Extended Time-Lapse Multimodal Microscopy for Tracking Cell and Tissue Dynamics in Skin

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Principles of Fluorescence Techniques
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Imaging technologies across size scales are needed to address complex biological questions.
Presentation Outline

• Introduction
  – Multi-modal, time-lapse intravital microscopy

• Multimodal imaging technique
  – Integrated optical coherence tomography (OCT), multiphoton microscopy (MPM), and fluorescence lifetime imaging microscopy (FLIM)
  – Technological advances (optical source, coherence curvature)

• In vivo murine skin
  – Non-rigid image registration method
  – Time-lapse imaging of wound healing

• In vivo human skin

• Future directions and Conclusions
Introduction to Intravital Microscopy in Cell Biology Research

Intravital microscopy
- The most relevant experimental setting for studying cellular dynamics
- An indispensable tool in cell biology

What are the current limitations?
- Limited contrast
  - Minimize the use of contrast agents
  - Live tissue has many cellular and extracellular components
- Limited time window
  - Continuous imaging limited to a few hours
  - Difficult to co-register time-lapse data at cellular resolutions
Integrated optical coherence tomography (OCT), multiphoton microscopy (MPM), and fluorescence lifetime imaging microscopy (FLIM)

**OCT**
- Scattering intensity: - tissue structure
- Phase sensitivity: - vasculature

**MPM**
- Two photon excited fluorescence: - endogenous proteins, fluorescent molecules
- Second harmonic generation: - collagen structure

**FLIM**
- Fluorescence lifetime: - metabolic function, molecular dynamics
Optical Coherence Tomography (OCT)

Analogous to ultrasound imaging, but with near-infrared light

- Cellular-level resolution
- Real-time volumetric imaging
- Digital computational analysis
- Contrast based on optical scattering properties

3-D Volume Acquisition

Temporal Acquisition
Comparison with Confocal Microscopy

### Confocal Microscopy

- Photomultiplier Tube
- Point Source
- Objective Lens
- Focal Plane
- Sample

### Optical Coherence Tomography (OCT)

- Source
- Detector
- Electronics
- Computer
- Reference Mirror
- Objective Lens
- Sample

**Spatial Rejection of Out-of-Plane Light**

**Spatial and Coherent Rejection of Out-of-Plane Light**

OCT rejects multiply-scattered light that is no longer coherent with the light in the reference arm.
Multi-Photon Microscopy

**Advantages:**
- Longer wavelength excitation
- Deeper penetration
- Less absorption/photodamage
- Less autofluorescence
- Higher resolution
- Easier ex/em separation

**Disadvantages:**
- Complexity
- Expensive laser
- Fewer probes
- Weaker signal
Intrinsic properties of isolated molecules

To probe molecular changes in biological environment (pH, ion concentration, protein conformation, protein-protein interaction, etc.)

Fluorescence Lifetime Imaging Microscopy (FLIM)

\[ I(t) = I_0 \cdot \exp\left(\frac{-t}{\tau}\right) \]

Integrated Microscope Combining MPM, OCM, and FLIM

- **MPM**: two-photon autofluorescence, second harmonic generation (SHG)
- **OCM**: high resolution OCT, scattering and phase information
- **FLIM**: fluorescence lifetime imaging of cell metabolism

**System overview**
- Co-registered data
- Dual spectrum laser source
  - Tunable Ti-Sapphire laser (MPM)
  - Super-continuum generation (OCT)
- Interchangeable sample arm optics
  - High & low numerical aperture (NA)

**System specifications**
- NA: 0.85 (MPM), 0.05 (OCT)
- 920 nm center wavelength
- OCT spectral bandwidth: 80 nm
- CCD max line rate: 32 kHz

Nonlinear Fiber – 2 m LMA-8 Fiber (Crystal Fibre)  
Tunable Ti:sapphire (MaiTai HP, Spectra-Physics)  
Noise: Less than 1.1 dBm/Hz difference between pump and broadened beam over 1 Hz to 25 MHz

Graf, et al., J Biomedical Optics, 14:034019, 2009
Coherence Gate Curvature Correction in High Numerical Aperture Optical Coherence Imaging

Simplified non-telecentric beam-scanning system
2-D en face plane shows curved coherence gate
Maximum pathlength difference across FOV (blue)
Rayleigh range of focused beam (red)

Sample arm schematic
Spectral interference pattern and unwrapped phase from mirror
Image mapping of optical delay from curved coherence gate
FFT of raw spectrum and after multiplying by conjugate of spectral phase profile

USAF resolution chart
Silicon gel & 50 nm iron-oxide nanoparticles
In vivo human epidermis

Top Row: Standard imaging
Bottom Row: Corrected curvature

Architectural Morphology and Histology of Skin

OCT and Histology of Murine Skin

OCT and Histology of Engineered Skin
Multiphoton Microscopy of Hair Follicles in Murine Skin
Multimodality 3-D Imaging of Murine Skin

MPM Stacks

OCM Stacks

Collagen Network, Single Hair Shaft and Follicle to Depth ~ 150 µm
3-D Multimodality Imaging of Murine Skin
Time-Lapse Multimodal Microscopy of Cutaneous Wound Healing in Mice

