Preclinical Oncology Studies Using Multimodal Optical Imaging

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While *in vivo*, multimodal optical imaging is now commonly used in a range of research disciplines including infection, inflammation, and metabolic diseases, it was first extensively applied in preclinical studies of oncology. Optical imaging is particularly well suited for cost efficient, sensitive, and high throughput *in vivo* tumor detection, monitoring and follow-up. Bioluminescence imaging (BLI) and fluorescence imaging (FLI) have been widely used to detect and quantify tumor burden in subcutaneous (SQ) and orthotopic tumor models. Longitudinal *in vivo* optical imaging has facilitated studies in tumor biology and cancer therapeutics. Additionally, *in vivo* FLI is used extensively to detect molecular markers (MMs) of tumor biology as well as in studies of probe development. Direct radionuclide imaging (DRI) has been particularly useful in evaluating candidate small-molecule oncology tracers and in discovering throughput biodistribution and *in vivo* targeting of novel therapeutic compounds.

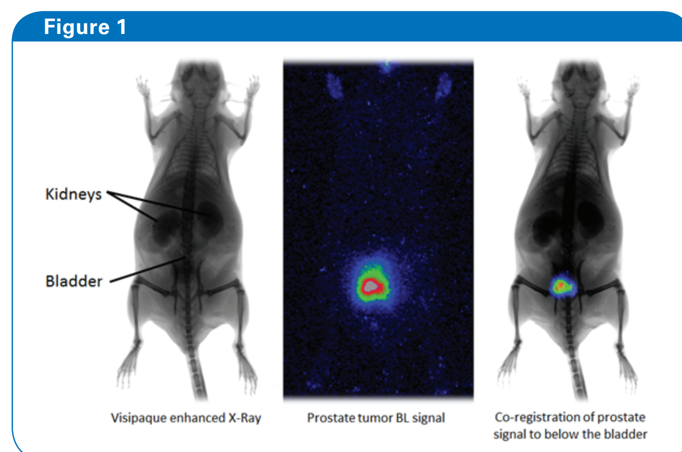
The Bruker In-Vivo Multispectral FX PRO and In-Vivo Xtreme systems provide multimodal BLI, FLI, DRI, CLI (Cherenkov luminescense imaging), and X-ray imaging capabilities. These systems have been utilized in numerous preclinical oncology studies for *in vivo* tumor detection/monitoring and for the detection of cancer molecular markers. This review presents a set of representative preclinical oncology studies performed using the Bruker In-Vivo optical imaging systems. The selected studies used BL (e.g. firefly luciferase), FL (e.g. GFP, RFP, and NIR fluorophores), and/or DRI (e.g. ^{99m}Tc and ^{125}I) reporters, and have advanced developments in tumor reporter systems and probes, cancer therapeutics, and have provided insights in to basic tumor biology. The note may also serve as a general primer to optical imaging methods and reporters used in preclinical oncology.

Multimodal Optical Imaging in Preclinical Oncology

Tumor Imaging

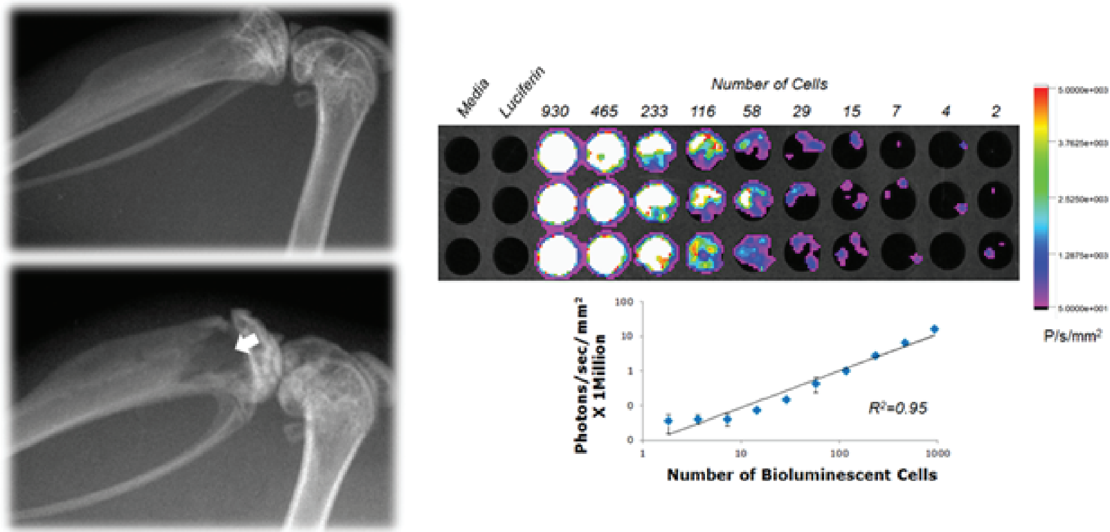
BLI, FLI, DRI, CLI, and X-ray imaging modalities each provide relative strengths for visualizing and characterizing *in vivo* tumors. Depending on the model and experimental objectives, researchers may preferentially utilize BLI, FLI, DRI and/or X-ray imaging analysis. X-ray imaging is frequently performed as complementary multimodal imaging for anatomical context of functional reporters. Figure 1 displays a multimodal BLI/X-ray image with contrast agent enhanced X-ray imaging

