In Vivo Imaging in the Preclinical Evaluation of Disease Progression, Treatment Efficacy, and Safety

Technical Focus: Optical Imaging and Ultrasound

Introduction

In the clinic, increasingly sophisticated imaging techniques (e.g., Ultrasound, CT, MRI/S, PET) are a critical part of the diagnosis and treatment of disease. Because of their direct application in clinical settings, these imaging techniques are considered translational technologies. Preclinically these modalities, as well as non-translational imaging techniques such as bioluminescence (BLI) and near infrared fluorescence (NIRF), are becoming an integral part of the drug discovery process. Anatomical, molecular, and pharmacologic imaging can provide valuable information to help understand the activity of compounds in development, supporting studies evaluating compound activity (mechanism of action, efficacy) as well as safety and toxicity. Combined with advances in analytical tools and the use of systems that are able to image not only small animals but larger species such as dogs and nonhuman primates, imaging is increasingly used in large molecule (antibodies, proteins) evaluation and to investigate abnormal findings in late stage toxicity studies.

There are a variety of imaging modalities, a number of which can provide similar information. The decision on which tool to use depends on the specific question and the stage of development of the project. Consideration of throughput and cost, for example, would be different early in the flow scheme where the need is to identify a lead compound, compared to later in drug development when the interest is in translatable biomarkers for the drug candidate. These decisions require a close interaction between therapeutic area scientists and imaging experts.

This technical bulletin focuses on optical imaging, the key non-translational modality used in preclinical studies, and ultrasound, a high throughput translational modality. Case examples have been included to highlight the types of drug development questions that can be answered using these modalities.

Optical Imaging

Although specific applications using near infrared fluorescent (NIRF) labeled probes may be used in surgical applications for defining tumor margins in clinical settings, optical imaging modalities have limited application in the clinical setting. In contrast to the clinic, optical imaging is extensively used in early preclinical research because of its relatively low cost, high throughput, and lack of radioactivity. These features make this an ideal imaging tool for early drug development where a number of compounds are screened to identify a lead candidate.

Optical imaging is primarily used for studies in mice and rats and is generally used to identify or characterize gross structures
or morphology (anatomical imaging). The most common
application of optical imaging uses tumor cell lines which have
been transfected with genes which either produce a fluorescent
protein (e.g., GFP, RFP) or an enzyme (firefly luciferase) which
produces light upon injection of its substrate, luciferin.

These cell lines are injected orthotopically into specific sites (e.g.,
lung tumor to lung, prostate tumor into prostate). Growth and
metastasis are evaluated longitudinally, based on the amount
of light emitted from the animal (Figure 1). Depending on the
system, both 2D and 3D images can be generated.

Optical imaging provides a distinct advantage over traditional
tumor models, given that relatively homogeneous populations
of tumor bearing animals can be identified at study start (e.g.,
ensuring only tumor-bearing animals are on study, randomizing
animals based on tumor size, etc), allowing for smaller groups
and tighter data (greater sensitivity).

In addition to anatomical imaging, optical imaging can now
be used to evaluate changes in cellular activity or status,
and to evaluate physiologic and/or cell responses to injury
or various stimuli, utilizing recently developed techniques
with specialized reagents. For example, compounds can
be evaluated for their potential to induce apoptosis. One
approach uses a near infrared fluorescent (NIRF) labeled
annexin V that binds to phosphatidyl serine which is
expressed on cells undergoing programmed cell death. A
second approach uses cells that have been transfected to
express an attenuated recombinant luciferase reporter
molecule that is cleaved to an activated form by functional
caspase 3, an enzyme that is upregulated in apoptotic cells.

Compound-induced activation or up regulation of cells can also
be evaluated with optical imaging techniques. For example, there
is a specific luciferin that has been engineered to be inactive until
processed by caspase 3 and NIRF probes that label enzymatically
active caspsases. Using similar approaches, activation of tumor
or inflammatory cells can be detected in disease models using
a variety of optical probes that are activated by cathepsins,
metalloproteinase, or myeloperoxidase (Figure 2). These same
techniques can also be used ex vivo, in example for examination
of atherosclerotic plaque. Further, targeted fluorescent agents are
available that bind to bone or integrins.

Optical imaging can also be used to assess vascular integrity.
One technique relies on the use of NIRF probes. NIRF-labeled
blood pool agents remain localized in the vasculature for various
periods of time to enable imaging of disease vasculature, vascular
permeability and angiogenesis (Figure 3).

In addition to vascular integrity, optical imaging can also be used to address drug development questions concerning vasodilation and blood flow, and in some models, indirectly assess wound healing. Laser Doppler Imaging (LDI) is a specialized optical technique, primarily used to measure blood flow. In LDI, a monochromatic laser beam is directed at the tissue surface. Light that is reflected off stationary tissue undergoes no shift while light that is reflected off cells with velocity (like red blood cells) undergoes a Doppler shift. The degree of Doppler shift is proportional to the velocity of the cell into which it collided. This light is randomly reflected back out of the tissue and onto a photodetector which calculates the average velocity of cells within the tissue. Laser Doppler is used extensively in rodents and nonhuman primates to measure the activity of migraine drugs on vasodilation (Figure 4), and has also been used to measure blood flow in cortical bone to detect changes during remodeling and fracture repair in rodents.