- **Bone marrow cells in wound healing**
  - Traditionally associated with coagulation and inflammation
  - Recently demonstrated to contribute stem cells
    - Differentiation into keratinocytes, fibroblasts and endothelial cells
    - Poorly understood and inconclusive evidence

- **Time-lapse OCT-MPM of wound healing**
  - Comprehensive view of wound healing processes
  - Correlation with bone-marrow-derived cell dynamics

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http://en.wikipedia.org/wiki/Wound_healing
Methods: Green Fluorescent Protein (GPF) Bone Marrow Transplanted Mice

• GFP bone marrow mouse model
  – Wild-type mice given bone marrow transplants from donors with global GFP expression
  – Bone marrow cells and their progeny express GFP

• Imaging information
  – Mice anesthetized with isoflurane gas
  – Ear or skin flap mounted on coverslip for imaging
  – Glycerol used for index matching
OCT Imaging of GFP Bone Marrow Transplanted Mouse Ear Skin

• Dual mode OCT
  – Intensity provides structural information
  – Phase variance visualizes vasculature

• OCT image characteristics
  – Resolution: 10 µm transverse, 3 µm axial
  – Field of view: 2 mm
  – Penetration depth: ~500 µm
MPM Imaging of GFP Bone Marrow Transplanted Mouse Ear Skin

- Two MPM channels
  - GFP fluorescence (bone marrow cells)
    - Autofluorescence from hair is removed using segmentation
  - SHG (collagen fibers in dermis)

- MPM image characteristics
  - Resolution: 0.8 µm transverse, 2 µm axial
  - Field of view: 370 µm
    - Mosaics acquired with motorized stage enable 2.5 mm FOV
  - Penetration depth: ~150 µm
Co-Registered OCT and MPM Images from GFP Bone Marrow Transplanted Mouse Skin

En face view of the superficial dermis (OCT variance-SHG-GFP overlay)

Graf, et al., Technology, 2013

Skin cross section (OCT-SHG overlay)

En face view of a skin wound (OCT intensity-SHG-GFP overlay)

wound bed

capillaries

collagen

GFP cells
Co-Registered OCT and MPM Images from GFP Bone Marrow Transplanted Mouse Skin

En face view

Cross-sectional view

Color Channel Labeling
- OCT intensity
- OCT phase variance
- GFP bone-marrow-derived cells
- SHG - collagen
Visualizing Long-Term Dynamic Changes with Intravital Microscopy

- Based on registration of images taken at different time points

- Rigid registration
  - Limited sample types
    - Firm tissues: brain, bone, eye, etc.
    - Skin fold chambers
  - Small field of view

- Non-rigid registration
  - Potentially enable a wider range of time-lapse studies
    - Wound healing, tumor growth, soft tissue deformation
  - More challenging problem

Rigid Registration (rotation, scaling, translation)

Non-rigid Registration (arbitrary deformation)
Long-Term Observation of Wound Healing Requires Non-Rigid Image Registration

• Non-rigid registration algorithm
  – Based on spatial patterns of hair follicle positions (as seen in SHG images)

• Challenges
  – Potentially highly non-rigid changes
  – Nearly identical landmarks (follicles)

• Need to determine the \textit{correspondence} of the follicles and the image \textit{transformation}
Hair follicle detection
- Based on Difference of Gaussian (DOG) filtering and radial symmetry assessment

Hair follicle matching
- Define a ‘neighborhood’ metric (locations of nearby follicles)
- Compare follicles between images
- Best matches define an initial transformation

Iterative transformation update and follicle matching update
- Neighborhood metric
- Consistency with the current transformation

Non-Rigid Image Registration Algorithm based on Hair Follicle Spatial Patterns
Non-Rigid Image Registration Example: Before and After Skin Wounding

SHG images:  day 0 – prior to wound (reference)
    day 1 – after wound (target)
Non-Rigid Image Registration Assessment

Image #1  Image #2
Hair follicle detection  Hair follicle detection
Hair follicle matching  Grid transformation
Registered Image #2

Image 1 (before wound)  Image 2 (after wound)

No Registration  Affine Registration  Non-Rigid Registration

Day 1  Day 2  Day 1 warped

Grid transformation

MI value = 0.37  MI value = 0.27  MI value = 0.75

100 µm
Integrated OCT-MPM and image registration enables multimodal time-lapse imaging of wound healing

**Experiment information**
- ~ 500 µm excisional skin wound in the ear
- Wound imaged with OCT-MPM periodically for 5 weeks
- Image dimensions: 2.5 mm by 2.5 mm by 0.15 mm (x, y, z)

**Video information (next slides)**
- MPM overlay 3D renderings
  - SHG (grey), GFP (green), segmented hair shafts (red)
- OCT magnitude *en face* sections
- Phase variance *en face* projections
Multimodal long-term time-lapse optical microscopy of \textit{in vivo} skin regeneration