Figure 1



Multimodal BLI/X-ray prostate tumor imaging. (Left) The X-ray contrast agent Visipaque™ (GE Healthcare) was administered prior to multimodal imaging providing enhanced kidney and bladder radiographic contrast. Middle) Prostate tumor cells transduced with firefly luciferase were imaged after administering luciferin via tail vein injection. (Right) BL image is registered with the X-ray for anatomical context. Images provided courtesy of Professor Bob Handa, Arizona State University, USA (Unpublished).

Figure 2



X-ray imaging in a bone metastasis model and a luciferase-tumor cell dilution. (Left) Bone metastasis model: MD-MBA-235 cells were applied via intracardiac injection. A control animal X-ray image is shown at top while the bone metastasis model is displayed below. The arrow highlights the tumor-cell derived bone lesion. Bone metastasis image provide by anonymous system user (2010, Unpublished). (Right) BLI of a luciferase expressing tumor cell line dilution series (930 to 2 cells), made in triplicate at top right, and photon flux (photons/second/mm^2) versus cell number (bottom right), imaged with the Bruker In-Vivo Xtreme. BLI cell dilution series data by Bruker BioSpin, PCI Lab (2014, Unpublished).

of soft tissue. Innate X-ray contrast of the lung (Madero-Visbal et al., 2012), skull cavity (Ali et al., 2012, Chu et al., 2014), bone and even other organs with appropriate contrast agents, can supply valuable anatomical reference for bioluminescense, fluorescence and radionuclide functional oncology reporters. Additionally, because changes in bone contrast may be apparent in radiographic images particularly in osteolytic models (Lu et al., 2007), and large tumor masses may be apparent especially when located at anatomical peripheral regions (Kolegraff et al., 2011), X-ray alone may serve as a highly informative, single imaging modality in certain cancer models. Figure 2 displays single modality X-ray imaging in an osteolytic bone metastasis model.

Both genetic bioluminescent and fluorescent optical reporters are used in preclinical oncology imaging studies (Edwards et al., 2009, Wu et al., 2009, Donigan et al., 2010, Madero-Visbal et al., 2012, Ullrich et al., 2014, von Wallbrunn et al.,2008). BLI is excellent for sensitive longitudinal tumor imaging owing to its potential for excellent signal-to-noise ratio (SNR). Most *in vivo* oncology BLI is conducted using firefly luciferase reporters. In Figure 1, X-ray imaging was also acquired as a useful, anatomical complement to the BLI oncology data. Madero-Visbal et al. (2012) observed in a lung tumor progression model (imaging luciferase transduced A549 cells over 64 days), that there was a strong positive correlation between measured BLI signal and actual tumor burden, as measured by histology. Such correlations point to *in vivo* BLI as a potentially quantitative tool in longitudinal tumor progression models. Figure 2 displays an *in vitro* luciferase-tumor cell dilution series imaged in triplicate. The dilution series shows excellent sensitivity and linearity in relation to tumor cell number.