Overall, in situations where it is applicable, optical imaging provides substantial information in a short period of time. Many of the imaging systems allow imaging of more than one animal at a time (up to 5 mice in some systems). Combination with other modalities, such as computed tomography (CT), makes this an even more valuable preclinical tool (Figure 5).

**Ultrasound**

Ultrasound imaging is another high-throughput, relatively low-cost imaging modality, but one which has many clinical applications. Preclinical species from small rodents to nonhuman primates and dogs can be assessed using ultrasound. Mice are most often used in preclinical studies as their small size makes them highly suitable for high frequency ultrasound which provides high resolution.

In ultrasound, there is a trade-off between resolution and depth of tissue penetration. During imaging, a transducer transmits ultrasound waves into the tissue. As these waves encounter various types of tissue, they are reflected back and picked up by the transducer. Images, both 2D and 3D can be reconstructed from the wave pattern. Image resolution increases as the wave frequency increases, however this increase in resolution is accompanied by a corresponding decrease in depth of tissue penetration. Optimizing this trade-off requires experience and training, therefore in ultrasound there may be significant operator variability. Despite these minor limitations, ultrasound is valuable for real-time morphological analysis in preclinical applications because of its low cost, speed, lack of radiation, portability and broad application.

Ultrasound can be used to evaluate many organ systems. Ultrasound is particularly good at imaging the cardiovascular system. Effects of compounds on neovascularization of tumors, detection of aortic aneurysms, quantification of cardiac hypertrophy (in rodents and nonhuman primates), and vascular regeneration have all been evaluated with ultrasound (Figure 6). Recently, the use of microbubbles, which provide additional contrast, has provided increased resolution. Further refinements include the use of markers to evaluate cell and tissue response. For example, labeling of the microbubbles.
with markers such as αvβ3 integrins can be done to identify areas of local vascular inflammation.

Since microbubbles can not extravasate, they are highly specific for detecting functional and molecular changes in the vasculature. This feature however, prevents their use for other tissues. However, similar to DCE-CT and DCE-MRI, with appropriate contrast agents, recent advances in ultrasound allow the measurement of changes in blood flow in tumors in response to treatment.

Finally, ultrasound can be used in a number of tumor models to evaluate both primary and metastatic sites. For example, in preclinical tumor models, ultrasound has been used to visualize and quantify liver metastases.

**Case Examples**

**Oncology** (Figure 1, page 2). Human ovarian cancer primarily metastasizes to the peritoneum. A preclinical model was needed to evaluate potential lead compounds for drug development. Using SKOV3_luc, an ovarian tumor cell line that expresses firefly luciferase, cells were injected into the peritoneum of nude mice and growth detected with bioluminescent imaging. Animals were recruited into groups 10 days after inoculation based on similar luminescence. The bar graph demonstrates how the use of this imaging tool enabled a uniform distribution of animals into the vehicle and treatment groups (similar total fluorescence (TF) values at start of the treatment phase). Treatment with Paclitaxel began on day 11. Significant inhibition of tumor growth was detectable one week after initiation of treatment. At necropsy, as shown in the scatter plot, luminescence correlated with harvested total tumor weight (cc=0.7545). This strong correlation demonstrates the utility of this imaging tool. The selected model illustrated here is capsaicin-induced vasodilation in a normal nonhuman primates (NHPs). This model may be directly translatable into the clinics as a pharmacodynamic assay as experimental reports using this tool in humans have been published. Capsaicin when applied to the skin, produces an increase in dermal blood flow.3 This response is caused by binding of the capsaicin to the TRPV1 receptor that is present on the sensory nerve endings. The release of calcitonin gene-related peptide (CGRP) is believed to be a major initiator of this response. Anesthetized nonhuman primates were treated with the calcitonin gene-related peptide antagonist BIBN and capsaicin applied within O-rings placed on the forearm. Vasodilation was captured by Laser Doppler imaging over multiple time points. As shown in the graph, a dose dependent effect of the compound was quantified. Within 5-10 minutes post-treatment with capsaicin, there was the expected increase in blood flow in vehicle treated nonhuman primates, compared to those given various concentrations of BIBN. Statistically significant differences were noted as early as 20 minutes, even with the relatively small group size. The small error bars show the uniformity of this response in this model.

**Arthritis** (Figure 2, page 2). In individuals with rheumatoid arthritis, macrophages recruited into the joints express activated proteases and matrix metalloproteinases (MMPs) that are involved in the development and maintenance of the inflammatory response. In a preclinical model of arthritis, DBA/1 mice are immunized intradermally with type II collagen in adjuvant, with a similar injection 21 days later. Inflammation develops in the paws and disease severity is typically measured using a subjective visual scoring system. Optical imaging with a NIRF-labeled probe detecting activated MMPs enables the quantification of arthritis incidence, development, and severity.

