Biological Imaging in the 1000-1700 nm NIR-II/SWIR Window

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Funding: NIH Director’s Pioneer Award DP1
Dai Lab: Carbon Based Nanoscience & Nanomedicine

- Carbon nanotubes and graphene nanoribbons: synthesis, physical properties, nanomedicine.

We Started NIR-II/SWIR Imaging with Carbon Nanotube Photoluminescence in the 1000-1700 nm range

Weisman & Smalley:

- Excited resonantly through $E_{ii}$ transitions in 500-900nm range.
- Fluoresce in NIR-II (1-1.7 µm) range; large Stokes shift from 808nm excitation.
The First In-Vivo **One-Photon** NIR-II/SWIR (1000-1700 nm) Fluorescence Imaging of Mice

InGaAs camera imaging of carbon nanotubes fluorescence in mouse blood vessels:

Excitation: 808 nm
Emission: 1000 nm – 1700 nm

Exposure time 0.2 s - 1 s


In vivo tumor vessel imaging
Various Ranges of Optical Wavelengths; Why NIR-II?

- 800 to 1000 nm: near infrared-I, NIR-I; ICG, IRDye800, etc.
- 1000-1700 nm: NIR-II or SWIR (short wavelength infrared)
- 1000-3000 nm: NIR-II or SWIR

Light absorption increases > 1300 nm; but imaging is not only limited by light absorption
Reduced Light Scattering in NIR-II Window

Scattering coefficient $\mu_s \propto \lambda^{-b}$:

- The reduced scattering, $\mu_s'$ vs wavelength ($\lambda$) depends on small-scale Rayleigh scatterers (size $<< \lambda$) and large-scale Mie scatterers (size $\geq \lambda$). Examples show average skin and breast scattering properties.

Steven L Jacques, *Physics in Medicine & Biology, Volume 58, Number 11, 2013*
Longer Light Attenuation/Penetration Length in NIR-II

Penetration depth/attenuation length vs. $\lambda$, greater in NIR-II:

\[
\text{Attenuation length } l = 1/\left[1/(l_{\text{scattering}} + 1/l_{\text{absorption}})\right]
\]

Various Sub-Windows of NIR-II

- InGaAs detectors/camera are sensitive in the 900 - 1700 nm range
Carbon Nanotubes for NIR-II Imaging of Mouse Model of Cardiovascular Diseases

NIR imaging of mouse hindlimb:

NIR-I (<900 nm): IRDye 800

NIR-II (>1000 nm): Nanotube fluorescence

NIR-II/SWIR Imaging: At the Interface of Nanoscience, Materials, Chemistry, Biology and Medicine

Developing a wide range of probes/fluorophores:

- nanotubes
- quantum dots
- conjugated polymer
- Rare earth nanoparticles
- molecular dyes

- NIR-II/SWIR bio-imaging publications since 2009: ~ 20,000 papers
A Donor-Acceptor-Donor Molecule for NIR-II Imaging

Donor and acceptor orbitals re-hybridize: reduced band-gap for long wavelength fluorescence

Excretable Molecules for NIR-II Imaging Towards Potential Human Use

- Renal excretion in 2-5 minutes post injection
- Rapid excretion could facilitate clinical translation
Targeted NIR-II Imaging of Tumor and Guided Tumor Resection Surgery

(a) Schematic representation of the targeted NIR-II imaging process. The diagram illustrates the conjugation of CH1055 with maleimide and the binding of Affibody to CH1055-ffbody.

(b) Fluorescence images showing the distribution of CH1055-Affibody and CH1055-Affibody + blocking in SAS (EGFR+) and U87MG (EGFR-) tumor models. The images demonstrate higher fluorescence in SAS tumors compared to U87MG tumors.

(c) Time-course images at 1 hr, 6 hr, and 24 hr post-injection of CH1055-Affibody and CH1055-Affibody + blocking. The images show increased fluorescence in ex vivo SAS tumors at 24 hr, indicating effective targeting.

(d) Graph showing the T/N ratio over time (1-6 hours) for CH1055-Affibody and CH1055-Affibody + blocking. The data suggests a significant increase in T/N ratio for CH1055-Affibody, indicating improved contrast.

