available that allows relatively rapid evaluation of gene function. The Flp-In system (Invitrogen) enables rapid and efficient stable integration of gene constructs in (tumor) cells without the need of time-consuming cloning procedures (80, 81). This method uses Flp recombinase to mediate homologous recombination between DNA sequences that carry Flp recombinase target sites. Flp recombinase will recognize the single Flp recombinase target sites in the genome and in the expression vector and will specifically and efficiently integrate the gene construct. This technology can also be used to stably express short hairpin RNA constructs to knock down specific genes (82). As a logical extension, it is possible to implement the Flp recombinase target technology described above in MDA-MB-231-Luc cells and other luciferase-expressing tumor cells. Using bioluminescence, the study of the role of specific genes that have been identified as being of interest in gene profiling studies in tumor progression and tissue-specific metastasis can be assessed in a rapid and cost-effective manner. Proof of concept has been shown in the OCM-1-luc-frt ulvea melanoma cells (83).

Going from Two-dimensional Qualitative Planar Imaging to Three-dimensional Quantitative Imaging and Multimodality Imaging

Optical imaging has been based on two-dimensional planar images, therefore, spatial resolution is poor and only semi-quantitative results are obtained. New developments may make it possible to extend bioluminescence and fluorescence imaging to three-dimensional, tomographic imaging that should provide better quantification of photon emission (79, 84). Fluorescence molecular tomography is leading this effort to resolve and quantify fluorochromes deep in tissues. The resolution achieved in optical tomographic methods depends on the depth and tissue dimensions and the optical properties of the tissue. In addition, fusing three-dimensional optical images with images obtained from the same animal using MRI or CT will allow one to obtain structural anatomic information that will enhance spatial resolution. Furthermore, structural tissue information obtained by CT and MRI, in combination with a mouse tissue atlas, can be used in an attempt to correct for tissue-dependent photon scattering and absorption. Multi-modality imaging is a way to register and relate different imaging data into a singular context (85).
Recent advances combining three-dimensional bioluminescence and microcomputed tomography provide integration of molecular and cellular imaging with the pathophysiology occurring in anatomic structures. This combination of images will generate critical anatomic, physiologic, and pathologic information superimposed with molecular and cellular level data as the source distribution inside the animal is reconstructed (Fig. 4A and B).

**Translation to the Clinic**

First, bioluminescence using firefly or *Renilla luciferase* as a reporter gene is not likely to be translated into clinic because these genes are not of human origin. In addition, the pharmacologic amount of luciferase substrate, either luciferin or coelenterazine, required to produce bioluminescence in a human would be large and costly, and potential toxic side effects are unknown. This makes bioluminescence not a likely candidate for optical imaging in humans. Second, as mentioned previously, the intensity of light exponentially decreases with increasing depth of the target, therefore to get better resolution, one has to correct for photon scattering and absorption. At the moment, near-infrared fluorescence imaging is the more likely choice because thenear-infrared region of the spectrum offers certain advantages, such as low autofluorescence and high photon penetration. Both organic and inorganic near-infrared fluorescence contrast agents are available for chemical conjugation to specific targeted molecules or as injectable nanoparticles. At the moment, however, noninvasive optical imaging using near-infrared fluorescence in humans is still restricted to more superficial tissues. Detection of fluorochromes from human tissues using planar imaging has already been shown for several applications, including the use of epi-illumination in skin and endoscopy for esophageal, cervical, lung, and other accessible epithelial cancers.

Using a combination of advanced photon and tomographic techniques, it may be possible to overcome some of the limitations of conventional optical imaging. These new technological advances, the use of near-infrared fluorescent probes that enable better penetration of light (to as deep as 15 cm in breast tissue) and independent characterization of the absorption and scatter of photons, will facilitate this translation to patient applications.

Several new approaches have been successfully applied in the clinic. Near-infrared fluorescence imaging using indocyanine green as a general blood pool agent to detect changes in blood flow in combination with tomography through the entire human breast has been applied for breast cancer detection (86, 87). Using a four-wavelength time domain optical imaging...
system (SoftScan, Advanced Research Technologies, Inc.) to do noninvasive near-infrared fluorescence measurements in the human breast. Intes et al. (88) showed that benign and malignant tumors can be noninvasively differentiated with optical imaging. Discrimination is based on differences in fluorescence of endogenous deoxyhemoglobin content between malignant and benign tumors.

With the development of new nontoxic fluorescence probes and nanoparticles, many of the optical technologies described earlier can be adapted for human imaging (i.e., intraoperative imaging for tumor margin and metastasis identification) in either endoscopic or laparoscopic applications, or even in open surgery. The latter has been shown by Frangioni et al. (89–93): they showed the utility of intraoperative near-infrared fluorescence imaging for sentinel lymph node mapping in various organs in large animals and also in a large animal model system with spontaneous cancer metastatic to regional lymph nodes. Therefore, near-infrared fluorescence imaging has the potential to facilitate human cancer surgery by providing sensitive, specific, and real-time intraoperative visualization of normal and disease processes and tissues. It will also provide new opportunities to optimize surgical removal of tumors and their (local or lymph node) metastases.

**Conclusions and Future Perspectives**

It is clear from the work presented in this review and from work by others that optical imaging is well suited to monitor gene expression in transgenic reporter mice and to detect and follow small numbers of cells noninvasively. However, caution must be exercised in the interpretation of the planar light signals obtained and must include a consideration of the depth of the light source, tissue penetration, and scatter, which can confound attempts at quantitation and comparisons between different light signals in the same animal as well as light signals from different animals. Nevertheless, this now enables researchers to follow the fate of tumor cells during tumor progression and metastasis in a semiquantitative manner. With the use of new three-dimensional tomographic optical imaging systems and software based on a tissue atlas of the animal that attempts to correct for tissue absorption and scattering, quantitative data may be obtained.

Optical imaging is a powerful tool in functional genomics of cancer development, progression, and metastasis and will allow us to identify in vivo molecular targets of cancer and their metastasis in small animals. The development of new smart luciferase-based reporter constructs, as well as new possibilities to create transgenic animals containing these reporter constructs, will enhance the use of noninvasive in vivo bioluminescence and fluorescence imaging in small animal models of human biology and disease. The application of bioluminescence in combination with new animal models for cancer will allow us to rapidly and inexpensively study the efficacy of new drugs and therapeutic approaches, such as gene therapy, stem cell therapy, metronomic low-dose chemotherapy, combination therapy, and antiangiogenic therapy. Successful optimization of the therapy in animals can be a first step toward clinical application.

Finally, the first applications of optical imaging in humans in a clinical setting have now started with the introduction of near-IR fluorescence–based breast scanners for breast cancer and new (smart or targeted) near-IR probes for intraoperative optical imaging–guided surgery. This will likely lead to an improvement in the success rate of total tumor removal as well as the removal of local (lymph node) metastasis in oncological surgery, which could lead to an improvement in survival and reduced morbidity.

**References**


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