**Oncology** (Figure 3, page 2). Tumor growth and progression requires the development of tumor vasculature. Anti-angiogenic drugs are an important anti-cancer treatment. In this example A549 tumor cells were injected intradermally into nude mice, and treatment with a VEGF inhibitor initiated. On the sixth day after inoculation mice were injected with a NIRF-labeled blood pool agent (Angiosense750) and imaged.

Compound treatment caused a significant dose dependent inhibition of neoangiogenesis and demonstrates the capability for rapid evaluation of potential inhibitors.

**Migraine** (Figure 4, page 3). Alterations in blood flow are considered key factors in the development of migraine. Preclinical models that can evaluate drug induced effects on vasodilation are important decision making tools. The selected model illustrated here is capsaicin-induced vasodilation in a normal nonhuman primates (NHPs). This model may be directly translatable into the clinics as a pharmacodynamic assay as experimental reports using this tool in humans have been published. Capsaicin when applied to the skin, produces an increase in dermal blood flow.3 This response is caused by binding of the capsaicin to the TRPV1 receptor that is present on the sensory nerve endings. The release of calcitonin gene-related peptide (CGRP) is believed to be a major initiator of this response. Anesthetized nonhuman primates were treated with the calcitonin gene-related peptide antagonist BIBN and capsaicin applied within O-rings placed on the forearm. Vasodilation was captured by Laser Doppler imaging over multiple time points. As shown in the graph, a dose dependent effect of the compound was quantified. Within 5-10 minutes post-treatment with capsaicin, there was the expected increase in blood flow in vehicle treated nonhuman primates, compared to those given various concentrations of BIBN. Statistically significant differences were noted as early as 20 minutes, even with the relatively small group size. The small error bars show the uniformity of this response in this model.

**Cardiovascular disease** (Figure 6, page 3). A number of mouse models of abdominal aortic aneurysm have been developed, including both genetic and chemically induced models. Figure 6 represents an image from a model of Angiotensin II-induced

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aneurysm in the mouse aorta. This represents a high frequency ultrasound longitudinal view of the abdominal aorta. The view demonstrates the bulging of the aorta and a dissection of the arterial wall producing a flap similar to that seen in the human setting. Ultrasound can be used to both monitor for development and staging of the aneurysm in the model, as well as to evaluate various treatment options.

**Conclusion**
Imaging can provide models that not only allow for longitudinal assessment of disease or therapy progression but that decrease animal use while providing more sensitive and uniform assays. Optical imaging is a “work horse” for early phase identification of lead candidates and can be used not only for anatomical imaging (e.g. tumors), but to assess cell activity, status or function; vascular integrity, vasodilation and blood flow. The relatively high throughput in optical imaging, in particular, lends itself to a variety of preclinical drug development (screening) flow schemes. High-resolution ultrasound is also a valuable high-throughput, low-cost preclinical tool which has shown direct translation into the clinical setting.

Both ultrasound and optical imaging are important tools in early target validation and screening of drugs for potential lead compounds. The pharmaceutical industry continues to leverage translational biomarkers such as imaging to get drugs on the market to meet medical needs. The longitudinal anatomical and molecular evaluation of compounds in relevant models can provide information on efficacy, mechanism of action, dosing, and safety that ensure that the molecules with the best chance for success move to the clinic.

**Note:** The images shown in this bulletin were generated by Covance Discovery and Translational Services scientists.

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**Covance Imaging Center**
Supporting Cancer, Neuroscience, Cardiovascular, Metabolic, Musculoskeletal, Inflammation and other therapeutic areas, Covance offers a number of small and large animal molecular, anatomical, and nuclear medicine imaging systems. These help identify clinically translatable and non-translatable biomarkers and assess the efficacy, mechanism of action, dosing and safety outcomes.

**Modalities include:**
- Bioluminescence/Fluorescence
- Computed Tomography (CT)
- Magnetic Resonance Imaging & Spectroscopy (MRI/MRS)
- Nuclear Medicine technologies (PET, Ex Vivo Autoradiography)
- Laser Doppler Imaging
- Ultrasound
- Thermal Imaging
- Dual Energy X-ray Absorptiometry (DEXA)
- Intravital Microscopy (through collaboration with INPhoton, LLC)
- Quantitative analytical tools enable translation of images to data used to make decisions
- Specialized image validation technologies, including: Immunohistochemistry, standard and near infrared fluorescence microscopy, specialized cryotomy, laser microdissection, unique bone histology

Covance scientists have expertise in a variety of imaging modalities using validated models to support drug discovery in multiple therapeutic areas as well as developing imaging tools for safety/toxicology. Our previous drug development experience allows for consultation to explore novel approaches to gaining answers. We have the ability to apply advanced technologies to accumulate more information on the integration of safety and efficacy which can lead to an increased chance of success in the clinical environment.