Max T/N in NIR-I
Imaging in NIR-IIb, 1500-1700 nm is Highly Advantageous

- InGaAs detectors sensitive up to 1700 nm
- Probes are scarce in NIR-IIb 1500-1700 nm
Bright PbS/CdS Core-Shell Quantum Dots Emitting at ~1600 nm (NIR-IIb) at 808 nm excitation

~ 40X brighter than CNTs

Mingxi Zhang et al., PNAS, 2018

Earlier QD-bio work: Alivisatos/Nie Bawendi

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Bright NIR-IIb Down-Conversion $\alpha$-phase $\text{NaYbF}_4$: Er, Ce, Zn Nanocrystals

Ex: 980 nm, Em: 1500-1700nm, NIR-IIb

Emission spectrum under 980 nm excitation:
Brain Imaging Through Intact Scalp/Skull with $\alpha$-phase Rare Earth Nanoparticles

Infrared makes a body more transparent

Excitation 980 nm / Emission $> 1500$ nm
Works with low power LED; Up to 100 frame per second
**Molecular Imaging of PD-L1 in Tumor for Cancer Immunotherapy**

- PD-L1 tumor to normal tissue ratio $T/NT$ reaches $> 40$
- Autofluorescence background $\sim 0$


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**Images**

- **ErNPs-aPDL1**
  - 5 mins p.i.
  - 12 hrs p.i.
  - 24 hrs p.i.
  - CT-26 tumor

- **free ErNPs**
  - CT-26 tumor

- **ErNPs-1/10<sup>th</sup>aPDL1 for 4T1**
  - 5 mins p.i.
  - 24 hrs p.i.

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*CT-26 tumor, 5 mins p.i.*
NIR-IIb 1500-1700 nm Fluorescence Life-Time of ErNP vs. PbS/CdS QDs

Log intensity (a.u.) vs. Time (ms)

- ErNPs-aPDL1 (1xPBS), $\tau = 4.3$ ms
- PbS-aCD8 (1xPBS), $\tau = 46$ $\mu$s
2-Plex Molecular Imaging of Immune Response: CD8+ T Cells Surrounding PD-L1+ Tumor Cells

- PbS QD imaging of CD8 with 808 nm /1600 nm Ex/Em, CW imaging
- ErNP imaging of PD-L1 with 980 nm /1600 nm Ex/Em, Er lifetime imaging
NIR-II Light Sheet Microscopy/Single Cell Imaging In Vivo?

Supplementary Figure 1. A schematic of the LSM in NIR-II Light Sheet Microscopy/Single Cell Imaging In Vivo?

• 1925, Ultramicroscopy, colloid based, Nobel prize, Zsigmondy
• 1993, Orthogonal-Plane Fluorescence Optical Sectioning, Voie, Burns, Spelman.
• 1994, Stelzer, Lindek
• 2002, thin LSM, Fuchs
• 2004, SPIM, Huisken, Stelzer
• Many groups……

Regular mode:

Oblique mode:

Feifei Wang, H. Dai et al., Nature Method, 2019
NIR-II Light Sheet Microscopy with up to 1319 nm excitation

Feifei Wang, H. Dai et al., Nature Method, 2019
**Ex vivo NIR-II Optical Sectioning of Mouse Brain Vasculatures**

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>785 nm</td>
<td>&lt; 1000 nm</td>
</tr>
<tr>
<td>785 nm</td>
<td>1100-1200 nm</td>
</tr>
<tr>
<td>785 nm</td>
<td>1500-1700 nm</td>
</tr>
</tbody>
</table>

Fixed, glycerol treated brain

Fixed 785 nm excitation

Feifei Wang, H. Dai et al., Nature Method, 2019
In Vivo Non-Invasive 1319 nm LSM of Mouse Head: Skin, Skull, Meninges, Cortex

- Vascular channels in the meninges observed in vivo, connecting brain and skull bone marrow, important to brain immune responses/defense (based on work by Nahrendorf group, Nature Neuroscience, 2018)

Feifei Wang, H. Dai et al., Nature Method, 2019
LSM with Structured Illumination Microscopy (LSM-SIM) in NIR-II

Excitation: 1540 nm
Emission: 1600-1700 nm

Cerebral cortex
Hippocampus

2692 x 1686 x 1492 µm³
1415 x 848 x 552 µm³

Prism
Cover glass
Mouse brain

Excitation:
808 nm
1319 nm
1540 nm

Scan

Normalized intensity
Distance (µm)

FWHM: 14 µm
13 µm
9 µm

808 nm - LSM
1319 nm - LSM
1540 nm - LSM

940 µm

x y z

3D data

F. Wang
H. Dai et al., PNAS 2021
In Vivo LSM-SIM imaging of CD8 and OX40 in Tumor Micro-Environment

CpG: an oligonucleotide that boosts immune response

- OX40 upregulated in tumor by CpG
- Little OX40 signal on CD8+ cells
Left tumor: CpG injected
Right tumor: No CpG

