Intravital Multiphoton Microscopy and Biomedical Research

Chen-Yuan Dong (董成淵)
Department of Physics
National Taiwan University
Histological Images of Liver Tumor
(HCC: hepatocellular carcinoma)

Courtesy: Dr. Hsuan-Shu Lee, NTUH
National Research Program for Genomic Medicine (NRPGM)

Optical Microscopy Core Facility for Genomic Research

- Mission Statement:
  To establish a state-of-the-art, interdisciplinary optical microscopy core facility to help life scientists in solving biomedical problems
  1. R&D
  2. Service
Research Approach

1. Technical developments of imaging modalities
   - non-linear optical microscopy
2. Ex-vivo spectral characterization of tissue morphology
3. Intravital investigations of biologically and medically relevant issues
   - intravital imaging chambers
Two-Photon Fluorescence Excitation

- UV or visible excitation
- Weakly Absorbed UV or visible excitation
- Near IR photon
- Fluorescent photon
- Intermediate state
- Excited state
- Fluorescent photon
- Near IR photon
Single photon excitation probability is proportional to the excitation intensity.

Two photon excitation probability is proportional to the square of the excitation intensity!
Multiphoton Microscopy

- Two-Photon Microscopy: observational and manipulation technology
  - point-like, non-linear fluorescence excitation
  - deeper penetration (3-D, tissue imaging)
  - easy accessibility to emission spectrum
  - 3-D control of photochemical reactions

- Harmonic Generation Microscopy
  - $D = \varepsilon_0 E + P$
  - $P_i = \chi_{ij}(1)E_j + \chi_{ijk}(2)E_jE_k + \chi_{ijkl}(3)E_jE_kE_l + \cdots$
  - Biological harmonic generators: collagen, muscle fibers, …
Multiphoton Microscope

- Pulsed laser
- fs ti-sa laser
- Excitation wavelength: 780 nm
- Objective: Nikon 60X WI N.A. 1.2
- Beam controlling optics
- X-Y scanner
- Computer
- PMT
- Beam expander
- Dichroic
- Sample
Multiphoton Microscopy
Applications

1. Dermatology
   - skin photoaging
2. Ophthalmology
   - minimally-invasive characterization of eye structures
   - corneal pathology (infection, keratoconus, scar)
   - collagen thermal denaturation
3. Tumor Physiology
   - multiphoton tissue characterization (BCC)
   - development of intravital imaging technologies
4. Hepatology
   - observation of hepatic metabolism
   - bile duct ligation
5. Tissue Engineering
   - scaffold imaging
   - chondrogenesis
6. Infectious Pathogens
   - fungi
Skin Structure (similar to eye)

- **Stratum corneum**
- **Basal cells**
- **Collagen/elastin fibers**
- **Fibroblast**
3-D Multiphoton Skin Movies

Epidermal-dermal junction  Dermal collagen and elastic fiber orientation  Elastic fiber projection
Skin Photoaging: Autofluorescence (AF) and Second Harmonic Generation (SHG) Imaging

20 years of age
40 years of age
70 years of age

Collagen
Elastin

SHG: blue
AF: green

Quantitative Determination Using SHG to AF Aging Index

Skin photoaging index = \frac{(SHG-AF)}{(SHG+AF)}
Porcine Cornea Structure

Teng et al., IOVS, 47: 1216-1224 (2006).
Porcine Cornea (Depth: 862μm)

Inner side of cornea

Corneal collagen

SHG: blue
AF: green
Porcine Limbus Structure

- Sclera
- Limbus
- Cornea
Multiphoton Scan of Porcine Limbus

Depth: surface

Depth: 50 μm

Sclera

Limbus

Corneal collagen

SHG: blue
AF: green

100 μm
Porcine Sclera Structure

Conjunctiva

Sclera collagen
Multiphoton Porcine Sclera Imaging (Depth: 50 μm)

SHG: blue
AF: green
Multiphoton Imaging Across an Entire GFP Mouse Cornea


SHG: blue
F: green

113 μm
Multiphoton Imaging of Normal Human Cornea

Corneal epithelium

Collagen

SHG: blue
AF: green

Collagen

Corneal epithelium
Multiphoton Imaging of Fungal (Fusarium solani) Infection
Acanthamoeba Keratitis

Bacterial co-infection (?)

