IN VIVO NIR-II IMAGING
A DISRUPTIVE FORCE FOR PRECLINICAL RESEARCH

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ABSTRACT

In vivo imaging in the second biological window (NIR-II) is in its earlier stages but will undoubtedly push many life science researchers back to the drawing board for their preclinical workflows.

Preclinical optical imaging suffers from the inability to localize signals due to light absorption, scattering and autofluorescence in living tissues. In vivo optical imaging can localize a signal well when it is at the surface but not when it is deep in the organism.

Preclinical biologists still strongly desire the ability to rapidly localize optical signals in vivo, but their discussions with imaging physicists often end up in a standstill. Biologists ask: can I use optical imaging to see my mCherry cancer cells in vivo? What about my luciferase cells? The answer is: it depends on many different factors such as the temperature of the animal, the optical properties of organs, how deep they are and how many photons come out.

NIR-II in vivo imaging is not impacted in the same way by drawbacks of light propagation in living tissues, thus enabling real-time imaging of optical probes much deeper in the organism and with much higher resolutions.

One of the breakthroughs in the field of in vivo SWIR imaging has been the demonstration that both NIR-I and NIR-II probes can work well for this application. There is an abundance of probes for the new imaging modality and many of them remain to be validated. The ball is back in the court for biologists to take. No longer will biologists need to accept the “oh well, I guess it depends” answer when asking an optical imaging physicist if it is possible to localize their probes in vivo.
A SHORT HISTORY OF SMALL ANIMAL OPTICAL IMAGING

Optical imaging of small animals is a widely used technique originating in the mid-90s’ when Chris and Pamela Contag together with David Beneron at Stanford University tracked optical signals in vivo by looking at bacterial infections in mice with a highly sensitive CCD camera. One of the key drivers for this early technology was its cost-effectiveness compared to PET and MRI imaging. It was also presented as a non-invasive and safe modality. It had visualization capabilities, high spatial resolution and rapid output. The in vivo imagers had a desktop feel and integrated a cooled CCD camera configuration that could be used at lab benches across the world. Moreover, everything was in vivo, reducing the cohort size for drug discovery and toxicology studies.

Thousands of these imaging systems are now installed at locations across the world and used every day to monitor the progression of disease models in laboratory animals.

Like other imaging modalities, in vivo optical imaging had advantages and disadvantages: it had great sensitivity allowing for early detection and monitoring of small changes for optical signals coming from the surface. However, the image resolution and sensitivity for signals deeper than a few millimetres was poor.

In the mid-2000s’, an attempt was made to help the recovery of signals deep in the organism with a modified configuration employing a trans-illumination mode of excitation to recover fluorescence signal depth and a profilometer to measure the height of animal for bioluminescent imaging. The newly developed imagers relied on algorithmic estimations to generate the first diffuse optical 3D tomography images in living systems. If successful, this would have the effect of further reducing cohort sizes by circumventing the need for ex vivo biodistribution studies in preclinical testing.

The new configuration of the next generation imagers provided the option of generating a 3D image but remained challenged by complications of light-diffusing in living tissues; if the estimation algorithms are off by 1 mm, the 3D reconstruction is off 10 fold [1]. Still today, most publications with 3D imagers only contain 2D images. More recently, CT and X-ray have been implemented in these systems to help with localization at depths but the fundamental issues of light propagation in living systems remain. CT and X-ray help but do not alleviate the fundamental physical limitations of optical tomography in the visible wavelengths.

THE NIR-II WINDOW, A SWEET SPOT FOR IN VIVO IMAGING

The second near-infrared spectral region (NIR-II) also referred to as shortwave infrared (SWIR) is usually defined as the wavelength range from 900 to 1700 nm. A full understanding of why this spectral window works so well for in vivo imaging remains to be fully elucidated [2], but a few principles are clear: SWIR emitters are excited by NIR-I light which has a better penetration, and there is less autofluorescence, scattering and absorption of light in the NIR-II wavelengths of 1000 to 1700 nm.
NIR-II imaging is poised to bring us an unprecedented combination of fast, deep and high-resolution imaging at a lower cost. This will have the effect of bringing preclinical imaging techniques such as visualizing tumours, vascular anatomy, and contact-free cardiography to institutes that do not have access to more costly micro-MRI or micro-CT imaging systems. Here is a review of some of the basic preclinical applications made possible by the new modality.