Genetic intrinsic fluorescent protein (FP) reporters enable imaging without substrate and without nearby metabolite requirements, and thereby also permit both *in vivo* and *ex vivo* imaging. However, largely due to the autofluorescent/scatter properties of tissue, genetic FP reporters have a less favorable *in vivo* SNR compared to bioluminescent reporters. While GFP reporters are useful for shallow and bright signal and have been used in several oncology studies (Mahmood et al., 2002, Cameron et al., 2010, Arbab et al., 2012), model variants including RFP, dsRed, mCherry, and tdTomato can provide improved SNR and have been developed and employed in optical *in vivo* oncology imaging studies (Cicek et al., 2007, Yang et al., 2010, Noh et al., 2010, Ullrich et al., 2014). For example, Ullrich et al. (2014) recently generated and validated a pheyochromocytoma (PHEO) cell line expressing mCherry. The cell line was validated *in vitro* and *in vivo* using FLI, and with X-ray imaging for anatomical reference. This cell line should serve as a useful tool in future studies of PHEO. Figure 3 shows an example of multimodal FL mCherry-tumor imaging in a bone metastasis model.

Figure 3

C-myc-BCL2 leukemia mouse and FL metastatic tumor imaging. Tumor cells engineered for fluorescent protein mCherry expression to facilitate in vivo FLI. Images courtesy of Dr. Sebastian Baeumer, Medical Clinic A, Molecular Hematology & Oncology, University Hospital Muenster, Germany and Dr. Christiane Geyer: IZKF/TRIC/IKR Muenster, University Hospital Muenster, Germany (Unpublished).

For short term, semi-quantitative FLI, tumor cells may be pre-labeled in culture using organic or inorganic fluorophores prior to administering or grafting *in vivo*. Quantum dots (QD), the lipophilic NIR (near-infrared) dye DiR (Invitrogen), and similar dyes have been used for this purpose (Wang et al., 2008, Gailhouste et al., 2010, Davison et al., 2013a). NIR fluorescent dyes are particularly useful for *in vivo* imaging due to the relatively low tissue scatter, absorption, and resulting auto-fluorescence properties of light in the NIR spectrum. Davison et al. (2013a) reported on a protocol using *in vivo* optical imaging and DiR pre-labeling to confirm MDA-MB-231 pulmonary implantation in a classic lung metastasis model. This protocol was subsequently employed in studies investigating the association of catalase and carcinoma associated fibroblasts in resulting tumor burden (Davison et al., 2013b; Weigel et al., 2014). These types of labeling approaches provide a simple means of labeling cells for sensitive optical imaging over a short time course that does not require genetic manipulation.

Optical Imaging of Oncology Molecular Markers

Malignant tumor development requires an accumulation of mutations. Some of these mutations are related to cell-cycle/apoptosis regulation, cell adhesion molecule (CAM) expression, and angiogenic and metastatic potential. Such mutations can result in the production unique molecular markers (MMs). Several studies have developed and/or employed optical probes that target oncology MMs (Moore et al., 2004, Medarova et al., 2005, von Wallbrunn et al., 2008, Büther et al., 2012, Hischemöller et al., 2012, Neesse et al., 2013, Coles et al., 2013). Advanced *in vivo* tumor characterizations have been factilited by angiogenic MM probes (Peng et al., 2006, Backer et al. 2007, Peng et al., 2008, Edwards et al., 2009, Akers et al., 2010, Ke et al., 2012, Alsibai et al., 2014), and metastasis and protease MM probes (Mahmood et al., 1999, 2003, Weissleder et al., 1999, Tung et al., 1999, Bremer et al., 2001a/b, 2002, Waschkau et al., 2013).While advances in probe development continue, many of the probes cited above are now available and can be utilized in additional tumor biology and therapeutic studies. Below we will briefly highlight some studies of cellular, angiogenic, metastatic and protease probe development.

Probes for optical imaging of oncology MMs employ a range of ligands including antibodies, minibodies, peptides, aptamers, and small molecules. MUC-1 is underglycosylated and disregulated in some cancers. Moore et al. (2004) reported on an uMUC-1 specific peptide ligand dual FL/MRI labeled nanoparticle probe. This probe was validated for *in vivo* imaging and in a later study was assessed as a potential imaging agent to evaluate candidate cancer therapeutics *in vivo* (Moore et al., 2004, Medarova et al., 2006). More recently, Hischemöller et al. (2012) evaluated a scFV MUC-1 specific probe with a fluorescent, upconversion nanoparticle reporter in an *in vivo* tumor model. A full review of recent reports on tumor cell MM probe research is beyond the scope of this note; however, for further reading, the reader is directed to reports on an HSP70 specific probe in a melanoma model reported by Coles et al. (2013), a Claudin-4 specific probe in a PDAC model by Neese et al. (2013), an endothelin receptor specific probe in a thyroid carcinoma model by Büther et al. (2012), a CD13/APN specific probe in a fibrosarcoma model by von Wallbrunn et. al. (2008), a uPAR specific probe in a pancreatic cancer model by Yang et al. (2009), and an alpha 3 integrin specific probe in both a ovarian tumor model and a glioblastoma model by Aina et al. (2005) and Xiao et al (2009), respectively. It is foreseeable that probes recognizing unique tumor cell MMs may facilitate future *in vivo* studies of tumor biology and therapeutics and may even serve as agents of personalized cancer diagnosis and treatment.