Acanthamoeba

SHG: blue
AF: green

Tan et al., *Journal of Biomedical Optics*, In press.
Acanthaamoeba Infection

- Culture: Acanthaamoeba castellanii + Pseudomonas aeruginosa
  - SHG: blue
  - AF: green
  - Bar: 50 μm
Keratotonus

Altered collagen fibers (toward apex)

Keratoconical apex

SHG: blue
AF: green

Tan et al., IOVS, 47(12):5251-5259.
Multiphoton Imaging of Corneal Scar

Disruption of basement membrane
Irregular growth of collagen

Blue: SHG; $\lambda = 380\text{nm}$; Green: Autofluorescence $\lambda = 490\text{nm}$

Teng et al., *Archives of Ophthalmology*, Accepted.
Multiphoton Imaging of Corneal Scar

- Irregular aligned collagen
- Granulation tissue
- Activated cells

Blue: SHG; $\lambda = 380\text{nm}$; Green: Autofluorescence $\lambda = 490\text{nm}$
Multiphoton Imaging of Corneal Scar

Parallel aligned collagen

Uvea adhesion
# Collagen Thermal Denaturation (SHG)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
<th>140</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
<tr>
<td>54</td>
<td></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>55</td>
<td></td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
</tr>
<tr>
<td>57.5</td>
<td></td>
<td><img src="image33.png" alt="Image" /></td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
<td><img src="image37.png" alt="Image" /></td>
<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
<td><img src="image40.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Large Area Intensity

Relative Intensity vs. Time (min)

- 40
- 50
- 53
- 54
- 55
- 57.5
- 60
- 70
SHG and Histological Images


Scale bar: 100 μm
Collagen Shrinkage and SHG Decay

- Sample: rat tail tendon
- 58° C treatment at different times

Rat Tail Tendon – SHG and Shrinkage

\[ \frac{L_d}{L_0} = 0.547 \frac{I_d}{I_0} + 0.525 \]
Basal Cell Carcinoma (BCC) Imaging

Histology

Multiphoton

Green: AF
Blue: SHG

Basal Cell Carcinoma (BCC) Imaging

Epithelium

dermis

Tumor masses

Tumor mass

200 μm
Quantitative Analysis of BCC

Tumor proximity index = \frac{(AF - SHG)}{(SHG + AF)}

Sample size: 3
Dorsal Skin Fold Chamber for In Vivo Oncology

6Al/4V ELI titanium alloy

1. Angiogenesis 2. Metastasis 3. Tumor treatment

Intravital Imaging of Mouse Physiology

In vivo leukocyte movement

GFP melanoma injection in vivo (day 1)

GFP melanoma dispersion in vivo (day 4 later)

SHG: blue
AF: green
Long Term Observation of In Vivo Tumor Growth

Top: 1 day
Bottom: 4 days

GFP human melanoma

Collagen

Blood vessel

Increasing imaging depth

Depth 0 um - 12 um - 24 um - 36 um - 48 um - 60 um

Increasing imaging depth

SHG: blue
F: green
In Vivo Hepatic Imaging Chamber

6Al/4V ELI titanium alloy

Liu et al., Journal of Biomedical Optics, Accepted.
In Vivo Hepatic Metabolism

Temporal snapshots

Metabolic Movie

Red: Rhodamine B isothiocyanate-dextran
Green: 6-CFDA (green)
Depth Resolved, In Vivo Imaging of Mouse Liver

Image size: 220 μm by 220 μm

Red: Rhodamine B isothiocyanate-dextran
Green: 6-CFDA (green)
Temporal Dependence of Carboxyfluorescein Intensity Profiles

Histoacryl: tissue adhesive
4011 (Henkel Loctite): for medical device
406 (Henkel Loctite): typical instant adhesive
Bile duct Ligation

Normal liver  
1 day post bile duct ligation

Image size: 660 μm by 660 μm
Bile duct Ligation Induced 6-CFDA Fluorescence Change

- 2days
- 1day-A
- 1day-B
- Control-A
- Control-B

Normalized intensity

Time (after 6-CFDA injection, min)
Large Area Multiphoton Imaging of Mouse Liver In Vivo

- Green: autofluorescence
- Red: rhodamine dextran
Multiphoton Porcine Cartilage Imaging

![Image of cartilage showing Collagen II fibers, Chondrocytes, and Lacunae](image)

- Collagen II fibers
- Chondrocytes
- Lacunae
### Multiphoton Imaging of Tissue Engineering Scaffolds (2-D)

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Autofluorescence</th>
<th>SHG</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>collagen scaffold</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>collagraft bone graft</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>matrix strip</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>OPLA</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>PGA</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>nylon</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
### Multiphoton Imaging of Tissue Engineering Scaffolds (3-D)