**Visualizing Blood Vessels and Mapping Blood Flows**

In vivo real-time visualization of the vascular system has great potential to improve our understanding of circulatory system-related pathologies and angiogenesis due to its ability to image vascular anatomy and blood flow. Current methods for assessing vascular structures such as micro-computed tomography (micro-CT), magnetic resonance imaging (MRI), have the advantage of unlimited penetration depth but suffer from long acquisition times and post-processing times thus complicating real-time imaging in the small animal. As Hongjie Dai’s group had shown in their 2012 paper [3], NIR-II imaging is also nicely suited to map arterial and venous blood flows even beyond the abilities of ultrasound at lower velocities. Such blood flow mapping could help to model tumour hemodynamics and oxygenation. Moreover, these capabilities are relevant for functional imaging of activity states such as muscle motion or brain response to stimuli, which are closely linked to perfusion.

Murine hindlimb models have been extensively used to look at the angiogenic effects of blood vessel growth modulation in healthy and diseased tissues such as animal models of peripheral artery disease. The high-resolution imaging of the blood vascular network will be useful for visualizing hindlimb ischemia and assessing the effectiveness of angiogenic therapies.
Assessing the effectiveness of angiogenic modulation is also important for cancer research. A solid tumour with a diameter of over 1 mm requires an adequate blood supply for growth and metastasis. Early detection and therapy research should also have an efficient and simple method to monitor tumour angiogenesis and NIR-II could represent the answer at an economical price point.

**Figure 2:** Data from an experiment mouse that had Ovarian tumour (SKOV-3) in the left flank following an IV injection of PbS QDots that have peak emission 1300 nm. The negative contrast in the tumour indicates a tumour barrier preventing contrast perfusion into the tumour. We also clearly see the blood vessels feeding the tumour. *Image courtesy of the Preclinical Imaging Laboratory of the National Research Center in Ottawa*

**Visualizing Tumours**

Early detection research will also benefit from seeing tumours earlier in the small animal. As previously mentioned, bioluminescence and fluorescence imaging have the advantage of sensitivity and detecting small changes in tumour biology but these techniques have replaced the calliper mostly in xenograft studies where the tumour is at the surface.

Visualization of tumours is possible with positive contrast caused by passive targeting (eg. EPR effect or targeting via lymph nodes), receptor binding, or probes activated by lysosomal enzymes. More recently, Bawendi’s group from MIT successfully imaged HER2-amplified breast cancer BT474 cells brain parenchyma following IV administration of Licor’s IRDye800 conjugated to trastuzumab (see figure 3). In addition, a PEGylated version of the Licor dye was used to show the blood vessels surrounding the tumour. [4]

**Figure 3:** Targeted SWIR imaging in vivo with IRDye 800CW of a nude mouse with a brain tumour from implanted human BT474 breast cancer cells. Three days after injecting IRDye 800CW–trastuzumab conjugate, fluorescence from the labelled tumour was imaged noninvasively through skin and skull on a SWIR camera. [4]
Contact-Free Measurements of Cardiography, Respiratory Rate and Intestinal Contractions

High-speed imaging made available by the fast frame rates of InGaAs cameras allows for contact-free cardiography and respiratory rate measurements in both anesthetized and awake animals. Research by Bawendi’s group at MIT using a blood pool contrast agent-based in micellar InAs–CdSe–CdS short-wave infrared quantum dots with high quantum yield generated sufficient signal-to-noise ratio for both anesthetized and awake mice showing how physiology is deeply influenced by anesthesia [5]. Such contact-free measurements of awake mice were obtained without the use of any restraining device. The monitoring of heart and respiratory rates of an anesthetized mouse was also recently observed by Photon etc. scientists in partnership with the Preclinical Imaging Laboratory of the National Research Center in Ottawa. This study also measured the mouse’s intestinal contractions, values that were corroborated by scientific publications (See figure 4).

Figure 4: Real-time imaging data on heartbeat, respiratory rate and intestinal contractions obtained on Photon etc.’s IR VIVO reflect values found in the literature. Image courtesy of Photon etc.

Real-Time Monitoring of In Vivo Targets for Therapeutics Development

NIR-II imaging has the potential to fundamentally impact the development of targeted therapeutics and drug discovery with real-time pharmacokinetic imaging of over a thousand targets simultaneously in a single mouse.

