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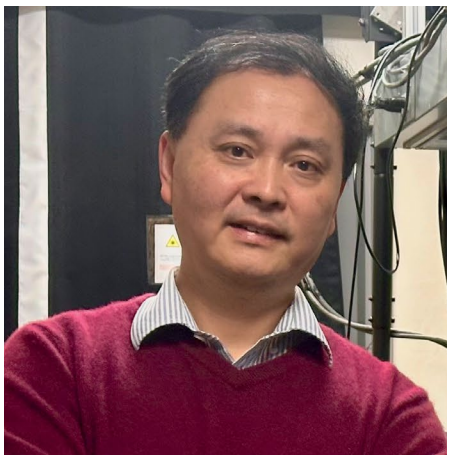
Seeing into deep tissue

Nature Photonics **spoke to Hongjie Dai from Stanford University about bioimaging in the second near-infrared (NIR-II) spectral window and its clinical potential.**



Why is deep tissue imaging important and how does the NIR-II spectral window help?

In an ideal world, we would hope to peek deep into living systems to glean information about biological structures and processes with high spatial resolution and molecular identification. Even better would be to do so with high temporal resolution to watch cellular behaviour and reactions in real time. Such capability is important to provide a basic understanding of life, disease and death at the ultimate length and time scales, and to devise effective therapeutic or surgical interventions.

While X-ray, magnetic resonance and positron-based imaging techniques have superior penetration depths, their spatial resolution is limited. Fluorescence imaging can achieve single-molecule resolution and ultrafast imaging speed. However, in vivo imaging has been limited to superficial layers due to light scattering by tissues and autofluorescence. In 2009, we showed in vivo fluorescence imaging in the second near-infrared (NIR-II) or short-wave infrared window (1,000–1,700 nm) by using single-walled carbon nanotubes as photoluminescent emitters. This greatly suppressed light scattering by biological tissue, allowing imaging of blood vessels through mouse tissues at millimetre depths, representing a substantial improvement compared with the ~100-micrometre depth achieved by previous fluorescence imaging in the ~900 nm spectral range. Many groups around the world are now carrying out biomedical research using NIR-II imaging for a wide range of preclinical studies of mouse models for investigating lymphatic systems, cardiovascular diseases such as peripheral ischaemia, stroke, traumatic brain injury and cancer immunotherapy. Clinical translations of NIR-II imaging are also emerging.

How is NIR-II fluorescence imaging typically performed?

There are several modes of NIR-II fluorescence imaging for non-invasive imaging of live animals in vivo or tissue samples ex vivo. One mode is widefield imaging, which employs an expanded 700–1,000 nm laser beam or LED light to excite NIR-II emitters in an animal, and detect light emission in the 1,000–1,700 nm range using a 2D InGaAs camera. This produces an image of spatially distributed NIR-II emitters projected onto a single plane. Another modality is NIR-II confocal microscopy for volumetric 3D imaging. In this case, we use spatial scanning of a focused 800–1,600 nm laser beam and detect emission point-by-point (or line-by-line) in the 1,000–2,000 nm range using either a photomultiplier tube, a linear array of InGaAs detectors or a superconducting nanowire single-photon detector. Light sheet microscopy (LSM) for volumetric 3D imaging can also be implemented by *z*-scanning a thin light sheet in the 800–1,300 nm range and imaging plane-by-plane in the 1,000–1,700 nm range using a 2D InGaAs camera. LSM in oblique and in structured illumination microscopy (LSM–SIM) mode have also been developed.

What are the current capabilities?

In vivo NIR-II fluorescence imaging of mice can reach depths of millimetres with spatial resolutions on the order of several micrometres, and up to one centimetre depth with lesser resolution. In terms of temporal resolution, real-time NIR-II imaging (above 30 frames per second) can be achieved to glean dynamical events in the body. For example, we can image mouse cerebral vasculatures with micrometre-scale resolution through intact skull and scalp, or we can perform real-time haemodynamic measurements of blood flows in the brain and mouse models of cardiovascular diseases.

In terms of molecular imaging, it is possible to perform in vivo through-tissue molecular imaging at the cellular level. This is done by imaging NIR-II-emitting probes conjugated with antibodies or other targeting ligands specific to cell surface markers on tumour or immune cells. For instance, volumetric 3D LSM–SIM imaging made it possible to identify certain lymphocytes in tumour microenvironments in response to cancer immunotherapy. Multiplexed NIR-II molecular imaging of several biomarkers in vivo has also been achieved using probes emitting at different wavelengths in the 1,000–1,700 nm range, or probes exhibiting different excited states lifetimes. In the NIR-IIc window (1,700–2,000 nm) it is also possible to image mouse lymph nodes without any surgery using 1,650 nm excitation. In this same window, confocal fluorescence imaging can also reach a millimetre imaging depth through an intact mouse head and enable non-invasive cellular-resolution imaging.

What are the advantages of NIR-II fluorescence over other forms of deep tissue imaging?