Spatially and temporally co-registered 3-D volumes

\textbf{OCM/OCT:} Tissue Structure

\textbf{Phase Variance:} Microvasculature

\textbf{TPEF:} GFP Bone marrow cells

\textbf{SHG:} Collagen

\textbf{Non-rigid transformation matrix:} Biomechanical changes

Time-lapse tracking from minutes to over several months

Graf, \textit{et al.}, Technology, 2013
Day 0  Day 1  Day 3  Day 8  Day 14  Day 22  Day 35  Day 44

Collagen synthesis
Bone marrow cell dynamics
Wound closure
Angiogenesis

Wound Contraction

Pixel area (µm²)

contraction expansion

Graf, et al., Technology, 2013
Quantification of Wound Contraction

Registration enables quantification of wound contraction

Warped mosaic  Warped grid  Pixel area of warped mosaic

Wound contraction vs. time

Wound contraction vs. time
Long-Term Imaging of Single Cells: Visualizing Activation of Langerhans Cells

- Langerhans cells: antigen presenting immune cells in the skin
- Following wounding in the vicinity of these cells we observed their activation and migration from the skin
Real-Time OCT of Human Skin

Human – Palmar & Dorsal

www.scf-online.com
OCM-MPM of *In Vivo* Human Skin - Superficial

Depth = 0 μm

Depth = 15 μm

Depth = 30 μm

- **OCM**
- **MPM**

- Nucleus-free keratinocyte
- Hair
OCM and MPM of *In Vivo* Human Skin Dermal-Epidermal Junction

Central tubular microstructures are dermal papillae viewed in *en face* sections.
Large-Area Mosaics for Cell Segmentation and Scattering Analysis

In Vivo Human Skin

Automated Nuclei Segmentation and Characterization

Real-Time Imaging of Capillary Loop Blood Flow

En face OCM of in vivo human skin

Quantitative Molecular Histopathology by Nonlinear Interferometric Vibrational Imaging (NIVI)

Spectral Decomposition of NIVI Signals from Skin

<table>
<thead>
<tr>
<th>$j$</th>
<th>$\Omega_j - \Omega_{j+1}$ (cm$^{-1}$)</th>
<th>Vibration</th>
<th>Contrast?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,842–2,865</td>
<td>CH$_2$ symmetric</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>2,865–2,890</td>
<td>CH$_2$ anti-symmetric</td>
<td>No</td>
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<tr>
<td>3</td>
<td>2,890–2,913</td>
<td>CH$_3$ symmetric</td>
<td>No</td>
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<tr>
<td>4</td>
<td>2,913–2,955</td>
<td>CH$_3$ symmetric</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>2,955–3,000</td>
<td>CH$_3$ asymmetric</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Chowdary, et al., Cancer Research 70:9562, 2010
Benalcazar, et al., Analytical and Bioanalytical Chemistry, 400:2817, 2011
Spectral Decomposition of NIVI Signals from Skin

Benalcazar, et al., Analytical and Bioanalytical Chemistry, 400:2817, 2011
Conclusions

Long-term time-lapse *in vivo* multimodal imaging provides a comprehensive view of wound healing and skin cell dynamics

<table>
<thead>
<tr>
<th>Image data</th>
<th>Contrast in skin</th>
<th>Wound healing or skin process</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT intensity</td>
<td>Tissue structure, skin layers</td>
<td>Structural repair; wound bed vs. dermis</td>
</tr>
<tr>
<td>OCT phase variance</td>
<td>Vasculature</td>
<td>Angiogenesis, blood and lymph flow</td>
</tr>
<tr>
<td>MPM (2-photon autofluorescence)</td>
<td>Cell structure, metabolism</td>
<td>Structural repair; metabolic activity</td>
</tr>
<tr>
<td>MPM (GFP fluorescence)</td>
<td>Bone marrow derived cells</td>
<td>Cell population dynamics; single cell migration</td>
</tr>
<tr>
<td>MPM (SHG)</td>
<td>Collagen; dermis structure</td>
<td>Collagen deposition; mechanical properties</td>
</tr>
<tr>
<td>FLIM</td>
<td>Cell metabolism; viability</td>
<td>Cell health and death; necrosis, apoptosis</td>
</tr>
<tr>
<td>Time-lapse registration</td>
<td>Time-dependent changes</td>
<td>Skin dynamics and wound contraction; Single-cell dynamics</td>
</tr>
<tr>
<td>MPM auto fluorescence</td>
<td>Epidermal and immune cells</td>
<td>Epithelialization; Langerhans cell dynamics</td>
</tr>
<tr>
<td>OCT intensity and phase variance</td>
<td>Lymphatic vessels</td>
<td>Lymph angiogenesis</td>
</tr>
</tbody>
</table>

*future work*
Acknowledgments

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Otolaryngology:

Ophthalmology:

Primary Care - Pediatrics:
Welcome

Located in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign, the Biophotonics Imaging Laboratory, directed by Professor Stephen Boppart, is dedicated to the development of novel optical biomedical imaging techniques.

News

- New position available in Neuro photonics!
- Check out the BIL Aquarium! Note: works only with Internet explorer.
- Prof. Boppart Honored with Hans Sigrist award
- BIL making headlines - Aberration Correction for 3-D Volumetric Imaging