The new NIR-II imaging technologies are now designed to offer the time profiling analysis for each pixel or a region of interest (ROI) in a single click, hence obtaining rapidly the real-time kinetic curves at several locations simultaneously on the mouse (for example, see figure 5). Then, a principal component analysis (PCA) may be applied to a time series of fluorescence imaging to precisely delineate major tissues and organs.
With one click spectrum extraction, it is possible to measure the kinetics of fluorescent intensity changes allowing scientists to determine the accumulation and elimination of the probes in selected regions or organs and to compare relative signal ratios between the organs. This information helps understand how the probes are metabolized by the biological system in detail and to obtain values from hepatobiliary elimination and gastrointestinal transit rates.

Such a wealth of biodistribution data from a single mouse should be of great interest to the probe development community. Historically, near-infrared imaging has been used with NIR dyes conjugated to anticancer agents for probe development. Such programs could be enhanced by simply shifting the detection technology to NIR-II and without relying on organ extraction to determine the biodistribution (see figure 6).

Using the same animal for multiple PK analyses will also help to further reduce cohort sizes for preclinical studies. NIR-II could start to play a fundamental role in compound screening and allow researchers to use in vivo imaging earlier in the process of probe validation.

One way NIR-II in vivo imaging will undoubtedly affect preclinical workflow is in study design, especially as it relates to the validation of probes in preclinical testing (second phase in figure 7). For example, with NIR-I in vivo imaging, it is not possible to determine the proportion of probes accessing various regions of an organism in real-time, and organ harvesting for ex vivo analyses is often employed to get biodistribution data. With NIR-II in vivo imaging, it is possible to look at very small concentrations of probes flow through different ROI's of an organism and to track minute changes in real-time.
Metabolic Imaging

PK analyses via high-speed NIR-II imaging is also a convenient way of obtaining metabolic information for the study of metabolic diseases. Different contrast agent techniques such as SWIR Qd nanosomes, the encapsulation of ICG in emulsions, or even standard ICG can be used to stage and monitor the progression of metabolic diseases by looking at how these time courses are modified as a result of adipose tissue physiology and/or hepatocyte metabolism.

In addition to measuring hepatobiliary elimination, Photon etc’s study with ICG was also able to measure the peristaltic movements of the intestine (see figure 8). To our knowledge, it is the first time that researchers have used in vivo imaging of small animals to measure intestinal contractions. Fewer studies of such gastrointestinal systems have been reported due to the limitations of breathing artifacts in modalities such as micro-CT imaging. Such a straightforward method to image the intestine could help make in vivo imaging accessible to scientists researching diseases such as Crohn’s disease or irritable bowel syndrome.

In yet another metabolic study with Photon etc.’s IR Vivo imaging system, scientists were able to detect lipid accumulation of non-alcoholic fatty liver disease (NAFLD) and showed that elevated lipid quantities in hepatic macrophages caused by a high-fat diet persist long after reverting to a normal diet. In this study, the researchers dynamically monitored endolysosomal lipid accumulation in vivo over time scales ranging from minutes to weeks, indicating its potential to accelerate preclinical research to stage and monitor the progression of fatty liver disease. [6]
TRANSLATIONAL RESEARCH

Perhaps one of the strongest advantages of preclinical imaging in the SWIR wavelengths is its translational potential. The InGaAs camera recently used in the first-in-human clinical study is the same model used by many labs for preclinical research. The clinical study also confirmed the advantage of working in NIR-II compared to NIR-I as it provided a higher tumour-detection sensitivity, a higher tumour-to-normal-tissue signal ratio and an enhanced tumour-detection rate. Similar to the preclinical story, these advantages are also made possible by the improved depth penetration and reduced background related to working in the NIR-II window. [7]

Such an ability to translate directly into the clinic is not found in most other imaging modalities, for example, a micro-CT system and a clinical computed tomography rely upon a different detection technology.

Translational research also pervades the imaging probes of the NIR-II; one of the major breakthroughs in imaging research in recent years has been the discovery that standard NIR probes are also SWIR emitters. As Carr et al. have suggested in their paper published in PNAS, preclinical research is only recently integrating NIR-II due to the lack of SWIR imaging hardware and the perceived need for probes with peak emission in NIR-II. Silicone-based spectrometers had not been giving us an accurate picture on the emission spectra since they did not have the required quantum efficiency in SWIR wavelengths to detect the long NIR-II emission tail of standard NIR-I dyes such as ICG or Licor’s IRDye 800CW. Carr et al. argue that the current in vivo benchmark for NIR-II imaging is ICG, a NIR-I dye FDA approved in 1957 and significantly brighter than commercially available NIR-II IR-E1050 dye. [8]