- Intra-tumor injection of CpG upregulated OX40 on CD4+ T cells, not on CD8+ cells

- In vivo molecular imaging of CD4, OX40, CD8 at single cell level for immune responses

- For immunotherapy, vaccine development… research
Where is the Limit?
Imaging in 1700-2000 nm NIR-IIc Window/Excitation up to 1700 nm

- InGaAs detectors not sensitive > 1700 nm
- Probes are scarce > 1700 nm
Imaging in 1700-2000 nm NIR-IIc Sub-Window

- Superconducting nanowire single-photon detector sensitive > 1700 nm
- Synthesized PbS/CdS emitting > 1700 nm

Two Color In Vivo Molecular Imaging of Lymph Nodes in ~1300 nm NIR-IIa and 1700-2000 nm NIR-IIc Sub-Windows

Two types of QDs conjugated to anti-CD169 and anti-CD3 respectively
- Red: aCD169-QDa, excitation: 808 nm, emission: 1200-1400 nm
- Green: aCD3-QDc, excitation: 1650 nm, emission: 1700-2000 nm
- Imaging of subcapsular sinus macrophage (CD169) and T cells (CD3) respectively
In Vivo NIR-IIc Confocal Lymph Node Imaging
Excitation up to 1650 nm, emission up to 1700-2000 nm

Wide-field image

Confocal microscopy


Green: aCD3-QDc, excitation: 1650 nm, emission: NIR-IIc. T cells

(Feifei Wang, Fuqiang Ren, H. Dai et al; Nature Nano, in press)
**In Vivo Lymph Node Imaging (One-Photon)**

Excitation up to 1650 nm, emission up to 1700-2000 nm

Red: aCD169-QDa, excitation: 808 nm, emission: 1200-1400 nm; anti-CD169 targets macrophages in subcapsular sinus.

Green: aCD3-QDc, excitation: 1650 nm, emission: NIR-lic. T cells

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In vivo non-invasive imaging in lymph node of mouse at the single-cell level.

Ex: 1650 nm  
Em: 1700-2000nm

In Vivo NIR-IIc Confocal Lymph Node Imaging

Excitation up to 1650 nm, emission up to 1700-2000 nm

- In vivo non-invasive imaging of lymph node of mouse
- Blood vessels: red color
- Molecular imaging of **high endothelial venules (HEV)** in a LN: green color labeled by NIR-IIc QD conjugated with anti-MECA79
- HEV are for immune cell trafficking between blood circulation and LNs.
### In Vivo One-Photon Imaging vs. Two- and Three-Photon Imaging

<table>
<thead>
<tr>
<th></th>
<th>Three-photon microscopy</th>
<th>NIR-IIc confocal microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excitation Wavelength</strong></td>
<td>1680 nm</td>
<td>1650 nm</td>
</tr>
<tr>
<td><strong>Emission wavelength</strong></td>
<td>~ 630 nm</td>
<td>1800-2000 nm</td>
</tr>
<tr>
<td><strong>Laser (laser cost)</strong></td>
<td>Femtosecond laser (~320k USD)</td>
<td>CW laser (~4k USD)</td>
</tr>
<tr>
<td><strong>Laser power</strong></td>
<td>3 – 35 mW</td>
<td>0.6 – 28.5 mW</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>XY: 0.9 μm, Z: 5.4 μm</td>
<td>Resolution estimated by imaging blood vessels in mouse brain ex vivo: XY: 2.0 ± 0.45 μm, Z: 7.1 ± 0.75 μm</td>
</tr>
<tr>
<td><strong>Objective</strong></td>
<td>Olympus XLPLN25XWMP2; 25×/1.05-NA</td>
<td>Resolution estimated by imaging blood vessels in mouse brain ex vivo: XY: 2.0 ± 0.45 μm, Z: 7.1 ± 0.75 μm</td>
</tr>
<tr>
<td><strong>Frame time (Area)</strong></td>
<td>8 – 20 s, (122 μm × 122 μm)</td>
<td>5 – 20 s, (550 μm × 550 μm)</td>
</tr>
<tr>
<td><strong>(Pixels per frame)</strong></td>
<td>(512 × 512)</td>
<td>(512 × 512)</td>
</tr>
<tr>
<td><strong>Imaging depth</strong></td>
<td>1400 μm (1680 nm excitation, scalp and skull removed, 10-week-old FVB/N mice)</td>
<td>1135 μm (through intact skin and skull, 3-week-old BALB/c mice)</td>
</tr>
<tr>
<td></td>
<td>700 μm (1319 nm, through intact skull with scalp removed, 12-week-old B6.Cg-Tg(Thy1-Brainbow1.0)H1i1ch/J mice)</td>
<td></td>
</tr>
</tbody>
</table>