Efforts to develop and validate angiogenic probes in cancer models are ongoing. The RGD peptide is probably the most widely employed angiogenic probe ligand (Edwards et al., 2009, Ke et al., 2012). In a study reported by Edwards et al. (2009) multimodal BLI, FLI, and scinitgraphy was used to evaluate a NIR/^{111}In RGD probe in an *in vivo* tumor (4t1-Luc) model. Throughput peptide screening techniques have been used to identify alternative candidate angiogenic MM ligands. Some of the ligands identified in screens have been evaluated as probes using *in vivo* NIR optical imaging (Peng et al., 2006, 2008). VEGF (another MM of angiogenesis) targeting by a VEGF specific sc NIR probe has also been evaluated *in vivo* (Backer et al., 2007). These probes may be used to evaluate candidate anti-angiogenic drugs and in studies of tumor biology.

The cellular and angiogenic MM probes referenced above and discussed until now employ simple ligand/receptor targeting. From 1999-2003 there were reports on auto-quenching probes to detect active proteases *in vivo* (Mahmood et al., 2003). These probes generally consist of a specific protease peptide recognition sequence flanked with auto-quenching NIR dyes that fluoresce only when the probe is cleaved. These probes are particularly “smart” because they show specificity for activated proteases. Protease (e.g. MMPs and Catepsin-B) activity is typically upregulated in many malignant tumors, and is associated with metastatic and angiogenic potential. *In vivo* validation for these new protease probes was performed in a range of tumor models (Mahmood et al., 1999, Weissleder et al, 1999, Tung et al., 2000, Bremer et al., 2001a). Additionally, Bremer et al. (2002) showed that *in vivo* protease signal correlated with phenotypes of tumor aggressiveness. Unfortunately, because the signal activity employed by these probes is based on fluorescent quenching, there is probably no direct path for clinical translation to whole body protease imaging. Recently however, Waschkau et al. (2013) reported on a ligand/receptor protease probe that recognizes

a pocket in activated MMPs that may have translation potential for imaging activated proteases because the fluorescent signal activity for this probe type could be converted for SPECT imaging by replacing the fluorescent reporter with a SPECT radionuclide.

Certain probes have more general or indirect mechanisms of tumor targeting and are not receptor specific. For example, the pHLIP NIR probe preferentially integrates into cancer cell membranes based on the characteristic acidic pH of tumors (Adreev et al., 2007, Reshetenyak et al., 2011). Additionally, there are reports on tumor detection/imaging with fluorescent nanoparticles (NP) based on tumor enhanced permeability and retention (EPR) (Park et al., 2007, Altinoğlu et al., 2008, Yang e tal., 2009, Perrault et al., 2010, Chou et al., 2012). Studies have evaluated the effects of NP size (typically 15 nm to 100 nm) and NP material (e.g. gold, calcium phosphate, hydrogel, nanolatex) on biodistribution and tumor uptake. Chou et al. (2012) employed multispectral imaging for dual *in vivo* imaging of different size gold NPs labeled with either X670 or Alexa Fluor 750, and represents an excellent example of *in vivo* NP imaging.

Indirect tumor imaging based on targeting of immune cells within tumors (Chen et al., 2005) and targeting of dying cells adjacent to tumors (Papagiannaros et al., 2010) have also been reported. Papagiannaros et al. (2010) recently reported on a nucleosome specific NIR probe for detecting dying cells in proximity to tumors. These probes with indirect specificity could facilitate *in vivo* studies of the tumor microenvironment.

Direct radionuclide imaging (DRI) can be a valuable component of multimodal oncology imaging, particularly imaging related to tracer development (Jain et al., 2005, Kularatne et al., 2009a/b, Colak et al., 2012, Orocio-Rodríguez, et al. 2015). In one study of small-molecule probe development, Kularatne et al. (2009a) validated a PSMA specific imaging compound DUPA-^{99m}Tc in a series of elegant *in vivo* DRI studies using PSMA+ tumors, PMSA- tumors, and blocking controls. A modified DUPA compound was also developed for possible therapeutic application (Kularatne et al., 2009b).