Traditional imaging modalities such as X-ray imaging, magnetic resonance imaging and positron emission tomography have superior penetration depths, but the spatial and temporal resolutions are inferior to fluorescence. Meaningful comparisons can be made with two-photon and three-photon imaging that are well-established techniques for deep tissue optical imaging with high spatial resolution. One-photon NIR-IIc imaging with 1,650 nm excitation is now on a par with the longest excitation wavelength used in multiphoton microscopy, thus suggesting a similar penetration depth of excitation light for the two techniques.

However, fluorescence emission in NIR-IIc one-photon excitation microscopy scales linearly with the excitation light intensity, while multiphoton fluorescence is nonlinear, thus decaying more rapidly with depth compared with the one-photon case. Consequently, through-skin NIR-IIc imaging can be done non-invasively with one-photon microscopy, whereas multiphoton imaging always requires

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surgical skin/skull removal. Also, one-photon NIR-II imaging employs cheaper equipment than multiphoton microscopy.

On the other hand, it is important to note that a major advantage of multiphoton imaging is the availability of genetically encoded fluorescent proteins emitting in the visible and far-red. Instead, fluorescent proteins emitting in the NIR-II window have been elusive. A promising direction could be to integrate NIR-II one-photon microscopy with multiphoton microscopy both employing ~1,650–1,700 nm excitation to maximize tissue penetration, while also multiplexing on imaging channels using a broader range of fluorescent probes.

Can NIR-II imaging be valuable for early detection and diagnosis of diseases?

NIR-II detection has applications for in vitro diagnostics due to high signal-to-background ratios with low interfering autofluorescence of biological samples and reagents in this spectral range. Point-of-care diagnostic devices integrated with NIR-II emitters have been launched and regulatory approval for detecting cancer, cardiovascular and inflammation/infection markers has been obtained. Preclinical in vivo molecular imaging using NIR-II-emitting probes conjugated with antibodies or other targeting ligands have detected cancer biomarkers in superficial tumours such as melanoma and in head and neck cancers. Preclinical NIR-II molecular imaging of PD-L1 [a transmembrane protein] expression in tumours has also reached superior tumour-to-normal-tissue signal ratios, showing potential for the diagnosis and prognosis for cancer immunotherapy.

How do you see NIR-II imaging being integrated into clinical practice?

For many years, the clinical use of NIR imaging in the ~900 nm spectral region has been facilitated by the FDA-approved indocyanine green dye. I believe that a new generation of clinical NIR imaging will emerge by employing NIR-II probes to increase signal:background ratios and improve penetration depths for assessing blood perfusion and vessel regeneration and for imaging of the lymphatic system. Further, targeted molecular imaging of tumours can be achieved in NIR-II with superior clarity of the tumour margin, allowing imaging-guided tumour resection with high precision. These and other intraoperative NIR-II imaging techniques are expected to play important roles in future clinical practices. I believe that the NIR-II emitters promising for clinical translation will include biocompatible small organic molecules, gold molecular clusters coated by biocompatible ligands, and rare-earth nanoparticles with stable, crosslinked hydrophilic coating layers.

What new promising developments are you excited about?

I am particularly excited about our recent development of one-photon in vivo 3D volumetric confocal imaging employing 1,650 nm excitation and NIR-IIc detection, as it pushes the limit of imaging depth on both the excitation and emission fronts. I expect much to be done and learned by imaging and monitoring immune trafficking and activities in lymph nodes at the single-cell level and in real time. I am also excited about utilizing NIR-II probes as bifunctional vaccine carrier and imaging probes, aimed at observing vaccine trafficking and developing new generations of effective vaccines for cancer and infectious diseases. Promising results have been obtained thus far.

In terms of future advances, another area of interest is to push one-photon imaging to the ~2,200 nm NIR-IId range, reaching the end of the NIR imaging window beyond which water absorption diminishes penetration depth. I am also very interested in advances in bioluminescence, genetically engineered fluorescent proteins and neuronal action potential sensors in the NIR-II window, which, in combination with molecular imaging probes, will further shift the paradigm of in vivo imaging with much enhanced resolution and functionality.

What are some of the current limitations of NIR-II imaging and how are they being addressed?

Current limitations include the relative scarcity of NIR-II fluorescent or luminescent agents. Despite significant progress made since 2009, starting from carbon nanotubes and including quantum dots, small molecules, rare-earth down-conversion nanocrystals and gold molecular clusters, much is left to be done. The lack of genetically engineered fluorescent probes in NIR-II has also limited the field. Fortunately, an increasing number of diverse scientists are joining forces to tackle these challenges, including chemists, materials scientists, biochemists, biologists and medical researchers from various disciplines with broad expertise.

Where do you see the field in 10 years from now?

I envisage that in 10 years a broader range of NIR-II emitters will be developed including up to ten colours in the 1,000–2,200 nm range for multiplexed molecule imaging in vivo, along with fluorescent proteins and bright probes with sensitivity to neuronal activities. NIR-II imaging will be widely used for preclinical research to peek deeply into living systems and visualize molecular-specific cellular features in real time. I hope NIR-II imaging will also find its way into clinical practice for intraoperative applications, such as guiding tumour resection, mapping lymph nodes for assessing cancer metathesis, and facilitating diagnosis and prognosis of immunotherapy.

Interviewed by Giampaolo Pitruzzello

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