<table>
<thead>
<tr>
<th></th>
<th>Autofluorescence</th>
<th>SHG</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>collagen scaffold</td>
<td><img src="collagen.png" alt="Image" /></td>
<td><img src="SHG_collar.png" alt="Image" /></td>
<td><img src="combined_collar.png" alt="Image" /></td>
</tr>
<tr>
<td>collagraft bone graft matrix strip</td>
<td><img src="collagraft.png" alt="Image" /></td>
<td><img src="SHG_collagraft.png" alt="Image" /></td>
<td><img src="combined_collagraft.png" alt="Image" /></td>
</tr>
<tr>
<td>OPLA</td>
<td><img src="OPLA.png" alt="Image" /></td>
<td><img src="SHG_OPLA.png" alt="Image" /></td>
<td><img src="combined_OPLA.png" alt="Image" /></td>
</tr>
<tr>
<td>PGA</td>
<td><img src="PGA.png" alt="Image" /></td>
<td><img src="SHG_PGA.png" alt="Image" /></td>
<td><img src="combined_PGA.png" alt="Image" /></td>
</tr>
<tr>
<td>nylon</td>
<td><img src="nylon.png" alt="Image" /></td>
<td><img src="SHG_nylon.png" alt="Image" /></td>
<td><img src="combined_nylon.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Multiphoton Chondeogenesis Imaging
(Tissue Engineering in PGA Scaffold)

0 week
AF + SHG

Lee et al., *Tissue Engineering*,
1 week
AF + SHG
2 weeks

AF + SHG
3 weeks

AF + SHG
Multiphoton Fungal Imaging

False-colored MPF images of fungi. (a) *Microsporum canis*  (b) *Aspergillus flavus*  (c) *Trichophyton mentagrophytes*  (d) *Fusarium solani*. 
What is Next?

- Multiphoton microscopy is effective for **label-free biopsy** in many tissue types.
- Clinical extension
  - Autofluorescence and second harmonic generation signal (**disease diagnosis**)
  - **Dermatology** and **ophthalmology** may be promising for real-time diagnosis.
- Applied to animal models for **intravital physiological processes**.
Conclusion

- Multiphoton microscopy is effective for **label-free imaging** of many tissue types
  - Clinical extension
    - **disease diagnosis**
    - **dermatology and ophthalmology** may be promising for real-time diagnosis
  - Structure/function relation of tissues (3-D)
  - Investigation of **intravital** physiological processes

- Theoretical description/modeling
  - 3-D self-assembly of fibrous network (collagen/elastin)
  - Cell-matrix interaction (migration, differentiation, assembly)
  - Cell/tissue metabolism
  - Infection
  - Tissue reorganization/genesis and (interaction of biomechanical and biochemical pathways)

- High **scientific** and **application** values
NTU Microscopic Biophysics Laboratory Group

- **PI**
  - C. Y. Dong

- **Postdoc**
  - W. L. Chen

- **Assistants**
  - Y. Sun
  - C. C. Lee
  - C. R. Kuo
  - M. C. Sung

- **Doctoral Students**
  - W. Lo
  - C. C. Wang
  - M. G. Lin
  - F. C. Lee

- **MS Students**
  - L. K. Wen
  - Y. Liu
  - C. N. Lee
  - C. K. Chou
  - W. P. Hong
  - C. C. Chang
  - Y. L. Chang
  - C. S. Huang
  - T. L. Sun

- **Undergraduates**
  - R. C. Wu
  - C. L. Yang
  - H. Y. Wu
Acknowledgment

Optical Molecular Imaging Microscopy Core Facility (A5)

- **NTU**
  - Dentistry
    - Dr. M. H. Chen (陳敏慧)
  - Dermatology
    - Dr. S. H. Jee (紀秀華), Dr. S. J. Lin (林頌然)
  - Internal Medicine
    - Liver:
      - Dr. H. S. Lee (李宣書)
    - Lung:
      - Dr. P. C. Yang, Dr. C. J. Yu (余忠仁)
  - Infection
    - Dr. S. C. Chang (張上淳), Dr. Y. C. Chen (陳宜君), Dr. J. L. Wang (王竣令)
  - Ophthalmology
    - Dr. F. R. Hu (胡芳蓉), Dr. W. L. Chen (陳偉勳), Dr. T. J. Wang (王宗仁)
  - Orthopedics
    - Dr. C. C. Jiang (江清泉), Dr. H. S. Chiang (江鴻生)

- **Chang-Gung Memorial Hospital**
  - Otolaryngology
    - Dr. P. Lou (娄培人)
  - Pathology
    - Dr. W. C. Lin (林維洲)

- **NTNU**
  - Chemistry
    - Dr. C. C. Chen (陳家俊)