- NIR-IIc One-Photon imaging: non-invasive/surgery free, longitudinal imaging
- NIR-IIc One-Photon imaging can perform molecular imaging through antibody targeting, which has not been done by 2P or 3P imaging thus far.
- NIR-IIc one-photon imaging has not achieved genetically encoded probes for imaging yet, an area multi-photon imaging has excelled.
Non-Invasive, Longitudinal NIR-II/SWIR In Vivo Imaging at Single Cell Level for Basic Research

Developing a wide range of probes/fluorophores:

- nanotubes
- quantum dots
- conjugated polymer
- Rare earth nanoparticles
- molecular dyes

- NIR-II/SWIR bio-imaging publications since 2009: ~ 20,000 papers
Nanoscience opened a new paradigm of molecular imaging in living mammals with single cell resolution at millimeters depth, for fundamental research and clinical translation.

Carbon nanotubes

Quantum dots;
Rare earth down-conversion nanoparticles

Encapsulated D-A polymers

Encapsulated D-A-D organic dyes

PEGylated D-A-D dyes

Sulfonated D-A-D dyes

Many available at Nirmidas Biotech. Inc
http://www.nirmidas.com/

Disclosure: H. Dai is a co-founder/share holder of Nirmidas Biotech Inc.
Small Animal NIR-II/SWIR Imager for Basic Research

- Small animal NIR-II/SWIR imaging system
- Multiple and customized lasers (808 nm and 975 nm as default)
- Whole body imaging and microscopy imaging modes
- Lifetime imaging capability
- Multi-color, multiplexed molecular imaging using organic and nanoparticles probes emitting up to 1700 nm

DeepVision of Nirmidas Biotech

Disclosure: H. Dai is a co-founder/share holder of Nirmidas Biotech Inc.
Towards Clinical Translation: Fluorescence Imaging for Cancer Surgery

• “Research efforts to develop targeted fluorophores for cancer imaging, coupled with the emergence of improved devices for fluorescence detection are set to revolutionize the field of oncological surgery.

• Surgery remains the cornerstone of treatment for solid tumours, but surgical techniques have not substantially changed over the past century.

• The presence of residual tumour tissue (defined as ‘positive margins’) after surgery has been reported in up to 30% of radical prostatectomies, 40% of pancreaticoduodenectomies, and 65% of high-grade glioma resections (in studies carried out in North America and Europe), highlighting the need for better intraoperative detection of malignant tissues.”
Previous NIR-I (< 1000 nm) Fluorescence Imaging Guided Tumor Resection Surgery

Molecular imaging of tumor with antibody-dye conjugate

Squamous Cell Carcinoma (SCC)

- Tumor-to-normal tissue ratio
- (T/NT) < 4, not high
- Tumor margin not clear/sharp

• 808 nm or 940 nm excitation;
• ~1000 nm or 1600 nm detection

F. Wang et al., *PNAS*, 2022
NIR-I (~ 900nm) vs. NIR-IIb (~ 1600 nm) Molecular Imaging of Tumor

IR800-aTRC105  |  IR800-aTRC105  |  ErNPs-aTRC105
NIR-I: 900-1000 nm  |  NIR-II: 1100-1300 nm  |  NIR-IIb: 1500-1700 nm

Anti-TRC105 targets angiogenesis/newly grown vessels in tumors

Ex: 808nm

Ex: 940nm

Normalized intensity vs. Distance (mm)

Wavelength vs. T/NT
**NIR-I Imaging Guided Surgery Using Antibody-IRDye800**

- 808 nm excitation;
- NIR-I 900-1000 nm detection

- T/muscle ratio $\sim 5$

**NIR-IIb Imaging Guided Surgery Using Antibody-Er Nanoparticles**

- 940 nm excitation;
- NIR-IIb 1500-1700 nm detection

- Clear tumor margin
- T/muscle ratio = 300 - 400
- Allow imaging of small numbers of cancer cells
NIR-IIb Imaging Guided Surgery Using Antibody-Er Nanoparticles