An optimal strategy for translational research may be to test FDA approved probes and also work to re-purpose some already existing NIR-I probes so as to increase their long tail emissions into the NIR-II. Dr. Xiaoyuan Chen and colleagues from the National Institutes of Health (NIH) have predicted that we will see NIR-I dyes used in translational and clinical SWIR imaging research. The reason for this is two-fold: On the one hand, NIR-I dyes have an off-peak NIR-II emission. On the other hand, the toxicology studies and investigational new drug (IND) applications have already been completed for these fluorophores. Therefore, achieving clinically significant emissions in the surgery room with these dyes will not need to go through all the same validation as newly developed NIR-II dyes. These authors also believe that the incorporation of InGaAs detectors into the current clinical NIR-Imaging hardware will improve surgical outcomes. [2]

An important part of this translational research will also be to continue efforts on optimizing quantum yields of dyes with peak emissions in the NIR-II and work to improve NIR-II emissions of NIR-I dyes with long emission tails. However, in the clinical, optical imaging modality will not compete with MRI and CT as it cannot access beyond centimetre depth. As such, it is important to combine NIR-II translational research programs with multimodal approaches such as the development of PET/Ultrasound/MRI probes conjugated to a NIR-I dye with a long emission tail.
THE ABUNDANCE OF CONTRAST AGENTS FOR NIR-II IMAGING

The discovery that long-tail emissions of NIR probes can be used for NIR-II imaging can now complement the already existing research programs for the development of probes with peak emissions in the SWIR wavelengths. In recent years, research groups around the world have developed NIR-II contrast agents such as small molecule-based dyes, single-walled carbon nanotubes, semiconductor quantum dots, graphene dots, nanoparticle alloys, down conversion rare-earth nanoparticles, semiconducting polymer-based nanoparticles (SPNPs), aggregation-induced emission (AIE) probes, etc [9] (see figure 9). These contrast agents take advantage of the superior penetration depth at these wavelengths. They each have unique properties that have shown great promises to generate new tools for diverse applications, going from surgery guidance to treatment efficacy assessment, theranostics, disease monitoring and diagnosis. [10]

![NIR Fluorophores](image)

Figure 9: Brief overview of the typical fluorophores for in vivo fluorescence imaging in the near-infrared region. Image courtesy of Photon etc.

Examples include carbon nanotube sensors that allow the detection of lipid or microRNA content. Depending on the cellular environment, the emission wavelength of the carbon nanotubes will be shifted and thus allow mapping of the fat content in real-time.

Pushing peak emissions further into the NIR-II may also have the effect of imaging deeper in the organism, detecting tumour lysosomal activity with greater precision and performing multispectral imaging with non-overlapping emissions spectra due to the narrow emission bands of some materials such as quantum dots and single-walled carbon nanotubes.

The ball is back in the court for biologists to take. No longer will biologists need to accept the “oh well, I guess it depends” answer when asking an optical imaging physicist if it is possible to localize their probes in vivo. The superior depth and clarity of NIR-II imaging enable researchers to tackle new applications. Researchers can take advantage of newly developed NIR-II probes as well as existing NIR-I dyes, which have already been validated and thoroughly tested by the preclinical research community.
MEET THE AUTHORS

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Stephen is a Life Science Accounts Manager at Photon etc. and the founder of MediLumine Inc, a company developing contrast agents for preclinical imaging. Stephen has a BSc. in biology from Concordia University with research experience in the physiology and biophysics at the University of Sherbrooke Faculty of Medicine. Stephen has worked in the field of small animal imaging for over 10 years developing various applications allowing scientists to monitor the progression of functional liver diseases, quantify tumour burdens, track cells in vivo and perform preclinical angiography.

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Jacob is the scientific communication and marketing expert at Photon etc. He graduated from Polytechnique Montreal in physical engineering with an international profile and sustainable development. He then pursued this branch going for a master’s degree in Sustainable Science at Universitat Politècnica de Catalunya in Barcelona. His transdisciplinary skills help him thrive in this sea of applications possible with Photon etc.'s technologies and know-how.

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Émilie is an Application Scientist and Researcher in Optics at Photon Etc. With over 10 years of experience in biophotonics, plus extensive exposure to varied applications in life science, Émilie strives to bring the latest innovations to the market. After her graduate studies at Polytechnique Montreal, Émilie worked for Nikon Instruments as a biosystems field support specialist and technical representative. She later joined the Tearney group at Harvard Medical School as an optical engineer for the design and manufacturing of endoscopes.

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REFERENCES

[1] Courtesy of MediLumine. Values obtained from measurements with the PRISM system.