While FLI provides significant value to preclininical oncology studies, DRI may be required for some applications. As noted by Kularatne (2009a/b), DRI is potentially translatable for clinical imaging. Additionally, radionuclides (RNs) are small (relative to fluorescent molecules) and therefore are less prone to modify small-molecule biodistributions. In addition to DRI, some RNs tracers such as ^{18}F-FDG can be imaged via Cherenkov luminescence (i.e., by the optical detection of RN-derived blue light emissions). Cherenkov imaging is sometimes used for PET tracers and is most commonly leveraged for ^{18}F-FDG tumor imaging. However, oncology tracer studies are probably most easily conducted using SPECT RNs, owing in part to the favorable availability, half-life, and labeling chemistry of SPECT RNs. SPECT tracer studies are typically conducted with non-Cherenkov RNI. While radioluminescence imaging for SPECT agents is theoretically possible in some environments, it has not been widely adopted. This is probably because SPECT RNs have weak radioluminescence emissions. DRI may provide superior sensitivity even for PET RNs, and particularly when the source is positioned deep within tissue (Professor Matthew Leevy, Notre Dame University, personal communication).

Therapeutics

Optical *in vivo* imaging is an ideal throughput screening tool for evaluating tumor progression which can be neatly leveraged for therapeutic response studies (Cicek et al., 2007, Madero-Visbal et al., 2012). FLI has also been used extensively to track the biodistribtion of traditional drug compounds and candidate drug delivery vehicles *in vivo* (van Vlerken et al., 2008, Thomas et al., 2009, Nurunnabi et al., 2010, Tacelosky et., 2012) and *ex vivo* (Kim et al., 2008, Hwang et al., 2008). Optical imaging may also be employed to detect specific molecular markers following therapy to verify a mechanism of action (Schwöppe et al., 2010). Using a fluorescent fibrinogen probe, Schwöppe et al. (2010) showed that a targeted tissue factor candidate therapeutic induced coagulation at target sites.

Evaluation of novel therapeutic agents including therapeutic DNA constructs may also be facilitated by *in vivo* imaging (Radkevich-Brown et al., 2010). Radkevich-Brown et al. (2010) employed a TetC/IL12 DNA candidate therapeutic construct with a firefly luciferase element to verify *in vivo* protein expression. Additionally, with the advent of inhibitory RNA (iRNA) knockdown (KD) technologies researchers can more readily assess the function of oncology genes *in vitro* and *in vivo*. These studies also help assess the suitability of specific genes as targets for therapeutic intervention. Frequently, KD is performed in culture, prior to grafting *in vivo* (Gondi et al., 2004, Gailhouste et al., 2010). For example, Gailhouste et al. (2010) showed that MEK1 KD performed in culture inhibited subsequent tumor growth *in vivo* as assessed using FLI. With an appropriate targeting and delivery scheme, iRNAs could ultimately be potent anti-cancer agents. Several studies have focused on validating specific iRNA delivery schemes *in vivo* mostly using specific reporter iRNA in xenograft tumor models. Frequently, targeted silencing of xenograft tumor cell FP genes and *in vivo* FLI is used to assess iRNA delivery (Medarova et al., 2007, Wu et al., 2009, Noh et al., 2010). In some studies, *in vivo* FLI has been used to track biodistibution and localization of the iRNA particles and delivery vehicles (Medarova et al., 2007, Kim et al., 2008, Thomas et al., 2009).

Conclusions

Techniques and reporters for *in vivo* multimodal oncology imaging have advanced significantly over the past decade. Preclinical *in vivo* imaging studies have included tumor progression analysis using bioluminescent and fluorescent reporters, probe development efforts, and studies of tumor biology and therapeutics. The Bruker In-Vivo optical imaging systems have been used in a wide range of studies, employing numerous reporter constructs. These systems provide for flexible BLI and FLI that can be leveraged for imaging of endogenous tumor reporters. The evaluation of such *in vivo* optical signal data can be further enhanced by X-ray anatomical references. Additionally, flexible FLI (including advanced multispectral imaging) and DRI capabilities, also combined with X-ray imaging, allow users to select the probes most suitable to their study design/objectives.

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