- 940 nm excitation;
- NIR-IIb 1500-1700 nm detection

F. Wang et al., PNAS, 2022
NIR-IIb Imaging and Histology of Resected Tumor Guided by Antibody-Er Nanoparticles

(a) Step 1: Intact tumor  
(b) NIR-IIb Imaging and Histology of Resected Tumor Guided by Antibody-Er Nanoparticles  
(c) Quantitative analysis of normalized intensity.  
(d) H&E staining of tumor and normal tissue.
NIR-IIb/SWIR Fluorescence Imaging Guided Tumor Resection Surgery

• Ultra-clear tumor margin
• Removal tumor down to single or few cancer cells
Summary

• One-photon fluorescence/ luminescence imaging with excitation in 700-1650 nm and emission in 1000-2000 nm range

• Nanoscience and chemistry can produce many probes/dyes in NIR-II/SWIR

• Deep tissue molecular imaging at single cell level for basic research: immunotherapy/vaccines; cardiovascular diseases; neuroscience

• Non-invasive, surgery free, longitudinal molecular imaging

• Clinical translations are exciting/challenging/rewarding

• Genetically engineered NIR-II probes are important
Cross-Linked Surface Coating for Rare Earth Nanoparticles: Biocompatible ‘P³ Coating’

Conjugation sites for antibodies e.g., anti-PD L1 for ‘molecular imaging’
P³-Coated NanoProbes Excretion/High Safety

Non-toxic P³ coated-nanoprobes:
- CNTs
- QDs
- Rare earth nanoparticles
- Magnetic nanoparticles

Z. Ma, H. Dai, et. al., Angew Chemie, 2020
<table>
<thead>
<tr>
<th>Detector:</th>
<th>InGaAs</th>
<th>SNSPD</th>
<th>SNSPD</th>
<th>SNSPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation:</td>
<td>808 nm</td>
<td>1319 nm</td>
<td>1540 nm ex</td>
<td>1650 nm ex</td>
</tr>
<tr>
<td>Emission:</td>
<td>NIR-IIb</td>
<td>NIR-IIb</td>
<td>NIR-IIc em</td>
<td>NIR-lic em</td>
</tr>
</tbody>
</table>

Depth of imaging in 5% Intralipid:

- 1.8 mm
- 2.3 mm
- 2.6 mm
- 3.2 mm
- 3.6 mm
- 4.4 mm

Scale: 500 μm
Skin

Objective

iLN

**Objective**

**Red:** p-FE (blood vessel)

**Green:** aMECA-79-QDc (HEV)

**Wide-field mouse image**

**Red:** p-FE (blood vessel)

**Green:** aMECA-79-QDc (HEV)

**Confocal microscopy**

**Red:** p-FE (blood vessel)

**Green:** aMECA-79-QDc (HEV)

**InGaAs Wide-field imaging**

**SNSPD Confocal microscopy**

1377 x 1264 x 500 μm³

1 day

4 day

**InGaAs Wide-field imaging**

1 mm

1 mm

200 μm
Intact mouse head

- Objective
- Cover glass
- D₂O
- 80% glycerol
- Scalp
- Skull
- Meninges
- Brain

Scalp
Skull
Meninges
Brain

550 x 550 x 810 μm³

500 x 528 x 1135 μm³

Depth (z):

- 400 μm
- 700 μm
- 900 μm
- 1100 μm

Detector:

- PMT, 1319 nm, NIR-IIb
- SNSPD, 1319 nm, NIR-IIb
- SNSPD, 1540 nm, NIR-IIc
- SNSPD, 1650 nm, NIR-IIc

Excitation (λ):

- 1319 nm
- 1540 nm
- 1650 nm

Emission (λ):

- NIR-IIb
- NIR-IIc

SBR

Normalized intensity

Distance (µm):

- 500
- 1000
- 1500
- 2000

FWHM = 3.4 µm
FWHM = 5.6 µm
FWHM = 6.2 µm
A Novel Coating P$^3$ (branched PEG-linear PAA-branched PEG) afforded rapid biliary excretion of various nanoparticles

Z. Ma, et al., Angew Chemie, 2020

NIR-IIb fluorescence imaging

Day 0  Day 1  Day 7

% Injection Dose

> 95% excretion in 2 weeks
Rapid-excreted magnetic nanoparticles with P$^3$ coating

P$^3$-iron oxide nanoparticles conjugated to IR-FEP

$\sim 90\%$ excretion in 2 weeks